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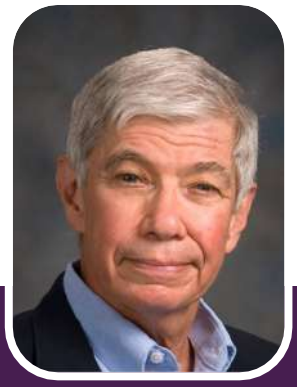

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Inhibition of Spermatogonial Differentiation by Testosterone

Review

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In this review we describe a variety of pathological conditions in rodents that result in seminiferous tubule atrophy, and which are characterized by the absence of all germ cells except for type A spermatogonia. In many such cases, suppression of gonadotropins and testosterone with gonadotropin-releasing hormone (GnRH) analogues restores spermatogonial differentiation and spermatogenic progression. In some cases, spermatogenesis is maintained after the cessation of hormonal treatment and fertility is restored. We and others have shown that the hormones normally responsible for the maintenance of spermatogenesis—testosterone, and in some cases, follicle-stimulating hormone (FSH)—actually inhibit spermatogonial differentiation in these conditions. This inhibitory function is a completely new role for androgens in the testis. It has long been known that systemic administration of low levels of testosterone can inhibit the completion of spermatogenesis (Steinberger, 1971) as a result of decreasing gonadotropin levels, thereby reducing testosterone production by the Leydig cells and actually reducing intratesticular testosterone (ITT) concentrations. In the pathological conditions we and others have described, however, the ITT concentration is not reduced and it is responsible for the inhibition of spermatogonial differentiation.

As will be described later, it is not clear whether the direct action of testosterone is to block an actual step of differentiation of the spermatogonia or to cause the apoptosis of the spermatogonia prior the step at which they would differentiate. However, throughout this review we will use the concept of ''inhibition of spermatogonial differentiation'' to encompass both possibilities.

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Blocks of Spermatogonial Differentiation

The stem spermatogonia, designated A_s, maintain their numbers by self-renewal, and some differentiate to form by sequential divisions A_{pr} (A-paired), and A_{al}-4 and A_{al}-8 (A-aligned) spermatogonia, which go on to produce A_1 spermatogonia.

This differentiation may be blocked in 3 ways. In one way, which is the focus of this review, undifferentiated spermatogonia proliferate but their numbers remain relatively constant because of apoptosis (Figure 1) (Allard and Boekelheide, 1996; Shuttlesworth et al, 2000). We will call this the proliferation-apoptosis (PAp) block to distinguish it from the other 2 ways. The second type of block in spermatogonial differentiation, which is caused by vitamin A deprivation, is characterized by spermatogonial differentiation to the A_{al} stage, but then proliferation ceases and spermatogonia can remain at this stage for a period of only several weeks (van Pelt and de Rooij, 1990) and is designated Ar (arrest). In the third kind of block, which is observed in several types of transgenic mice, including *bax*-deficient, or *bcl-2*-overexpressing, or glial cell line-derived neurotropic factor (GDNF)-overexpressing mice (Knudson et al, 1995; Furuchi et al, 1996; Meng et al, 2000), type A spermatogonia proliferate and accumulate but produce few differentiated cells, and is designated proliferation-accumulation (PAc).

The precise relationship between these 3 blocks in rodents and the clinical phenotype of spermatogonial arrest in humans, which is often the result of hypogonadotropism (Johnsen, 1970), is not known. However, the types of spermatogonia present in humans and their proliferative status have not been studied.

Conditions Causing the Proliferation-Apoptosis Block in Spermatogonial Differentiation

A variety of testicular toxicants produce similar testicular histology in rats consistent with the PAp type of block. These agents include hexanedione (Boekelheide and Hall, 1991), boric acid (Ku et al, 1993), radiation (Kangasniemi et al, 1996), procarbazine (Meistrich, 1999), dibromochloropropane (DBCP; Meistrich, unpublished results), and indenopyridines (Hild et al, 2001). The type A spermatogonia proliferate in atrophic tubules but they do not accumulate because they continue to be lost by apoptosis many months after the original acute or subchronic exposure. Atrophic tubules with actively dividing stem type A spermatogonia were also observed in testis cross-sec-

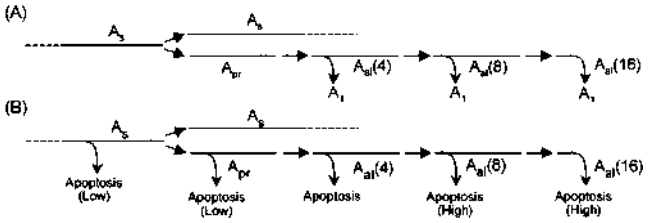

Figure 1. Outline of stem cell kinetics in **(A)** normal rodents and **(B)** rodents with a proliferation-apoptosis block in spermatogonial differentiation as described for toxicant-treated rats, cryptorchid mice, and some mutant mice. In normal rodents, no apoptosis is observed at these stages, and the A_{al} and some A_{pr} spermatogonia are induced to undergo differentiation into A_1 spermatogonia at stage VII–VIII of the cycle of the seminiferous epithelium. In the rodents with the PAp block, spermatogonia of all clonal sizes undergo apoptosis, with the probability of undergoing an apoptotic event, as opposed to a mitotic division, increasing with chain length.

tions from 27-month-old Brown-Norway rats (Schoenfeld et al, 2001). The failure of these cells to differentiate is in part responsible for the decline in spermatogenesis with age in these rats.

In contrast to that of rats, brief exposures to such toxicants do not induce such a block in spermatogonial differentiation in mice. Whereas 3.5 Gy of irradiation was sufficient to induce this block in $LBNF_1$ rats, mouse spermatogonia maintain their ability to differentiate even after doses of 12 Gy (Meistrich et al, 1978). But a block in spermatogonial differentiation can be induced by continuous elevation of temperature. In cryptorchid C57BL/6 mouse testes, spermatogenesis fails to progress past the A_{al} spermatogonial stage (Haneji et al, 1983); these cells actively proliferate but die by apoptosis (de Rooij et al, 1999). Similar blocks in the differentiation of A spermatogonia were also observed in *jsd* (juvenile spermatogonial depletion) mice (Beamer et al, 1988) and in Sl^{17H} mice, which have an altered form of stem cell factor in Sertoli cells (Brannan et al, 1992). In these mice, an initial wave of spermatogenesis is not maintained, so that the adult testis tubules contain only Sertoli cells and type A spermatogonia; the latter proliferate but die by apoptosis (de Rooij et al, 1999). In addition, certain other mouse mutants, including XO-Sxr^b (Sutcliffe and Burgoyne, 1989) and *Dazl* (Schrans-Stassen et al, 1999), have PAp blocks in spermatogonial differentiation but they differ from the above 2 models in that this condition is apparent by postnatal day 10 and there is no initial wave of spermatogenesis.

It was indeed surprising that such a wide variety of toxicant exposures, conditions, and genetic mutations produced such a similar phenotype. For example, some of these toxicants, such as irradiation, are believed to act directly on germ cells (Lee et al, 1999), whereas other toxicants, such as hexanedione, are believed to act on Sertoli cells. Furthermore, at least in some instances, the block to spermatogonial differentiation does not begin to develop for almost 6 weeks after the insult. Both these observations imply that the block to spermatogonial differentiation is not a direct consequence of the initial event, but that different initiating events produce a common outcome, which in turn, leads to the block.

Characterization of the Proliferation-Apoptosis Block of Spermatogonial Differentiation

Although the PAp blocks in spermatogonial differentiation caused by different agents have much in common (Figure 1), they show some quantitative differences in terms of the stage to which spermatogonia differentiate before undergoing apoptosis.

The type A spermatogonia in atrophic testes were first identified following exposure of either Sprague-Dawley or Fischer F344 rats to hexanedione (Boekelheide and Hall, 1991). Stem cells (isolated type A spermatogonia), although reduced in number from controls, still constituted a substantial proportion of these remaining A spermatogonia (Allard et al, 1995). The A spermatogonia were in active proliferation, but their numbers remained constant because they underwent apoptosis (Allard and Boekelheide, 1996). Calculations based on numbers of stem cells and total spermatogonia indicated that the cells were progressing to the A_2 or A_3 spermatogonial stage (Allard and Boekelheide, 1996).

In contrast, direct, whole-mounted tubule analysis of mitotic clones of A spermatogonia in irradiated $LBNF_1$ rats revealed that most of the clones were isolated or paired A spermatogonia and few had a clone size greater than 4, indicating that they were early progeny from the stem cells (Shuttlesworth et al, 2000). Very few clones progressed to become A_{al}-8 and A_{al}-16, which are the clone sizes that most often undergo differentiation in normal rats (Figure 1A) because the probability of apoptosis increased as clone size increased (Figure 1B). Thus, failure of spermatogonia to differentiate appeared to be a consequence of their undergoing apoptosis first.

In *jsd*, Sl^{17H}, and cryptorchid mice, the clones of A spermatogonia in whole-mounted tubules were arranged as 1 to 16 cells (de Rooij et al, 1999). There were appreciable and similar numbers of clones of A_{al}-8 and A_{al}-16 in all 3 models. These undifferentiated A spermatogonia were proliferating, but they did not accumulate, and the larger clones in particular underwent apoptosis. Because the clone sizes indicate that spermatogonia develop to the point at which the A_{al} cells should differentiate into A_1 spermatogonia, the failure to do so indicates the lack of a signaling system rather than prior apoptosis.

The difference in numbers and stage of development of spermatogonia between the irradiated rat and the mouse models appears to be real because the same methodology was employed. It is not known whether these differences are due to how mice and rats respond to

blocks at the spermatogonial level or whether differences in the cause of the blocks. The difference in stage at which the block was reported to occur in irradiated vs hexanedione-treated rats could be a result of the different analytical methods employed, in addition to the possible contributions of rat strain or the nature of the original toxic insult.

Hormone Levels During the Proliferation-Apoptosis Block to Spermatogonial Differentiation

As is typical in cases of testicular tubular atrophy, FSH and luteinizing hormone (LH) levels rise in most cases in which only type A spermatogonia remain in the tubules. FSH levels were elevated 1.5-fold to 2-fold and LH levels were elevated 2-fold to 4-fold after treatment of rats with hexanedione (Boekelheide and Hall, 1991), gamma radiation (Kangasniemi et al, 1996), procarbazine (Meistrich et al, 1999), indenopyridine (Hodel and Suter, 1978), boric acid (Ku et al, 1993), and DBCP (Meistrich, unpublished results) and in *jsd* mice (Shetty et al, 2001).

In all the cases studied, serum testosterone remained unchanged. It has been shown that when the germ cells in the testes are lost, testicular mass and, consequently, blood flow decline (Wang et al, 1983). The maintenance of serum testosterone levels is a result of the hypothalamic-pituitary axis acting to keep serum testosterone constant when there is a decline in testicular blood flow by adjusting LH levels accordingly. This results in a 2.5-fold to 3-fold increase in ITT concentrations, which was confirmed in irradiated, procarbazine-treated, and DBCP-treated rats and *jsd* mice. The greater proportion of Leydig cells (their numbers are not decreased) in the testis, the decreased clearance rate of newly produced testosterone from the testis, and the elevated LH levels are all responsible for the increase in ITT concentrations.

There were 2 exceptions to this pattern of hormone changes. First, in cryptorchid mice, FSH was elevated 1.5-fold, but LH was unchanged (Mendis-Handagama et al, 1990). Second, in aged rats, both serum and testicular interstitial fluid testosterone levels were depressed (Schoenfeld et al, 2001). This depression in testosterone levels may be a combined result of the general depression with aging in LH and Leydig cell function, which can no longer respond by increasing testicular testosterone production. Nevertheless, these results show that above normal levels of ITT are not necessarily required for inhibition of spermatogonial differentiation, which will be discussed later.

GnRH Analogue Treatment Reverses Proliferation-Apoptosis Blocks in Spermatogonial Differentiation

We first demonstrated the stimulation of recovery of spermatogenesis in rats using hormone treatment given after irradiation (Meistrich and Kangasniemi, 1997). All previous studies had focused on the possible protective effect of giving the suppressive hormones before the toxicant exposure (Ward et al, 1990). However, we ruled out many possible mechanisms (Meistrich et al, 1997) by which the hormone treatment could have protected the survival of the spermatogonia and concluded that the only explanation that fit the data was that the hormonal treatment given before the toxic insult helped somatic cells to support sustained recovery of spermatogenesis from surviving stem cells after the toxicant exposure (Meistrich et al, 2000). In all subsequent work we have focused on giving the GnRH analogue treatment after toxicant exposure, although others have given the hormones before and after the toxicant.

In our initial study (Meistrich and Kangasniemi, 1997), the tubule differentiation index (TDI; the percentage of tubule cross-sections containing differentiated cells) was only 37% at 10 weeks after 3.5 Gy irradiation in the absence of hormone treatment. When GnRH agonist treatment was started immediately after irradiation, the TDI at 10 weeks was dramatically increased to 91%. However, because GnRH analogue treatments suppress testosterone, which is required for spermatid differentiation, there is histological recovery to the round spermatid stage, but no sperm are produced. The production of sperm after cessation of a transient GnRH analogue block will be discussed below. We also showed that systemic exogenous administration of testosterone, which suppresses ITT concentrations, also maintains spermatogonial differentiation after irradiation.

In other cases involving a toxicant-induced PAp block to spermatogonial differentiation (Table 1) maintenance or recovery of spermatogenesis was enhanced by giving GnRH analogues after the toxicant treatment (Table 2). These include hexanedione, procarbazine, or DBCP.

In some other cases, GnRH analogue treatment has also proved beneficial to the maintenance or recovery of spermatogenesis after exposure to a toxicant for which the blocks in spermatogonial differentiation were not well characterized. Treatment with GnRH agonist for about 12 weeks after exposure to the anticancer agent busulfan significantly increased the TDI at week 18 (Udagawa et al, 2001). However, a 4-week hormone treatment prior to busulfan injection was ineffective. The irreversible loss of spermatogenic function that occurred after a single dose of heat to rat testes was likewise counteracted by GnRH analogue posttreatment (Setchell et al, 2001), and treatment with GnRH agonist before heating was also effective (Setchell et al, 2002). Finally, prevention of the indenopyridine-induced block to spermatogonial differentiation was achieved when GnRH analogues were given both before and after drug treatment (Hild et al, 2001). However, in a subsequent study using a GnRH antagonist, only prior, but not subsequent, treatment with the GnRH

Table 1. *Examples of pathological conditions causing a proliferation-apoptosis block in spermatogonial differentiation in rodents*

Species	Treatment* or Gene	Duration of Exposure	Reference
Rat	Hexanedione	Subchronic (5 wk)	(Boekelheide and Hall, 1991)
Rat	Radiation (gamma)	Acute	(Kangasniemi et al, 1996)
Rat	Radiation (neutron)	Acute	(Wilson et al, 1999)
Rat	Procarbazine	Acute	(Meistrich 1999)
Rat	Indenopyridine (CDB-4022)	Acute	(Hild et al, 2001)
Rat	Boric acid	Subchronic (9 wk)	(Ku et al, 1993)
Rat	DBCP	Acute	Meistrich, unpublished
Rat	Aging	Chronic	(Schoenfeld et al, 2001)
Mouse	*jsd* mutation	Permanent (genetic)	(Beamer et al, 1988; de Rooij et al, 1999)
Mouse	Heating (cryptorchid)	Chronic	(Nishimune et al, 1978; de Rooij et al, 1999)
Mouse	Sl^{17H} mutation	Permanent (genetic)	(Brannan et al, 1992; de Rooij et al, 1999)
Mouse	X*Sxr*^{b}O (*Eif2s2y* mutation)	Permanent (genetic)	(Sutcliffe and Burgoyne, 1989)
Mouse	*Dazl* −/− mutation	Permanent (genetic)	(Schrans-Stassen et al, 2001)

* In all cases, except for indenopyridine treatment, spermatogonia proliferated but were lost by apoptosis.

analogue was effective at restoring recovery of spermatogenesis following indenopyridine treatment (S.A. Hild, personal communication).

GnRH analogue treatment also enhanced the stimulation of recovery of spermatogenesis from stem cells following spermatogonial transplantation. When mouse testicular cells were transplanted into busulfan-treated mouse recipients, the efficiency of differentiated germ cell production from transplanted stem cells in the recipient tubules was enhanced with GnRH analogue treatment (Ogawa et al, 1998; Dobrinski et al, 2001). However, a significant benefit was derived only from pretreatment with GnRH analogue, indicating that the hormone treatment may be important for the stem cells to attach in their proper niche in the seminiferous tubules, but not for the initiation of differentiation. The importance of suppressing ITT levels with either GnRH agonist or exogenous testosterone treatment was also demonstrated in studies in which rat or mouse spermatogonia were transplanted into busulfan-treated rat hosts (Ogawa et al, 1999).

When GnRH treatment is given relative to the toxic exposure is important. Data from irradiated and hexanedione-treated rats showed that treating immediately after exposure to a toxicant was more effective than delayed treatments in the restoration of spermatogonial differentiation (Meistrich et al, 1999). However, there has not been a strict comparison between the effects of pretreatments and posttreatments in any of the models in which both treatments are effective.

Fertility can be restored in these pathological situations by GnRH analogue treatment. When a 10-week GnRH agonist or GnRH antagonist treatment was started immediately after 3.7-Gy irradiation, fertility was maintained at week 20 in the GnRH agonist and GnRH antagonist treated rats at normal and nearly normal levels, respectively, whereas none of the irradiated-only rats were fertile (Meistrich et al, 2001b). When treatment was initiated 10 weeks after 5 Gy irradiation, at which point spermatogenesis had completely declined, fertility was restored at week 30 to subnormal levels in 83% of GnRH agonist and 50% of GnRH antagonist treated rats. Thus we conclude that normal fertility can be restored by GnRH treatment after irradiation, although that may depend on initiation of the GnRH analogue treatment soon after a toxicant exposure that is not too severe. We have also demonstrated that GnRH analogue posttreatment significantly increases recovery of fertility in rats after procarbazine treatment (Meistrich et al, 1999). In contrast in the *jsd* mice, a transient increase in spermatogonial and spermatocyte differentiation was produced by the GnRH antagonist treatment; testicular sperm extraction and intracytoplasmic sperm injection (ICSI) were both required to produce offspring (Tohda et al, 2002).

Maintenance of Spermatogenesis After Reversal

Although the TDI in rats receiving 3.5 Gy of radiation and GnRH agonist for 10 weeks was 91%, testicular sperm head counts were only 0.1% of controls because the hormone treatment suppressed spermiogenesis. However, when additional time without further GnRH treatment was allowed before the rats were killed, the TDI recovered to 100%, and sperm counts reached about 50% of normal control levels at 6.5 weeks after stopping treatment and were maintained at this level for at least another 3.5 weeks.

The maintenance of spermatogenesis in irradiated rats after GnRH analogue treatment is stopped depends on the toxicant dose and time of initiation and duration of the hormone treatment. For example, when a 7-week GnRH analogue treatment was initiated at week 15 after 6 Gy of irradiation, the TDI was elevated from 0% in irradiated-only rats to 95% at week 24 (2 weeks after stopping the GnRH treatment), but then declined to 50% at week 36 (14 weeks after stopping GnRH; G.A. Shuttlesworth and M.L. Meistrich, unpublished data). Thus permanent progression and maintenance of spermatogenesis is not

Table 2. *Treatments or mutations in which the block of spermatogonial differentiation can be reversed with hormone treatment*

Species	Treatment or Mutation*	Hormone treatment and timing relative to inducing event			References
		Before	Immediately After	After a Delay	
Rat	Hexanedione*		GnRH-agonist	GnRH-agonist	(Blanchard et al, 1998)
Rat	Radiation (gamma)*	T+E2	GnRH-agonist, GnRH-antagonist, testosterone	GnRH-agonist, GnRH-antagonist, testosterone, androgens	(Meistrich and Kangasniemi, 1997; Meistrich et al, 2000, 2001b; Shetty et al, 2000; Shetty et al, 2002)
Rat	Procarbazine*	GnRH-agonist + flutamide, GnRH-antagonist + flutamide, T, T+E2	GnRH-agonist		(Parchuri et al, 1993; Kangasniemi et al, 1995a,b; Meistrich, 1999)
Rat	Indenopyridine (CDB-4022)*	GnRH-agonist†	GnRH-agonist†		(Hild et al, 2001)
		GnRH-antagonist	{GnRH-antagonist}‡		S.A. Hild, personal communication
Rat	DBCP*		GnRH-agonist	GnRH-agonist	M.L. Meistrich, unpublished
Rat	Acute heating	GnRH-agonist + flutamide	GnRH-agonist + flutamide		(Setchell et al, 2001)
Rat	Aging*			GnRH-agonist (at 27 mo)	(Schoenfeld et al, 2001)
Rat	Busulfan	{GnRH-agonist}‡	GnRH-agonist		(Udagawa et al, 2001)
Rat	Busulfan, spermatogonial transplant	GnRH-agonist†	GnRH-agonist†		(Ogawa et al, 1999)
Mouse	*jsd* mutation*		GnRH-antagonist (at age 5 weeks)	GnRH-antagonist (at age 10 weeks)	(Matsumiya et al, 1999; Shetty et al, 2001)
Mouse	Busulfan, spermatogonial transplant	GnRH-agonist	{GnRH-agonist}‡		(Ogawa et al, 1998; Dobrinski et al, 2001)

* Instances in which it has been shown that type A spermatogonia are present in the atrophic tubules but do not undergo differentiation.
† Hormone treatment was given both before and after the toxicant treatment.
‡ The hormone in brackets {hormone} indicates the treatment was tried but was unsuccessful.

assured by this technique. Although no time course studies were done, extensive recovery of spermatogenesis in tubules after hexanedione treatment was observed 9 weeks after the end of a 10-week GnRH agonist treatment, and the degree of recovery was inversely correlated with the dose of hexanedione (Blanchard et al, 1998).

In contrast to the toxicant-treated rat models, spermatogenesis degenerated rapidly in *jsd* mice after withdrawal of the GnRH antagonist. Whereas a 6-week GnRH antagonist treatment increased the TDI from 11% in nonhormone treated mice to 95%, 5 weeks after cessation of the treatment the TDI progressively declined to 78% and to 8% after 13 weeks (Shetty et al, 2001). Although one wave of late spermatids was produced from the differentiating spermatogonia and spermatocytes that developed during the GnRH antagonist treatment, the maximum percentage of tubules that contained elongated spermatids was only 20% at week 4 after the hormone treatment was stopped (Tohda et al, 2002). However, these elongated spermatids were used in ICSI to effect a pregnancy.

The difference between the maintenance of spermatogenesis in the irradiated rat model and *jsd* mice is that the former likely involves an epigenetic change, whereas the latter is a genetic alteration. The epigenetic change caused by irradiation to render spermatogonial differentiation sensitive to inhibition by testosterone can be largely reversed by hormonal treatment. But the underlying defect in a genetic disorder manifests itself again as soon as the hormone treatment is stopped.

Role of Testosterone in Block of Spermatogonial Differentiation

Because the GnRH analogues that were used to stimulate or maintain spermatogonial differentiation in the various cases described above generally suppress LH, FSH, and testosterone, these hormones were implicated in the inhibition of spermatogonial differentiation. Using irradiated rat and *jsd* mouse models, we and others investigated the roles of these hormones in the regulation of spermatogonial differentiation.

One study involved the administration of exogenous LH to GnRH antagonist–treated *jsd* mice (Tohda et al, 2001). Whereas the GnRH antagonist restored spermatogonial differentiation, the addition of exogenous LH inhibited it. However, other experiments with *jsd* mice (Shetty et al, 2001; Tohda et al, 2001) and with irradiated rats indicated that it was the testosterone production stimulated by the LH, and not the LH itself, that inhibited spermatogonial differentiation. For example, GnRH agonist treatment of $LBNF_1$ rats did not suppress LH levels, but it did suppress ITT, serum testosterone, and FSH levels and stimulated spermatogonial differentiation (Meistrich and Kangasniemi, 1997; Meistrich et al, 1999). In another study, when irradiated rats treated with GnRH agonist were given exogenous testosterone, spermatogonial differentiation was inhibited despite a suppression of LH levels (Shetty et al, 2001). This led us to further investigate the precise roles of testosterone and FSH in the inhibition of spermatogonial differentiation after irradiation.

Various studies have indicated that testosterone had an inhibitory effect. Because there is a major increase in the ITT concentration in mice between 30 and 40 days of age (Jean-Faucher et al, 1978), the large decline in the numbers of B spermatogonia in *jsd* testes, which occurs between 6 and 7 weeks of age (Kojima et al, 1997), could very well be a consequence of the increase in ITT. In addition in these mice, the stimulation of spermatogonial differentiation by suppression of testosterone with GnRH antagonist was reversed by exogenous testosterone (Shetty et al, 2001). Furthermore, that inhibition by testosterone was reversed by treatment with the androgen-receptor antagonist flutamide.

In irradiated rats, we have shown that testosterone dose-dependently reduced the GnRH antagonist-stimulated spermatogonial differentiation. (Shetty et al, 2000, 2002). Further, the stimulatory action of low-dose testosterone alone, which reduces ITT concentrations, was also reduced with increasing doses of testosterone that increased both ITT and serum testosterone concentrations. The TDIs and the serum and ITT levels were similar for each given dose of testosterone, with or without the GnRH antagonist, showing that the testosterone levels in the testis or the serum, or both, limit the ability of spermatogonia to differentiate. The inhibition of spermatogonial differentiation by testosterone was further confirmed by showing that flutamide reversed the inhibition induced by exogenous testosterone in GnRH antagonist–treated, irradiated rats (Shetty et al, 2000). Further support for our hypothesis that it is indeed testosterone acting through the androgen receptor and not a nonandrogenic metabolite of testosterone that inhibits spermatogonial differentiation was obtained by showing that various androgens, including 5α-dihydrotestosterone (a 5α-reduced androgen), 7α-methyl-19-nortestosterone (a non-5α-reducible androgen but one that can be aromatized), and methyltrienolone (a nonmetabolizable androgen) also suppressed spermatogonial differentiation in GnRH antagonist–treated irradiated rats (Shetty et al, 2002). In the same study, we showed that estradiol (E_2) was not inhibitory.

When testicular testosterone levels in irradiated rats treated with various GnRH analogues and testosterone combinations were compared with the TDI, an excellent negative correlation was observed (Figure 2) with only 1 point deviating significantly from each of the fitted curves (Figure 2, B and D). Although a general negative trend

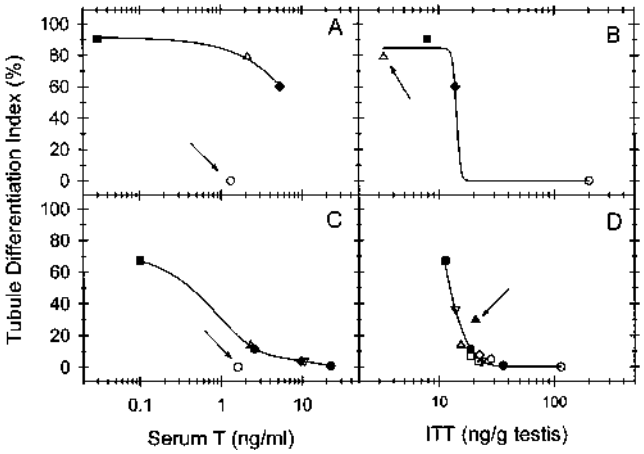

Figure 2. Correlation between serum testosterone **(A, C)** and ITT **(B, D)** during hormone treatment and the levels of recovery of spermatogenesis at the end of experiment. **(A, B)** Data from combinations of testosterone with GnRH agonist, given during weeks 0–10 after 6 Gy irradiation. TDI analysis was performed on testicular histological sections prepared on week 20. **(C, D)** Data from combinations of testosterone with GnRH antagonist, given during weeks 3–7 after 5 Gy irradiation. TDI analysis was performed on testicular histological sections prepared on week 13. Equivalent symbols in **(A** through **D)** are from the same treatments. Regression curves were fitted to the data with the exception of the deviant points (open circles) in **(A** and **C).** Arrows indicate discrepancies from complete correlations. Data from 2 reports (Shetty et al, 2000, 2002) were combined.

was also noted for serum testosterone vs TDI, there was a very significant deviation (Figure 2, A and C, arrow and open circles) in which the irradiated rats with a moderate amount of serum testosterone showed no differentiation. However, irradiated rats treated with GnRH analogues and testosterone (Figure 2, A and C, open upward triangles and filled diamonds) showed higher serum testosterone, but a significantly higher percentage of the tubules contained differentiating cells. This led us to conclude that ITT is the major factor, as the irradiated-only rats (open circles) had much higher ITT concentrations than those also treated with GnRH analogues and testosterone (Figure 2, B and D, open upward triangle and filled diamond). However, there were some small but significant discrepancies in the correlation between ITT and TDI. For example, GnRH agonist–treated, irradiated rats (Figure 2B, filled square) showed a higher TDI but also higher ITT than a similar group of rats that also received testosterone implants (Fib. 2B, open triangle). Because the former group had much lower serum testosterone levels (Figure 2A), we suggested that although the ITT was the major factor inhibiting spermatogenic recovery, serum testosterone seemed to have a minor additive inhibitory role. The point that deviated from the curve in Figure 2D (filled triangle) was a result of treatment of irradiated rats with GnRH antagonist and daily injections of testosterone proportionate, which may result in varying levels of testosterone throughout the course of treatment.

In all these situations, ITT concentrations in the normal range (about 50 ng/g testis) seem to inhibit the differentiation of spermatogonia. Figure 2D shows that even ITT concentrations of 15–30 ng/g of testis inhibited spermatogonial differentiation. Further, the observed block in the spermatogonial differentiation in aged rats that had ITT concentrations below normal and spermatogonial differentiation was stimulated by further suppression of ITT with a GnRH agonist show that above normal levels of ITT are not necessarily required for the inhibition of spermatogonial differentiation. Rather, in these circumstances, spermatogonial differentiation becomes sensitive to physiological levels of testosterone.

Based on the concept that testosterone inhibited spermatogenesis in toxicant-treated rats, hexanedione-exposed rats were treated with ethane dimethane sulfonate (EDS), which specifically eliminates Leydig cells, followed by GnRH agonist, which prevented Leydig cell regeneration (Richburg et al, 2002). Even though EDS reduced testosterone levels to undetectable levels, the EDS treatment inhibited the recovery of spermatogonial differentiation that the GnRH agonist would normally induce. Although the results of this study seemed to contradict the hypothesis that testosterone inhibits spermatogonial differentiation, that hypothesis could still be valid if a Leydig cell factor is required for the stimulation of spermatogenic recovery in the atrophic testis and this factor were eliminated by EDS, but not by GnRH analogue treatment.

Role of FSH in Block of Spermatogonial Differentiation

The elevated FSH levels in these pathological models of testicular atrophy could contribute to the inhibition of spermatogonial differentiation. Although as shown above, testosterone appears to be an inhibitory factor, it is necessary to determine whether FSH also has a role.

The possible contribution of serum testosterone to inhibiting spermatogonial differentiation suggests that testosterone may act at an extratesticular site. One such likely site is the pituitary, where it could act by altering gonadotropin levels. We have already ruled out LH as having a significant contribution to the inhibition of spermatogonial differentiation, so we focused on a possible role for FSH. However, testosterone has a complex action on pituitary production of FSH. When testosterone is given to rats or mice that have normal GnRH production and action, it suppresses FSH levels by having a combined action on the hypothalamus and pituitary. However, when testosterone is given to GnRH antagonist–treated rats, but not mice (Shetty et al, 2001), it reverses the GnRH antagonist-induced reduction of FSH levels in these rats by direct up-regulation of FSHβ gene transcription in the pituitary (Perheentupa et al, 1993). The levels of FSH in the presence of exogenous testosterone appear

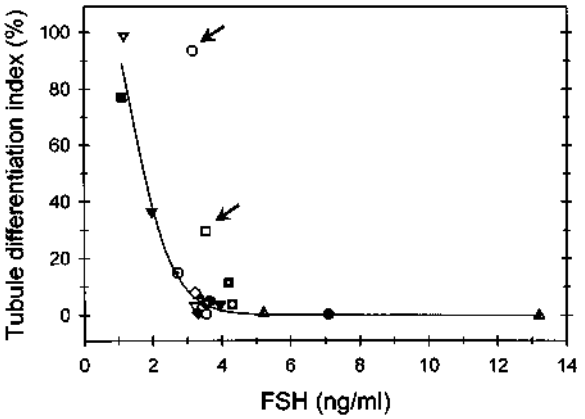

Figure 3. Correlation between serum FSH levels during hormone treatment and the subsequent levels of recovery of spermatogenesis in irradiated rats treated with various combinations of GnRH antagonist, 1 of the androgens, and the antiandrogen flutamide. The points indicated by arrows deviated from the curve fitted to the other points. The deviant points are from rats treated with GnRH antagonist, testosterone, and flutamide (open circle), or GnRH antagonist and daily injections of testosterone propionate (open square). Data from 2 reports (Shetty et al, 2000, 2002) were combined.

to be independent of whether or not a GnRH antagonist is also given (Shetty et al, 2000).

There was good inverse correlation between TDI and FSH levels (Figure 3), which could be due in part to the concomitant rise in FSH when testosterone was given. Several points did deviate from this correlation curve (Figure 3, arrow). Although TDI and ITT were even more closely correlated, (Figure 2), a role for FSH in inhibition of spermatogonial differentiation could not be ruled out. We directly tested the role of FSH by giving exogenous FSH to irradiated rats while suppressing levels and actions of androgens with GnRH antagonist and flutamide. Exogenous FSH significantly inhibited the tubule differentiation stimulated by GnRH antagonist-flutamide treatment, although not as drastically as did androgens (G. Shetty, unpublished data). From these data and the overall relationship between hormone levels and TDI (Figure 4), we conclude that primarily ITT, but also FSH, which is regulated by serum testosterone, inhibits spermatogonial differentiation in irradiated rats.

In contrast to the results with rats, administration of exogenous FSH in *jsd* mice during suppression of gonadotropins did not inhibit spermatogonial differentiation (Tohda et al, 2001). Further confirmation of the inability of FSH to inhibit spermatogonial differentiation in *jsd* mice was shown by the lack of correlation between FSH levels and the TDI (Shetty et al, 2001) and the lack of correlation in timing of the rise in FSH levels, which reaches near adult levels during the first 2 weeks after

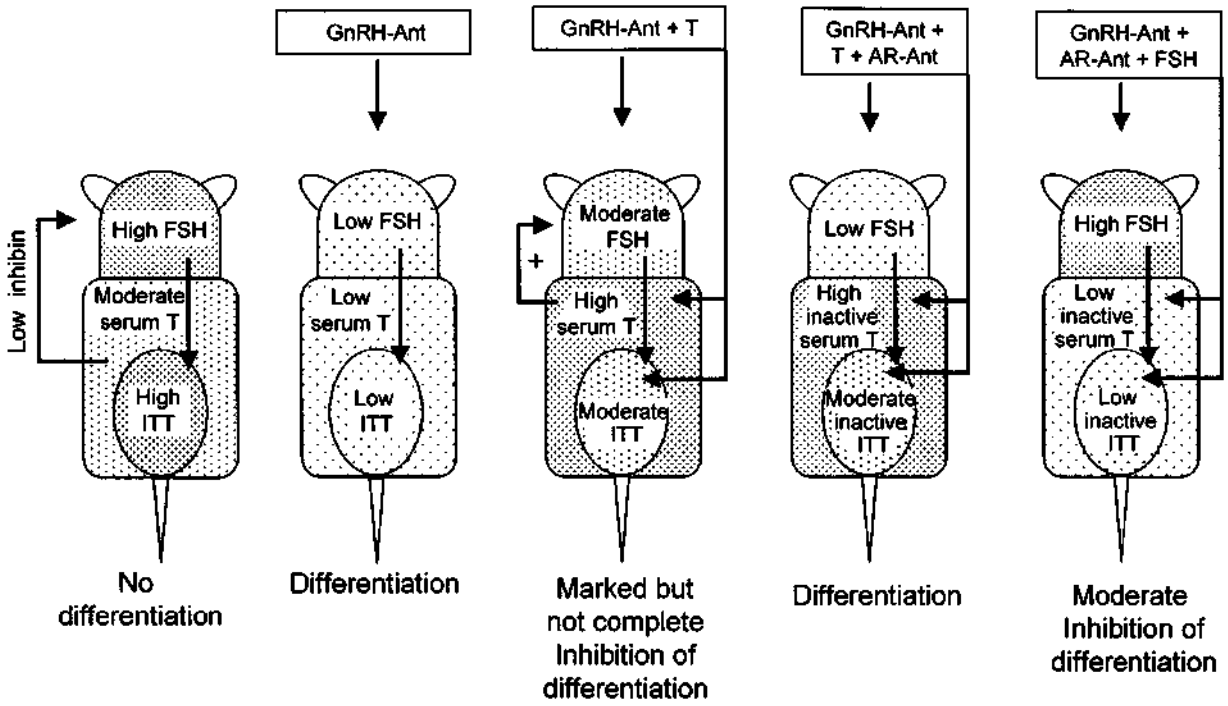

Figure 4. Schematic of observations in irradiated rats showing the different levels of FSH and testosterone in the serum and testis during various hormone treatments and the resulting changes in the differentiation of spermatogonia. Whereas no spermatogonial differentiation is observed in irradiated rats not treated with hormones **(left panel),** likely due to the high levels of ITT and FSH, differentiation is induced by suppression of testosterone and FSH **(second panel).** The fact that there is some differentiation in the third panel indicates that either ITT or FSH are inhibitory. The fourth panel indicates that testosterone is acting through the androgen receptor, although it could be acting at the pituitary or testis. The fifth panel (compare with the second panel) shows that FSH has an inhibitory role.

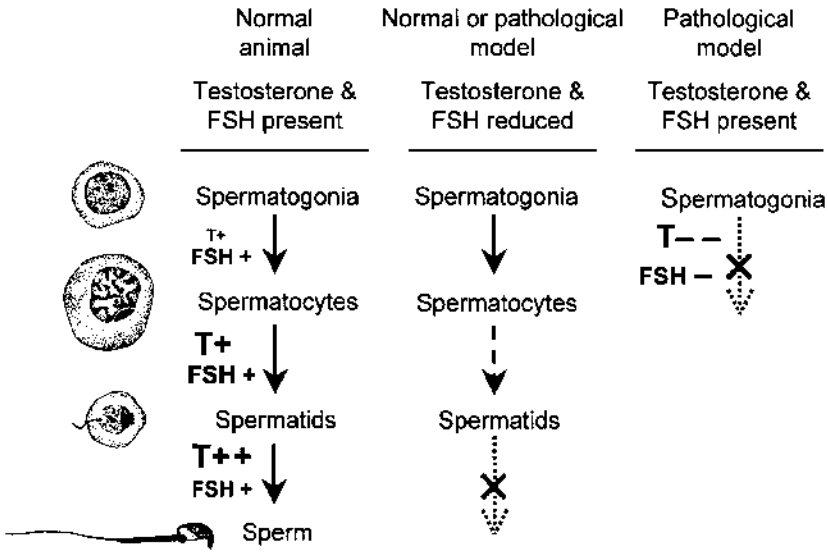

Figure 5. Roles of testosterone and FSH in normal spermatogenesis and in pathological models involving a proliferation-apoptosis block in spermatogonial differentiation. Stimulatory and inhibitory roles are indicated by pluses and minuses, respectively, with the strength of the stimulatory or inhibitory action indicated by the numbers of pluses or minuses and the font size.

birth (Slegtenhorst-Eegdeman et al, 1998), and the major block in spermatogonial differentiation, which does not occur until between weeks 6 and 7 after birth (Kojima et al, 1997).

Relationship to Roles of Hormones in Normal Spermatogenesis

In the various pathological conditions we have described, it appears that testosterone and FSH may act additively to inhibit the differentiation of spermatogonia, whereas in normal spermatogenesis they act additively to support survival and differentiation of spermatocytes and spermatids. Thus the differences between the action of the hormones in the 2 situations involves not only the direction of action but also their targets during spermatogenesis (Figure 5). In spermatocyte and spermatid differentiation, the normal requirement for primarily testosterone, but also with some additive effects of FSH (O'Donnell et al, 1994; El Shennawy et al, 1998), appears not to be altered in the pathological situation, in which differentiation does not proceed past the spermatocyte or early spermatid stage during suppression of testosterone and FSH. However, in these pathological models the hormones act at an additional checkpoint. Spermatogonial survival and differentiation, which in normal rats can proceed in the absence of testosterone and FSH but is augmented by these hormones (Huang and Nieschlag, 1986; Meachem et al, 1999), becomes, in these pathological models, sensitive to inhibition by testosterone and in some cases, to inhibition by FSH as well. Possible mechanisms for the development of this checkpoint will be described in the next section.

Possible Mechanisms for Block in Spermatogonial Differentiation

Although testosterone and FSH have effects on spermatogonial differentiation in these pathological models, spermatogonia are not known to have receptors for these hormones. According to currently accepted dogma, in normal animals, FSH receptors (FSHRs) are localized exclusively in the Sertoli cell (Kliesch et al, 1992) and androgen receptors (ARs) are localized in a variety of somatic cell types, including Sertoli, Leydig, peritubular myoid, and vascular smooth muscle cells (Bremner et al, 1994). Furthermore, normal development of germ cells that lack an AR is possible (Johnston et al, 2001). Because germ cells lack AR and FSHR, these hormones must act via paracrine or juxtacrine routes between the cells that contain the receptors for these hormones and the spermatogonia.

The model chosen to explain the apparent contradiction, that testosterone inhibits spermatogonial differentiation after some pathological insults or genetic defects but not in normal spermatogenesis, depends on whether the pathology directly alters the spermatogonia or the androgen-responsive somatic cell. In most cases the target is not known. Although certain toxicants are believed to act primarily on Sertoli cells (eg, hexanedione; Boekelheide, 1988) or germ cells (eg, radiation; Lee et al, 1999), it is not possible to prove that the long-term effects are due to action on these cells. Hence, Figure 6, which lays out our model, is divided into two parts, A and B, which assume the defect lies in the spermatogonia, whereas Figure 6C and D, assume it lies in the Sertoli cells. The Sertoli cell was used as the example of the androgen-

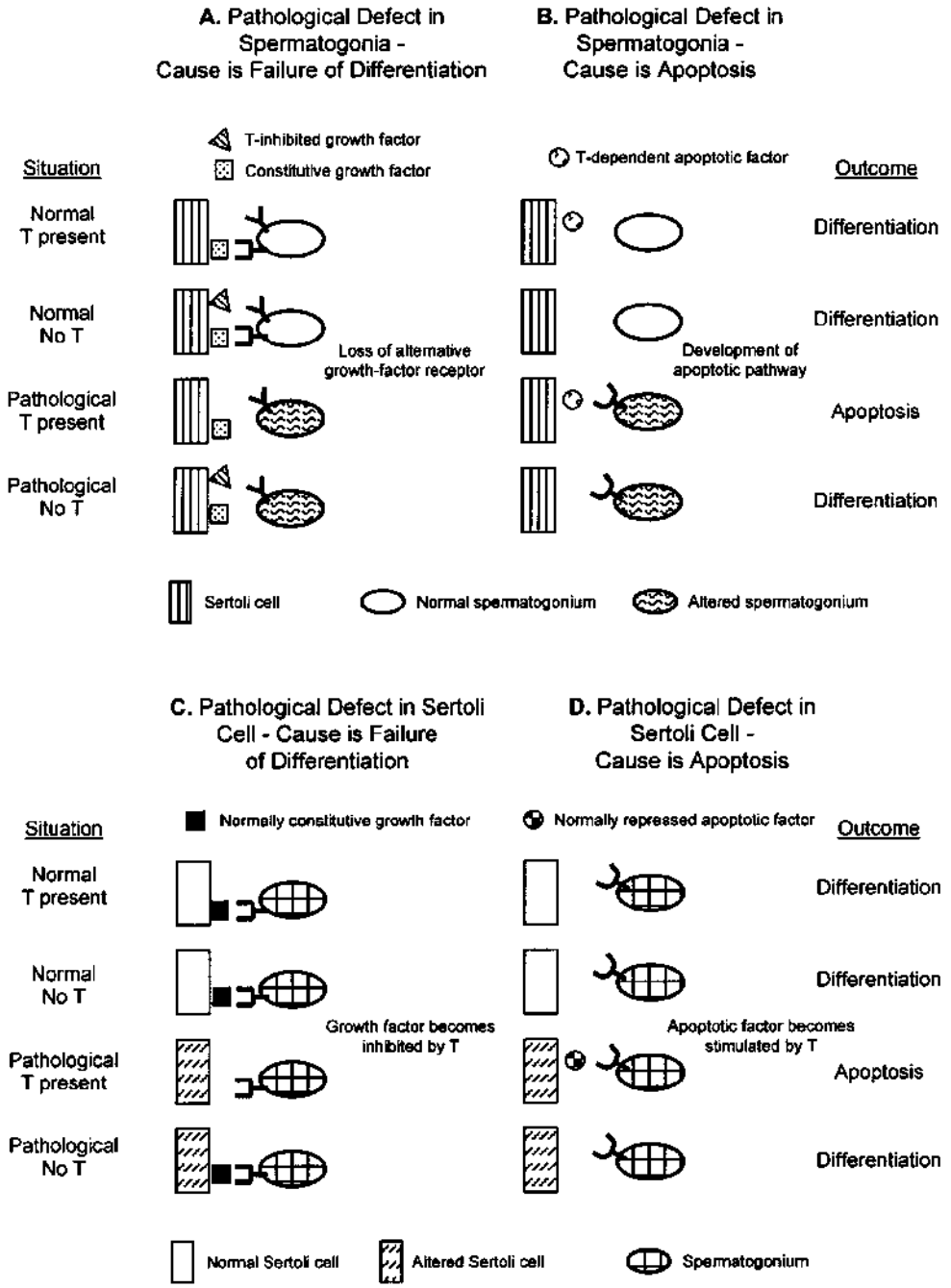

Figure 6. Models to explain testosterone-dependent inhibition of spermatogonial differentiation in pathological situations in mice and rats but not normal rodents. **(A)** It is assumed that the defect is in spermatogonia and they do not differentiate because a growth, survival, or differentiation factor is missing. Normal spermatogonia could have 2 pathways that support this step, but altered spermatogonia lack one receptor (square symbol) (or intracellular component of signal-transduction pathway) and therefore require the second receptor, the ligand (triangle), which is suppressed by the presence of testosterone. **(B)** It is assumed that the defect is in spermatogonia and they do not differentiate because they are killed by apoptosis. Sertoli cells could secrete an apoptotic effector (circle) in the presence of testosterone but normal spermatogonia lack the receptor or pathway for this ligand. The altered spermatogonia possess this receptor and therefore become sensitive to apoptosis in the presence of testosterone. **(C)** It is assumed that the defect is in somatic (Sertoli) cells and the reason spermatogonia do not differentiate is that a growth or differentiation factor is absent. Normal Sertoli cells make this growth factor constitutively, but in the altered Sertoli cells, it could be inhibited by testosterone. **(D)** It is assumed that the defect is in somatic (Sertoli) cells and the reason spermatogonia do not differentiate is that they are killed by apoptosis. Whereas normal Sertoli cells do not make an effector for this apoptotic process, the altered Sertoli cells could make this effector, but only in the presence of testosterone.

responsive somatic cell because it is most likely, but we cannot rule out the possibility that the peritubular, Leydig, or vascular smooth muscle cells are instead involved in some cases. In Sl^{17H} mice the defective gene, stem cell factor, is indeed specifically produced by Sertoli cells. However, for the *jsd* mutation, transplantation experiments have shown conclusively that the defect is expressed in the spermatogonia, not in the somatic cells (Boettger-Tong et al, 2000; Ohta et al, 2001).

The appropriate choice of model also depends on whether the cause of the block is spermatogonial apoptosis (Figure 6, B and D), or the lack of a functional signal for the spermatogonia to differentiate (Figure 6, A and C). In the first case, the failure to differentiate is a secondary consequence of the failure of the cells to survive to an appropriate stage. In the second instance, the observed apoptosis would be a secondary consequence of cells remaining undifferentiated for too long.

Alterations in spermatogonia could make these cells either more sensitive to testosterone-induced proapoptotic factors from the Sertoli cell (Figure 6B) or more dependent on testosterone-suppressible growth and differentiation factors from their surroundings (Figure 6A). Alternatively, testosterone could act on somatic cells to induce proapoptotic factors (Figure 6D) or to inhibit normally secreted growth factors (Figure 6C). In any case, all of these models predict that there should be at least one gene or gene product specifically regulated by testosterone in the target somatic cell.

Possibilities for Clinical Application

The above animal models may be applicable to 4 areas of human infertility or fertility control: idiopathic male infertility involving spermatogenic arrest, infertility due to treatment of cancer and autoimmune diseases with chemotherapy or radiotherapy, infertility due to environmental or occupational exposures, and development of a male reversible contraceptive.

Many cases of male infertility involve testicular disorders with arrest at various stages of spermatogenesis, including arrest at the spermatogonial stage in 10% of such cases (Skakkebaek et al, 1973). If testosterone inhibits spermatogenesis as was the case with *jsd* mice (Tohda et al, 2002), the late spermatids might be produced with intermittent testosterone suppression and be used for ICSI.

Chemotherapy or radiotherapy induces prolonged or permanent azoospermia in 3000 men of reproductive age in the United States each year (Meistrich et al, 2001a). Azoospermia also results from cyclophosphamide treatment for autoimmune diseases (Watson et al, 1985). Although some of these treatments may kill all of the stem cells, sometimes stem spermatogonia do survive but they fail to differentiate, as was observed in the rodent models. This is evidenced by the spontaneous reinitiation of spermatogenesis in some patients after many years of azoospermia (Meistrich et al, 1992). There are also histological examples of failure to differentiate past the spermatogonial (Kreuser et al, 1989) or the spermatocyte (Meistrich and van Beek, 1990) stages during the azoospermic period. The trigger for the spontaneous recovery is not known. Although several earlier attempts to enhance recovery of spermatogenesis by treatment with GnRH analogues before and during chemotherapy or radiotherapy were unsuccessful (Morris and Shalet, 1990), low-dose systemic testosterone to suppress intratesticular testosterone levels did induce recovery of spermatogenesis in all men treated with cyclophosphamide (Masala et al, 1997). However, there has been only one trial of the use of hormonal suppression after the completion of chemotherapy, and in that trial, no recovery was observed (Thomson et al, 2002). It should be noted that all the patients had been treated before puberty with high doses of procarbazine or radiation, which likely led to a complete loss of stem cells. A study using GnRH analogues for adult patients whose azoospermia resulted from lower doses of cytotoxic agents should be conducted next.

Environmental and occupational exposures to toxicants that block spermatogonial differentiation in rats may also produce similar effects in men. Boric acid is in widespread commercial and consumer use. Hexanedione is the active metabolite of the widely used solvent n-hexane. As yet there are no reports of effects of these chemicals on human spermatogenesis. However, DBCP, which is now banned, produced azoospermia in all highly exposed workers involved in its production (Whorton et al, 1979), and many thousands of agricultural workers who were exposed to DBCP appear to have an increased incidence of azoospermia (Slutsky et al, 1999). That azoospermia in men following exposure to moderate doses of DBCP may be spontaneously reversible years later (Potashnik and Porath, 1995) indicates that the stem cells may have survived and that DBCP may cause azoospermia by producing a prolonged block in spermatogenic differentiation.

The ability to reversibly block the differentiation of spermatogonia has potential for use as a male contraceptive. Compounds such as indenopyridines, in a single dose produce apparently irreversible sterility in rats, mice, and dogs without other toxicity (Cook et al, 1995), but their development as contraceptives is limited by the irreversibility of the spermatogenic block. However, the presence of type A spermatogonia in the tubules of indenopyridine-treated rats suggests that it could be reversed. Although spermatogonial differentiation and recovery of spermatogenesis was enhanced by treating rats with GnRH analogues before and after indenopyridine treatment (Hild et al, 2001), further studies showed that

only the treatment before indenopyridine was effective (S.A. Hild, personal communication). It is now important to determine whether spermatogonial differentiation can be reinitiated by hormonal or other forms of treatment given after the induction of a block to differentiation by the indenopyridine.

Although GnRH analogues and gonadal steroids have similar actions in humans and rodents, we do not know whether they will stimulate recovery of spermatogenesis in men with genetic or toxicant-induced blocks in spermatogonial differentiation because we do not know whether the mechanisms of the block are the same in the different species. Preliminary analyses of studies in irradiated monkeys show that GnRH antagonist treatment failed to prevent or reverse the reductions in spermatogenesis produced by radiation (A. Kamischke, personal communication; Richburg et al, 2002). Therefore, it is important to elucidate the mechanism by which testosterone inhibits spermatogonial differentiation in rodents to evaluate its application to men. Mechanistic knowledge can be used to find targets downstream from the initial action of androgen to develop restorative treatments that allow maintenance of androgen levels.

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Rat Models of Post-Irradiation Recovery of Spermatogenesis: Interstrain Differences

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Abstract

Recently we reported large differences between rat strains in spermatogenesis recovery at 10 weeks after 5-Gy irradiation suggesting that there are interstrain as well as interspecies differences in testicular radiation response. To determine whether these interstrain differences in sensitivity might be a result of the particular dose and time-point chosen, we performed dose-response and time-course studies on sensitive Brown-Norway (BN) and more resistant spontaneously hypertensive (SHR) and Sprague-Dawley (SD) rats. Type A spermatogonia were observed in atrophic tubules at 10 weeks after irradiation in all strains indicating that tubular atrophy was caused by a block in their differentiation, but the doses to produce the block ranged from 4.0 Gy in BN to 10 Gy in SD rats. Although the numbers of type A spermatogonial were unaffected at doses below 6 Gy, higher doses reduced their number, indicating that stem cell killing also contributed to the failure of recovery. After 10 weeks, there was no further recovery and even a decline in spermatogonial differentiation in BN rats, but in SHR rats, sperm production returned to control levels by 20 weeks after 5.0 Gy and, after 7.5 Gy, differentiation resumed in 60% of tubules by 30 weeks. Suppression of testosterone and gonadotropins after irradiation restored production of differentiated cells in nearly all tubules in BN rats and in all tubules in SHR rats. Thus the differences in recovery of spermatogenesis between strains were a result of both quantitative differences in their sensitivities to a radiation-induced, hormone-dependent block of spermatogonial differentiation and qualitative interstrain differences in the progression of post-irradiation recovery. The progression of recovery in SHR rats was similar to the prolonged delays in recovery of human spermatogenesis after cytotoxic agent exposure and thus may be a system for investigating a phenomenon also observed in men.

Keywords

ionizing radiation; spermatogenesis; rat strains; spermatogonia

INTRODUCTION

The mammalian testis is sensitive to ionizing radiation: low doses can temporarily reduce sperm production, moderate doses can cause prolonged reductions in sperm count, and high

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doses can result in permanent azoospermia. In humans, the testis appears more sensitive and exhibits longer delays before spermatogenesis recovers than in most rodent models. Single doses as low as 0.15 Gy cause temporary reductions in spermatogonial numbers and sperm count that can last as long as 6 months (Clifton & Bremner, 1983; Paulsen, 1973; Rowley *et al.*, 1974). Higher doses can produce azoospermia that lasts from about 8 months after 0.5 Gy to about 2 years after 6 Gy, and it then takes several years for sperm production to return to normal. The delays indicate that there are surviving spermatogonial stem cells that are blocked at some point in their differentiation, but the mechanisms of the block and subsequent recovery of spermatogenesis in human are not known. The fractionated radiation therapy used in cancer treatment is more toxic to the testis than the single doses (Meistrich & van Beek, 1990) and can result in no recovery of spermatogenesis occurs even 5 years after treatment (Hahn *et al.*, 1982; Sandeman, 1966; Speiser *et al.*, 1973), suggesting that all stem cells may have been killed.

An animal model that simulates the response of the human testis to radiation is needed to improve our understanding of this process. Non-human primates (macaques) show the most similarities to human including the histological types of spermatogonia (Ehmcke & Schlatt, 2006) and drastic declines in spermatogonial numbers and sperm count after 2 or 4 Gy lasting 6 months before recovery begins, and incomplete recovery even after 18 months (Foppiani *et al.*, 1999; Kamischke *et al.*, 2003; van Alphen *et al.*, 1988). However, studies on primates are limited by theirs cost and lack of genetic tools. Rodent models are inexpensive, have more detailed literature, inbred lines, and genetic tools, and are most amenable to laboratory studies but they have not so far shown the delayed recovery phenomenon.

The recovery from toxic effects of radiation in mice is much more rapid and robust than in humans. The stem spermatogonia surviving irradiation begin to differentiate almost immediately after doses even as high as 6 Gy and restore spermatogonial numbers to control levels after only 2 weeks (Erickson & Hall, 1983). Sperm production begins to increase within 7 weeks after 2 Gy and within 11 weeks after 6 to 12 Gy and reaches 60% of control values within 23 weeks after 6 Gy (Meistrich *et al.*, 1978; Meistrich & Samuels, 1985; Searle & Beechey, 1974). Killing of stem spermatogonia first becomes significant at a dose of 4 Gy (de Ruiter-Bootsma *et al.*, 1976; Erickson, 1981), and their numbers are further reduced with higher doses, with 9 Gy resulting in only 25% of tubules recovering production of differentiated cells within 5 weeks (Lu *et al.*, 1980; Withers *et al.*, 1974). Since a negligible number of the atrophic tubules contain spermatogonia (Kangasniemi *et al.*, 1996a), these atrophic tubules are primarily due to stem cell killing and not a block in spermatogonial differentiation, although there is some reduction in the yield of later differentiated cells after high doses (van den Aardweg *et al.*, 1983).

The rat testis appears somewhat more sensitive to damage produced by irradiation than the mouse and shows less recovery. However in Sprague-Dawley (SD) rats, the most widely-studied and most resistant strain, spermatogonial numbers recovered after 3 Gy to control levels within 5 weeks (Dym & Clermont, 1970), and epididymal sperm counts to 40% of control after 19 weeks (Jégou *et al.*, 1991). But after 6 Gy, recovery was far from complete at 16 weeks, as testis weights were only 52% of control and 44% of the tubules had incomplete spermatogenesis (Erickson & Hall, 1983). Following 9 Gy of radiation, less than 10% of tubules showed differentiating cells at 8 weeks (Delic *et al.*, 1986), and not until 26 weeks did sperm production reach 10% of control (Pinon-Lataillade *et al.*, 1991). Other strains of rats, such as LBNF1 (F1 hybrids of Lewis and Brown-Norway), were much more sensitive and, despite the survival and maintenance of stem spermatogonia, the testis showed progressive failure of recovery (Kangasniemi *et al.*, 1996b; Shetty *et al.*, 2000). Although some recovery of differentiated cells was transiently observed at 6 weeks after irradiation, this declined progressively to zero at 60 weeks after 3.5 Gy and by 10 weeks after 5 Gy, thus

indicating a permanent failure of spermatogenic recovery. The only other rats previously studied, various Wistar substrains, did show some recovery after 5 Gy, but to levels below that of Sprague-Dawley (Delic *et al.*, 1986; Delic *et al.*, 1987).

To systematically characterize these strain differences, we directly compared the recovery of spermatogenesis at 10 weeks after 5-Gy irradiation in seven rat strains and observed dramatic differences (Abuelhija *et al.*, 2012). There was no recovery of differentiating germ cells in the Lewis and Brown Norway (BN) stains despite the presence of type A spermatogonia in many tubules. Thus they showed the complete block in spermatogonial differentiation as had been previously observed in the LBNF1 hybrids (Kangasniemi *et al.*, 1996b). In contrast, in two Wistar-derived inbred strains, Wistar-Kyoto and spontaneously hypertensive rats (SHR), recovery of spermatogenesis was observed in 55% and 94% of the tubules, respectively. Sperm production was still markedly reduced, as it was only 3% of control levels in both Wistar strains. SD rats showed the best recovery of spermatogenesis, as 98% of tubules showed recovery and sperm production was 6% of controls. Nevertheless, the atrophic tubules in all strains contained type A spermatogonia, indicating that the tubular atrophy observed after 5 Gy was due primarily to a block in spermatogonial differentiation and not stem cell killing.

However, questions remain regarding the differences in sensitivity between strains. For example, is not known whether the different strains would show the same qualitative patterns of recovery, but differ in quantitative doses to produce blocks in recovery, and could we find a strain and dose that results in recovery after a delay. Furthermore, the role of testosterone in blocking spermatogenic recovery in different strains needs to be investigated to determine whether the recovery in the resistant strains is a result of their insensitivity to the action of testosterone, which we have previously shown is responsible for the block in spermatogonial differentiation in LBNF1 rats (Shetty *et al.*, 2000). In addition, we must determine whether the presence of tubules at different stages of differentiation at 10 weeks after irradiation represented a block at a later stage of differentiation or just a delay in initiation of differentiation. Finally we wanted to identify a strain showing some characteristics of the transient block in spermatogenic cell differentiation and the delayed recovery process observed in human testes.

To address these questions, we performed a dose-response, time-course, and hormone-effect study of the recovery of spermatogenesis after irradiation in three strains of rats. We performed all studies comparing BN and SHR since these strains are most amenable to future genetic studies as recombinant inbred rats between these two strains are already available to identify quantitative trait loci responsible for the differences in radiation sensitivity (Tabakoff *et al.*, 2009) and their genomes have been sequenced (Atanur *et al.*, 2010; Gibbs *et al.*, 2004). In addition, dose-response studies were performed on SD rats as this strain is the most resistant and is most widely used in toxicological studies.

MATERIALS AND METHODS

Animals and Irradiation Exposure

Brown Norway (BN/SsNHsd) and Sprague-Dawley (Hsd:Sprague Dawley SD) rats were obtained from Harlan Laboratories; SHR (SHR/NCrl) rats were obtained from Charles River Laboratories. We obtained the rats at 7 wk of age and allowed them to acclimatize in our facility for 1 wk prior to use. Rats were housed under standard lighting (12 h light, 12 h dark) and were given food and water *ad libitum*. All procedures were approved by the University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Rats were irradiated as described previously (Shetty *et al.*, 2000). Briefly, they were anesthetized and affixed to an acrylic board with surgical tape; then the lower part of the body was irradiated by a ^{60}Co gamma ray unit (Eldorado 8; Atomic Energy Canada Ltd., Ottawa, ON, Canada). The field extended distally from a line about 6 cm above the base of the scrotum. Different doses (2.7 Gy to 12.5 Gy) were given at a dose rate of approximately 1 Gy/min; dose ranges were chosen for each strain based on the sensitivity observed previously (Abuelhija *et al.*, 2012). Testis tissue was harvested at various times between 10 and 40 weeks after irradiation (Table 1). Each dose and time point represents the mean and standard error of between 3 and 10 rats.

Hormone treatment

Hormone suppressive treatment was performed with the GnRH antagonist (GnRH-ant) acyline (National Institute of Child Health and Human Development) and the androgen receptor-antagonist flutamide starting immediately after radiation and continuing until tissue harvest, Acyline was dissolved in water and administered as weekly subcutaneous injections of 1.5 mg/kg (Porter *et al.*, 2006). Flutamide was administered by subcutaneous implantation of four 5-cm-long Silastic capsules calculated to deliver 20 mg/kg/day (Porter *et al.*, 2009). Each treatment group (time and dose point) consisted of a minimum of 4 rats.

Intratesticular interstitial fluid and tissue processing

Rats were killed by an overdose of a ketamine-acepromazine mixture. Each testis was surgically excised and weighed with the tunica albuginea intact. The right testis was fixed overnight in Bouin's fluid.

Interstitial tubule fluid was collected from the left testis as we had done previously (Abuelhija *et al.*, 2012) using a modification of methods described earlier (Porter *et al.*, 2006; Rhenberg, 1993). Briefly, the testis was suspended by silk sutures and centrifuged for 30 min at 60 × g at 4°C, and the weight of the fluid collected was determined. The remaining weight of the testis parenchymal tissue was measured after removing the tunica albuginea. The tissue was then homogenized in water for sperm head counts.

Evaluation of Spermatogenesis

For histological analysis, the fixed right testis was embedded in glycol methacrylate plastic (JB4, Polysciences Inc., Warrington, PA), and 4-μm sections were cut and stained with periodic-acid Schiff's (PAS) and hematoxylin. To evaluate the recovery of spermatogenesis from irradiation, we scored a minimum of 200 seminiferous tubules in one section from each animal for the most advanced germ-cell stage present in each tubule. Unless otherwise stated, we computed the tubule differentiation index (TDI), which is the percentage of tubules containing 3 or more cells that had reached type B spermatogonial stage or later (Meistrich & van Beek, 1993). To obtain a more complete description of the stages of differentiation present in the testis, we also determined the percentages of tubules with 3 or more cells reaching the leptotene spermatocyte stage or later (TDI-spermatocyte) or the round spermatid stage or later (TDI-spermatids), or with 10 or more cells reaching the elongating or elongated spermatid stage (TDI-late spermatids).

We counted all type A spermatogonia, which includes the stem, chains of undifferentiated, and differentiating spermatogonia to type A_4 (Chiarini-Garcia *et al.*, 2003) and Sertoli cells, in atrophic seminiferous tubule cross-sections of irradiated rat testes at 1000× magnification (n=3-7/group). For samples with almost complete seminiferous tubule atrophy, cells were counted using systematic random sampling (Stereo Investigator version 8.0 software, MicroBrightField, Inc., Williston, VT), by counting A spermatogonia and Sertoli cells in 300 randomly selected 100 μm × 80 μm fields. In samples with few atrophic seminiferous

tubules, these tubules were identified visually using light microscopy, and all cells in the tubules were counted. A minimum of 500 Sertoli cells were counted per testis. Results were presented as A spermatogonia per 100 Sertoli cells.

Testicular sperm production was evaluated by counting sonication-resistant sperm heads, which represent nuclei of step 12–19 spermatids, in testicular homogenates. An aliquot of the homogenate of the left testis was sonicated, and the sperm heads were counted in a hemacytometer using phase contrast optics (Meistrich & van Beek, 1993).

Hormone Assays

Serum testosterone and interstitial fluid testosterone (IFT) concentrations were measured using a coated-tube radioimmunoassay kit (Coat-A-Count Total Testosterone, Cat No. TKTT1, Siemens, Los Angeles, CA) similar to procedures described previously (Porter *et al.*, 2006; Shetty *et al.*, 2000). Rat serum follicle-stimulating hormone (FSH) was measured by radioimmunoassay, and luteinizing hormone (LH) was measured by a sensitive two-site sandwich immunoassay. Both FSH and LH were measured by the University of Virginia, Center for Research in Reproduction, Ligand Assay and Analysis Core, using previously described methods (Gay *et al.*, 1970).

Statistical analysis

Results were presented as either mean ± SEM calculated from untransformed data or, in the case of sperm head counts, testosterone, and LH as the mean ± SEM calculated from log-transformed data obtained from individual rats. The statistical significance of differences between two groups was determined using the *t*-test with $P < 0.05$ being considered significant.

RESULTS

Spermatogenesis at 10 Weeks after Irradiation (Dose-Response)

To identify the doses that induce the declines in spermatogenic recovery and the accumulation of testicular interstitial fluid, which had been correlated with the block to recovery (Porter *et al.*, 2006), BN, SHR, and SD rats were given different ranges of doses of radiation depending on the sensitivity of the strain, and tissue was harvested 10 weeks later. Radiation reduced the testicular parenchymal weights in all the strains in a dose-responsive manner, with a steep initial decline, corresponding to the major phase of germ cell loss, followed by a shallower slope reaching 15–20% of control at high doses (Fig. 1A). The steep decline occurred in BN rats at doses below 3 Gy, but 5 to 6 Gy were required in SHR and SD rats to complete the steep decline.

As shown previously (Abuelhija *et al.*, 2012), there were large increases (~0.2 g) in testicular interstitial fluid in BN rats at 5 Gy of irradiation but small or negligible increases in SHR and SD rats. In all strains, the increases in interstitial fluid were dose-responsive (Fig. 1B). The increase reached a maximum at 3.3 Gy in BN, but 5.7 Gy and 10 to 11 Gy were required in SHR and SD, respectively, to reach maximal levels, which were less than that observed in BN.

To assess whether the radiation-induced decline in spermatogenesis was quantitatively different in the different strains, the dose-response of the recovery of spermatogenesis from surviving stem cells was assessed by the percentage of tubules showing differentiated cells (tubule differentiation index, TDI) in histological sections (Fig. 2A) and the numbers of late spermatids produced (Fig. 2B). Control rats showed differentiation in 100% of the tubules and 2×10^8 late spermatids per testis. Both parameters showed dose-responsive declines, with

the BN rats being most sensitive to irradiation, SHR showing intermediate sensitivity, and SD displaying the most resistance. It was noted that doses that reduced the TDI to between 30% and 60% of control reduced late spermatid counts to between 0.05% and 1% of control.

The atrophic tubules showing no differentiated cells at 10 weeks after irradiation were then examined to determine whether the absence of differentiated cells was a consequence of killing of all stem spermatogonia or a block in spermatogonial differentiation. In the sensitive BN strain, atrophic tubules were observed at all doses tested, but in the resistant strains they could only be observed after 5.0 Gy in SHR and after 6.5 Gy in SD rats. At the lower doses of radiation, atrophic tubules in all the strains contained between 2.5 and 2.8 type A spermatogonia per 100 Sertoli cells (Fig. 3A). Increasing the radiation exposure produced a dose-responsive reduction in the numbers of type A spermatogonia in the three strains resulting in about 0.5 type A spermatogonia per 100 Sertoli cells after 10 Gy of irradiation. This reduction suggests that stem cells were killed at these higher doses of radiation. However, the presence of some type A spermatogonia in atrophic tubules demonstrated that a block in spermatogonial differentiation (Meistrich & Shetty, 2003) also contributed to the failure of spermatogenesis to recover.

Recovery of Spermatogenesis after Irradiation (Time-Course)

To identify strains with permanent or reversible blocks in spermatogonial differentiation, we examined recovery at times longer than 10 weeks. In BN rats there was no significant histological recovery of spermatogonial differentiation between 10 and 20 weeks after irradiation with the doses (≥3.3 Gy) that were tested (Fig. 4C). The numbers of late spermatids remained low ($\leq 10^5$) (Fig. 4E); values in the 10^4–10^5 range were occasionally observed despite the lack of histological evidence of differentiation in the testis and may have represented sperm heads retained in the testis. The lack of recovery can be attributed to a continued block in spermatogonial differentiation and not a loss of stem cells, as the numbers of type A spermatogonia did not show any decrease between weeks 10 and 20 (Fig. 3B)

In contrast, SHR rats showed progressive recovery at all doses up to 7.5 Gy. With doses up to 5 Gy, sperm production approached control levels by 15 weeks after irradiation (Fig. 4F). After higher doses, the percentage of tubules with differentiated cells was less than 7% at 10 weeks after irradiation, but steadily increased reaching 60% by 20 weeks after 6.5 Gy and by 30 weeks after 7.5 Gy (Fig. 4D). Although most tubules showed differentiation, it was generally only to the B spermatogonial or spermatocyte stages (Fig. 5). After 6.5 Gy appreciable differentiation to the round or late spermatid stages was observed in only one rat at 15 weeks (out of 4 examined) and one at 20 weeks (out of 5). After 7.5 Gy, few tubules progressed to the round spermatid stage and almost none to the late spermatid stage, indicating a block at a later stage of differentiation. Sperm production measured in the contralateral testis, surprisingly, appeared to slightly increase at 15 weeks after 7.5 Gy but then remained low at later times (Fig. 4F). Furthermore, after 7.5 Gy (Fig. 5B), there was no further histological recovery of spermatogenesis between 30 and 40 weeks.

Hormone analyses

To determine whether differences in testosterone or FSH levels might be related to the differential induction of the block in spermatogonial differentiation in the strains, hormone analyses were performed on the three strains of rats before and after irradiation. Serum testosterone showed a modest trend toward reduction at 10 weeks after irradiation in all strains (Fig. 6A), but this was only significant in SHR and SD rats. Interstitial fluid testosterone (IFT) levels were unaffected by the radiation in all 3 strains (Fig. 6B). Serum FSH levels significantly increased by about 2-fold 10 weeks after radiation in all strains

(Fig. 6C) as expected owing to the germ cell loss that occurs. LH levels (data not shown) also appeared to be elevated by irradiation.

The levels of serum testosterone, interstitial fluid testosterone, and serum FSH levels in SHR rats were significantly higher than the corresponding values in BN rats both in unirradiated rats and after nearly all dose (Fig.6) and time points (Fig. 7). The values in SD rats were generally intermediate between those of the other two strains (Fig. 6A–C). Although testosterone and FSH were previously shown to contribute to the spermatogonial differentiation block in LBNF1 rats (Shetty *et al.*, 2006), the greater sensitivity of BN rats than of SHR or SD to induction of a spermatogonial block by radiation cannot be attributed to higher levels of testosterone or FSH.

Suppression of hormone levels and spermatogenesis recovery

To confirm that the action of testosterone and/or FSH was involved in the radiation-induced block of spermatogonial differentiation in these strains, we examined the effects of hormone suppression on spermatogenic recovery in BN and SHR rats at different times after 7.5 Gy (Fig. 7) and at 10 weeks after 5 and 10 Gy (data not shown). Hormone suppression decreased serum testosterone to below the limits of detection in both strains (Fig. 7A). IFT levels were reduced in BN rats to ~1 ng/ml and were reduced even more in SHR rats (Fig. 7B). However, these residual levels of intratesticular testosterone would not be expected to have significant effects on spermatogenesis because the rats were also treated with flutamide. The suppressive treatment also markedly reduced serum FSH levels to about 1 ng/ml in all groups of rats (Fig. 7C) and reduced LH to undetectable levels (not shown).

Although hormonal suppression in control and treated rats markedly decreased testicular parenchymal weights to about 7% of control in both strains at the various dose and time points (Fig 8A,D), which was also evident by the decrease in tubule diameter (compare Fig. 9A and C), it induced differentiation in a high percentage of tubules in irradiated rats of both strains (Figs. 8B and 9C). In BN rats, irradiation with 5 Gy and above almost completely eliminated the differentiating spermatogenic cells (TDI < 2%); nevertheless, hormone suppression starting immediately after irradiation with 5 Gy restored the production of differentiated cells in 100% of tubules; however, with the low testosterone and FSH levels, differentiation could only proceed to the spermatocyte stage (Fig. 9D). There was incomplete recovery of spermatogonial differentiation at 10 weeks after 7.5 Gy, as only 88% of tubules showed differentiating cells, but recovery progressed with time so that by week 20 100% of tubules were differentiated (Fig. 8E). The higher dose of 10 Gy reduced the percentage of tubules showing differentiation at 10 weeks to 48%. In SHR rats, after the 7.5- and 10-Gy doses, which blocked all spontaneous recovery at 10 weeks after irradiation, hormonal suppression stimulated the production of differentiated cells in 100% and 90% of tubules, respectively.

Hormone suppression completely reversed the large increase in interstitial fluid accumulation observed in BN rats (Fig 8C,F). The modest increases in interstitial fluid observed in SHR rats after irradiation were also reversed by the hormone suppression.

DISCUSSION

The human testis is characterized by high sensitivity to and delayed recovery of spermatogenesis after moderate doses of radiation. Here we compared 3 rat strains and found that the BN rats were also very sensitive to the gonadotoxic effects of radiation, but they showed no recovery of spermatogenesis. SD rats displayed the most resistance to radiation as high doses were required to produce severe gonadotoxic effects. However, SHR rats showed marked and prolonged gonadotoxic effects to doses of about 6 Gy, and may

indicate that Wistar-derived rats might have some of the sensitivity characteristics similar to human testes.

The present study clarifies the question of whether the data of our previous study (Abuelhija *et al.*, 2012), showing that at 10 weeks after 5-Gy irradiation there was no recovery of spermatogenesis in BN rats, whereas in SHR and SD rats nearly all of the tubules contained differentiating germ cells, are a result of qualitative or quantitative differences between the strains. The dose-response studies showed that at 10 weeks after irradiation, BN rats failed to show recovery even after low doses (4 Gy) (Fig. 2), similar to the sensitivity described previously in LBNF1 rats (Kangasniemi *et al.*, 1996b), which are F1 hybrids of Brown-Norway and Lewis, another very sensitive strain (Abuelhija *et al.*, 2012). At these low doses, the atrophic tubules are almost exclusively due to a block in spermatogonial differentiation, as type A spermatogonia were present and their numbers were maintained in the atrophic tubules. In the resistant SHR and SD strains, low to intermediate doses (≤5 Gy) did not produce a significant block in spermatogonial differentiation. However higher doses of irradiation induced radiation-induced blocks in spermatogonial differentiation, similar to that observed in BN rats at the lower doses, in SHR and SD rats after 6.5 Gy and 8 Gy, respectively. Thus the major contribution to the differences in recovery of spermatogenesis between strains is the quantitative difference in their sensitivities to a radiation-induced block of spermatogonial differentiation.

The time-course studies addressed whether this block was reversible at later times in the different strains. The block was not reversible at all between 10 and 20 weeks in BN rats even at doses as low as 3.3 or 4 Gy (Fig. 4A). Based on results with LBNF1 rats, which were followed for 60 weeks to demonstrate the permanence of the block, we suggest that no recovery will occur with BN rats even after longer periods of time (Kangasniemi *et al.*, 1996b). Furthermore, in LBNF1 rats, the incomplete block produced at 3.5 Gy became even more severe between 10 and 60 weeks, with spermatogonial differentiation steadily declining to a complete block; a similar decline occurred in BN rats after 3.3 Gy (Fig. 4C).

In contrast in the more resistant SHR rats, doses above 5 Gy were required to produce a block in spermatogonial differentiation at 10 weeks after irradiation. This block that was observed after doses of 6.5 and 7.5 Gy was reversible, as demonstrated by the progressive increase in the number of spermatogonia in the atrophic tubules at 15 and 20 weeks (Fig. 3B), and in differentiating tubules at 20 and 30 weeks after irradiation (Fig. 4D). Thus there is a qualitative difference between strains, as the more resistant strains, like SHR, showed a delayed but progressive recovery of spermatogonial differentiation, whereas the block in spermatogonial differentiation in the sensitive strains like BN and LBNF1 was permanent.

Although the block in spermatogonial differentiation in SHR rats was reversed at later post-irradiation times, there still was a prolonged decrease in spermatogenesis as exemplified by the reduction in testis weights, later differentiated cells, and sperm production at doses >5 Gy (Figs. 4B,F, 5). The reversible, but incomplete recovery in the SHR strain appears to be similar to that previously reported in SD rats (see Introduction). In SHR rats, the presence of appreciable numbers of tubules containing B spermatogonia and spermatocytes at 20 weeks after 7.5-Gy irradiation, but almost no spermatids at weeks 30 and 40, demonstrates that the absence of late stage germinal cells is a result of a decreased efficiency or even a block in development to later differentiation steps, and not just a result of the delay in the initiation of spermatogonial differentiation. It is highly unlikely that these differentiated germ cells were arrested in development since the spermatogonia were mitotically active and, when present, the later cells were arranged according to the stages of the cycle of the seminiferous epithelium. We believe that a damaged somatic environment, as previously observed to produce the block in spermatogonial differentiation in LBNF1 rats (Zhang *et al.*, 2007), is

unable to properly support spermatogenic cell differentiation. Hence the recovery observed between 15 and 30 weeks may be due to restoration of a favorable somatic environment, like that which occurs when hormones are suppressed, but a mechanism for this spontaneous recovery is not known.

Since doses of ≥5.7 or 6.5 Gy were necessary to produce a block in spermatogonial differentiation in resistant strains like SHR and SD, respectively (Fig. 2A), the possible role of stem cell killing could also be considered as a cause of the atrophic tubules at higher doses. But as the numbers of type A spermatogonia were still maintained in the atrophic tubules at doses up to 5.7 Gy or 6.5 Gy for SHR and SD respectively (Fig. 3A), the block in spermatogonial differentiation must be the principal cause of tubular atrophy at these doses. However at higher doses, there was a decline in the numbers of A spermatogonia (Fig. 3A), suggesting that stem cell killing is also a cause of tubular atrophy, but cannot be the only cause since A spermatogonia were still observed. These results are consistent with direct counts of isolated type A spermatogonia, the putative stem cells, in SD rats, which indicated that although there was a transient loss of these cells after doses as low as 2 Gy, 6 Gy was required to cause a more prolonged loss of the these stem cells for 26 days (Erickson, 1976).

The block in spermatogonial differentiation in irradiated LBNF1 rats was previously shown to be mediated by the action of testosterone and also to some extent by FSH (Shetty *et al.*, 2006). This inhibitory action of the hormones is in contrast to the situation in normal rats, in which spermatogonial differentiation is qualitatively independent of both testosterone and FSH (Huang & Nieschlag, 1986). Here we show that hormones were also responsible for the spermatogonial block in BN rats, as the production of differentiated cells in all tubules could be restored by hormone suppression for 10 weeks after 5-Gy irradiation (Fig. 8B). The lack of sensitivity of SHR or SD rats to the radiation-induced block in spermatogonial differentiation at 5 Gy cannot be a result of lower levels of testosterone and FSH since irradiated rats of these strains actually had higher levels of these hormones than did BN (Fig. 6), or of the absence of the hormone-dependence of the block in spermatogonial differentiation, which was demonstrated in 7.5-Gy irradiated SHR rats (Fig. 8B). Thus the differences between the sensitive and resistant strains appear to be a result of differences in the dose required to render the testis sensitive to this block of spermatogonial differentiation.

Another factor that may be involved in this block in spermatogonial differentiation appears to be the accumulation of testicular interstitial fluid as irradiation of LBNF1 rats dramatically increased testicular interstitial fluid at the time the block in spermatogonial differentiation occurred and hormonal treatments to restore spermatogonial differentiation reduced interstitial fluid (Porter *et al.*, 2006) However, other sensitive (Lewis) or intermediate (Wistar-Kyoto) strains also had low interstitial fluid accumulation, indicating that fluid accumulation could not be the cause of the block in those strains (Abuelhija *et al.*, 2012). In the present study dose-responsive increases in interstitial fluid levels (Fig. 1B), although lower in magnitude in SHR and SD than in BN, occurred in all 3 strains at doses corresponding to the decline in spermatogonial differentiation (Fig. 2A). This result and the reversal of the radiation-induced fluid increase in BN and SHR rats with hormone suppression (Fig. 8C,F) further support a correlation between increases in interstitial fluid and the block in spermatogonial differentiation in these strains.

Finally, the demonstration of a delay in the recovery of spermatogonial differentiation (15–20 weeks) in SHR rats irradiated with 7.5 Gy (Fig. 4D) and an even longer delay in the production of late spermatids in the testis (30–40 weeks) appears to provide a rat model for the prolonged delays in recovery of human spermatogenesis after radiation and other cytotoxic exposures. The relative roles of stem cell renewal/spermatogonial differentiation, which show differences between rodents and primates (Ehmcke & Schlatt, 2006), and the

changing ability of the somatic environment to support spermatogenic cell differentiation in the delayed recovery phenomenon is not known. Further studies at longer times are needed in SHR, other Wistar-derived strains, or SD rats (Pinon-Lataillade *et al.*, 1991) to determine if the recovery continues to progress and will lead to increases in epididymal sperm counts and to investigate the mechanisms underlying the delay or block.

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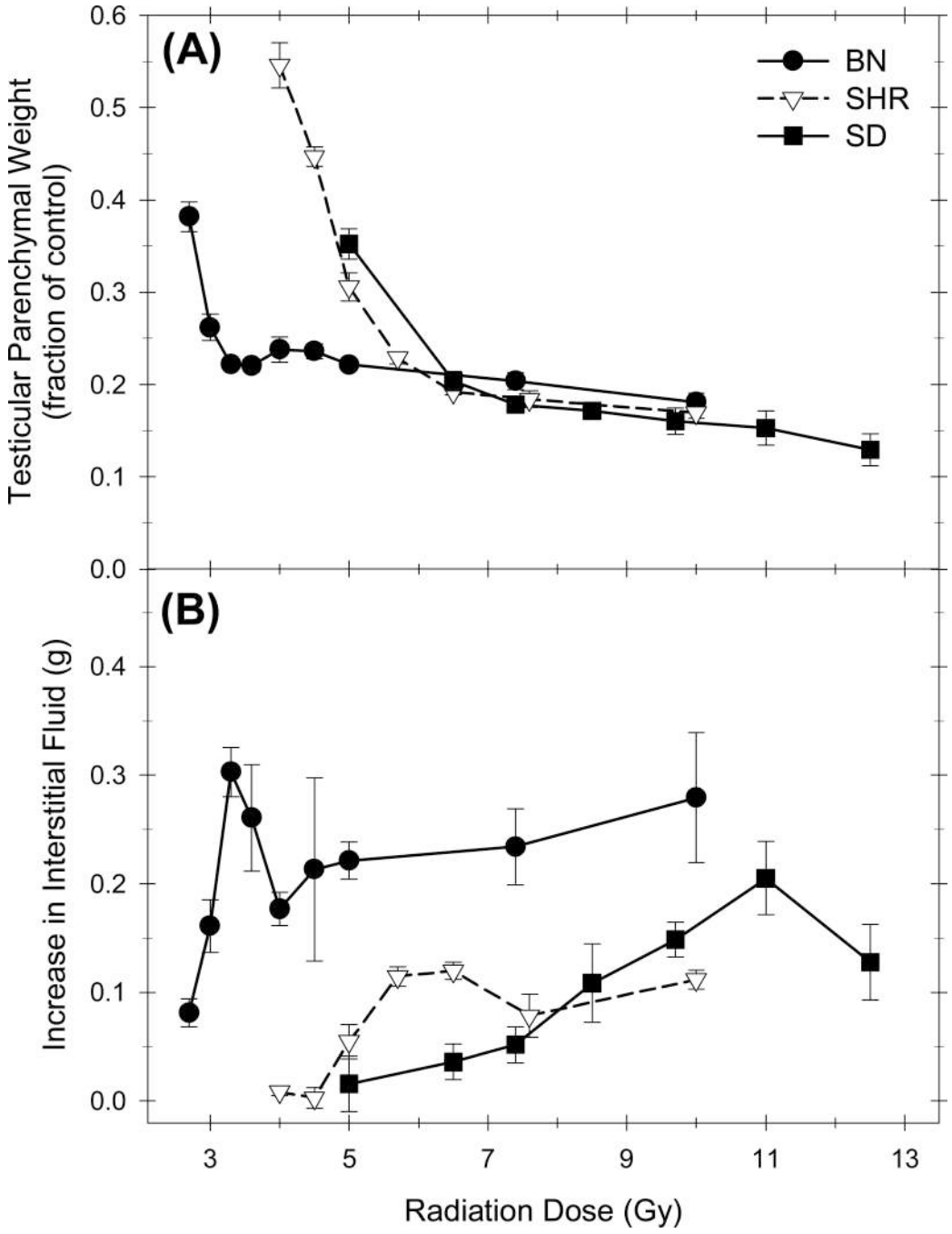

FIG 1.

Weights of testis parenchymal tissue and interstitial fluid of BN, SHR and SD rats 10 weeks after irradiation. (A) Testis weights relative to those of unirradiated controls of same strain. Control values were 1.50 g, 1.27 g, and 1.66 g for BN, SHR, and SD, respectively. (B) Increase in interstitial fluid weights from unirradiated control levels.

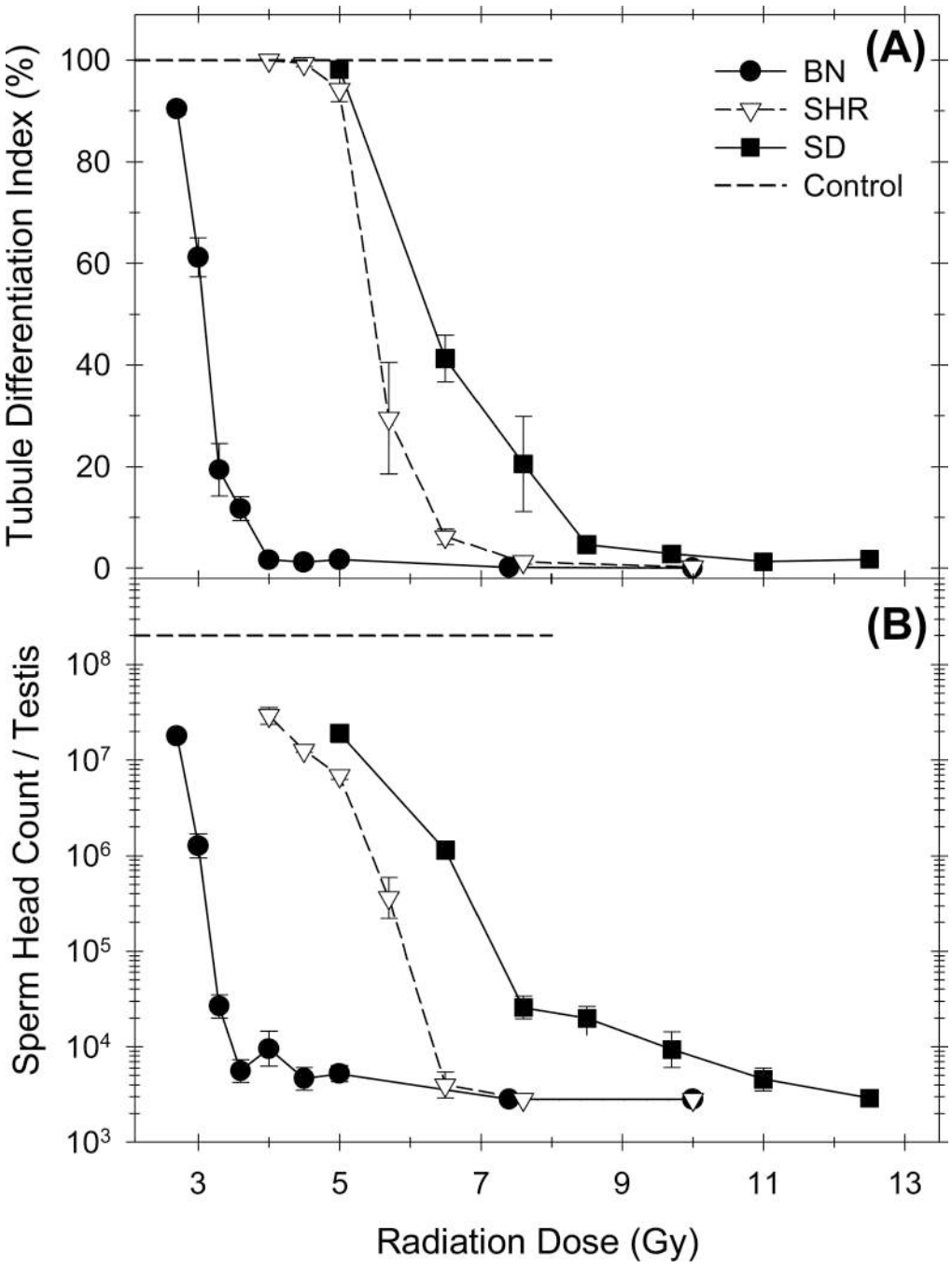

FIG 2.

Recovery of spermatogenesis at 10 weeks after various doses of radiation. (A) Tubule differentiation index (TDI), defined as percentage of tubules differentiating to the B spermatogonial stage or beyond. (B) Testicular sperm production: numbers of sonication-resistant late spermatids per testis. The dashed lines indicate the control values.

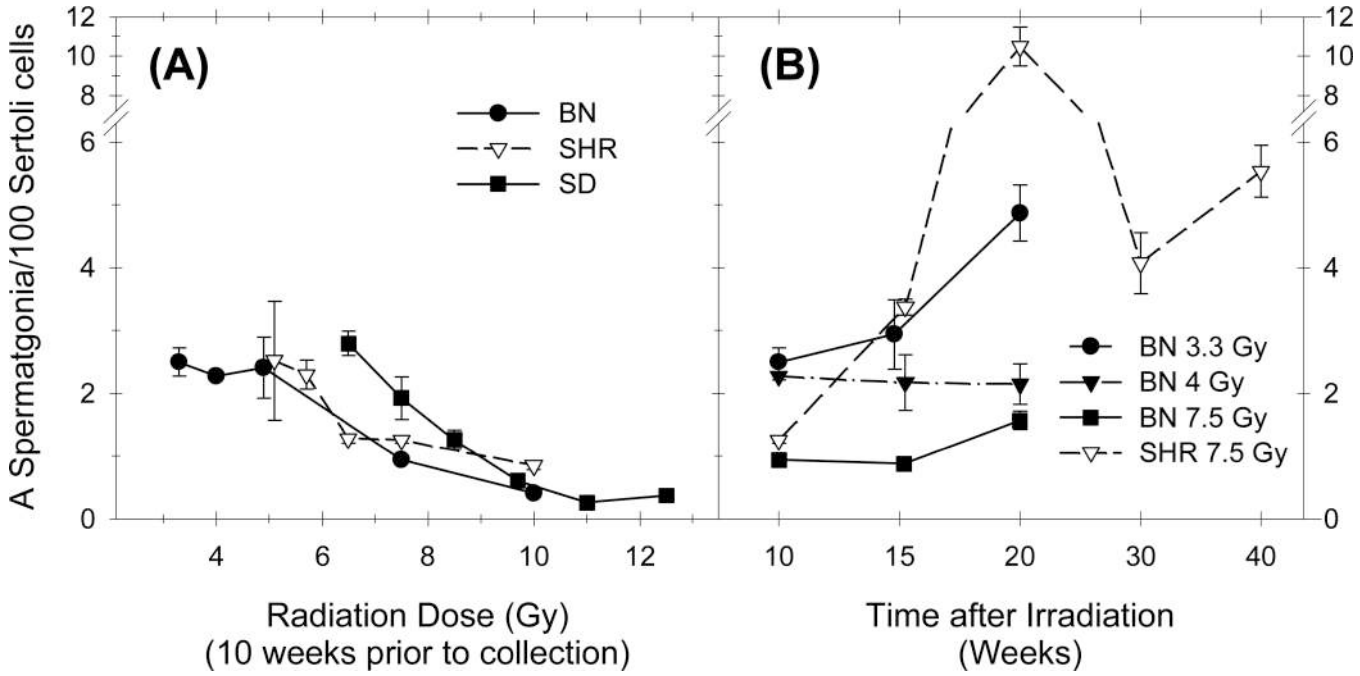

FIG 3.

Numbers of type A spermatogonia per 100 Sertoli cells in nonrepopulating tubules of (A) BN, SHR, and SD rats 10 weeks after irradiation (dose-response), and (B) BN and SHR rats at longer periods of time after different doses of irradiation (time course).

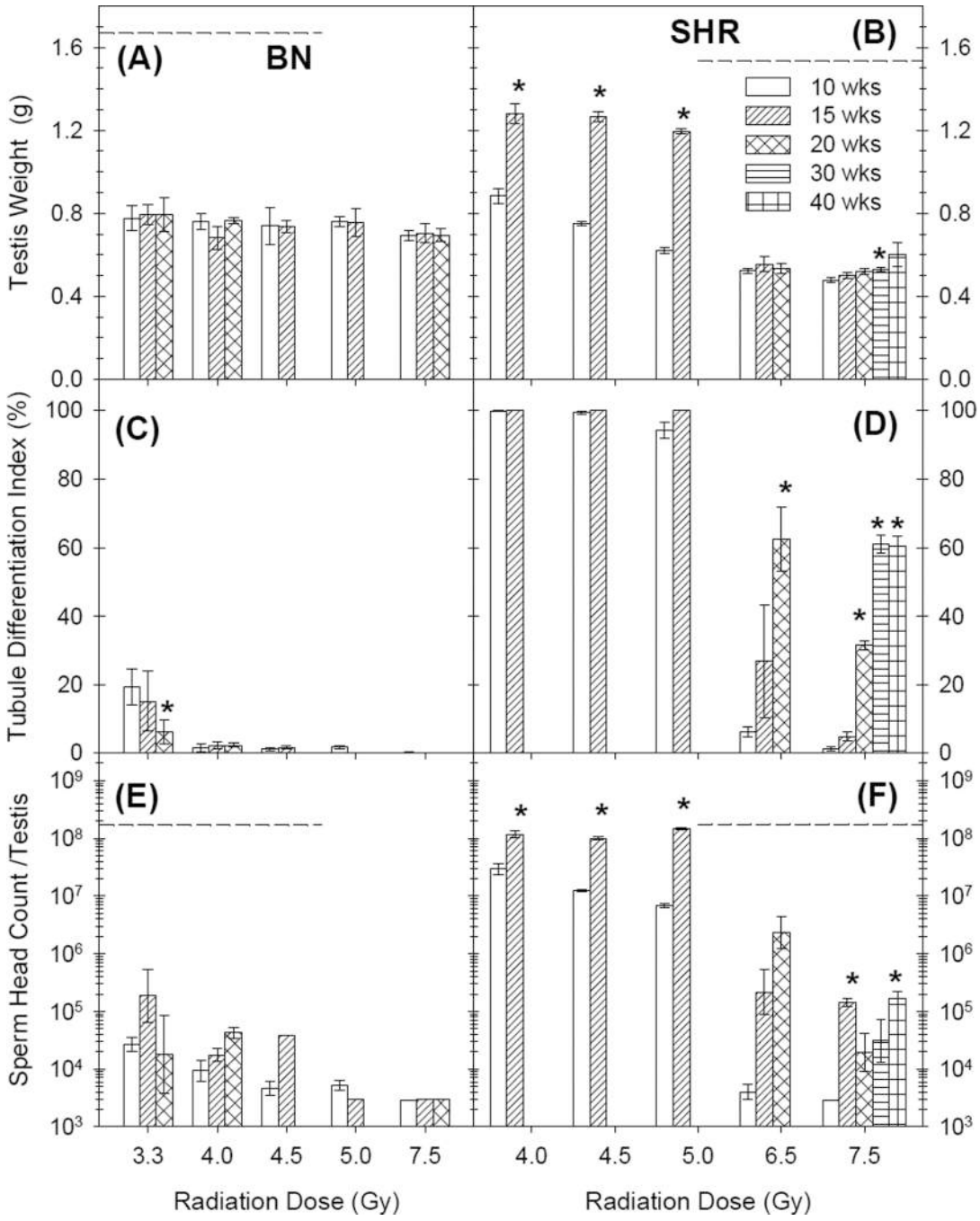

FIG 4.

Time courses of changes in (A,B) absolute testis weights, (C,D) tubule differentiation indices, and (E,F) sperm head counts of BN (A,C,E) and SHR (B,D,F) rats after different doses of radiation. The dashed lines indicate the control values. (*) indicates significantly different from value at 10 weeks ($P<0.05$, *t*-test).

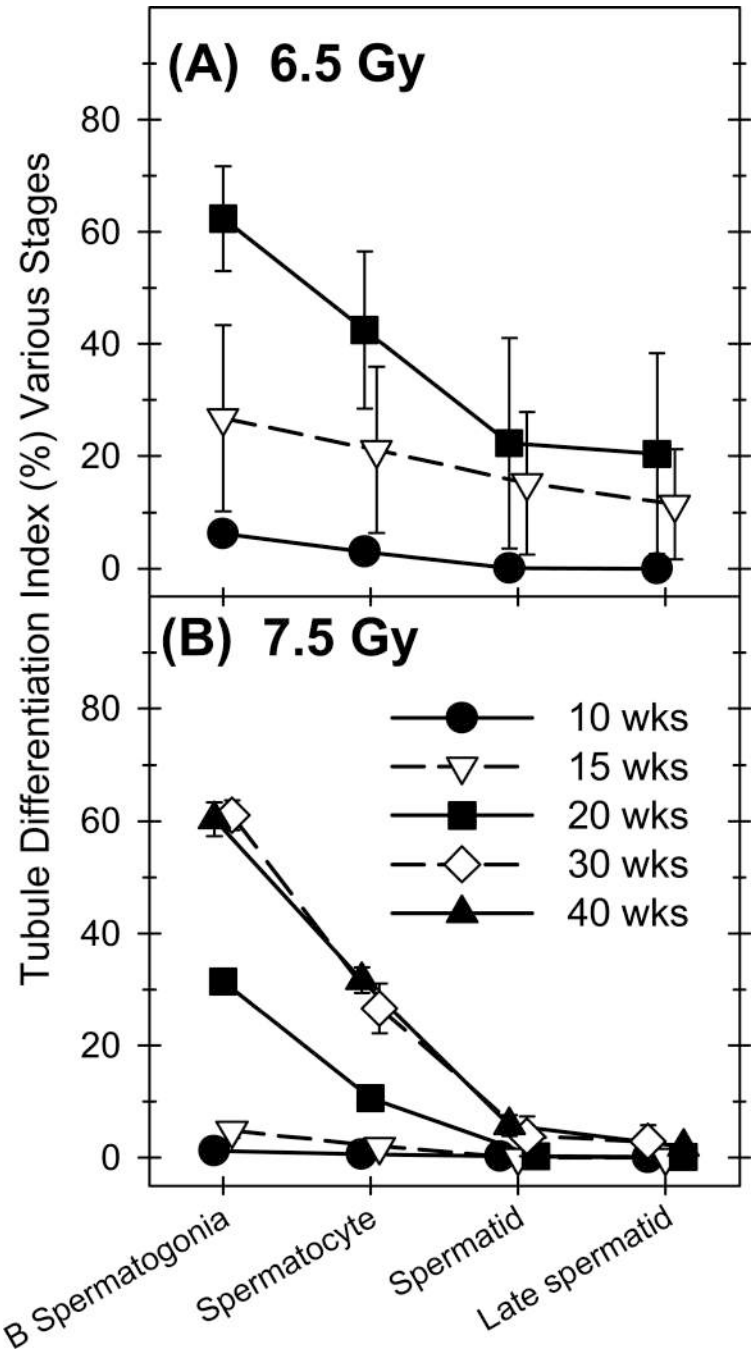

FIG 5.

Recovery of progression of spermatogenesis as measured by the percentage of tubules with morphologically differentiated cells reaching indicated stage of differentiation or beyond for SHR rats at various times after (A) 6.5 Gy or (B) 7.5 Gy.

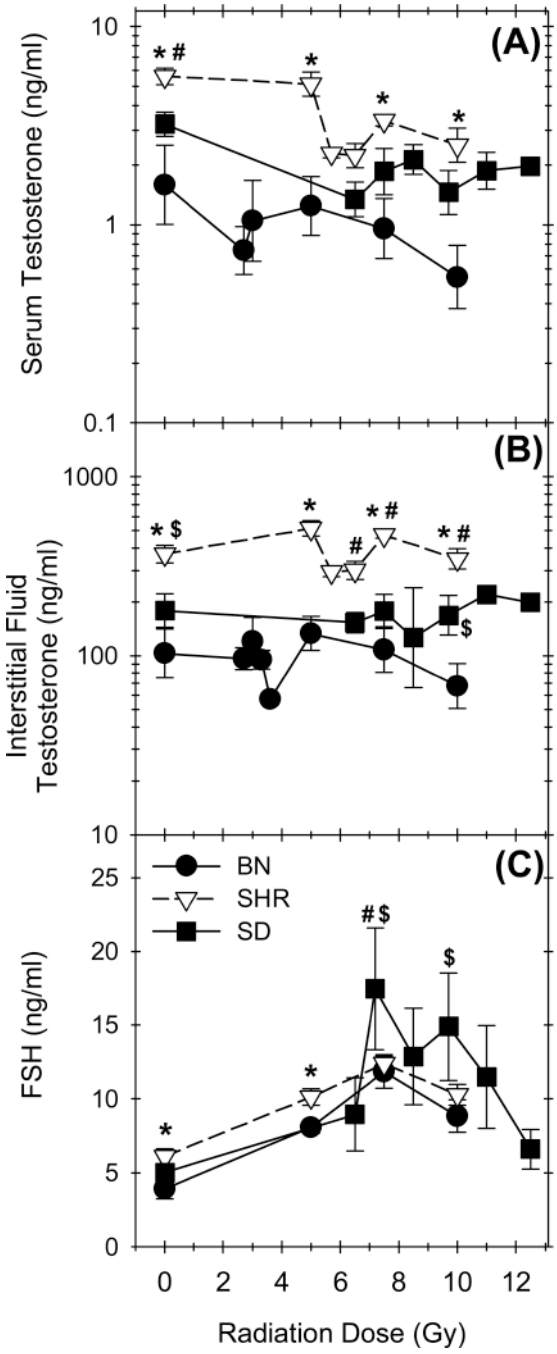

FIG 6.

Hormones levels in BN, SHR, and SD rats measured 10 weeks after different doses of radiation. (A) Serum testosterone. (B) Intratesticular fluid testosterone. (C) Serum FSH. (*) indicates values in SHR are significantly different from those in BN. (#) indicates values in SHR are significantly different from those in SD. ($) indicates values in SD are significantly different from those in BN ($P<0.05$, t-test).

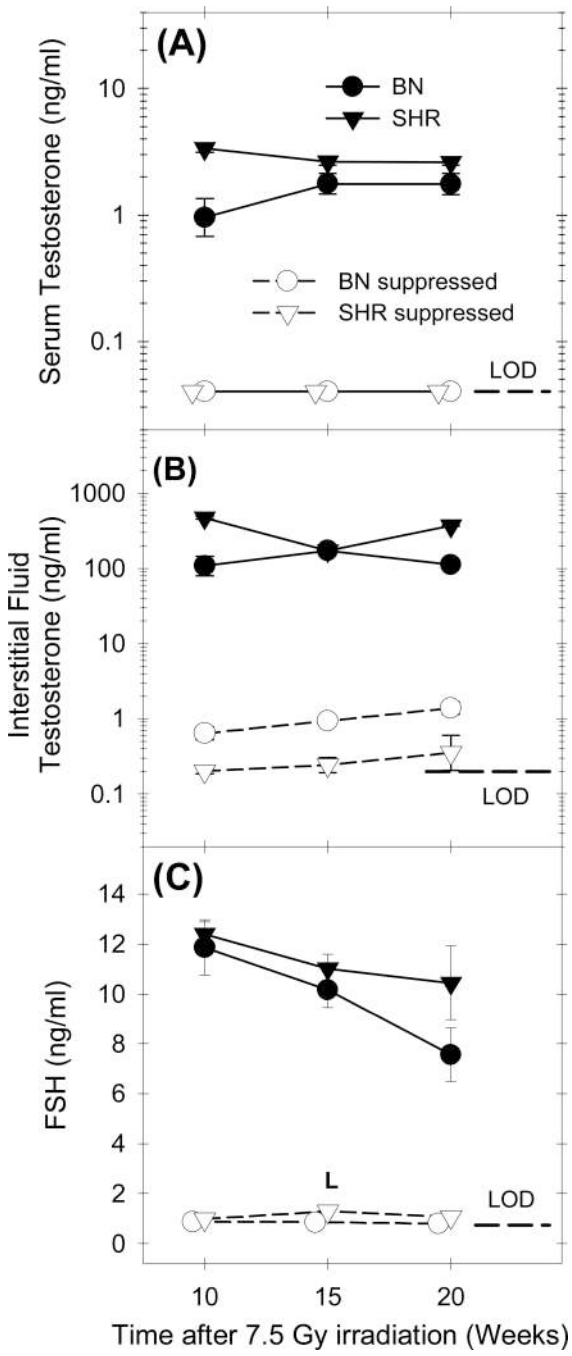

FIG 7.

Hormone levels in BN and SHR rats without hormone suppression (filled symbols) and after hormone suppression (open symbols) at different times after 7.5 Gy irradiation. (A) Serum testosterone. (B) Intratesticular fluid testosterone. (C) Serum FSH. LOD indicates limit of detection of the assay, (L) Indicates undetectable values of some but not all samples in the group.

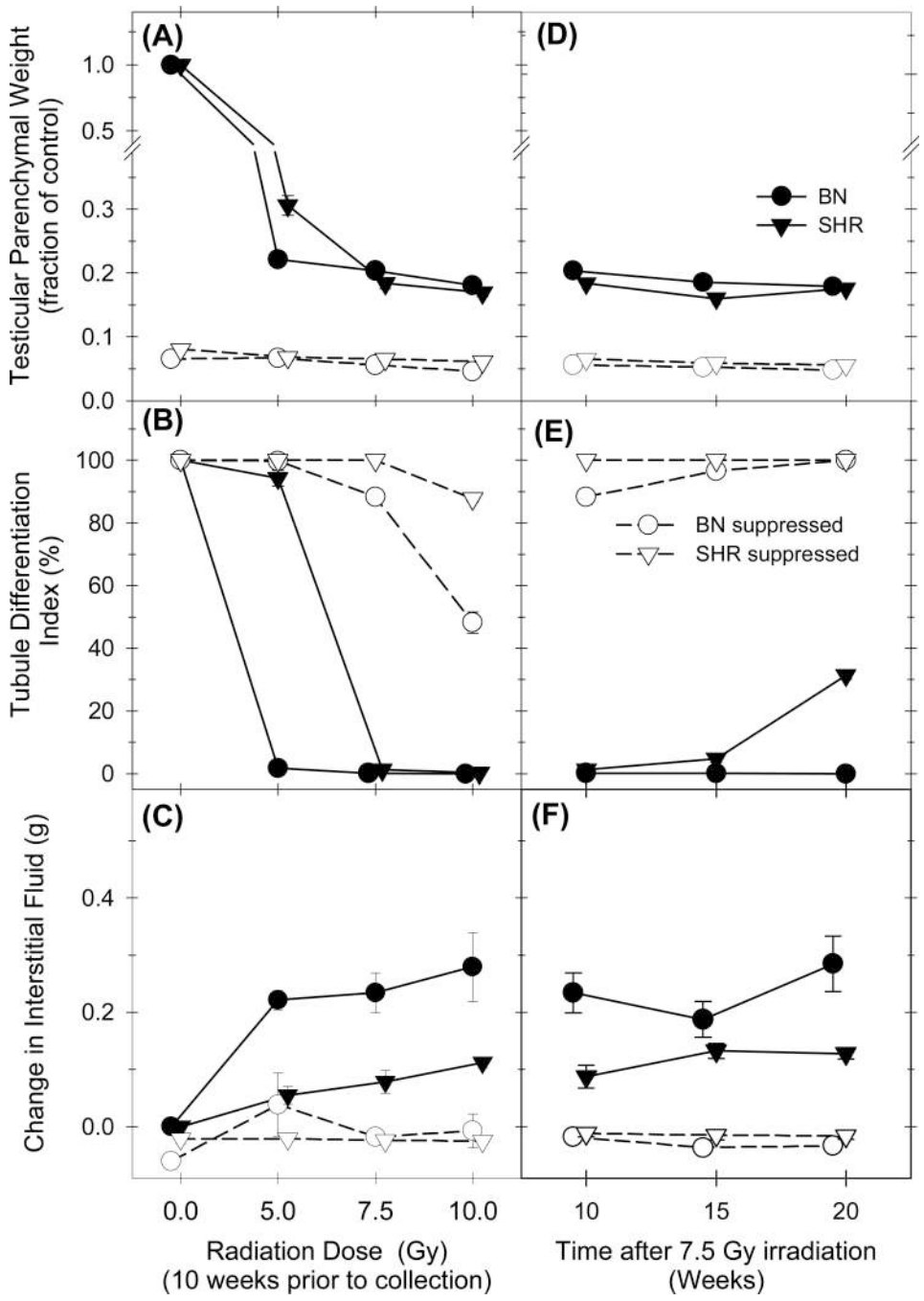

FIG 8.

Dose-response and time-course of changes in testis weights, differentiation in tubules, and interstitial fluid in BN and SHR rats without hormone suppression (filled symbols) and after hormone suppression (open symbols). (A,D) Testis weights relative to unirradiated controls of same strain. (B,E) Percentage of tubules with differentiated cells. (C,F) Change in interstitial fluid weights from unirradiated control levels.

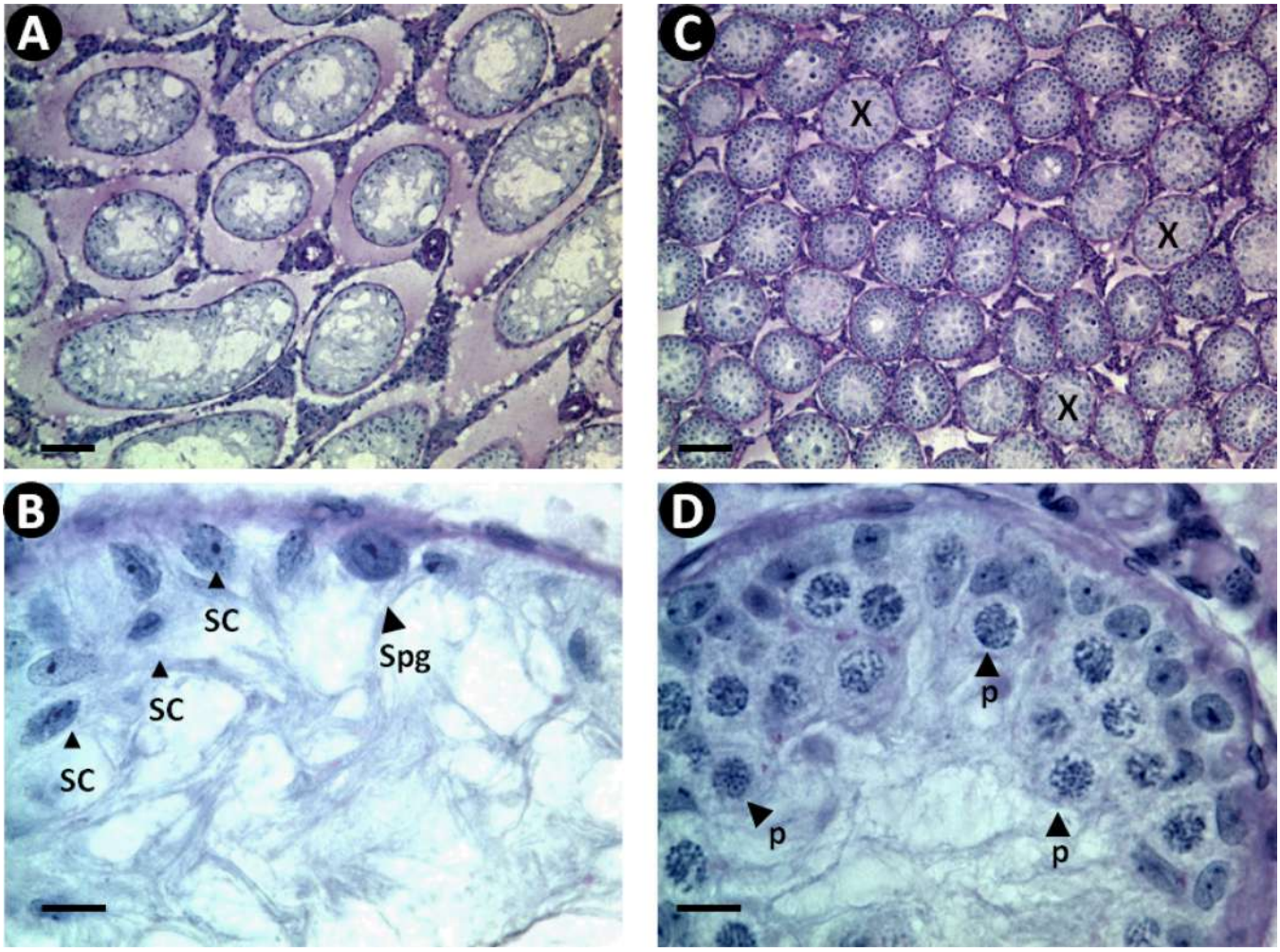

FIG 9.

Histology of BN rat testes 10 weeks after irradiation with 7.5 Gy without (A,B) or with (C,D) hormone suppression. (A) Irradiation produced atrophic tubules and interstitial edema. (B) Most tubules contained only Sertoli cells (SC) but some contained a few type A spermatogonia (Spg). (C) Hormone suppression after irradiation induced recovery of spermatogenesis in nearly all tubules, except those marked with. (X). (D) The recovering tubules showed development to only the pachytene spermatocyte stage (p). (A,C) bar: 100 μm, (B,D) bar: 10 μm.

Table 1

Radiation Doses, Analysis Times, and Strains Analyzed

Rat Strain	Doses (in Gy) used and time of tissue harvest after irradiation				
	10 weeks	15 weeks	20 weeks	30 weeks	40 weeks
BN	2.7, 3.0, 3.3, 3.6, 4.0, 4.5, 5.0, 7.5, 10.0	3.3, 4.0, 4.5, 5.0,7.5,	3.3, 4.0, 7.5		
SHR	4.0, 4.5, 5.0, 5.7, 6.5, 7.5, 10.0	4.0, 4.5, 5.0, 6.5, 7.5	6.5, 7.5	7.5	7.5
SD	5.0, 6.5, 7.5 8.5, 9.7, 11.0, 12.5				

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Hormonal Suppression Restores Fertility in Irradiated Mice from both Endogenous and Donor-Derived Stem Spermatogonia

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Irradiation interrupts spermatogenesis and causes prolonged sterility in male mammals. Hormonal suppression treatment with gonadotropin-releasing hormone (GnRH) analogues has restored spermatogenesis in irradiated rats, but similar attempts were unsuccessful in irradiated mice, monkeys, and humans. In this study, we tested a stronger hormonal suppression regimen (the GnRH antagonist, acyline, and plus flutamide) for efficacy both in restoring endogenous spermatogenesis and in enhancing colonization of transplanted stem spermatogonia in mouse testes irradiated with a total doses between 10.5 and 13.5 Gy. A 4-week hormonal suppression treatment, given immediately after irradiation, increased endogenous spermatogenic recovery 1.5-fold, and 11-week hormonal suppression produced twofold increases compared with sham-treated irradiated controls. Furthermore, 10-week hormonal suppression restored fertility from endogenous surviving spermatogonial stem cells in 90% of 10.5-Gy irradiated mice, whereas only 10% were fertile without hormonal suppression. Four- and 11-week hormonal suppression also enhanced spermatogenic development from transplanted stem spermatogonia in irradiated recipient mice, by 3.1- and 4.8-fold, respectively, compared with those not given hormonal treatment. Moreover, the 10-week hormonal suppression regimen, but not a sham treatment, restored fertility of some 13.5-Gy irradiated recipient mice from donor-derived spermatogonial stem cells. This is the first report of hormonal suppression inducing recovery of endogenous spermatogenesis and fertility in a mouse model treated with anticancer agents. The combination of spermatogonial transplantation with hormonal suppression should be investigated as a treatment to restore fertility in young men after cytotoxic cancer therapy.

Key Words: **irradiation; spermatogenesis; spermatogonial transplantation; fertility; hormonal suppression; mice.**

Radiation and chemotherapy, as testicular toxicants, can lead to temporary or permanent sterility in mammals. Indeed, cancer therapy has induced prolonged or permanent azoospermia in many thousands of men (Meistrich *et al.*, 2005). The continued increase in long-term survival and cure following cancer treatment makes the preservation and restoration of reproductive function of increasing importance (Meistrich *et al.*, 2005).

The prolonged depletion of mature germ cells by radiation or chemotherapy is generally believed to be because of the killing of stem spermatogonia. Although a small number of surviving stem spermatogonia could regenerate spermatogenesis, it usually takes long times for spontaneous recovery to the level required for fertility (Meistrich *et al.*, 1978; Pryzant *et al.*, 1993).

Although testosterone is necessary for normal sperm production, it appears to be associated with the failure of recovery of spermatogenesis from surviving stem cells in some pathological situations (Meistrich and Shetty, 2003, Review). Consequently, transient hormonal suppression has been employed in attempts to protect the testis and/or stimulate recovery of spermatogenesis following radiation or chemotherapy-induced germinal damage (Meistrich *et al.*, 2005). It has been demonstrated repeatedly in rats that the suppression of intratesticular testosterone levels induced by treatment with steroids or gonadotropin-releasing hormone (GnRH) analogues protects against prolonged damage to spermatogenesis if given before radiation or chemotherapy or stimulates recovery if given after the cytotoxic damage; as a consequence, subsequent fertility is increased (Meistrich and Kangasniemi, 1997; Meistrich *et al.*, 2001; Udagawa *et al.*, 2001). Suppression of testosterone has also been shown to enhance the recovery of rat spermatogenesis after damage induced by numerous environmental male reproductive toxicants (Meistrich and Shetty, 2003, Review).

However, the results differ between species (Shetty *et al.*, forthcoming). Although the treatments improve fertility in rats, previous attempts using hormonal suppression to protect or simulate recovery of spermatogenesis in men (Meistrich and Shetty, 2008, Review) and primate model systems (Boekelheide *et al.*, 2005; Kamischke *et al.*, 2003) treated with irradiation and/or cytotoxic drugs have been unsuccessful, with the exception of one report in humans (Masala *et al.*, 1997). In mice, pretreatment reductions of gonadotropins with GnRH analogues or genetic mutations also failed to protect against the

radiation- or chemotherapy-induced disruption of spermatogenesis (Crawford *et al*., 1998; da Cunha *et al*., 1987; Kangasniemi *et al*., 1996a; Nonomura *et al*., 1991), and no study has been performed to examine the stimulation of recovery by posttreatment hormonal suppression.

The studies in rats have also shown that after cytotoxic exposure, a significant population of surviving stem spermatogonia are blocked in their differentiation (Kangasniemi *et al*., 1996b; Meistrich and Shetty, 2003). But in human (Kreuser *et al*., 1989) or monkey (Boekelheide *et al*., 2005; van Alphen *et al*., 1988) testis, such a radiation or chemotherapy-induced block in spermatogonial differentiation is only transient or rare. In mice, the spermatogonia that survive irradiation actively proliferate to produce colonies containing differentiating cells, and very few of the atrophic tubules contain undifferentiated spermatogonia (Kangasniemi *et al*., 1996a). Because the pathophysiological profile in the irradiated mouse testis is more similar to primates than is that of the rat, stimulation of spermatogenic recovery in the mouse by hormonal suppression may be a more appropriate model than rat for future applications to human.

To overcome the loss of stem spermatogonia resulting from cytotoxic therapies, spermatogonial transplantation may also be used to supplement this cell population. When donor stem spermatogonia are introduced into germ cell–depleted seminiferous tubules of host testes, they are able to colonize and undergo complete spermatogenesis. Furthermore, hormonal suppression significantly enhanced spermatogenesis from transplanted spermatogonia in recipient rat testes treated with irradiation (Zhang *et al*., 2007) or busulfan (Ogawa *et al*., 1999) and in recipient mouse testes (Dobrinski *et al*., 2001; Kanatsu-Shinohara *et al*., 2004; Ogawa *et al*., 1998; Ohmura *et al*., 2003). Although hormonal suppression's ability to improve the success of spermatogonial transplantation was dramatic in rat testes, the effects in mice were only moderate and variable from different studies and seemed to be strongly associated with the timing of treatment.

We hypothesized that a more effective hormonal suppression regimen, such as prolonged suppression using both a GnRH antagonist (GnRH-ant), which is more effective than GnRH agonists, and an antiandrogen can efficiently stimulate spermatogenesis from transplanted spermatogonia in mice. Moreover, we examined whether this treatment regimen could also promote the recovery of endogenous spermatogenesis and fertility in irradiated mice.

MATERIALS AND METHODS

Animals. Adult C57BL/6Law male mice at 8–12 weeks of age, bred at The University of Texas, M. D. Anderson Cancer Center, were used in irradiation experiments and as transplantation recipients. Donor mice were obtained by breeding C57BL/6-Tg(CAG-EGFP)1Osb/J mice ubiquitously expressing green fluorescent protein (GFP) (Jackson Laboratory, Bar Harbor, ME) with C57BL/6Law mice. The animals were maintained on a 12-h light 12-h dark cycle and were allowed food and water *ad libitum*. All animal procedures were approved by The University of Texas M. D. Anderson Cancer Center Animal Care and Use Committee.

Experimental design. Four experiments were conducted as outlined in Figure 1. The radiation doses and timing of assays used were based on earlier studies in which recovery of spermatogenesis in mice was measured (Meistrich *et al*., 1978). Total doses of 9–12 Gy resulted in gradual recoveries of sperm counts over the course of 45 weeks, with the mice regaining fertility at about 28 weeks after 9 Gy and failing to recover after 12 Gy. The durations of hormone-suppressive treatments were based on studies in rats, which showed that 4 weeks of GnRH-ant treatment, given after irradiation, with or without flutamide, was able to stimulate spermatogenic recovery (Shetty *et al*., 2000), 10 weeks of GnRH-ant treatment was able to stimulate both recovery of spermatogenesis and fertility (Meistrich *et al*., 2001), and that 13 weeks of suppression stimulated differentiation of transplanted spermatogonia (Zhang *et al*., 2007). In experiment (Exp.) 1, we examined effects of hormonal suppression regimens with GnRH-ant given for different time periods on spermatogenic recovery in mice treated with three different irradiation doses. In Exp. 2, we determined the effect of hormonal suppression on differentiation of endogenous stem cells and colonization of transplanted stem cells in the same irradiated mice with two different irradiation doses. In Exp. 3, we further examined whether hormonal suppression was able to restore fertility by improving recovery of endogenous spermatogenesis after a total dose of 10.5 Gy, the irradiation dose that demonstrated favorable response to hormonal suppression treatment in Exp. 1. In Exp. 4, we used a higher dose of irradiation (13.5 Gy) to destroy nearly all the endogenous spermatogenesis and primarily examined whether hormonal suppression could enhance donor cell colonization and donor-derived spermatogenesis and thereby restore fertility.

Irradiation. Mice were restrained in plastic chambers and then placed into a metal shield module with a 3-cm diameter hole, so that only the lower abdominal and scrotal area of the animal was irradiated by a ^{137}Cs gamma-ray unit. The radiation was delivered as an initial 1.5-Gy dose and followed by a variable second dose given 24 h later as indicated in Figure 1. The fractionated radiation regimen has been shown to be more effective than a single dose in depleting germ cells and produce less unwanted adverse effects (Creemers *et al*., 2002). The radiation doses are presented as the total dose of the two fractions throughout the text. Doses were chosen based on the recoveries of spermatogenesis and fertility after different single doses of irradiation (Meistrich *et al*., 1978).

Hormonal suppression treatment. Hormonal suppression treatments were initiated immediately after irradiation and maintained for 4, 10, or 11 weeks in different experiments, as indicated in Figure 1. The GnRH-ant, acyline (obtained from the Contraceptive Development Branch of National Institute of Child Health and Human Development, North Bethesda, MD), was prepared in sterile water and sc injected at an initial dose of 20 mg/kg body weight and followed by maintenance doses of 10 mg/kg body weight given every other week. For Exps 3 and 4, in which fertility tests were performed, a lower dose of 6 mg acyline/kg body weight was given in the last injection at week 8 to allow quicker recovery of hormonal levels. Flutamide, an androgen receptor antagonist, was delivered by implanting two 2-cm Silastic brand silicone capsules filled with the drug. We used two 2-cm length flutamide capsules based on our previous experiments that a total length of 4-cm flutamide is effective in suppressing the testosterone action on the normal testis (Shetty *et al*., 2006b). The effect was similar to that observed previously with pellets releasing 1.2 mg of flutamide/day (Kangasniemi *et al*., 1996a). The flutamide capsules were implanted right after completion of irradiation (within 30 min) and were removed after 4, 10, or 11 weeks for 4-week, 10-week, or 11-week treatment groups, respectively. The controls were sham treated by injection of sterile water and implantation of empty capsules. In Exp. 1, the flutamide implants were found lost because of the sealing staples not being fastened well after implantation and were removed from the housing cages within the first few days. We thus considered the hormonal-suppressive treatment to be GnRH-ant only in that study.

Transplantation. Immature heterozygous GFP mice at 14–17 days of age were used as donors, except for 12-Gy group in Exp. 2, in which 19- to 27-day-old

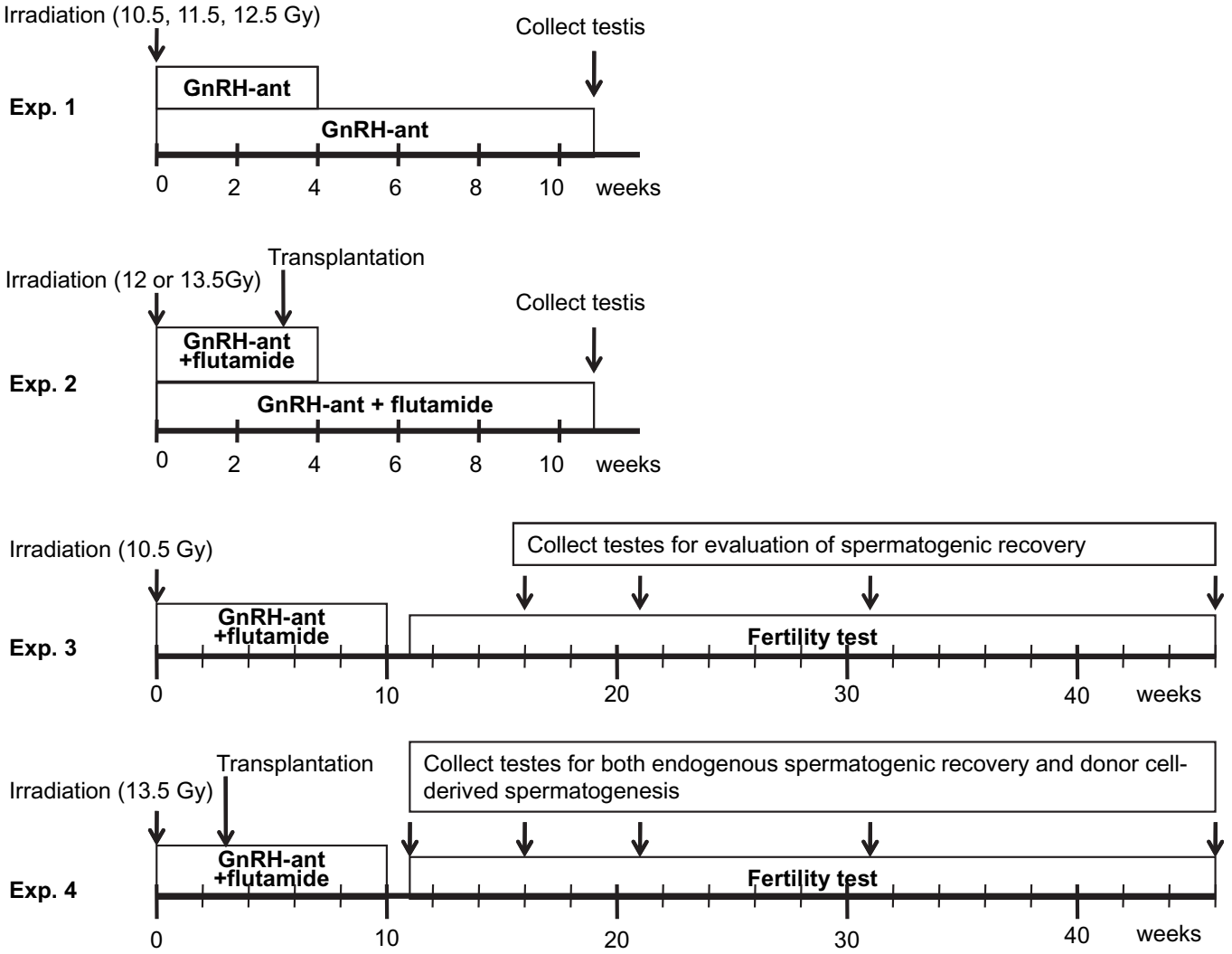

FIG. 1. Schematics of the four experimental protocols used. Mice were irradiated at week 0 with total doses as indicated. Hormonal suppression treatment was started immediately after irradiation and continued for 4, 10, or 11 weeks. In Exps 2 and 4, transplantation was performed at 3 weeks after irradiation.

mice were used. The stem cell spermatogonia donor cells were prepared as previously reported (Zhang *et al*., 2006). Briefly, after the tunica was removed, testicular tissue was sequentially digested, first with 0.05% type IV collagenase for 20 min and then with combined 0.05% type IV collagenase and 0.05% hyaluronidase for 20 min in modified Dulbecco's Modified Eagle Medium (DMEM)/F12 solution containing 100 μg/ml DNase at 35°C in a shaking water bath. The tubules were washed in Dulbecco's PBS (GIBCO, Carlsbad, CA) and then incubated in 0.1% trypsin in D-PBS containing magnesium, 100 μg DNase/ml, and 1mM ethylene glycol tetraacetic acid. After neutralization of trypsin with serum and filtration through a 35-μm nylon screen, the cell suspensions were centrifuged and resuspended in DMEM/F12 solution containing 100 μg DNase/ml. Trypan blue (Invitrogen, Grand Island, NY) was added to a final concentration of 0.04%, and the cell suspension was kept on ice until transplantation. The average of viability of cells was 93%.

Mice irradiated with 12 or 13.5 Gy, as indicated in Figure 1, were used as recipients for spermatogonial transplantation 3 weeks after irradiation. The lower abdomen was opened, and the testis was withdrawn from the body cavity. The efferent duct was identified, and surrounding fat was dissected away under a microscope. The donor cells were transplanted into seminiferous tubules through efferent duct injection using a glass micropipette controlled by a FemtoJet microinjector (Brinkmann Instruments Inc., Westbury, NY). The average injection volume was 8.2 μl, and an average of 3.1×10^5 cells per testis was injected. The success of the injection was monitored by observing the distribution of the Trypan blue dye. In Exp. 4, the transplantation control groups were injected with media instead of cells.

Evaluation of spermatogenesis. The mice were euthanized at different times after irradiation as indicated in Figure 1. The weights of the body, testis, and seminal vesicle (SV) were recorded in all experiments, and in Exps 3 and 4, the epididymis weights were also recorded.

For histological evaluation of endogenous spermatogenic recovery, testes were fixed in Bouin's solution (both testes in Exp. 1, left testis in Exp. 3, and in sham-transplanted groups of Exp. 4), embedded in paraffin, and sectioned at 5-μm thickness. The testicular cross sections were stained with hematoxylin and periodic acid-Schiff reagent and then examined under light microscopy. Spermatogenic recovery was evaluated as previously described (Shetty *et al*., 2001) by the tubule differentiation index (TDI), which is defined as the percentage of tubules that contain three or more differentiating germ cells at the B spermatogonial stage or beyond.

For evaluation of spermatogenic recovery in the recipient testes (Exp. 2 and transplanted groups of Exp. 4), both testes were removed and fixed in 4% paraformaldehyde at 4°C for up to 24 h and embedded in paraffin. After deparaffinization and rehydration, the testicular sections were subjected to

antigen retrieval and nonspecific antibody-binding blocking. The sections were then incubated with rabbit monoclonal anti-GFP (Cell Signaling, Danvers, MA) at 1:300 dilution at 4°C overnight, followed by a biotinylated anti-rabbit immunoglobulin G and an avidin-biotin-peroxidase complex reagent (Vectastain Elite kit, Vector Laboratories, Burlingame, CA). The immunoreactivity was visualized by incubation with the peroxidase substrate diaminobenzidine (Vector Laboratories). The slides were counterstained with hematoxylin.

The progress of spermatogenesis in the recipient testes was evaluated by the TDI as described above. GFP staining was used to differentiate whether the germ cells originated from the donor cells (GFP positive) or from the endogenous spermatogonia (GFP negative). The TDI for donor cells was corrected for injection of different cell numbers by normalization to the average cell numbers injected in each experiment.

The lengths of the donor cell colonies were measured in the 12-Gy study of Exp. 2. After removal of the tunica from one testis of three or four mice from each treatment group, the seminiferous tubules were separated using a fine forceps under a dissecting microscope. GFP-positive colonies were identified and imaged under a fluorescence microscope. The length of the colonies was measured by using the image processing software Axiovision version 4.6 (Carl Zeiss Microimaging, Inc., Göttingen, Germany).

To assess the recovery of sperm production, sperm heads were counted in the testes and sperm were counted in the cauda epididymis. The tunica albuginea was removed from one testis of a mouse, and the testis was weighed, homogenized, and sonicated. The sperm heads were counted in a hemacytometer (Meistrich and van Beek, 1993). For epididymal sperm counts, both cauda epididymis were minced in 1 ml PBS and incubated at 37°C for 30 min, and the suspension was passed though a 80-μm pore size metal filter. Sperm were counted using a hemacytometer.

Serum testosterone measurement. Blood was collected from the axillary vein of mice under anesthesia at euthanasia. The serum was separated by centrifugation and stored at −20°C until measurement of testosterone. Serum testosterones were determined as described earlier (Shetty *et al.*, 2000) by using a coated tube radioimmunoassay kit (DSL-4000, Diagnostic Systems Laboratories, Webster, TX).

Fertility test. The fertility of mice was tested starting at 11 weeks after irradiation. Each male was housed with two ND4 Swiss Webster virgin females (Harlan Laboratories, Indianapolis, IN) until the time assigned for euthanasia of the males. The recovery of fertility for each mouse was defined as the date of conception of first litter, 20 days prior to the birth date. The number and size of litters were recorded. A group of unirradiated adult male mice were used as positive controls for the fertility test. The pups from the transplanted mice were examined for the expression of the GFP transgene to determine if the return of fertility was from endogenous stem spermatogonia or from donor cells. Because GFP heterozygous donors were used, we expected that half of pups would be GFP positive if the sperm were derived from donor stem cells.

Statistical analysis. Simple comparisons of tissue weights, sperm counts, and TDI among groups were performed using two sample *t*-tests (for two group comparison) or ANOVA with Student-Newman-Keuls post hoc pairwise comparisons (for three or more groups). Statistical analysis of testicular sperm count data was performed on log-transformed data because the transformed distributions are closer to normal. In a few cases that the TDI data were not normally distributed, nonparametric analyses were performed, as indicated in the figure legends, using the Wilcoxon-Mann-Whitney test for two group comparisons and the Kruskal Wallis test for three or more group comparisons. Time to fertility recovery was analyzed by the Kaplan-Meier estimator. Difference between treatment groups was considered significant when $p < 0.05$. These statistical analyses were performed using the SPSS version 16 statistical software package (SPSS Inc., Chicago, IL).

We used linear mixed models (Verbeke and Molenberghs, 2000) to examine the effects of hormone suppression treatment, source of stem cells, radiation level, stem cell transplantation, and sacrifice time on the TDI. For this analysis, the TDI of the hormonal-suppressed groups was normalized against the no-hormone–treated controls by dividing all their TDI values by the mean TDI of the mice that received no hormonal suppression with the same combination of radiation dose, source of stem cells, and sacrifice time point, and is referred to as the TDI ratio. To assess whether the effect of 4-week hormonal suppression differs from that of 11-week hormonal suppression and whether the differences between the two treatment regimens are dependent on the source of stem cells, we fitted the linear mixed model on all data points from Exps 1 and 2. The model included fixed effects of hormone suppression (11 weeks vs. 4 weeks), source of stem cells (endogenous vs. donor), the interaction between hormone suppression and source of stem cell, transplantation, and radiation nested within transplantation, and a random effect of mice (Model 1). To assess whether the effect of 10 weeks of hormone suppression on endogenous TDI irradiated with 10.5 Gy varies over time, we fitted a linear model with a fixed effect of sacrifice time (four levels: 16, 21, 31, and 46 weeks) on all the data points from Exp. 3 (Model 2). Finally, to assess whether the effect of 10-week hormone suppression on TDI is greater toward donor cells than endogenous cells and whether the effect is time dependent, we fitted a linear mixed model on the data points from the mice receiving stem cell transplantation from Exp. 4, with fixed effects of source of stem cells (endogenous vs. donor), sacrifice time (five levels: 11, 16, 21, 31, and 46 weeks), the interaction between source of stem cell and sacrifice time, and a random effect of animal (Model 3). All linear models were performed in SAS v 9.1 (SAS Institute, Cary, NC).

RESULTS

Hormonal Suppression (Exp. 1 and Exp. 2)

In Exp. 1, mice were irradiated with total radiation doses of 10.5, 11.5, or 12.5 Gy followed by 4- or 11-week treatments with GnRH-ant (acyline) and euthanized at 11 weeks after irradiation. Body weights were slightly, but reversibly, reduced in the mice under hormonal suppression treatment (Table 1). There was a marked decrease in testis weights, SV weights, and serum testosterone concentration with the 11-week treatment. The reduction in SV weight with 11-week treatment clearly confirms that testosterone activity was suppressed during the treatment.

In a subsequent experiment (Exp. 2), mice were irradiated with total radiation doses of 12 or 13.5 Gy followed by 4- or 11-week treatments with GnRH-ant (acyline) plus antiandrogen and spermatogonial transplantation. Alterations of body and tissue weights were similar to those in Exp. 1. In addition, in a group of mice euthanized at the end of the 4-week treatment time, both testis and SV weights were reduced (Table 1). Although the concentration of serum testosterone was not suppressed after 4-week treatment, the action of testosterone was clearly reduced in those mice at 4 weeks because the SV weights were significantly decreased. The reduced SV weights in the absence of serum testosterone level reductions at 4-week treatment is likely because of the effect of antiandrogen flutamide that blocks the action of testosterone. Note that the tissue weights in these mice returned to nearly the levels of control mice at 11 weeks, suggesting that the suppression of testosterone was reversible after the treatment ceased. With 11-week treatment, both serum testosterone levels and SV weights were significantly reduced, showing that both testosterone level and action were effectively suppressed in those animals.

TABLE 1
Effects of Hormone Suppression on Weights and Testosterone Levels

Experiment and irradiation dose	Hormonal suppression	Analysis time (weeks after irradiation)	Body weight (g)	Testis weight (mg)	SV weight (mg)	Serum testosterone concentration (ng/ml)
Exp. 1						
10.5 Gy	Sham	11	29 ± 1[a]	25 ± 1[a]	300 ± 12[a]	2.8 ± 0.8[a]
11.5 Gy	4 weeks GnRH-ant	11	27 ± 1[a]	24 ± 1[a]	260 ± 7[b]	3.2 ± 0.9[a]
12.5 Gy	11 weeks GnRH-ant	11	25 ± 1[b]	14 ± 1[b]	22 ± 2[c]	0.5 ± 0.1[b]
Exp. 2	Sham	11	28 ± 0[d]	29 ± 1[d]	256 ± 12[d]	1.2 ± 0.3[d]
12 Gy	4 weeks GnRH-ant + flutamide	4	25 ± 0[e]	14 ± 0[e]	21 ± 3[e]	2 ± 0.9[d]
13.5 Gy	4 weeks GnRH-ant + flutamide	11	27 ± 0[d]	27 ± 1[d]	241 ± 7[d]	0.9 ± 0.2[d]
	11 weeks GnRH-ant + flutamide	11	25 ± 0[e]	11 ± 0[f]	10 ± 1[e]	0.1 ± 0.0[e]

Note. The data of three irradiation doses from Exp. 1 were pooled because ANOVA analysis showed no significant difference among them. The data from the two irradiation doses from Exp. 2 were pooled because *t*-test analysis showed no significant difference between them except for the SV weights of 11-week treatments (9 ± 0 mg at 12 Gy vs. 13 ± 2 mg at 13.5 Gy), both of which were dramatically lower than those in control mice. *N* was between 9 and 29 for weights and between 5 and 12 for serum testosterone concentrations. For a given parameter, values that are significantly different from each other ($p < 0.05$) within each experiment are indicated by different letters.

Effect of Hormonal Suppression on Recovery of Endogenous Spermatogenesis (Exp. 1 and Exp. 2)

The hormonal suppression treatment stimulated the recovery of endogenous spermatogenesis. In mice irradiated with 10.5 Gy, there was a significant increase in the percentages of tubules with differentiated germ cells after 11-week hormonal suppression, as compared with sham-treated mice (Fig. 2A). Four-week treatment also showed a consistent trend of an increase compared with controls, but the effects were generally less than those with the 11-week treatment. Similar results were observed with the recovery of endogenous spermatogenesis in mice irradiated with 12 and 13.5 Gy and then also subjected to spermatogonial transplantation, with significant increases in recovery after the 11-week hormonal suppression and a significant increase of spermatogenic recovery with the 4-week treatment in mice irradiated with 12 Gy (Fig. 2B).

The results from Model 1 revealed that there were no significant effects of transplantation ($p = 0.61$) or radiation dose (nested within transplantation) ($p = 0.43$), though the stimulatory effect of hormone suppression was marginally lower in Exp. 1, in which the flutamide capsules were lost, than in Exp. 2. The 4-week hormone suppression treatment appeared to increase the TDI of endogenous cells over that observed with no hormone suppression by 1.5-fold ($p = 0.10$), whereas the 11-week treatment significantly increased the TDI by twofold ($p = 0.025$). Although the overall effect of the 11-week hormone suppression appeared greater than that of the 4-week treatment, the difference was not statistically significant ($p = 0.25$).

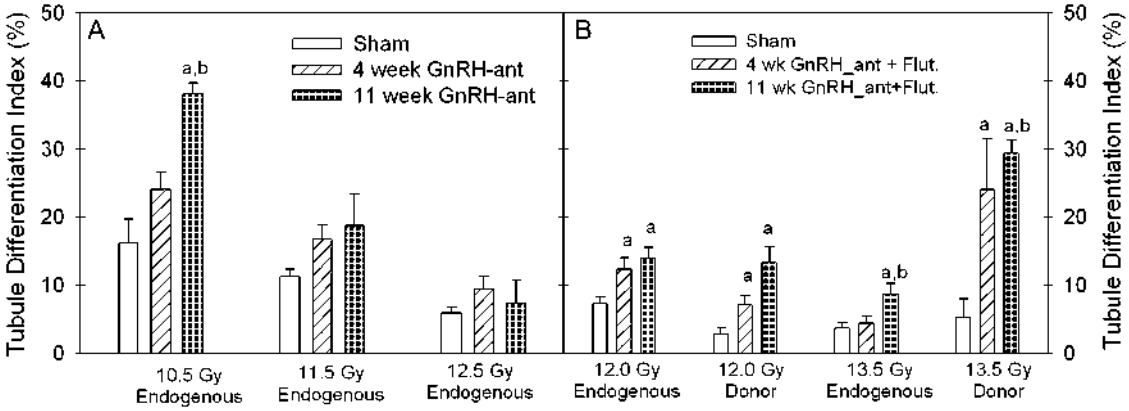

FIG. 2. Hormonal suppression improved spermatogenic recovery from both endogenous and donor-derived stem spermatogonia in irradiated mouse testis in Exp. 1 (A) and Exp. 2 (B). The TDI is the percentage of tubule cross sections with more than three differentiated germ cells. ''Endogenous'' indicates the differentiated germ cells that were derived from surviving stem spermatogonia of the recipient. ''Donor'' indicates the differentiated germ cells that were derived from GFP-positive transplanted stem spermatogonia. The statistical analyses of 12- and 13.5-Gy donor TDI data in panel (B) were conducted by a nonparametric method because the data are not normally distributed. $N = 3$–4 (panel A) and $N = 4$–18 (panel B). ''a'' and ''b'', $p < 0.05$ versus the sham- or 4-week–treated groups, respectively.

Effect of Hormonal Suppression on Colony Development from Transplanted Stem Spermatogonia (Exp. 2)

Gross examination of recipient testes under fluorescence microscopy showed that transplanted spermatogonia successfully colonized the testes in all treatment groups (Figs. 3A–C). Immunohistochemical staining for GFP was then performed on tissue sections (Figs. 3D–F) to determine which tubules were repopulated with spermatogenic cells derived from donor versus endogenous stem cells. We noted that the overall efficiency of donor-derived stem cell colonization was lower in the 12-Gy study than that in the 13.5-Gy study (Fig. 2B). This discrepancy is attributable to the older age of the donor animals used in the 12-Gy study as previous observations showed that the efficiency of colonization by cells from 28-day-old donors was only half that of cells from 12-day-old donors (McLean *et al.*, 2003).

When testosterone was suppressed for either 4 or 11 weeks in the irradiated recipient mouse testes, the recovery of spermatogenesis from transplanted stem spermatogonia was

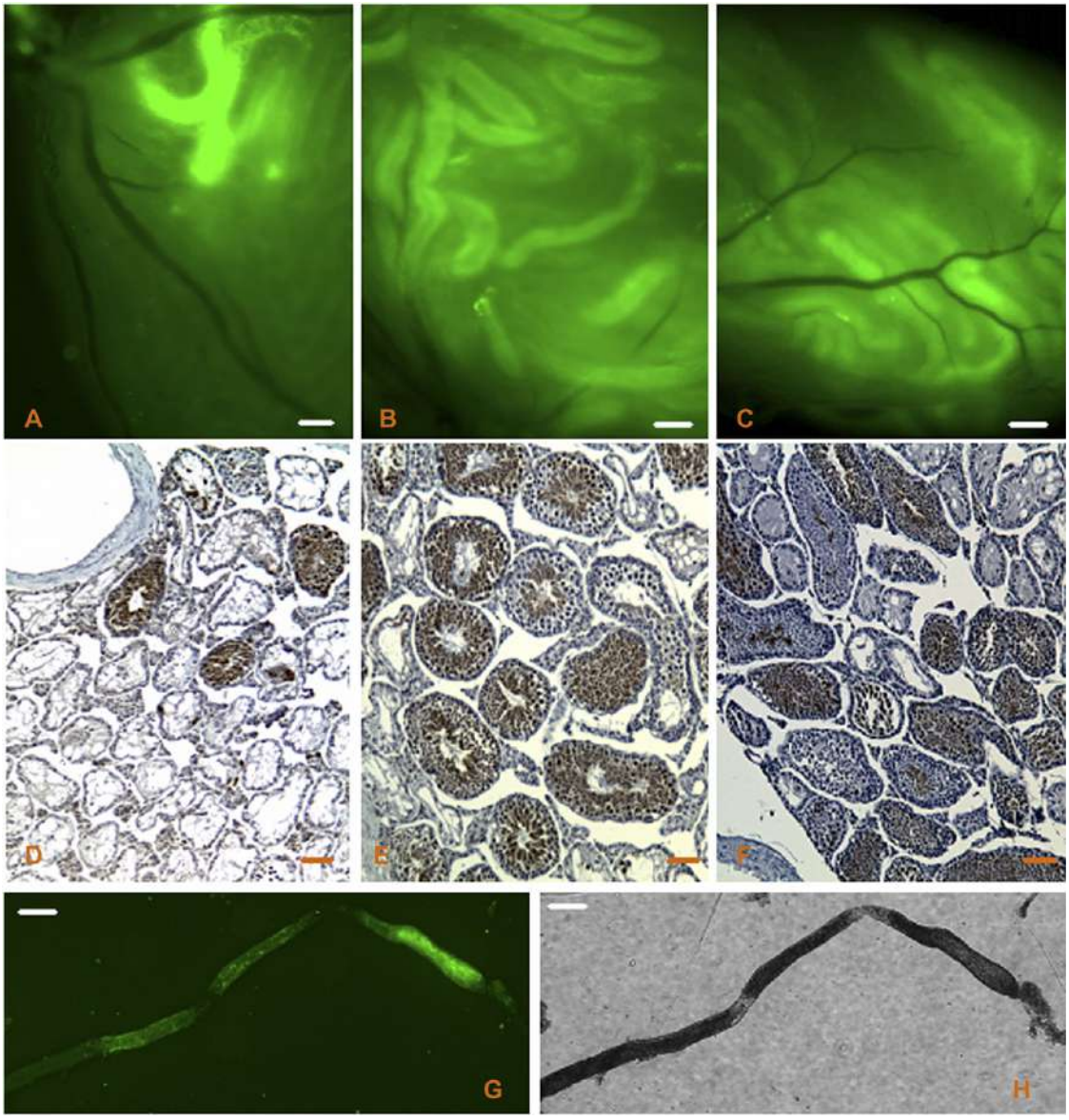

FIG. 3. Evaluation of the recipient testes. GFP-expressing donor cell colonization was visualized at 11 weeks after transplantation by fluorescence microscopy in irradiated recipient testes that were sham treated (A) or given 4-week GnRH-ant plus flutamide (B) or 11-week GnRH-ant plus flutamide (C) treatments. Immunohistochemical staining for GFP in fixed tissue cross sections from (D) sham-treated mice, (E) 4-week GnRH-ant plus flutamide treatment, or (F) 11-week GnRH-ant plus flutamide treatment was performed to distinguish tubules with donor-derived spermatogenesis (brown) from those with endogenous spermatogenesis (blue). Note that there is some nonspecific staining of cytoplasm of the late spermatids. GFP-positive tubules were separated from recipient testis and imaged by fluorescence (G) for measurement of colony length or by bright field (H) to clearly show the tubule. Bars, 200 μm (A, B, C, G, and H) and 100 μm (D, E, and F).

consistently improved in two separate studies with different doses of irradiation (Fig. 2B). The results from Model 1 showed that the 4-week hormonal suppression treatment significantly ($p < 0.0001$) increased the TDI of donor cells over that observed with no hormonal suppression by 3.1-fold, whereas the 11-week treatment significantly ($p < 0.0001$) increased the TDI of donor cells by 4.8-fold. Furthermore, the increase in TDI with 11-week therapy was greater than that with the 4-week treatment ($p = 0.002$). The effects of hormonal suppression appeared to be greater toward donor than toward endogenous stem cells (p for interaction term $= 0.07$), and the fold increases in TDI with both 4- and 11-week hormonal suppressions were greater toward the donor than toward the endogenous cells ($p = 0.002$ and $p < 0.0001$ for 4- and 11-week hormonal suppression, respectively).

Because the relationship between the TDI, which is the percentage of tubule cross sections with differentiating germ cells, and the actual number of developing donor germ cell colonies is dependent on the length of those colonies, we measured the colony lengths (Fig. 3G) after the different hormonal treatments. The length of donor cell colonies (12-Gy dose, Exp. 2) was 512 ± 12 μm ($n = 9$ colonies from three testes) in sham-treated mice, 498 ± 14 μm ($n = 36$ from four testes) in mice receiving 4-week hormonal suppression, and 464 ± 34 μm ($n = 26$ from four testes) in mice receiving 11-week hormonal suppression. These values were not significantly different from each other. These results demonstrate that the TDI values were proportional to numbers of colonies and that the higher TDI in the 11-week hormonal suppression group was actually because of increased colony numbers.

Effect of Hormonal Suppression on Regeneration of Reproductive Tissues and Spermatogenesis (Exp. 3 and Exp. 4)

Because hormonal suppression was able to stimulate the initiation of spermatogenesis from both endogenous and donor-derived stem spermatogonia, we tested whether the spermatogenic recovery was maintained after treatment and whether spermatozoa were produced.

We first examined the time course of the recovery of the androgen-dependent reproductive accessory organs after either 10.5-Gy irradiation (Exp. 3) or 13.5-Gy irradiation (Exp. 4). Because androgen is required for the development of spermatozoa and mating behavior, we monitored the reversibility of testosterone suppression by SV weights. In the mice receiving GnRH-ant (acyline) and flutamide for the first 10 weeks after irradiation, the SV weights remained low at 16 weeks but returned to levels observed in the non–hormone-suppressed mice by 21 weeks (Figs. 4A and 4F). Note that the epididymal weights, which were reduced primarily by hormonal suppression, although sperm numbers may also have some effect, took between 31 and 46 weeks to recover to the levels observed in the irradiated mice that did not receive hormonal suppression (Figs. 4B and 4G).

Spermatogenesis gradually recovered in 10.5-Gy irradiated testes not receiving hormonal suppression treatment, as indicated by gradual increases in testis weights, testicular sperm head counts, and TDIs (Figs. 4C and 4D and 5A). The suppression of testosterone significantly improved the progress of spermatogenic recovery, as shown by the significant increases of testis weights (Fig. 4C), testicular sperm head count (Fig. 4D), and TDI (Fig. 5A) at the 16-, 21-, and 31-week postirradiation time points. Model 2 showed that the TDI ratio was overall significantly greater than 1 ($p < 0.0001$); it appeared to be highest at early time points (week 16, 1.78-fold) and showed a downward trend ($p = 0.08$) with time. The cauda epididymal sperm counts were lower in the hormone-suppressed mice than in controls at week 16 as a result of the residual effect of the hormonal suppression. However, they were significantly increased in the previously hormone-suppressed group by week 21 and were significantly higher than those in the sham-treated controls on weeks 31 and 46 (Fig. 4E). However, it should be noted that epididymal sperm counts are more variable than testicular sperm head counts, can be affected by sperm storage or hormone levels because of changes in transport rate, and show an attenuated response to changes after irradiation (Meistrich and Samuels, 1985).

We then examined the time course of restoration of spermatogenesis in mice irradiated with a higher dose (13.5 Gy) to deplete endogenous spermatogenesis and subjected to the hormonal suppression treatment and/or germ cell transplantation (Exp. 4); four treatment groups: sham, hormonal suppression only, transplantation only, and the combination of hormonal suppression and transplantation were analyzed. In 13.5-Gy irradiated testes in which only sham transplantation was performed, hormonal suppression improved the spermatogenic recovery from endogenous spermatogonia, as indicated by elevated sperm head count in the testis (Fig. 4I) and increased endogenous TDIs (Figs. 5B and C) at nearly all time points, with the differences being statistically significant at several of these time points.

Transplantation of germ cells alone, without hormonal suppression, resulted in the formation of donor colonies in up to 10% of the tubules at the 21-, 31-, and 46-week time points (filled squares, Fig. 5C), but these donor colonies did not produce enough cells to significantly increase testis weights (filled inverted triangles, Fig. 4H). However, in these transplanted mice, 10-week suppression of testosterone markedly enhanced recovery of spermatogenesis as measured by significant increases in testis weights during weeks 16–31 after irradiation (open inverted triangles, Figs. 4H and 4I). Testicular sperm head counts, which were only measured at week 46, were also increased (triangles, Fig. 4I). This was clearly a result of enhanced spermatogenesis from transplanted spermatogonia, compared with mice receiving transplantation but not hormonal suppression, as shown by significant increases of donor TDI (open squares, Fig. 5C) at all time points.

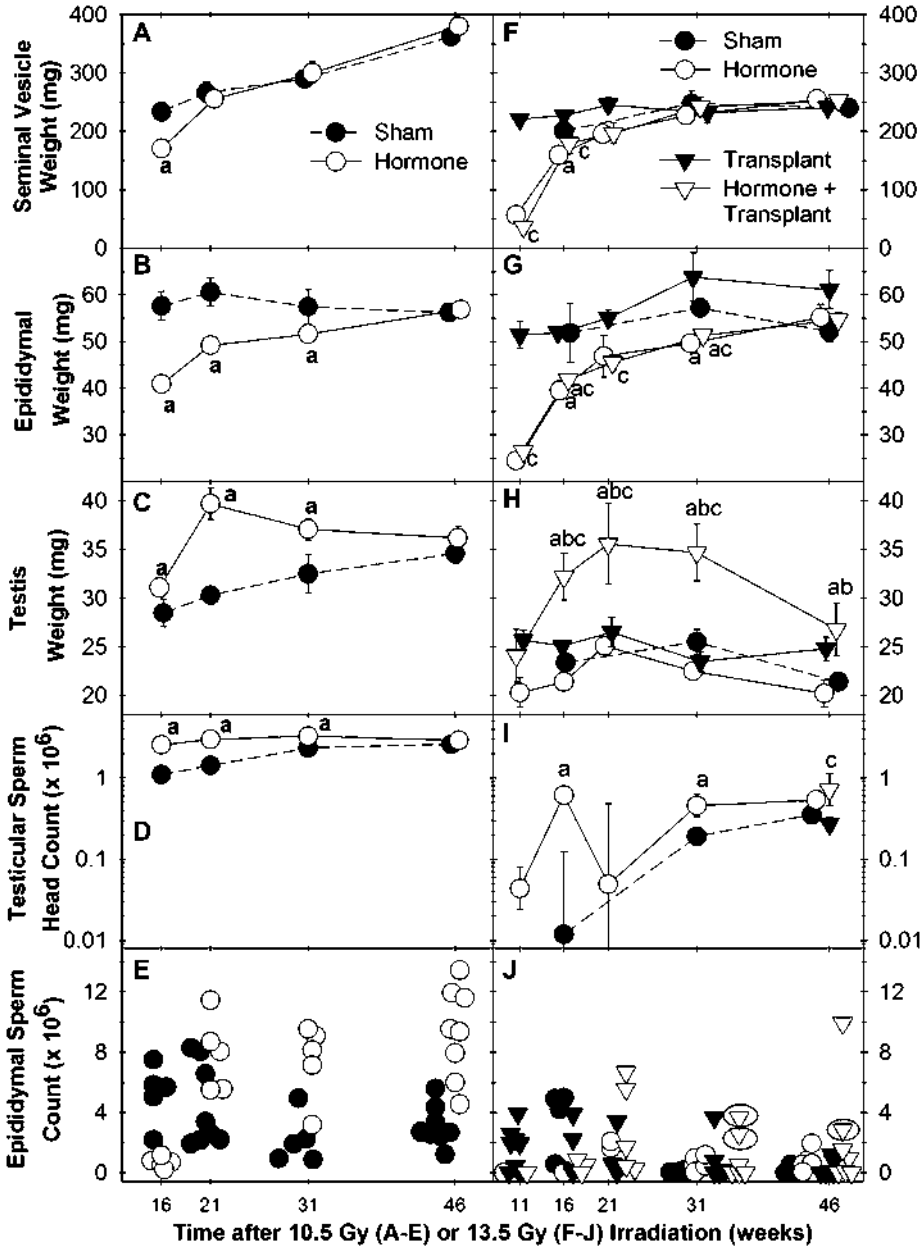

FIG. 4. Time course of accessory sexual organ or spermatogenic recovery in mice irradiated with 10.5 Gy (A–E) from Exp. 3 or 13.5 Gy (F–J) from Exp. 4, as measured by SV weight (A and F), epididymal weight (B and G), testis weight (C and H), testicular sperm head count (note log scale) (D and I), and cauda epididymal sperm count (E and J). Groups are designated as Sham, Hormone (GnRH-ant + flutamide only, no transplantation), Transplant (transplantation only), Hormone + Transplant (GnRH + flutamide and spermatogonial transplantation). $N = 10$ for 46-week time point data and 5 for all other time points for both irradiation doses. ''a,'' ''b,'' and ''c'', $p < 0.05$ versus values in the sham group, hormone group, and transplant group, respectively. Ellipses in (K) identify those mice that were fertile.

Model 3 showed that the 10-week hormone suppression significantly increased the TDI ratio for both donor and endogenous stem cells in this experiment and that the TDI ratio for donor stem cells was greater than for endogenous stem cells ($p = 0.0003$). Although effect of time of assessment was not statistically significant, the increases in donor TDI ratios were between 4.7-fold (at the 11-week time point) and approximately sevenfold (later time points).

In general, the spermatozoa numbers in cauda epididymis in all treatment groups were relatively low and variable from animal to animal (Fig. 4J). However, during the 21- to 46-week time period, most of the mice with the highest sperm counts

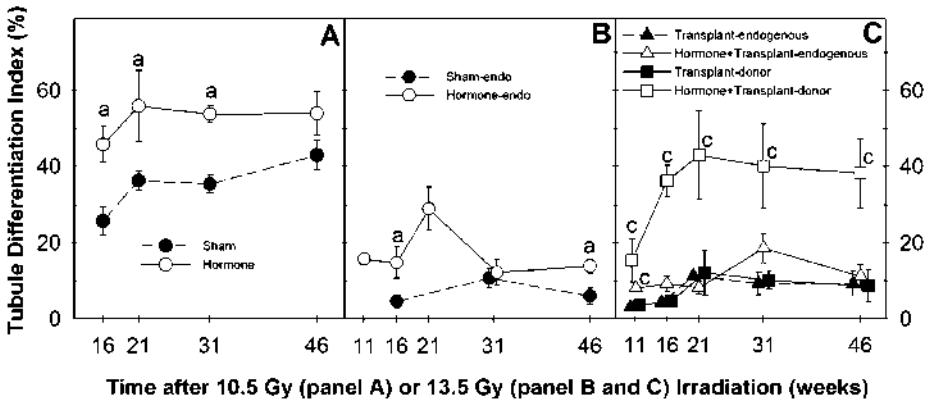

FIG. 5. TDIs in testes of mice irradiated with 10.5 Gy (A) from Exp. 3 or 13.5 Gy (B, endogenous and C, transplanted) from Exp. 4, with and without hormonal suppression. Furthermore, in the transplanted testes, the TDI of the endogenous cells and donor cells were scored separately. $N = 10$ for 46-week time point group and 5 for all other time point groups for both irradiation doses. The statistical analysis of 46-week TDI data in panel (B) was conducted by a nonparametric method because the data are not normally distributed. ''a'' and ''c'', $p < 0.05$ versus values in the sham group and transplant-only group, respectively.

were those treated with both hormonal suppression and germ cell transplantation.

Effect of Hormonal Suppression on Restoration of Fertility (Exp. 3 and Exp. 4)

The recovery of spermatogenesis by hormonal suppression treatment observed in Exp. 3 was indeed translated into function as fertility was restored in 90% of the treated mice during 20–40 weeks after irradiation. This was significantly higher than the recovery of fertility in the mice without hormonal suppression, in which only 10% recovered ($p < 0.001$) and the recovery did not occur until week 37 (Fig. 6A). Although a high level of fertility was restored in the irradiated, hormonally suppressed mice, their fecundity was only at most a quarter of that of unirradiated, non-hormonally treated control mice (Fig. 6C). During the 21- to 46-week postirradiation time periods, only 83% of the treated males were fertile, litter size was 5.2 compared with 8.9 in normal controls, and the number of litters they produced per 5-week period was only half that of the controls.

Although mice irradiated with 13.5 Gy (Exp. 4), even if they were treated with either hormonal suppression or transplantation, were sterile up to 46 weeks after irradiation, combined treatment with hormonal suppression and transplantation successfully restored fertility of three mice between 15 and 30 weeks after irradiation (Fig. 6B) ($p = 0.09$). However, it is not clear why there was a trend of declining fecundity and also testis weights (Fig. 4H) at week 46, but not decreases in donor cell TDI (Fig. 5C). In the hormonally treated, transplanted mice, the fecundity was only 5% of controls (Fig. 6D), as at most 20% of mice were fertile during any time period; average litter sizes were only 6.9; and the frequency of litters was 40% of that in controls.

In every litter produced by the transplanted, hormonally suppressed mice, both GFP-positive and GFP-negative pups were present, and of the total of 76 pups from 11 litters, 43 were GFP positive. Because the donor animals we used were hemizygous for GFP, we expected that half of spermatozoa from donor stem spermatogonia would carry the GFP transgene. Thus, nearly all the spermatozoa in those fertile mice must have developed from transplanted stem spermatogonia.

DISCUSSION

In the current study, for the first time, we demonstrated that hormonal suppression given after irradiation successfully accelerated the recovery of spermatogenesis and significantly shortened the time to return of fertility in irradiated mice. Moreover, the suppression regimen also enhanced the efficiency of transplanted cell colonization in irradiated mouse recipient testes and resulted in the production of progeny mice derived from the donor cells.

The duration, timing, and degree of hormonal suppression all appeared to be important for successful recovery from spermatogenic injury in the irradiated mouse testis. In previous attempts to stimulate spermatogenic recovery following cytotoxic damage in mice, hormonal suppression was generally given prior to or during irradiation or chemotherapy (da Cunha *et al.*, 1987; Kangasniemi *et al.*, 1996a; Nonomura *et al.*, 1991). The durations of hormonal suppression treatment were generally short, being less than 4 weeks in these studies. Two studies (da Cunha *et al.*, 1987; Nonomura *et al.*, 1991) used treatment with a GnRH agonist, which is not as effective as GnRH-ant at hormonal suppression (Meistrich *et al.*, 2001). In a third study, hypogonadal mice with a null mutation in the GnRH gene were used (Crawford *et al.*, 1998), but there was still some basal production of testosterone from the Leydig cells in these mice (Singh *et al.*, 1995). Thus, the androgen ablation in most of the studies was incomplete. Only one of the studies employed GnRH-ant and antiandrogen to produce total

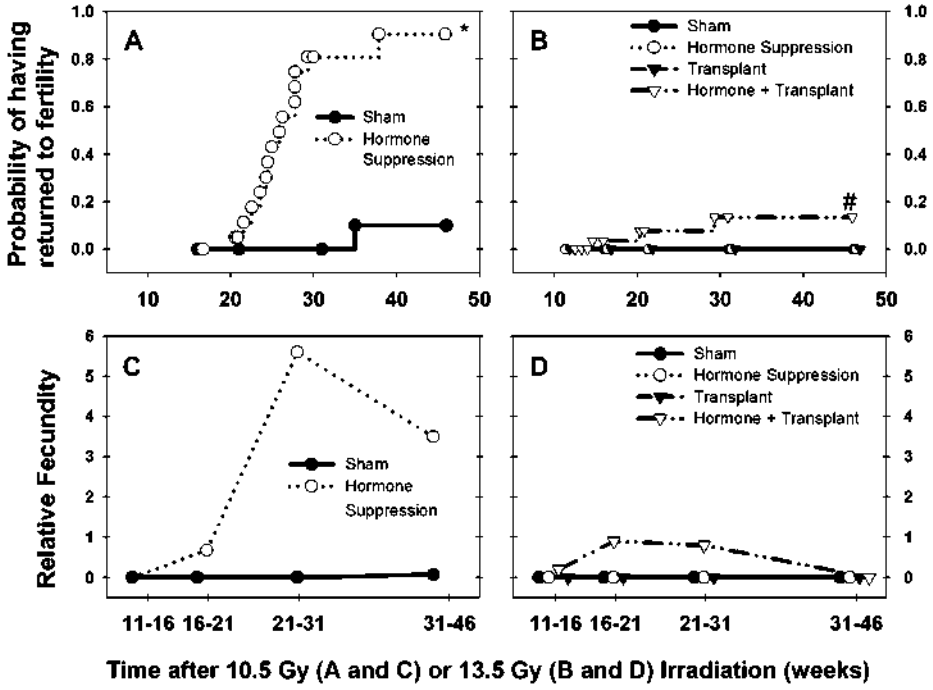

FIG. 6. Time course of fertility recovery expressed as probability of having returned to fertility for male mice irradiated with 10.5 Gy with or without hormone suppression (A) or irradiated with 13.5 Gy with or without hormone suppression or spermatogonial transplantation (B) by Kaplan-Meier survival analysis. Relative fecundity (fraction of males that were fertile × litters per fertile male per 5-week time period × average litter size) for male mice irradiated with 10.5 Gy, with or without hormone suppression (C), or irradiated with 13.5 Gy, with or without hormone suppression or spermatogonial transplantation (D). Note, for comparison, that the calculated Relative fecundity for our unirradiated, non-hormonally treated control mice was 21.4. Symbols in panels (A and B) represent times at which individual mouse became fertile or were censored (i.e., the time of euthanasia before they became fertile). *$p < 0.05$ compared with values of sham-treated group. #$p < 0.05$ compared with values of sham, hormonal suppression, or transplant groups.

androgen ablation, but the duration of treatment was only 2 weeks (Kangasniemi *et al*., 1996a). The success of our current treatment strategy is likely attributable to the suppression of testosterone immediately in the postirradiation period, a relatively prolonged treatment period, and perhaps the use of an antiandrogen to completely inhibit the action of the residual testosterone.

In comparison to the less than twofold stimulation of endogenous colony formation observed here in the mouse, hormonal suppression in the rat using GnRH-ant alone for as short as 4 weeks (Shetty *et al*., 2000) or GnRH-ant plus flutamide for 2 weeks, either prior to or after cytotoxic therapy (Kangasniemi *et al*., 1995; Shetty *et al*., 2006a), produced much greater stimulation of endogenous colony formation. Because hormonal suppression stimulates recovery of spermatogenesis in irradiated rats by reversing the block to spermatogonial differentiation (Meistrich and Shetty, 2003; Meistrich *et al*., 2000), this interspecies difference in stimulation appears to be related to differences in the magnitude of the differentiation block that is produced by the cytotoxic injury.

The duration, timing, and degree of hormonal suppression are also of importance in enhancing colonization of transplanted spermatogonia in recipient mouse testes. In the present study, the hormonal suppression was always started 3 weeks before transplantation. When the hormonal suppression was continued for only 1 week more after transplantation, there was an average of a 3.1-fold stimulation of the TDI (Exp. 2), and when it was continued for 7 or 8 weeks (Exp. 4 or Exp. 2, respectively) after transplantation, the TDI was increased significantly to 4.8-fold. Analysis of three previous studies using hormonal suppression with GnRH agonists (Dobrinski *et al*., 2001; Ogawa *et al*., 1998; Ohmura *et al*., 2003) given for 4 weeks indicates a consistent enhancement of donor spermatogenesis by about 1.9-fold. GnRH agonist suppressive treatments lasting 8–10 weeks stimulated increases in colonization by about threefold (Dobrinski *et al*., 2001; Kanatsu-Shinohara *et al*., 2004). By comparison, our hormonal suppression strategy with the GnRH-ant and antiandrogen appears to be somewhat more effective.

Several processes are needed for the development of spermatogenesis from transplanted stem spermatogonia. Homing involves moving to the basement membrane of seminiferous tubules to already existing niches or formation of new niches. Individual stem cells must then proliferate, undergoing self-renewing divisions to increase their numbers (de Rooij, 2001). Finally, the stem cells must reach a steady state of self-renewal and differentiation divisions to produce later

spermatogenic cells while maintaining their populations. The use of two different hormonal suppression times and the evaluation of both endogenous and donor-derived spermatogenesis in the same animals allowed us to determine the relative effect of hormonal suppression on homing versus proliferation/differentiation.

The greater stimulation of tubule repopulation by transplanted cells, which were introduced after 3 weeks of hormonal suppression, than by endogenous stem cells must have been due primarily to an increase in the homing efficiency of the donor cells. The 3.1-fold stimulation by the 4-week hormonal suppression treatment was primarily because of enhancement of the homing step. The additional stimulation to 4.8-fold from continuing the treatment to week 11 can be attributed to effects on proliferation/differentiation. Previous observations showing that hormonal suppression prior to but not after transplantation stimulates recovery (Dobrinski *et al*., 2001) are consistent with the effect on the homing step but not the additional stimulation we observed with continuing the hormone suppression after transplantation.

The enhancement of the abilities of stem cells to both produce differentiated cells and maintain their numbers, independent of the homing process, by hormonal suppression must be responsible for the stimulatory effect of the 4-week suppression on the endogenous TDI and the greater effectiveness of the longer than the shorter treatment on both endogenous and donor TDI. Previously, a 1.8-fold increase in donor cell colony number was observed even when the hormonal-suppressive treatment was not started until 4 weeks after transplantation (Ohmura *et al*., 2003).

The molecular and cellular mechanisms by which hormonal suppression facilitates homing of transplanted stem cell spermatogonia and enhances their proliferation and/or differentiation are unclear. Because cellular alterations produced by irradiation that inhibit the differentiation of both endogenous and transplanted spermatogonia in the rat are in the somatic environment (Zhang *et al*., 2007), which contains the cells that have the receptors for testosterone and the gonadotropic hormones, it is most likely that hormonal suppression enhances germ cell development from spermatogonial stem cells by action on the supporting somatic environment. Recently, β1-integrin, expressed in both the stem spermatogonia and the Sertoli cells, has been identified as an essential adhesion receptor for the homing of mouse transplanted stem spermatogonia (Kanatsu-Shinohara *et al*., 2008). However, analysis of gene expression changes in irradiated rat testes (Zhou *et al*., 2010) and SCARKO mouse testes (Wang *et al*., 2009) (Zhou, Wang, Small, Liu, Weng, Yang, Griswold, and Meistrich, submitted) showed that neither β1-integrin nor its targets, the various laminin genes, is upregulated by hormonal suppression or abrogation of androgen action on Sertoli cells and therefore do not appear to be involved in the enhancement of homing. Further studies of the hormonal regulation of stem spermatogonial homing and proliferation/differentiation are necessary to understand this phenomenon.

The combined treatment of hormonal suppression and spermatogonial transplantation appears to be necessary for promoting recovery of spermatogenesis and fertility, especially after relatively high radiation doses. Although hormonal suppression alone restored fertility after 10.5-Gy irradiation, it did not increase endogenous spermatogenesis to the level necessary for fertility in 13.5-Gy irradiated mice. Spermatogonial transplantation supplemented the recipient testes with stem spermatogonia, providing an additional source for repopulation of the testes.

Our results demonstrated that functional spermatozoa developed after hormonal suppression and/or transplantation. The fecundity was low compared with wild-type mice, as expected because the testicular sperm counts were below or barely above the cutoff of 2×10^6 (15% of control) necessary for recovery of fertility after irradiation (Meistrich *et al*., 1978). This result also illustrates the interspecies difference, as in irradiated rats hormonal suppression for 10 weeks restored endogenous testicular sperm production to 86% of control and nearly completely restored fecundity (Meistrich *et al*., 2001).

The application of the hormonal suppression treatment and spermatogonial transplantation can be considered as treatment for men exposed to testicular toxicants. Although clinical trials of hormonal suppression alone have not been very successful, it is likely in several of these studies that the gonadal damage was so severe that there were too few stem cells to yield significant recovery (Meistrich and Shetty, 2008). Combined treatment of hormonal suppression and spermatogonial transplantation should be more promising because hormonal suppression more significantly stimulated homing of the transplanted germ cells than it did recovery of endogenous spermatogenesis. The development of methods for improvement of transplantation efficiency are most important for fertility preservation in prepubertal boys who are subjected to gonadal damage from a cytotoxic cancer treatment but are too young to be able to produce sperm for storage. The mouse may be a better model than the rat for preclinical trials because the stimulation of recovery occurs without reversal of a major block of spermatogonial differentiation as occurs in the rat.

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Mini-Review

Mini-Review

Update on Novel Hormonal and Nonhormonal Male Contraceptive Development

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Abbreviations: 11β-MNTDC, 11β-methyl-nortestosterone dodecylcarbonate; ADAM, A Disintegrin And Metalloproteinase; ADAMTS, ADAMs with Thrombospondin motifs; ALDH, aldehyde dehydrogenase; BET, bromodomain and extraterminal; DMAU, dimethandrolone undecanoate; EPPIN, epididymal peptidase inhibitor; HIPK4, homeodomain-interacting protein kinase 4; mAb, monoclonal antibody; MENT, 7α-methyl-19-nortestosterone; NES, Nestorone®; NICHD, National Institute of Child Health and Human Development; RA, retinoic acid; RARα, retinoic acid receptor α; RISUG, reversible inhibition of sperm under guidance; T, testosterone; TSSK, testis-specific serine/threonine kinases; TU, testosterone undecanoate.

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Abstract

Background: The advent of new methods of male contraception would increase contraceptive options for men and women and advance male contraceptive agency. Pharmaceutical R&D for male contraception has been dormant since the 1990s. The Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) has supported a contraceptive development program since 1969 and supports most ongoing hormonal male contraceptive development. Nonhormonal methods are in earlier stages of development.

Content: Several hormonal male contraceptive agents have entered clinical trials. Novel single agent products being evaluated include dimethandrolone undecanoate, 11β-methyl-nortestosterone dodecylcarbonate, and 7α-methyl-19-nortestosterone. A contraceptive efficacy trial of Nestorone®/testosterone gel is underway. Potential nonhormonal methods are at preclinical stages of development. Many nonhormonal male contraceptive targets that affect sperm production, sperm function, or sperm transport have been identified.

Summary: NICHD supports development of reversible male contraceptive agents. Other organizations such as the World Health Organization, the Population Council, and the Male Contraception Initiative are pursuing male contraceptive development, but industry involvement remains limited.

Key Words: contraception, male contraception, nonhormonal contraceptive development, sperm

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Despite a variety of contraceptive options available to women, the unintended pregnancy rate in the United States remains at approximately 45% (1). Male condoms and withdrawal are the only reversible contraceptive methods available to men; however, with typical failure rates of 13% and 20%, respectively, these methods are much less reliable than available female methods like combined hormonal contraceptives (pills, rings, patches), hormonal injections or implants, hormonal intrauterine systems, and the copper intrauterine device (2). Studies across cultures, countries, and continents indicate that >50% of men would be interested in using a reversible method, if available (3), and many women would be willing to rely on their partner to use a contraceptive (4). Lack of acceptable male-controlled methods contributes to perceptions of men having limited ability to participate in reproductive decision making (5). Currently, 21% of contraceptive use in the United States involves male condom or vasectomy (6). Novel reversible male contraceptives offer the opportunity for greater reproductive agency for the male partner. Computational modeling suggests that the unintended pregnancy rate in the United States could fall by 3.5% with only 10% uptake of a male contraceptive pill among potential users and 5.3% with 15% uptake (7).

Content

Hormonal Male Contraception

Studies as early as the 1970s have demonstrated that hormonal male contraceptives can be as effective as female methods (8). Hormonal male methods build on knowledge of how hormonal methods function in women. Exogenous progestins inhibit production of gonadotropins that regulate synthesis of sex hormones (estrogen in ovaries, testosterone [T] in testes) that are needed for development of an egg or sperm. Similarities in male and female reproductive biology provide the basis for designing effective hormonal contraception for men.

High intratesticular T concentration is required for spermatogenesis. In healthy men, testicular T levels are maintained 40- to 100-fold higher than serum T levels. Below a threshold amount of testicular T, sperm production does not occur. Studies show that exogenous steroid hormone administration, an androgen alone or in combination with a progestin or a gonadotropin-releasing hormone agonist or antagonist, suppresses testicular T production through feedback inhibition of the hypothalamic–pituitary axis. However, other androgen-dependent functions such as libido, erection, ejaculation, and maintenance of muscle mass, require adequate serum androgen levels. Therefore, exogenous androgens must be added back to maintain sufficient serum levels to support those functions while keeping testicular T below the threshold to initiate sperm production.

The cutoff for normal fertility is 15 million sperm/mL; an average ejaculate contains >60 million sperm. Early efficacy studies showed that the overall pregnancy rate attributable to men with sperm concentrations ranging between 0 and 3.0 million sperm/mL was 1.4 per 100 person-years (9). Achievement of severe oligozoospermia (sperm concentration ≤1 million per mL) is associated with a pregnancy risk of approximately 2% per year (10), which is on par with highly effective female methods and the rationale for accepting 1 million/mL as the goal for sperm suppression for male hormonal methods (11). Proof-of-concept studies of combinations of progestins and T derivatives have demonstrated sperm suppression to levels that could reliably reach this goal (12-18). Sperm suppression rates of 89% to 100% have been achieved in these studies. It is unclear why some men fail to suppress. In contraceptive efficacy studies of potential male methods, confirmation of sperm suppression has been required prior to allowing men to rely on the product for contraception. It is anticipated that regulatory approval would require this confirmation; however, there is the potential for home assessment of sperm concentration with commercially available sperm assessment kits.

The search for the "male pill" has been stymied by lack of a safe, effective oral androgen, which is necessary to provide hormone addback when testicular T production and spermatogenesis are suppressed. Unfortunately, oral testosterone is cleared too rapidly to be effective as a single daily dose regimen even in combination with a progestin. Multiple doses of oral testosterone per day would be impractical for contraception. Although 17-methyltestosterone has better oral bioavailability, long-term use has been associated with hepatotoxicity. Oral testosterone undecanoate (TU) recently has been approved in the United States, but dosing is twice per day.

The Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) has supported development of new androgens that bind to both androgen and progesterone receptors and potentially may serve as single-agent male contraceptives (19). Two lead candidates in development are dimethandrolone undecanoate (DMAU) and 11β-methyl-nortestosterone dodecylcarbonate (11β-MNTDC) (20, 21). The compounds are not aromatizable, which may lower serum estradiol levels if endogenous T synthesis is inhibited. Potential long-term effects on bone health are unknown, but increases in P1NP, a serum marker of bone formation, were seen in a 28-day study of oral DMAU (22). When administered orally or intramuscularly, DMAU is hydrolyzed to active drug, dimethandrolone, a derivative

of 19-nortestosterone, which binds to both androgen and progesterone receptors. DMAU has been evaluated in phase I clinical trials in the NICHD's Contraceptive Clinical Trials Network and was well tolerated (23). In a 28-day trial of 200 mg and 400 mg daily oral DMAU, serum gonadotropins were suppressed to low levels in most subjects (24). No serious adverse effects were seen. Most participants (80%) were satisfied with the method; 54% reported they would use it as their primary method of birth control if available (25). A first-in-man clinical trial of 11β-MNTDC at single doses of 200, 400, and 800 mg showed the drug was well tolerated without serious adverse effects (26). A 28-day trial of 200 mg and 400 mg daily oral 11β-MNTDC showed marked suppression of gonadotropins over the treatment period, again without serious adverse effects (27). Longer term evaluation of these progestogenic androgens is necessary to determine if they are safe and can effectively suppress sperm production.

Another synthetic progestogenic androgen, 7α-methyl-19-nortestosterone (MENT), is currently under evaluation as a possible male contraceptive (28). Initial evaluations of MENT implants to suppress sperm production were comparable to initial studies with TU, with about two-thirds of men showing dose-dependent spermatogenesis suppression (29). Improvements of the MENT implant resulting in sustained levels of MENT release are in development but require further validation.

Transdermal or injectable androgens may provide an alternative to an oral product. Testosterone gels are widely used in the United States to treat hypoandrogenism. Several trials have evaluated the ability of injectable testosterone enanthate or TU alone or in combination with a progestin to achieve sperm suppression with promising results (12-14, 16, 30). A trial combining T gel and injections of the progestin, depomedroxyprogesterone acetate, a female contraceptive product, resulted in effective sperm suppression in 90% of subjects (31). Notably, this method involved 2 Food and Drug Administration–approved products, albeit used for off-label indications.

Few regimens have been evaluated in contraceptive efficacy studies (18, 30, 32-34) (Table 1). The most recently completed contraceptive effectiveness trial built on the success of injectable TU by adding a progestin to achieve sperm suppression. This phase II multisite international clinical trial sponsored by World Health Organization and CONRAD, evaluated contraceptive efficacy and safety of separate intramuscular injections of a long-acting progestin, norethisterone enanthate, and the long-acting androgen, TU, at 8-week intervals (18). Couples (n = 320) were enrolled; 266 men suppressed to low sperm counts and the couples entered the efficacy phase. The study was terminated early per recommendation of an external safety review committee due to the frequency of reported mood changes, depression, injection site pain, and increased libido. Despite this, the combined method failure rate, including sperm nonsuppression by the end of the suppression phase, sperm rebound during the efficacy phase, and pregnancy during the efficacy phase, was 7.5%. For comparison, typical use failure rates for women using birth control pills are estimated at 7% to 9% (2). Importantly, >75% of participants said they would be willing to use the method if available.

Another regimen in development includes daily applications of a gel containing T and the progestin, Nestorone® (NES). In the proof of concept trial, use of T gel (100 mg) and NES gel (8 mg) suppressed sperm concentration to <1 million/mL or azoospermia in 89% of men compared with only 23% of men using T gel and a placebo gel (17). Suppression of serum gonadotropins (luteinizing hormone and follicle-stimulating hormone) occurred rapidly. Gonadotropin hormone concentrations that were >1 IU/L after 4 weeks of treatment predicted treatment failure (sperm concentration >1 million/mL) with 97% sensitivity (35). Most failure was due to inconsistent or nonuse of the products rather than to nonresponse to the drug regimen. When asked about acceptability of the regimen, over half of participants reported being satisfied or extremely satisfied with the method (36). A contraceptive efficacy study to evaluate combined NES/T in a single gel preparation for use as a primary method of contraception in couples is currently underway in the NICHD Contraceptive Clinical Trials Network.

Hormonal male contraceptive methods have proven effective in clinical trials. Regulatory approval of a new contraceptive drug for women usually requires 20 000 cycles of safety and contraceptive efficacy evaluation for at least 1 year of use. Long-term safety of a male method will need to be demonstrated before a drug would pass regulatory approval. Calculation of potential risk/benefit of a male contraceptive drug is challenging because men do not face medical risks associated with pregnancy and childbirth; any systemic product for men must have a strong safety profile. However, consideration of a shared risk model of the benefits of pregnancy prevention from a biologic and psychosocial context for both partners is important (37). The goal of identifying additional health benefits for male methods is especially attractive. Realistically, long-term trials in sufficient numbers of couples will require years before a product could reach the market. Regulatory agencies will need to provide guidance on what is required for approval of this new class of drugs. Additionally, pharmaceutical investment will be critical to achieve this goal.

Table 1. Male hormonal contraceptive efficacy trials

Regimen	N enrolled	N entering/ completing efficacy	Pregnancy/Failure rate per 100 couple years	Author/Sponsor
Testosterone enanthate weekly injection	271	157/119	1 0.8 (0.0 to 4.5)	WHO 1990
Testosterone enanthate weekly injection	357	268/209	4 1.4 (0.4 to 3.7)	WHO 1996
Testosterone undecanoate monthly injection	308	296/280	1 2.3 (0.5 to 4.2)	Gu et al. 2003
Testosterone undecanoate monthly injection	1045	855/733	9 1.1 (0.4 to 1.8)	Gu et al. 2009
Testosterone implant every 4-6 months + depomedroxyprogesterone acetate injection q 3 months	55	53/28	0 0 (0 to 8)	Turner et al. 2003
Testosterone undecanoate injection + norethisterone enanthate injection every 8 weeks	320	266/111[a]	4 2.2 (0.8 to 5.8)	WHO/CONRAD; Behre et al. 2016
Testosterone + Nestorone® transdermal gel applied daily	ongoing	ongoing	ongoing	NICHD

[a]Study stopped early.

Nonhormonal Male Contraception

In contrast to hormonal male contraception, where the mechanism of action is to stop sperm production through feedback inhibition of the hypothalamic–pituitary axis, the goal of nonhormonal contraceptives is to avoid the hypothalamic–pituitary axis and thereby potentially avoid side effects associated with hormones. Nonhormonal male contraceptive development is largely still in the preclinical phase and involves targeting proteins that impact either sperm production or sperm function. The number of potential targets is large and growing. Approaches to control sperm production or function vary widely; each approach will have to be evaluated to demonstrate a high degree of safety in addition to effectiveness as a contraceptive. Data from animal models suggest that such targets may prove effective if specificity is enhanced to limit off-target effects. Efforts using iterative screening, structural biology, computational modeling, and designer chemistry are being employed to move forward several potential nonhormonal male contraceptives. While measurement of sperm suppression may not provide the appropriate mechanism to evaluate effectiveness of these methods, ultimately regulatory approval will require demonstration of contraceptive effectiveness.

First studied and recognized in male rats, vitamin A (retinol) deficiency and its physiologically active metabolite, all-trans retinoic acid, have long been recognized for their role in male sterility (38). Male retinoic acid receptor α (RARα) knockout mice are infertile. The retinoic acid (RA) pathway, including conversion of retinol to retinal and finally to RA, provides opportunities for inhibitors or antagonists to stop RA synthesis and thereby stop spermatogenesis. A clinical study of a bisdichloroacetyldiamine analog in the RA pathway, WIN18,446, was used to treat over 60 men for 1 year (39). The drug was well tolerated and efficacious at inhibition of spermatogenesis. However, development of the drug was halted after finding that consumption of alcohol with the drug induced a severe disulfiram reaction, due to off-target inhibition of a liver enzyme, aldehyde dehydrogenase (ALDH)-2, which detoxifies aldehyde during alcohol metabolism. A different aldehyde dehydrogenase subfamily, ALDH1A, is involved in synthesis of RA and a testis-specific member includes ALDH1A2. Covalent and noncovalent small molecule inhibitors of ALDH1A2 recently have been developed. Ternary x-ray cocrystal structures of the inhibitors provide the structural framework for design of potent, selective inhibitors of ALDH1A2 (40).

An alternative approach in the RA synthetic pathway is inhibition of RARα. An RARα variant is essential for spermatogenesis and mouse knockouts are infertile (41). A study with the panretinoic acid receptor antagonist, BMS-189453, demonstrated reversible spermatogenesis inhibition in a mouse model (38). Structure Based Drug Design, with iterative screening, is being employed to develop potent, specific antagonists to inhibit RARα activity in the RA synthetic pathway to inhibit sperm production.

BRDT, a testis-specific bromodomain protein, is another nonhormonal target. A subfamily of bromodomain and extraterminal (BET) proteins consists of 4 members: BRD2, BRD3, BRD4, and BRDT. Testis-specific BRDT is critical for chromatin remodeling during spermatogenesis (42, 43). Male mice with homozygous BRDT null mutations are sterile (44). One study showed that JQ1, a small molecule inhibitor of BRDT, was able to cross the blood–testis barrier and cause complete, reversible contraceptive activity in male mice (45). Although effective for

contraception, JQ1 had off-target binding to other BRD proteins. Efforts are underway with different chemical scaffolds to develop optimized inhibitors of BRDT. A study entailing virtual screening, analytical testing, structure-activity relationship evaluation and compound optimization via x-ray cocrystal has resulted in different chemical scaffolds with potent BRDT inhibitory activity (46). Each BET protein has 2 bromodomain modules, and the second module (BD2) may be a target for enhancing specificity. Focused library screening and subsequent optimization has produced potent BET inhibitor candidates selective for BD2 (47).

Mechanistically different drug candidates that target Sertoli–germ cell adhesion and cause release of immature spermatids from the seminiferous epithelium have been identified. CDB-4022, an indenopyridine, inhibits mature sperm production in primates and stallions. Cessation of drug treatment results in full reversibility of sperm production with no apparent side effects (48-50). Additional drug candidates targeting Sertoli cell–germ cell interaction are indazole carboxylic acid derivatives: Gamendazole, H2-gamendazole, and Adjudin. Rats treated with oral doses of H2-gamendazole showed inhibition of fertility (51). Although the effect was reversible at low doses of the drug, at higher doses, the fertility effect was irreversible. Targeting a drug to Sertoli cells is particularly difficult due to the challenge of crossing the blood–testis barrier. To overcome this challenge, Adjudin was conjugated to a recombinant follicle-stimulating hormone–binding fragment to target the testis germ cell–Sertoli cell junction (52). However, the increase in specificity was offset by reduced oral bioavailability. Safety and reversibility of these candidates needs to be demonstrated in higher mammals to determine if they truly are candidates for development in humans.

Numerous ion channel and kinase protein targets affect sperm motility, many of which are expressed in the sperm tail region. Ions channels CatSper (a calcium ion channel) and KSper (a potassium ion channel) are sperm specific and required for male fertility (53). Animal models incorporating mutations and deletions of these genes have shown male infertility without apparent systemic effects. An in vitro study with HC-056456, an inhibitor of the calcium ion channel, demonstrated that the drug prevented hyperactivation of sperm (54). A sperm-specific potassium channel, SLO3 (also known as KCNU1) controls calcium entry through CatSper. Genetic deletion of SLO3 causes male infertility in mice (55).

Under normal function, CatSper causes sperm tail hyperactivation when progesterone binds and activates α/β hydrolase domain-containing protein 2, causing depletion of endocannabinoid 2-arachidonoylglycerol from the spermatozoa plasma membrane. Removal of 2-arachidonoylglycerol by α/β hydrolase domain–containing protein 2 releases CatSper inhibition and causes calcium influx leading to sperm activation (56). Physiologically, the cumulus–oocyte complex secretes progesterone after ovulation. Following intercourse, sperm enter the tubal isthmus through the uterotubal junction and form a reservoir (57). Sperm can remain viable in the isthmus for several days until progesterone and other triggers signal them to swim toward the tubal ampulla where fertilization may occur. A recent study demonstrated that the steroidal inhibitor, RU1968, causes dysfunction of CatSper's progesterone-mediated motility response. The inhibitor is nontoxic to human sperm and inhibits hSLO3 with approximately 15-fold lower potency than CatSper (58). It is unclear if this approach would be more appropriate for female use since it impacts progesterone function in the oviduct.

ADCY10, a major enzyme that generates cAMP in sperm, was discovered in 1999 (59). ADCY10 is the only soluble adenylyl cyclase among adenylyl cyclase family proteins of ADCY1-10. ADCY10 knockout mice have a severe defect in sperm motility and are infertile (60). Tissue analysis and subsequent tissue enrichment profile showed ADCY10 is expressed in many nonreproductive tissues (www.proteinatlas.org). More recent whole exome sequencing of 2 infertile men showed that their condition of asthenozoospermia, a condition that affects progressive motility of sperm, was due to homozygous variant upstream of nucleotide binding site of ADCY10 leading to premature termination (61). These men lead normal lives except for infertility phenotype and the potential for developing calcium oxalate kidney stones. However, the association of ADCY10 with dominant absorptive hypercalciuria has not diminished the discovery and optimization effort for ADCY10 inhibitors as new nonhormonal contraceptives (62).

ADCY10 and a sperm Na^+/H^+-exchanger form a complex critical for sperm motility (63). *Nhe8*$^{-/-}$ male mice are infertile due to disruption in acrosome formation (64). The sperm Na^+/H^+-exchanger in human sperm is mainly localized to the principal piece of the tail, and the expression pattern suggests a role in regulation of sperm motility (65). The Na^+/K^+-ATPase (sodium pump) is important in sperm motility and capacitation (66). These ion channels are found in many tissues, but the α4-subunit of the Na^+/K^+-ATPase is sperm specific and appears necessary for sperm function. The α4-subunit knockout male mice are completely infertile (67). Known inhibitors for Na^+/K^+ pumps are cardenolide analogs which have been used clinically to treat congestive heart failure. Ouabain, a cardenolide analog, has higher affinity for Na^+/K^+-ATPase α4 (ATP1A4) isoform than other somatic forms (α1, α2, and α3) in both mice and humans.

Optimization using the ouabain scaffold as a starting point may yield derivatives with specificity for the α4-subunit (68, 69). Ouabain derivatives modified at the glycone (C3) and lactone (C17) domains show picomolar inhibition for the α4 isoform with an excellent selectivity profile against α1, α2, and α3. Decrease in sperm motility in vitro and in vivo has been demonstrated for new ouabain triazole analogues (70).

Several testis-specific serine/threonine kinases (TSSKs) are important for spermatogenesis and function. In the human kinome, TSSKs belong to a 5-member testis-specific serine/threonine kinase family: TSSK1, TSSK2, TSSK3, TSSK4 (also known as TSSK5) and TSSK6. Male double TSSK1/TSSK2 knockout mice display infertility (71, 72). Stable, enzymatically active recombinant human TSSK2 protein production represents a key achievement in progress towards targeting TSSKs in humans (73). Additionally, mutation screening in 494 men with azoospermia or severe oligozoospermia compared with 357 fertile controls indicate that single nucleotide polymorphisms of the TSSK2 gene are associated with idiopathic male infertility (74). High-throughput screening of TSSK2 assays have revealed potent inhibitors (<100 nanomolar) that show promise for targeting TSSKs with small molecule inhibitors (75, 76).

The homeodomain-interacting protein kinase 4 (HIPK4) plays a role in later stages of sperm maturation and is another potential nonhormonal target (77). Studies have demonstrated that HIPK4 is expressed predominantly in round and early elongating spermatids. HIPK4 mutation in sperm display reduced oocyte binding and incompetence for in vitro fertilization (77). HIPK4 knockout mice are infertile but are otherwise healthy (78).

Sperm surface protein EPPIN (epididymal peptidase inhibitor) is another potentially druggable contraceptive target. This protein is expressed only in male reproductive tissues, testis, and epididymis (www.citdbase.org). An early study of immunization of male nonhuman primates with human EPPIN resulted in high anti-EPPIN antibody titer leading to infertility (79). Another preclinical nonhuman primate study showed that intravenous infusion of a small molecule inhibitor of EPPIN, EP055, resulted in dramatic reduction of sperm motility to ~20% of pretreatment levels. EP055 is thought to affect sperm motility by causing rapid decrease in sperm internal pH and calcium levels (80).

Another path for contraceptive discovery is via inhibition of serine protease from ejaculate, which disrupts sperm motility to reduce fertility. As such, panserine protease inhibitor, 4-(2-aminoethyl) benzenesulfonyl fluoride, inhibits semen liquefaction in vivo and drastically reduces the number of sperm in the oviduct, demonstrating inhibition of sperm transportation. Female mice treated with 4-(2-aminoethyl) benzenesulfonyl fluoride demonstrated lower fertilization rates and had significantly fewer pups per litter (81). Analysis of the semen liquefaction mechanism identified kallikrein-related peptidase 3 as another potential nonhormonal contraceptive target (82).

Numerous protein targets that affect sperm function have been identified. Several members of A Disintegrin And Metalloproteinases (ADAMs) family of proteins are expressed exclusively or predominately in testes or epididymis (83). Additionally, related members of ADAMs with Thrombospondin motifs (ADAMTS) are proposed to participate in sperm-egg adhesion (84). An ADAMTS-like protein from sea urchin is proposed to mediate species-specific sperm–egg adhesion (85). A systematic study to identify sperm membrane alloantigens in swine found more than 20 potential unique sperm membrane and 5 sperm raft proteins. Among these, ADAM1, 2, and 3 were dominant sperm membrane alloantigens (86). In ADAM3 knockout mice, sperm were unable to enter the oviduct (87). However, it is unclear if human sperm have the same requirement for ADAM3. Numerous ADAM proteins form complexes that are required for sperm–egg binding (83). Another potential candidate, Izumo1, a sperm surface protein that binds to JUNO (Izumo1R) on the egg, is required for sperm–egg fusion (88).

The absolute requirement for sexual reproduction is the process of sperm–egg recognition and subsequent fusion of the 2 gametes during fertilization. This requires fusion of gamete membranes; however, prior to this event, protein–protein recognition and interaction between egg and sperm must occur. A milestone in sperm–egg interaction was achieved when the cocrystal complex of the sperm surface protein IZUMO1 and the egg protein JUNO(IZOMO1R) was solved (88, 89). Knockdown of either protein results in infertile but otherwise healthy mice. The 2 studies provide structural insight into sperm–egg interaction at a molecular level. Although the crystal structure and other studies reveal that IZUMO1–JUNO interaction could be responsible for sperm–oocyte membrane adhesion, underlying membrane fusion steps are more complex. Studies have shown that sperm proteins, SOF1, TMEM95 (90), SPACA6 (91), and FIMP (92), are required for sperm–oocyte fusion in mice (93). Mouse knockouts of these proteins are completely infertile or severely subfertile. Discovery of proteins like CRISP2, found in male reproductive tract (94) and GLIPR1L1, an IZUMO binding protein (95), shed more light on our understanding of sperm proteins and the complexity of sperm-oocyte fusion and fertilization.

A monoclonal antibody (mAb) technology platform used against 2 sexually transmitted pathogens, HIV1 and HSV2, is being developed as an antisperm mAb (96). The antisperm antibody, H6-3C4, used in this platform was originally isolated from the blood of an infertile

woman. The antibody recognizes a carbohydrate epitope on a glycosylphosphatidylinositol-anchored glycoprotein, CD52g, found in abundance on the surface of human sperm (97). This contraceptive mAb is currently waiting for regulatory approval for a phase I clinical trial (96). A different technology upstream in the same thread but using the host to make and deliver the antibody is another attractive contraceptive model. Synthetic mRNA technology, such as recently used in the COVID-19 vaccine development effort, has been utilized to express PGT121, a well-established HIV-neutralizing antibody. In the HIV antibody work, inoculation of the female sheep reproductive tract with synthetic mRNA results in high levels of antibody expression (98). This new paradigm of antibody delivery to the female reproductive tract causing significant antibody production may be applied to contraceptive development. This may be facilitated by the recent synthesis of mRNA encoding antisperm mAb (96).

Gossypol is a polyphenolic aldehyde–containing compound isolated from the cotton plant (99, 100). Gossypol was implicated in causing infertility in the 1950s and 1960s when peasants in rural areas of China began to press uncooked cotton seeds for cooking oil. Women and men who consumed the raw untreated oil became infertile. Subsequently, a gossypol-free diet resulted in eventual recovery for women. However, some men did not recover from their infertility and impotency. This indicated that quantity and duration of cotton oil consumption impacted the rate of recovery, likelihood of permanent infertility, and led to the idea that gossypol could be used as a male contraceptive (99). Clinical studies of gossypol for male contraception were initiated in China in the 1970s and 1980s. In total, more than 8000 volunteers participated in these studies. Gossypol was highly efficacious as a male contraceptive; however, the narrow therapeutic window and frequent association with hypokalemia and irreversible sterility caused termination of further clinical development (101, 102). A more recent animal study in ewes investigating consumption of a gossypol-rich diet during the critical period of fetal development and early neonatal life in offspring showed significant arrest of growth and testis weight. Reduced testosterone levels and a significantly altered testis transcriptome were seen in male offspring. Many of these altered testis transcriptomes are implicated in testis development and sperm biology (103).

Extract from *Trypterigium wilfordii* Hook. f., commonly called Thunder God Vine, has been used in Chinese herbal medicine for many years. For more than 50 years, the refined extract was used to treat rheumatoid arthritis, chronic nephritis, chronic hepatitis, and various skin disorders (104, 105). Triptolide, a major component of the extract, belongs to the class of chemicals called diterpene epoxides (106). Indication of potential contraceptive potential for triptolide was discovered when rheumatoid arthritis patients treated with the extract developed necrospermia or azoospermia (105, 107, 108). Subsequent studies in rats showed that *T. wilfordii* extracts containing diterpene epoxides cause severe decrease in epididymal sperm count and motility in male rats (104, 105, 108-112). Similar to gossypol, prolonged exposure was associated with irreversible infertility in rodents (110, 112). Unfortunately, triptolide's immunosuppressive properties likely would prevent it from being developed as a contraceptive.

A commonly used plant in Jamu preparation, an Indonesian traditional medicine, *Justicia gendarussa* has been used as a male contraceptive in Papua. Additionally, this plant is used to treat a variety of ailments including arthritis, cancer, as an anti-inflammatory, antibacterial, and antifungal. In particular, gendarusin A and B, flavonoid scaffold analogs, are thought to be active metabolites responsible for eliciting the contraceptive effect, possibly by decreasing human sperm hyaluronidase activity (113). An unpublished clinical trial performed in Indonesia reported contraceptive efficacy of extract of the *J. gendarussa* plant if ingested by the male partner daily for at least 20 days before having intercourse during the female's ovulatory period (114). Fertility was restored within 30 days after last usage and minimal side effects were reported. Additional study on its mechanism of action and evaluation of longer duration of use would be required prior to regulatory approval.

Focusing on a local versus systemic approach, development of a nonhormonal method to reversibly block the vas deferens began in the 1970s in India. The procedure, called RISUG (reversible inhibition of sperm under guidance), involves injection of the polymer styrene maleic anhydride mixed with the solvent dimethyl sulfoxide into the vas deferens (115). The polymer is thought to damage sperm, making them ineffective. The procedure was used in the first human in 1989. By 2000, RISUG was evaluated in a phase III clinical trial in India with promising results. However, an inspection of the Indian facilities by World Health Organization raised concerns that studies were not done according to international standards, and further development was curtailed. Intellectual property rights to RISUG were acquired by the Parsemus Foundation, a nongovernmental organization, in 2010, who then developed Vasalgel™, also a styrene maleic anhydride acid polymer dissolved in dimethyl sulfoxide. Vasalgel™ is purported to act as a mechanical barrier to sperm passage. It is thought that sperm flow can be restored by flushing the vas with an injection of sodium bicarbonate solution. Studies in rabbits and monkeys have been completed (116, 117). Similar technology was used in China in the mid-1980s,

in which a polyurethane elastomer plug was injected into the vas deferens to achieve azoospermia in 96% of men, but these results were seen 24 months after injection (118, 119). Reversibility and return to fertility was demonstrated through surgical removal of the plug (120). A small company, Contraline, is developing a hydrogel, ADAM™, for injection into the vas deferens to block the flow of sperm but not other fluids (121). The gel ideally could be dissolved to restore fertility.

The Parsemus Foundation also has supported development of the "clean sheets" pill that allows for orgasm without ejaculation. They developed initial drug prototypes based on side effect profiles of 2 therapeutic drugs, thioridazine and phenoxybenzamine, which inhibit semen emission without affecting erection or orgasm. Further optimization of the prototypes is on hold pending a funding source.

A contraceptive infertility target database was established in 2018 as a tool to identify male and female reproductive tissue specific transcriptome and proteome targets. This database, Contraceptive Infertility Target Data Base (CITDBase: https://www.citdbase.org), is a curation of publicly available transcriptomic, proteomic, and immunohistochemistry (antibody-Ab) data from human tissues. Filters are applied for adjusting the degree of separation between reproductive and nonreproductive tissues in mining of gene/protein targets. This website allows investigators to mine transcriptomic and proteomic resources to identify high quality contraceptive/infertility targets.

Conclusion

Although some nonhormonal inhibitors and natural products have been evaluated in humans, nonhormonal male contraceptives are in early stages of development. In addition to protein targets and small molecule inhibitors described above, active research is ongoing for nonhormonal contraceptive target discovery and validation. Numerous laboratories are engaged in discovery and optimization of small molecule inhibitors. It is hoped that some of these small molecule contraceptive agents would enter preclinical development and advance into clinical development.

Introduction of an effective reversible male contraceptive method has potential to substantially reduce unplanned pregnancy rates. It likely would represent a new market opportunity rather than creating a significant reduction in the use of existing female contraceptive methods and would provide an opportunity for men to better engage in reproductive decision making. How a possible risk to 1 individual may be mitigated by prevention of potential health consequences in another individual provides an interesting regulatory consideration for evaluation of systemic male contraceptive agents. At the current pace of drug development, regulatory approval for a new male product in the United States likely would not occur until at least 2030. This timeline potentially could be shortened with increased resources and investment into the development pipeline.

Additional Information

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REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY

RESEARCH Open Access

A unique view on male infertility around the globe

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Abstract

Background: Infertility affects an estimated 15% of couples globally, amounting to 48.5 million couples. Males are found to be solely responsible for 20-30% of infertility cases and contribute to 50% of cases overall. However, this number does not accurately represent all regions of the world. Indeed, on a global level, there is a lack of accurate statistics on rates of male infertility. Our report examines major regions of the world and reports rates of male infertility based on data on female infertility.

Methods: Our search consisted of systematic reviews, meta-analyses, and population-based studies by searching the terms "epidemiology, male infertility, and prevalence." We identified 16 articles for detailed study. We typically used the assumption that 50% of all cases of infertility are due to female factors alone, 20-30% are due to male factors alone, and the remaining 20-30% are due to a combination of male and female factors. Therefore, in regions of the world where male factor or rates of male infertility were not reported, we used this assumption to calculate general rates of male factor infertility.

Results: Our calculated data showed that the distribution of infertility due to male factor ranged from 20% to 70% and that the percentage of infertile men ranged from 2·5% to 12%. Infertility rates were highest in Africa and Central/Eastern Europe. Additionally, according to a variety of sources, rates of male infertility in North America, Australia, and Central and Eastern Europe varied from 4 5-6%, 9%, and 8-12%, respectively.

Conclusion: This study demonstrates a novel and unique way to calculate the distribution of male infertility around the world. According to our results, at least 30 million men worldwide are infertile with the highest rates in Africa and Eastern Europe. Results indicate further research is needed regarding etiology and treatment, reduce stigma & cultural barriers, and establish a more precise calculation.

Keywords: Male infertility, Global health, Fecundity, Worldwide

Background

Infertility is a worldwide problem, and according to Sharlip et al, it affects 15% of couples that have unprotected intercourse [1]. Although this statistic is commonly cited, it is an amalgamation of numbers taken from around the world and thus does not reflect rates in specific countries and regions. On a global scale, accurate information regarding rates of male infertility is acutely lacking, and has not been accurately reported.

Calculating regionally based male infertility rates is challenging for a number of reasons. First, population surveys generally interview couples or female partners of a couple who have unprotected intercourse and wish to have children. This is a very specific population. As such, data from a significant number of infertile individuals is never included, which may bias the data.

Second, unlike female infertility, male infertility is not well reported in general but especially in countries where cultural differences and patriarchal societies may prevent accurate statistics from being collected and compiled. For example, in Northern Africa and Middle East, the female partner is often blamed for infertility. Men, therefore, do not usually agree to undergo fertility evaluation, resulting in underreporting of male infertility. Furthermore, polygamy is a common practice in many cultures [2]. One of the reasons for polygamy is to overcome infertility and increase the probability of having children. Additionally, in some African countries, the tradition of "Chiramu" allows an infertile male to bring in a brother or a relative to impregnate his wife [2]. In this way, the man retains his masculine identity and status in his community's eyes.

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A third challenge stems from the fact that male infertility has never been defined as a disease, which has resulted in sparse statistics. Additionally, demographic and clinical studies vary in epidemiological definition of infertility. While many clinical studies have examined infertility over the course of a year, several demographic studies examine infertility over a five-year projection [3]. Finally, while some studies only examine females, others only examine the men presenting to infertility clinics, which are generally small groups who are not representative of the larger population of infertile men.

Without accurate, region-specific data, it is not possible to identify and comprehensively treat infertile men. Therefore, to bridge this gap in knowledge, we have consolidated current data and, where recent information is lacking, estimated rates of male infertility using pre-existing data on female infertility in areas around the world. We focused especially on North America, Latin America and the Caribbean, North Africa and the Middle East, Sub-Saharan Africa, Europe, Eastern Europe, Central Asia, Eastern Asia, the Pacific, and Australia. The developing world has much less data available, which is why the above regions were selected.

Therefore, the goal of this commentary is to consolidate the large breadth of information available on male infertility and provide answers to the following two questions: How does the rate of male infertility vary in the different regions across the world? Can accurate estimates of male infertility be captured globally while identifying potential socio-economic and cultural reporting barriers that skew the results?

Methods

We limited our literature search to include only systematic reviews and meta-analyses (where possible) of mainly population-based studies. For factors that did not generate a result for meta-analysis or systematic review using the Boolean terms: "factor" AND systematic review" or "factor" AND meta-analysis,", a search was done using that particular factor, e.g. "factor" AND male infertility" to elicit an original study that looked into the effect of that factor on any aspect of male infertility. We searched PubMed, Web of Knowledge, MEDLINE, EBSCOhost and Google Scholar using the following keywords: epidemiology, male infertility, and prevalence (Figure 1).

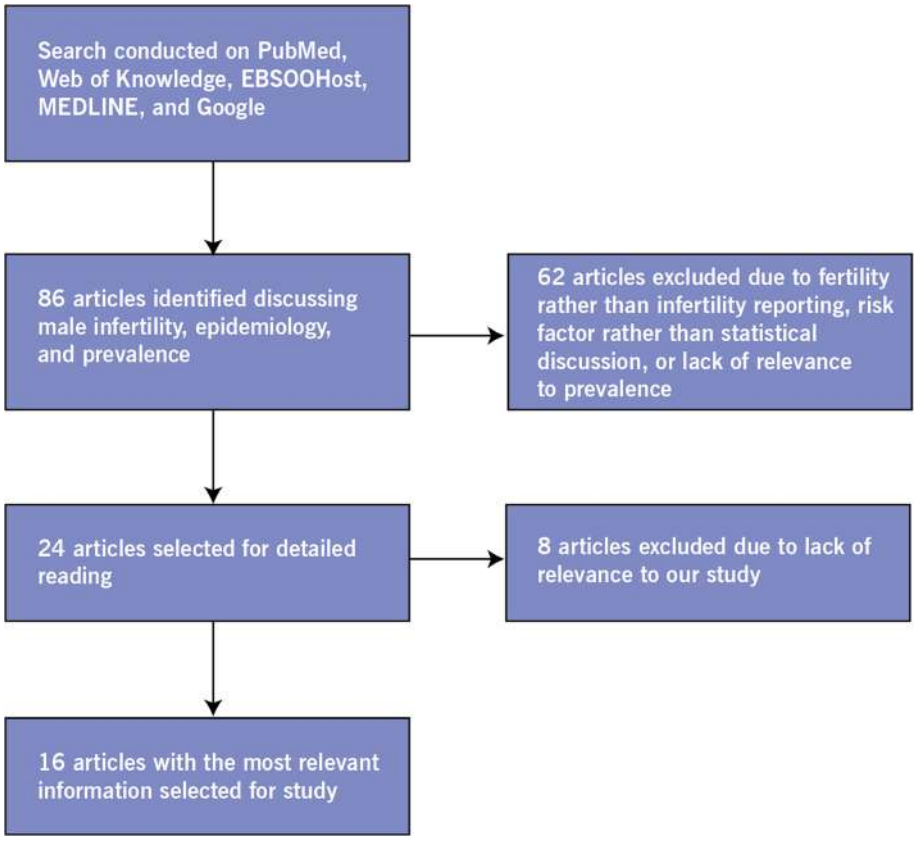

Figure 1 Flow chart demonstrating methodology. This figure is a representation of our methods, including number of articles found and filtered, and inclusion and exclusion criteria for the final article selection.

We initially identified 86 relevant articles in our multiple searches. Of those, 62 articles were excluded due to reporting of fertility rather than infertility, discussion of risk factors rather than statistics regarding overarching infertility, or lack of information regarding prevalence of infertility. From the remaining 24 articles, we further shortened the list to 16 articles that contained the most relevant information for our study. Most of these articles looked at a one-year definition of infertility. Mascarenhaas and colleagues used a 5-year demographic definition of male infertility in their study [3]. Our report uses the definition of infertility pertaining to one year. However, we report the numbers used by Mascarenhas and colleagues as well.

Statistical analysis

It has been stated that 48.5 million couples that have unprotected intercourse suffer from infertility worldwide [4]. However, this statistic does not clearly define infertility by geographic region. Additionally, many clinical studies do not begin to explore infertility until a couple attempts to get pregnant for at least one year according to the World Health Organization (WHO). Demographic studies, on the other hand, look at infertility over a five-year projection [3]. In general, according to Sharlip, 50% of infertility cases are due to a solely female factor, pure male factor accounts for 20-30% of the problem, and the remaining 20-30% is due to a combination of both male and female factors [1]. We used the "Sharlip factor" as a basis for calculations because it was the most widely cited and reported statistic regarding male infertility. Further, a more accurate statistic is as of yet, unavailable. Therefore we used the same parameters to calculate the statistics found in this report. In regions where the prevalence of male infertility was not reported, we calculated male infertility statistics utilizing female infertility rates. This statistic was calculated by using the reported rate of infertility in that region. We applied Sharlip's estimate that approximately 20-30% of the total infertile couple population could be attributed to male infertility [1]. We calculated percentages, such as each region's total infertile male population. Using a combination of these two numbers, we were able to calculate an estimated number of infertile men. To further explain how our numbers were calculated, we provide an example. Data was taken from the WHO regarding infertility rates as reported by female partners in regions of the world. In Sub-Saharan Africa, 14.2% of women reported infertility. From this, we assumed with couples infertility at 14.2%, then female factor infertility would be 7.1%. Since the other 50% is assumed to be a combination of male factor and combined factor infertility, we calculated 20-30% of 7.1% to arrive at solely male factor infertility and 40-55% of 7.1% to arrive at any situation when the male factor is involved in any way.

Results

The calculated global data shows that the percent of infertility that is attributable to males ranged between 20-70% (Table 1). Additionally, the percentage of infertile males in these countries varied from 2.5-12% (Table 1). The largest pockets of male infertility occurred in Central and Eastern Europe (8% to 12%) and Australia (8% to 9%). North America demonstrates rates of male infertility 4.5-6% [4]. While a calculated percentage reveals 4.5-6% of North American males are infertile, the Centers for Disease Control (CDC) estimates that 9.4% of males in the United States are infertile (Table 1) [4]. Sub-Saharan Africa is typically thought to have high rates of infertility; however, possibly due to underreporting, the rates shown in Table 1 appear low. The CDC and the WHO do not use the Sharlip calculation when reporting their data. Rather, their rates are based upon in-person interviews of representative populations, whereas Sharlip's

Table 1 This table shows male infertility, based on various studies reporting male or female infertility globally

	Males that are reported infertile	Couples that are reported infertile	Couples in which the male factor is one of multiple factors involved
North America	4.5-6%[a]	15%	50%
Middle East	Unknown	Unknown	60%-70%[b] [23]
Sub-Saharan Africa	2.5%-4.8%[a]	12.5%-16% [22]	20-40% [22]
Europe	7.5%[a] [11]	15% [11]	50% of all infertile couples
Australia	8%; 9%[b] [10]	15%	40% [17]
Central/Eastern Europe	8%-12% [6,14]	20% [14]	56% [6]
Asia	Unknown	Unknown	37% [19]
Latin America	Unknown	Unknown	52% [19]
Africa	Unknown	Unknown	43% [19]

[a]Percentages were calculated from data reported on female infertility, using the assumption that 50% of infertility cases are due to females only, and 20-30% are due to male factor only.
[b]Study states that 60-70% of all men presenting to IVF clinics in the Middle East have some involvement in the cause of infertility.

data is based upon previously reported data from a classic French study. What makes our data novel is the fact that we use the data that is representative of the population examined. For example, when calculating current infertility rates in North America, we use information from the CDC, which is representative of the North American population. However, this same data is not representative of the Sub-Saharan population. We therefore used female data from the WHO combined with the representative rates reported by Sharlip to calculate male infertility in those regions.

Table 2 takes data from a WHO study conducted from 1994-2000. North and West Africa had the highest rates of infertility, which ranged from 4.24%-6.35%. Central and East Asia had the lowest rates of infertility, with 2·05%-3.07% of infertility cases due to male factor alone (Table 2). Cases of infertility due to both male and female factors ranged from 2.84% in Sub-Saharan Africa to 11.65% in Northern and Western Africa.

Table 3 shows infertility data in terms of regional populations. While most of our data was reported as percentages, we converted them into population absolute numbers in order to gain a broader understanding and perhaps more accurate estimate of the number of infertile men. The number of infertile men ranged from 5,000 to 18,000,000 with a worldwide estimate of 30,625,864 to 30,641,262 men who may be infertile. The highest number of infertile men was concentrated in Europe. According to this table, in any given region, at least 5,459 men may be infertile (Table 3).

Table 4 extrapolates male data from pre-existing female data reported in a systematic analysis conducted by the WHO. The rates of primary infertility, as reported by women, ranged from 1.5% to 2.6%, which were much lower than those reported over the course of 12 or more months. The male contribution to these rates of infertility ranged from 0.4% to 1.82% according to WHO estimates. Secondary infertility reported by women ranged from 7.2% to 18%, with the highest rates in Central and Eastern Europe, followed by South Asia at 12.2% and Sub-Saharan Africa at 11.65%. This data consolidated information between 1990 and 2010, providing a 5-year projection of infertility [3]. According to this data, the highest rates of infertility were concentrated through Africa and Central/Eastern Europe [3].

Table 5 shows male infertility data (reported in earlier studies) from France, Western Siberia, Nigeria, Mongolia, Poland/Eastern Europe, Egypt, Iran, and Sudan [5-9]. We found that 6.4% to 42.4% of infertility cases in these areas were due to a male factor.

Discussion

Global

Male infertility is a global population health concern. There are an estimated 48·5 million couples with infertility worldwide [3]. In the current study, we calculated rates of male infertility across the globe based on a review of the current literature (Figure 2). Since we do not know the actual rates of infertility, most of the numbers shown are based on self-report, thus cover a wide range. Overall, by examining the available literature and consolidating the information, our data indicates that global rates of male infertility range from 2.5% to 12%.

North America and Europe

North America, Europe and Australia are developed countries, which may explain why rates of infertility are reportedly believed to be more accurate when compared to less developed countries. In North America, 4·5%-6% of males are infertile (Table 1). This number is similar to that of Australia, where 8% of males are infertile and 9% of males over the age of 40 have visited an infertility clinic at some point (Table 1) and Europe, where 7.5% of males are infertile. These numbers are based upon data from the National Health Statistics Report (NHSR) from the CDC National Health Interview Survey, the Australian Institute for Health and Welfare (AIHW), and the European Association of Urology (EAU) guidelines for male infertility [4,10,11]. These three regions of the world were the only organizations with the most accurate reporting of data available. The estimation that 20-30% of infertility is due to a sole male factor helped calculate

Table 2 Calculated data taken from the WHO regarding infertile women, extrapolated to men, globally ranging from 1994-2000 [9]

	Total Infertility Rate as reported by female partners (women who have had sexual intercourse but no pregnancy, ages 15-49)	Male factor only (20-30%); Female factor not involved	Male factor Involved (40-55%)	Female factor involved (50%)
Sub-Saharan Africa	14.20%[a] [18]	2.84%-4.26%[b,c]	2.84%-5.68% [19]	7.1%
Central/East Asia	10.23%[a] [18]	2.05%-3.07%[b]	3.79% [19]	5.1%
North/West Africa	21.18%[a] [18]	4.24%-6.35%[b]	8.47-11.65% [19]	10.6%
Latin America/Caribbean	13.70%[a] [18]	2.74%-4.11%[b]	7.12% [19]	6.85%

[a]Female data reported by country; we used the mean of these countries' data to define the region's average reported infertility.
[b]Male data calculated based on the argument that while 50% of infertility is due to females, 20-30% is due to males. Specific male infertility rates for these regions are not well reported.
[c]Data calculated from a different source than Sub-Saharan Africa calculations in Table 1.

Table 3 This shows infertility reported as gross numbers, using global population estimates

	Total population of Region	Male population of Region	Total Male reproductive population (15-60y)	Male Infertility, %	Infertile Men
North America	347,388,982	171,213,918 (49.2%)	116,254,250[a]	9.4% [4]	10,927,899
Latin America/Caribbean	582,418	287634 (49.3%)	361,099	Unknown	Unable to Calculate
Sub-Saharan Africa	850,000	420,000 (49.4%)	218,348	2.5%-4.8%	5,459-10,481
Eastern Europe/Central Asia	399,110	190,718 (47.8%)	259,421	8-12% [14]	20,754-31,130
Europe	734,228,972	353,542,772 (48.1%)	248,187,025[a]	7.5% [11]	18,614,027
Asia/Pacific	3,653,257	1,875,094 (51.3%)	1,199,437	Unknown	Unable to Calculate
Oceania	35,162,670	17,699,546 (50.3%)	11,752,499[a]	9% [10]	1,057,725

Explanation of calculations:
These numbers are crude estimations and calculations. They were calculated from two sources: UNFPA Country Profiles and World Stat. Both sources provided total population, male population, and population less than 15 years and greater than either 60 or 65 years. The calculations were performed as follows. For example, in Sub-Saharan Africa, the total population amounts to 850,000, according to UNFPA. The male population was 420,000. This amounts to 49.4% of the total population. The population less than 15 years old was 43% of the total population, and the population greater than 60 years old was 5% of the total population. These percentages convert to 365,500 and 42,500, respectively. After calculating that 49.4% of the total population is males, we also assumed that 49.4% of the total population between the ages of 15 and 60 were also males. Therefore, [850,000 – (365,500 + 42,500)] × 49.4% = 218,348. This is the total male reproductive population between the ages of 15 and 60. This number was multiplied by the percentage of male infertility prevalent in this population. 218,348 × (2.5% to 4.8%) = 5,459 to 10,481 infertile males present in Sub-Saharan Africa.
Unfortunately, some statistics were unable to calculate, due to the lack of reporting on these regions of the world.
[a]Population reported as Male, age 15-64.

numbers in the developing world, providing the most conclusive report of male infertility around the world. Quantifying the available information gives us insight into where the greatest need is for further research into underlying etiology and treatment.

When comparing regions with another, Europe reports similar population estimates as the United States, with 15% of European couples and 7.5% of men reported infertile [11]. Olsen and colleagues found that infertility varied across Europe. After 12 months, 51.1%, 43.2%, 37.9%, 19.1%, and 43.2% of couples sought help for infertility in Denmark, Germany, Italy, Poland, and Spain, respectively, with approximately 40% seeking help across the sample [12]. A classical French study completed by Thonneau and colleagues in 1991 examined 1686 infertile couples and found that in a small region of France to find that abnormal infertility was present in males 20% of the time, and present in females 34% of the time, and in both males and females 38% of the time [13]. This region is different from the whole of Europe, and statistics are sparse. However, Sanocka and colleagues state that Poland's population is considered representative of Eastern Europe [14]. That study stated that 20% of couples are infertile in Poland, and 40-60% of those couples' cases are due to male factor alone, whereas a more recent study by Bablok and colleagues states that 56% of infertility cases are due to an involved male factor [6,14]. The most interesting part of our manuscript references the fact that all these numbers reported are so different. We conclude that the large varieties in these numbers are largely due to cultural differences. In the United States and Europe, infertility is a problem that men often feel comfortable addressing with their physician. This allows the problem to both get addressed and reported statistically.

Table 4 A 5 year extrapolation as reported by a Systematic Analysis of 277 Health Surveys on Female infertility [11]

	Primary infertility		Secondary infertility	
	Total 5 year infertility rate as reported by females[a,b]	5 year Male factor infertility rate (calculated)	Total 5 year infertility rate as reported by females[a,b]	5 year Male factor infertility rate (calculated)
Latin America	1.5% [3]	0.78%[c] [19]	7.5%	3.9%[c] [19]
North Africa/Middle East	2.6% [3]	1.56-1.82%[c] [23]	7.2%	4.32-5.04%[c] [23]
Sub-Saharan Africa	2% [3]	0.4-0.8%[c] [22]	11.6%	2.32-4.4%[c] [22]
Central/Eastern Europe	2.2% [3]	1.23%[c] [6]	18%	10.03%[c] [6]
South Asia	2.2% [3]	0.81%[c] [19]	12.2%	4.51%[c] [19]
East Asia/Pacific	1.5% [3]	0.56%[c] [19]	11%	4.07%[c] [19]
World	1.9% [3]	0.38-0.57%[d]	10.5%	2.1-3.15%[d]

[a]Percentage of child-seeking women.
[b]Measured in 2010.
[c]Male data was calculated based on the various reported rates of male factor contribution to infertility cases in multiple studies (cited above).
[d]Male data for world was calculated based on the argument that while 50% of infertility is due to females, 20-30% is due to males.

Table 5 Infertility around the world[a], [12] reported from previous studies examining male infertility to summarize previous research

	Population	Author, year	Female factor	Male factor	Combination
French Regions (1988-1989)	1686 Couples	Thonneau et al. 1991 [13]	30%	20%	39%
Western Siberia	2000 Married women; 186 couples	Philippov et al. 1998 [27]	52.70%	6.40%	38.70%
Southeastern Nigeria	314 couples	Ikechebelu et al. 2003 [19]	25.80%	42.40%	20.70%
Mongolia	430 Couples	Bayasgalan et al. 2004 [28]	45.80%	25.60%	18.80%
Poland/Eastern Europe	Unreported	Sanocka and Kurpisz 2003 [14]; Bablok et al. 2011 [6]	Unreported	40-60%[b] [14]; 56% [6]	Unreported
Egypt	190 Women	Inhorn, Buss 1994 [7]	82%	13%[c]; 46%[d] [7]	Unreported
Yazd Province of Iran	5200 Couples	Aflatoonian et al. 2009 [8]	57.5%	25.3% [8]	8%
Sudan	710 couples	Elussein et al. 2008 [9]	49.3%	36.2% [9]	Unreported

[a]Table has been adapted from Winters and Walsh [12].
[b]This number was from Sanocka et al., which stated that 20% of couples are infertile, and 40-60% of those cases are due to male factor infertility [6]. This calculation amounts to 8-12% of men overall are the reason for these infertility cases.
[c]In Inhorn and Buss, in 11/87 (13%) of evaluated cases, male factor infertility was the sole cause of infertility [13].
[d]In Inhorn and Buss, in 40/87 (46%) of cases, male factor was involved [13].

Australia

We see that Australia's rates are similar to those in North America and the United States, at 8-9%; additionally, 40% of infertility cases in Australia are due to male factor involvement (Tables 1 and 3; Figure 1) [10,15]. While the Australian Institute for Health and Welfares (AIHW) statistics data is on males aged 40 and older, the AIHW states that 8% of males have reported trying to have children unsuccessfully and 9% are being evaluated for infertility [10].

Africa and the infertility belt

The rates in North Africa, Sub-Saharan Africa, and Eastern Europe are close to some of the higher percentages of male infertility estimated worldwide (Table 2) [16]. Male factor involvement for Table 2 was calculated using the statistics

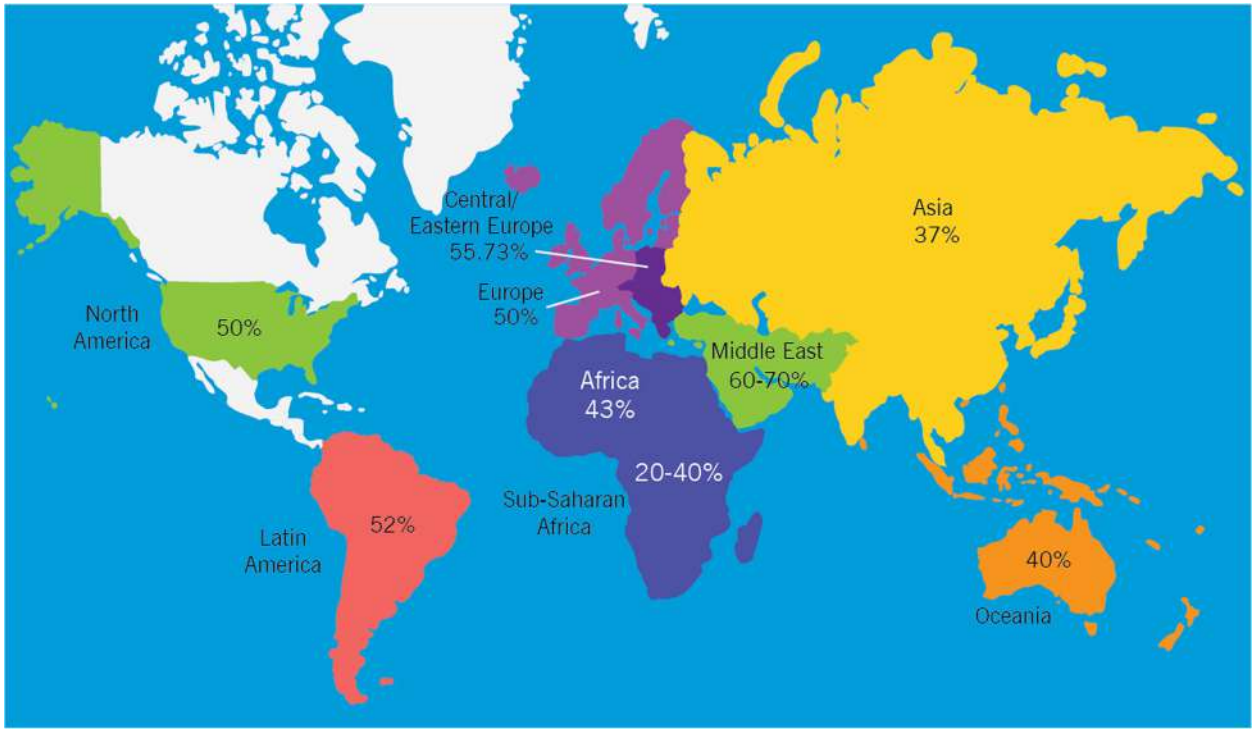

Figure 2 World map containing percentages of infertility cases per region that are due to male factor. This figure demonstrates rates of infertility cases in each region studied (North America, Latin America, Africa, Europe, Central/Eastern Europe, Middle East, Asia, and Oceania) due to male factor involvement.

found by Cates, Farley, and Rowe in 1985 [17]. With the discovery that male infertility is most prevalent throughout this region, this may be where marketing for assisted reproductive therapy, treatment for infection, and efforts for WHO research can be concentrated.

The highest numbers relate to a region known as the "African Infertility Belt," which stretches east to west across central Africa from Gabon to the United Republic of Tanzania [18]. This region of the world has very high rates of infertility in women, and as men are involved in up to 43% of the problem, the argument follows that male infertility is also high in this region [17,19,20]. Male factor contribution to infertility is also extremely high in the close geographical region of the Middle East [21]. We also noticed that primary infertility rates were much lower than secondary infertility. This may result from the high amount of child marriage and young pregnancy occurring in developing countries, and the later development of sexually transmitted diseases (STDs) and pelvic infections [22]. However, these numbers are of questionable significance due to the scant nature of their collection. Additionally, the population of subsaharan Africa grows yearly. This does not imply that the rates of male infertility may not be high, but rather that the population may be growing in other ways. Typically, in regions of Africa and other societies, the male is seen as the dominant individual in both the community and the family structure. Therefore, men, especially in Africa and the Middle East do not report their infertility, as they believe it is emasculating to be unable to impregnate a woman. As a result of this, the men in these societies especially tend to blame females for the lack of child and do not get help.

Other diseases

The "African Infertility Belt" also has high rates of STDs such as N. gonnorrhoeae and C. trachomatis, which may have some correlation and relationship with the high rates of infertility in this region of the world [23]. Collet and co-workers discovered that a tubal factor was present in 82.8% of females presenting to infertility clinics and frequently positive endocervical cultures for N. gonorrhoeae and C. trachomatis [23].

Total absolute numbers calculated

We have drawn on the arguments that approximately 50% of cases are due to women, and 20-30% of cases are due to men. The remaining 20-30% of infertility cases is due to a combination of male and female factors. In Table 1, multiple reports state an infertility rate of anywhere from 2.5% to 12% [6,10,11,14,20,21]. Total numbers of infertile men worldwide may amount from 30,625,864 to 30,641,262 (Table 3). This number does not include estimates from Latin America or Asia (the most populous continent on the planet), due to underreporting there. These numbers indicate over 30 million more men and their female counterparts who could benefit from assisted reproductive technology (ART) and treatment for infertility. Additionally, regardless of the lower rates of infertility in North America, Europe, and Australia, these regions should not be neglected in the research for future treatment options. These regions also make up a part of the worldwide infertility phenomenon. While there may be regions of Africa and Asia attracting more urgent attention, this same consideration should be extended globally.

Updated WHO guidelines

In 2010, the WHO changed their guidelines for semen analysis for the diagnosis of the infertile male [24]. In doing so, they established reference values that were much lower than their previous ones, resulting in more men qualifying as "normal" [6]. Now, a man with reference values of greater than 15 million sperm, greater than 5% normal morphology, and 40% progressive motility would be considered normal. [25] With the new guidelines, more men would be considered fertile, while there may be an unnoticed rise in the number of infertile men. Therefore, a recent study involving our group advises caution when interpreting the new WHO reference values because they have not yet been accurately defined to discriminate fertile from infertile men [25].

Limitations of our study

One major limitation of our study is the number of infertile couples who have never participated in intercourse. Following this limitation, we therefore cannot estimate the number of infertile men who have never participated in unprotected sexual intercourse. Additional limitations of any epidemiological study regarding infertility and sexual activity include that the quality of data varies from very poor to very good. Reproductive information is private and couples may not be inclined to be truthful in surveys [26]. Many men may not be willing to participate in semen studies [26]. Another limitation included the difference between one-year infertility rates and the five-year infertility rates reported by Mascarenhas et al. [3]. This difference in rates over a five-year projection may be due to the fact that over five years, the cases of infertility may either resolve, these couples may have found an alternative to traditional conception, or the study could have suffered from attrition. A major limitation of this study is that much of our data are based on WHO studies from the 1900's and that the definition of a male factor in these studies was not well defined. Male factor infertility was based on both abnormal semen analyses and on associated factors like varicoceles and urogenital infections, and STDs in men with normal semen analyses.

In countries with an accurate registration of diseases, the prevalence of both male infertility and male factor leading to couples' infertility is lower than that in developing countries. Rates from developing countries are more likely due to a problem with definition of male infertility and lack of accurate reporting rather than a true reflection of male infertility in those regions. Finally, the biggest limitation was that we based our calculations on Sharlip et al. and applied these numbers for female infertility to that of men.

Conclusions

According to our results, at least 30 million men worldwide are infertile with the highest rates in Africa and Eastern Europe. However, due to the varying credibility and older nature of many of the articles analyzed, it is quite difficult to make a definite conclusion on the nature of these infertility rates.

The main message of these findings is that male infertility is a global health issue that has not been researched or studied to truly understand its magnitude and prevalence. This information provides insight into where the greatest need is for further research into underlying etiology and treatment. The major recommendations of this manuscript are:

1. As a society, we must reduce barriers from stigmas associated with infertility due to religious and cultural beliefs.
2. We must create a globally accepted population-based calculation in order to understand the prevalence and magnitude of male infertility.
3. Much work is needed to raise awareness about male infertility.

With broad and accurate understanding, we can both treat infertility by managing underlying conditions.

Abbreviations

ART: Assisted reproductive technology; AIHW: Australian institute for health and welfare; CDC: Centers for disease control; EAU: European association of urology; NHSR: National health statistics report; STDs: Sexually transmitted diseases; WHO: World Health Organization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AA conceived the idea, supervised the study, and edited the article for submission. AM conducted the literature review, writing of the manuscript, data analysis and calculations, and prepared the article for submission. AH and MC helped with the reviewing and editing of the manuscript. All authors read and approved the final manuscript.

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Differences in Radiation Sensitivity of Recovery of Spermatogenesis Between Rat Strains

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Previous studies with Lewis/Brown-Norway (BN) F1 hybrid rats indicated that spermatogenesis was much more sensitive to ionizing radiation than in the widely studied outbred Sprague Dawley stock, suggesting that there were genetically based differences; however, the relative sensitivities of various inbred strains had not been established. As a first step to defining the genes responsible for these differences, we compared the sensitivities of seven rat strains to radiation damage of spermatogenesis. Recovery of spermatogenesis was examined 10 weeks after 5-Gy irradiation of seven strains (BN, Lewis, Long-Evans, Wistar Kyoto, spontaneously hypertensive [SHR], Fischer 344, and Sprague Dawley). The percentages of tubules containing differentiated cells and testicular sperm counts showed that BN and Lewis were most sensitive to radiation (< 2% of tubules recovered, $< 2 \times 10^5$ late spermatids per testis), Long-Evans, Wistar Kyoto, Fischer, and SHR were more resistant, and Sprague Dawley was the most resistant (98% of tubules recovered, 2×10^7 late spermatids per testis). Although increases in intratesticular testosterone levels and interstitial fluid volume after irradiation had been suggested as factors inhibiting recovery of spermatogenesis, neither appeared to correlate with the radiation sensitivity of spermatogenesis in these strains. In all strains, the atrophic tubules without differentiated germ cells nevertheless showed the presence of type A spermatogonia, indicating that their differentiation was blocked. Thus, we conclude that the differences in radiation sensitivity of recovery of spermatogenesis between rat strains of different genetic backgrounds can be accounted for by differences in the extent of the radiation-induced block of spermatogonial differentiation.

Key Words: **ionizing radiation; spermatogenesis; rat strains; spermatogonia.**

Identification and quantification of risks that particular toxicants will damage the human male reproductive system are based on results from animal model systems. Rodents have been the primary model system used in reproductive toxicology because they are small, inexpensive, and genetically well characterized. However, it is important for qualitative extrapolation to human that the mechanisms of the toxicity in the test species have the same characteristics as in the human. For quantitative extrapolation, it also is necessary to consider the doses to produce equivalent effects (Meistrich, 1992). Within a test species, the strain chosen is important because there may be quantitative differences in the response with different strains. Furthermore, there might be qualitative differences in mechanisms with different strains.

For many decades, the rat had been the primary rodent model used for reproductive toxicology. However, the mouse has been used increasingly in recent years because more genetic tools are available in this species to elucidate mechanisms, and there have been numerous studies of strain differences in effects of toxicants on spermatogenesis in mice (Bianchi *et al.*, 1985; Meistrich *et al.*, 1984; Spearow *et al.*, 1999). In contrast, there have been very few reports characterizing strain differences in sensitivities of various of rat strains (Delic *et al.*, 1987; Parchuri *et al.*, 1993; Sotomayor *et al.*, 1996), and some of these studies often included outbred rat stocks rather than inbred strains. However, the genetic knowledge and techniques in the rat are now progressing with the sequencing of the genome (Gibbs *et al.*, 2004), the existence of sets of recombinant inbred lines (Tabakoff *et al.*, 2009; Voigt *et al.*, 2008), and the ability to produce gene knockouts (Jacob *et al.*, 2010), so that studies of strain differences in rats have the potential to lead to discovery of gene function.

Previously, we reported that there were dramatic interstrain differences in the recovery of spermatogenesis in rat testes from the chemotherapy drug procarbazine (Parchuri *et al.*, 1993). Whereas spermatogenesis in most outbred Sprague Dawley rats was nearly completely resistant to prolonged effects of multiple injections of procarbazine on the testis, about 25% of the rats were quite sensitive to that treatment. In contrast, both Lewis and LBNF1 (F1 hybrids of Lewis and Brown-Norway [BN] inbred strains) were extremely sensitive to the same doses of procarbazine. With chemical treatment, it is not known whether the differences in sensitivity were due to

target organ sensitivities as opposed to differences in pharmacokinetics or systemic effects.

To more specifically examine differences in target organ sensitivities, we compared the data on radiation sensitivities of different strains. Radiation is highly toxic to the human testis and 4–6 Gy can produce total loss of sperm production for about 2 years (Clifton and Bremner, 1983).

The most sensitive targets for radiation damage to the testis are the proliferating differentiating spermatogonia (A_1–A_4, intermediate, and B spermatogonia) (Erickson, 1976). The loss of these cells results in a progressive depletion of differentiating germ cells (Dym and Clermont, 1970). The spermatogonial stem cells (undifferentiated type A) are more resistant and can survive moderate radiation doses, and, if the dose is not too high, they can eventually produce complete recovery of spermatogenesis in resistant strains (Dym and Clermont, 1970). However, at high doses or in sensitive strains, the recovery may be incomplete or permanent testicular atrophy may occur (Kangasniemi *et al*., 1996). For example, LBNF1 rats were much more sensitive to prolonged spermatogenic damage from irradiation (Kangasniemi *et al*., 1996) than were Sprague Dawley rats, based on comparison of similar doses and endpoints gathered from the literature (Delic *et al*., 1987; Erickson and Hall, 1983; Huckins, 1978). Whereas LBNF1 rats showed atrophic seminiferous tubules with only A spermatogonia, indicating a block in their differentiation, Sprague Dawley rats showed progressive recovery of spermatogenesis at similar doses. A block in spermatogonial differentiation after exposure to a variety of therapeutic and environmental toxicants, including hexanedione and dibromochloropropane, has been observed in Sprague Dawley and Fischer 344, in addition to LBNF1, rats (Meistrich and Shetty, 2003).

The radiation-induced block in spermatogonial differentiation in LBNF1 rats was not due to damage to the stem cells as they differentiated into spermatozoa after transplantation into the depleted testes of nude mice (Zhang *et al*., 2006). It was also not due to failure of the stem cells to proliferate, as they were actively cycling in atrophic tubules of several models with spermatogonial blocks induced by irradiation, hexanedione, or age, but was rather due to apoptosis of these cells when they began to differentiate (Allard *et al*., 1995; Schoenfeld *et al*., 2001; Shuttlesworth *et al*., 2000). This block was due to damage to the somatic environment as transplanted spermatogonia from normal immature rats failed to differentiate in the irradiated testis tubules (Zhang *et al*., 2007). The cause of the block is not known but several candidate genes whose expression changes in somatic cells of LBNF1 rats after radiation have been identified (Zhou *et al*., 2010, 2011).

To extend these anecdotal observations, we directly compared the sensitivities of seven different strains or stocks of rats treated with the same doses of radiation. Strains were chosen on the basis of their usefulness in toxicological or endocrine studies, previous indications of strain differences, and the existence of recombinant inbred lines to facilitate identification of loci contributing to the phenotype. We identified very marked differences in the sensitivity of the strains to radiation.

MATERIALS AND METHODS

Animals and irradiation exposure. We examined seven strains of rats to measure the recovery of spermatogenesis after irradiation. These included five inbred strains: BN (BN/SsNHsd) and Lewis (LEW/SsNHsd) obtained from Harlan Laboratories; Fischer 344 (F344/NCrl), Wistar Kyoto (WKY/NCrl), and SHR (SHR/NCrl) obtained from Charles River Laboratories; and two outbred stocks: Long-Evans (Crl:LE) from Charles River and Sprague Dawley (Hsd:Sprague Dawley SD) from Harlan. We obtained the rats at 7 weeks of age and they were allowed to acclimatize in our facility for 1 week prior to use. Rats were housed under standard lighting (12-h light, 12-h dark) and were given food and water *ad libitum*. All procedures were approved by the University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee, and the housing facilities were approved by the American Association of Laboratory Animal Care.

Rats were anesthetized with a mixture of ketamine (0.72 mg/kg) and acepromazine (0.022 mg/kg) im and affixed to an acrylic board with surgical tape; then, the lower part of the body was irradiated by a ^{60}Co gamma ray unit (Eldorado 8; Atomic Energy Canada Ltd., Ottawa, ON, Canada). The field extended distally from a line about 6 cm above the base of the scrotum. A single dose of 5 Gy was given at a dose rate of approximately 1 Gy/min (Shetty *et al*., 1989). Rats were euthanized 10 weeks after irradiation; serum was collected for hormone measurements, and the testis tissue was harvested for analysis as indicated below because all tubules in Sprague Dawley rats showed recovery of spermatogenesis at this dose, another group of Sprague Dawley rats were given 6.5 Gy of irradiation, and testis tissue was harvested 10 weeks later.

For each strain/stock, (n = 5–10) irradiated rats and (n = 3) age-matched unirradiated controls were analyzed.

Intratesticular interstitial fluid and tissue processing. Rats were killed by an overdose of the ketamine-acepromazine mixture. Each testis was surgically excised and weighed with the tunica albuginea intact. The right testis was fixed overnight in Bouin's fluid.

Interstitial tubule fluid was collected from the left testis using a modification of previously described methods (Porter *et al*., 2006; Rhenberg, 1993). Briefly, a silk suture was attached to the caput end of the testis with a wound clip. Four 1-mm incisions that did not intersect were cut into the caudal end of the testis. The testis was suspended inside a 10-ml syringe barrel by the attached suture, which was taped to the outside of the syringe. A preweighed silicone-coated microcentrifuge tube was attached to the luer lock tip of the syringe. The syringe assembly was centrifuged inside a 50-ml tube for 30 min at 60 × g at 4°C, and the weight of the microfuge tube containing the fluid was determined. The remaining weight of the testis parenchymal tissue was measured after removing the tunica albuginea. The tissue was then homogenized in water for sperm head counts.

Evaluation of spermatogenesis. For histological analysis, the fixed right testis was cut in half, testis section was taken from the middle from one of the two pieces and then embedded in glycol methacrylate plastic (JB4; Polysciences Inc., Warrington, PA), and 4-μm sections were cut and stained with periodic acid Schiff and hematoxylin. To evaluate the recovery of spermatogenesis from irradiation, we scored a minimum of 200 seminiferous tubules from the whole testis cross section from each animal for the most advanced germ cell stage present in each tubule. Unless otherwise stated, we computed the tubule differentiation index (TDI), which is the percentage of tubules containing three or more cells that had reached type B spermatogonial stage or later (Meistrich and van Beek, 1993). To obtain a more complete

description of the stages of differentiation present in the testis, we also determined the percentages of tubules with three or more cells reaching the leptotene spermatocyte stage or later (TDI-spermatocyte) or the round spermatid stage or later (TDI-spermatids) or with 10 or more cells reaching the elongating or elongated spermatids stage (TDI-late spermatids).

Although there are multiple subtypes of A spermatogonia in the rat testis (Chiarini-Garcia *et al*., 2003; van Bragt *et al*., 2008), they cannot be reliably distinguished in Bouin's-fixed methacrylate-embedded sections. Therefore, we counted all type A spermatogonia and Sertoli cells in atrophic seminiferous tubule cross-sections of irradiated rat testes at ×1000 magnification ($n = 3$–7 per group). For samples with almost complete seminiferous tubule atrophy, cells were counted using systematic random sampling (Stereo Investigator version 8.0 software; MicroBrightField, Inc., Williston, VT), by counting A spermatogonia and Sertoli cells in 300 randomly selected 100 × 80 μm fields. Results were presented as A spermatogonia per 100 Sertoli cells. In samples with few atrophic seminiferous tubules, these tubules were identified visually using light microscopy, and all cells in the tubules were counted. A minimum of 500 Sertoli cells was counted per testis.

Testicular sperm production was evaluated by counting sonication-resistant sperm heads, which represent nuclei of step 12–19 spermatids, in testicular homogenates. An aliquot of the homogenate of the left testis was sonicated and the sperm heads were counted in a hemocytometer using phase contrast optics (Meistrich and van Beek, 1993).

Hormone assays. Serum testosterone and intratesticular fluid testosterone concentrations were measured using a coated-tube radioimmunoassay kit (Coat-A-Count Total Testosterone; Cat No. TKTT1; Siemens, Los Angeles, CA) similar to procedures described previously (Porter *et al*., 2006; Shetty *et al*., 2000). Rat serum follicle–stimulating hormone (FSH) was measured by radioimmunoassay, and luteinizing hormone (LH) was measured by a sensitive two-site sandwich immunoassay. Both FSH and LH were measured by the University of Virginia, Center for Research in Reproduction, Ligand Assay and Analysis Core, using previously described methods (Gay *et al*., 1970).

Statistical analysis. Results were presented as either mean ± SEM calculated from untransformed data or, in the case of sperm head counts, testosterone, and LH, as the mean ± SEM calculated from log-transformed data obtained from individual rats. The statistical significance of differences between two groups was determined using SPSS version 19 software (Lead Technologies, Chicago, IL) using one-way ANOVA and the Student-Newman-Keuls *post hoc* test with $p < 0.05$ being considered significant.

RESULTS

Recovery of Spermatogenesis After Irradiation

Ten weeks after 5-Gy irradiation, rats were killed and testis tissue was harvested and serum removed for hormone analysis. Whereas the control testis parenchymal weights ranged from 1.04 g (Lewis and Fischer 344) to 1.74 g (Long-Evans), radiation markedly reduced testicular weights in all strains to between 0.29 g (Lewis) and 0.59 g (Sprague Dawley) (Fig. 1A). Expressing the parenchymal weight as a fraction of the control for each strain showed small but significant differences between strains. BN, Lewis, and Long-Evans appeared most sensitive as testicular weights decreased to between 24 and 28% of control. Fischer, Wistar Kyoto, and SHR had testicular weights of about 30% of control. Sprague Dawley was most resistant, with a testicular weight of 36% of control.

Interstitial fluid weights of control rats ranged from 0.06 g in Lewis to 0.11 g in Sprague Dawley, but no significant differences between strains were observed. Interstitial fluid weights were measured after irradiation (Fig. 1C) and showed negligible increases of only 0.01 g from the control in the Wistar Kyoto and SHR strains, marginal increases of 0.04–0.06 g in the Lewis, Fischer, and Sprague Dawley strains, but large significant increases of 0.15 g in Long-Evans and 0.22 g in the BN rats (Fig. 1D).

Despite only small differences in testis weights, the histological appearances of the testes were markedly different. Some strains, such as BN and Lewis, showed complete tubular atrophy with no differentiated germ cells present in any of the seminiferous tubules (Figs. 2A and B). However, the two strains differed in that there were large cellular interstitial spaces in BN, indicative of interstitial edema corresponding to the fluid accumulation in this strain (Fig. 1C), but not in the Lewis strain (not shown). Other strains such as SHR and Sprague Dawley showed recovery of spermatogenesis in essentially all tubules (Fig. 2C). Although late spermatids were observed in some tubules, other tubules showed incomplete recovery only to the spermatocyte or round spermatid stage (Fig. 2D).

The recovery of spermatogenesis was quantified by calculation of the TDI in histological sections (Fig. 3A). BN and Lewis were the most sensitive with less than 2% of tubules having evidence of differentiated germ cells, Long-Evans, Wistar Kyoto, and Fischer had between 50 and 75% of tubules with differentiated cells, whereas SHR and Sprague Dawley were more resistant, with evidence of differentiation in nearly all tubules. Long-Evans rats showed high variation in tubule differentiation (standard deviation: 31%), whereas the inbred strains and the outbred Sprague Dawley rats had standard deviations of < 10%.

The atrophic tubules were examined to determine whether they were a result of killing of stem spermatogonia or a block in their differentiation as previously observed with LBNF1 rats (Kangasniemi *et al*., 1996). The atrophic tubules observed in 5-Gy irradiated BN, Lewis, Long-Evans, Wistar Kyoto, Fischer 344, and SHR rats contained between 2.2 and 3.9 type A spermatogonia per 100 Sertoli cells (Table 1, Fig. 2C), indicating that the stem cells were not killed but their differentiation was blocked. Although residual A spermatogonia in Sprague Dawley rats exposed to 5 Gy could not be counted because less than 2% of tubules were atrophic, at 6.5 Gy, there were atrophic tubules and they did contain 2.8 type A spermatogonia per 100 Sertoli cells.

Among the recovering tubules, there was heterogeneity in the stages to which differentiation was observed. For example, of the 54% of the tubules showing differentiation in irradiated Long-Evans rats, 1% recovered only to the B spermatogonial stage, 17% reached the spermatocyte stage, 32% recovered to the round spermatid stages, and only 5% of the tubules reached the late spermatid stage. We used these data to plot the percentages of tubules reaching each stage of differentiation or beyond (Fig. 3B). These plots revealed differences between strains in the ability of differentiating tubules to progress. For

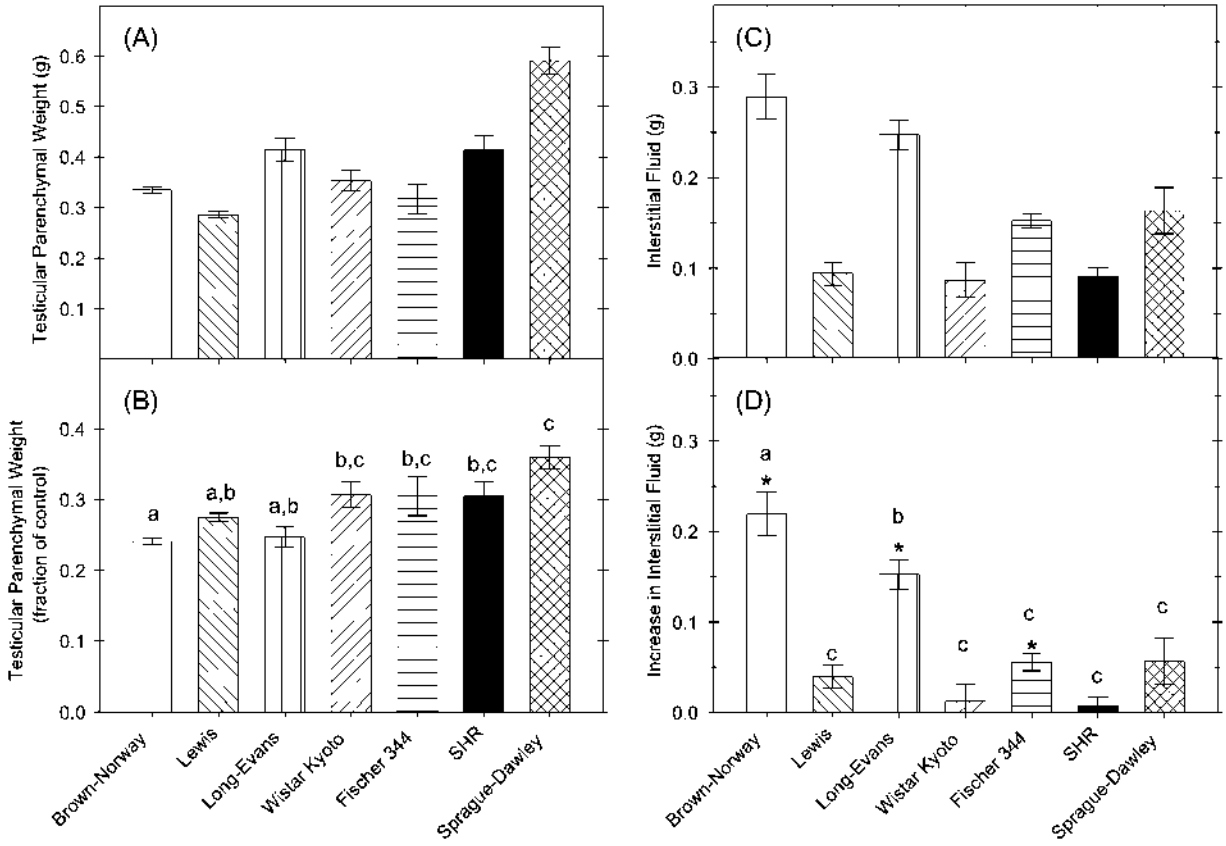

FIG. 1. Weights of testis parenchymal tissue and interstitial fluid for rats of seven different strains 10 weeks after irradiation with 5 Gy. (A) Absolute testis weights. (B) Testis weights relative to unirradiated controls of same strain. (C) Absolute testis interstitial fluid weights. (D) Increase in interstitial fluid weights from unirradiated control levels. In (B) and (D), the values for groups of irradiated rats with different letters (a, b, and c) are significantly different from each other ($p < 0.05$) and groups with the same letter are not. In (D), * is used to indicate strains that showed significant increases ($p < 0.05$) in testicular interstitial fluid resulting from irradiation.

example, whereas in both SHR and Sprague Dawley rats germ cell differentiation reached the spermatocyte stage or beyond in over 90% of tubules, in SHR only 20% of tubules recovered to the late spermatid stage but in Sprague Dawley 42% showed late spermatids.

Although the histological data provide an indication of sperm production, a more quantitative measure is the number of sonication-resistant sperm heads per testis. Irradiated rats showed huge differences between strains, with over 1000-fold differences in testicular sperm production, varying from about 10^4 in BN and Lewis to almost 2×10^7 in Sprague Dawley (Fig. 4A). As there were some differences in control values, ranging from 1.4×10^8 in Lewis to 2.4×10^8 in Long-Evans, the counts were normalized to the control values (Fig. 4B). BN and Lewis were the most sensitive rat strains with more than a 10,000-fold reduction in sperm production, and Sprague Dawley was most resistant with sperm count remaining at 9% of control. Long-Evans rats were more resistant than Lewis and BN but more sensitive than Wistar Kyoto, Fischer, and SHR; they also showed the largest standard deviation in the counts. These strain differences were consistent with the percentages of tubules with late spermatids in the histological sections (Fig. 3B).

Hormone analyses were performed on one sensitive strain, BN, and one resistant strain, SHR. Serum testosterone, interstitial fluid testosterone, and serum FSH levels in control and irradiated SHR rats were significantly higher than the corresponding values in BN rats, and serum LH levels were significantly higher in control SHR rats than BN rats (Fig. 5). Although there were no significant changes in serum testosterone and LH levels in either BN or SHR rats as a result of irradiation, interstitial fluid testosterone levels were significantly increased after radiation in SHR rats by1.4-fold, and serum FSH levels significantly increased after radiation in both SHR (1.7-fold) and BN rats (2-fold).

DISCUSSION

In this study, we directly compared the recovery of spermatogenesis at 10 weeks after 5 Gy of irradiation in seven different strains or stocks of rats. The results showed that the recovery of spermatogenesis was incomplete in all strains of rats analyzed. Even in the most resistant strain, Sprague Dawley, sperm counts had not even recovered to 10% of control levels.

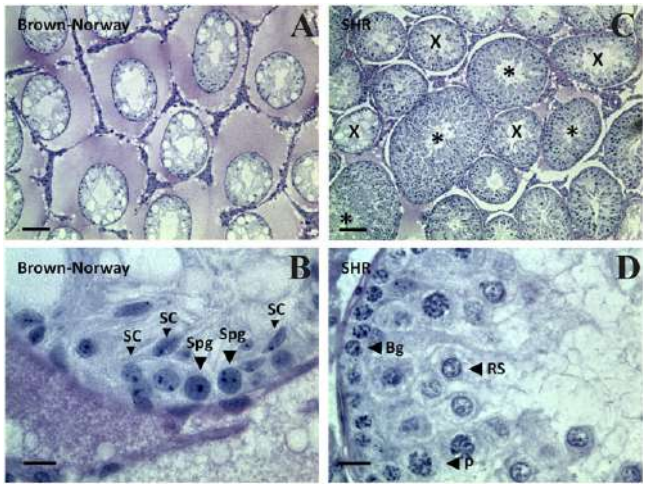

FIG. 2. Histology of rat testes 10 weeks after irradiation with 5 Gy. (A) BN testis showing atrophic tubules and interstitial edema. (B) The tubules in BN contained mostly Sertoli cells (SC) but some contained a few type A spermatogonia (Spg). (C) SHR testis showing recovery of spermatogenesis in nearly all tubules. Some tubules in SHR testes (*) showed complete spermatogenesis; other tubules (X) only showed development to the early spermatid stage. (D) Higher magnification image of tubule from irradiated SHR rat showing development to only the early spermatid stage. (Bg) Type B spermatogonia, (P) pachytene spermatocyte, and (RS) round spermatid. Scale (A, C) bar: 100 μm, Scale (B, D) bar: 10 μm.

TABLE 1
Number of Type A Spermatogonia per 100 Sertoli Cells in Nonrepopulating Tubules at 10 Weeks After 5-Gy Irradiation[a] in Different Rat Strains (n = 3–7 per Group)

Strain[a]	Spermatogonia per 100 Sertoli cells[b]
BN	2.4 ± 0.5
Lewis	2.2 ± 0.3
Long-Evans	3.4 ± 0.3
Wistar Kyoto	3.9 ± 0.3
Fischer 344	2.5 ± 0.4
SHR	2.5 ± 0.9
Sprague Dawley[a]	2.8 ± 0.2

[a]There were insufficient (< 2%) nonrepopulating tubules in 5-Gy irradiated Sprague Dawley rats to perform these counts, so the group irradiated with 6.5 Gy was used for these counts.
[b]No significant differences were observed between different rat strains.

The contribution of the block in spermatogonial differentiation, previously described in LBNF1 rats, to the failure of recovery was assessed. The atrophic tubules observed in all strains of rats contained similar numbers of type A spermatogonia (Table 1). These results indicate that the failure of recovery was not due to loss of stem cells but rather to treatment-induced block in the ability of the spermatogonia in these tubules to differentiate and that the major component of the difference in sensitivity between the strains was in the percentage of tubules with evidence of a block in spermatogonial differentiation at a given dose (Fig. 3). Thus, the radiation-induced block in spermatogonial differentiation is a characteristic of all strains but had not been observed before in Sprague Dawley rats either because the radiation doses were low (Dym and Clermont, 1970; Erickson and Hall, 1983; Huckins, 1978) or the spermatogonia in the atrophic tubules were not noticed in paraffin-embedded tissues (Delic *et al.*, 1987).

Even in the tubules showing differentiated germ cells, there was heterogeneity in the ability to differentiate into the various

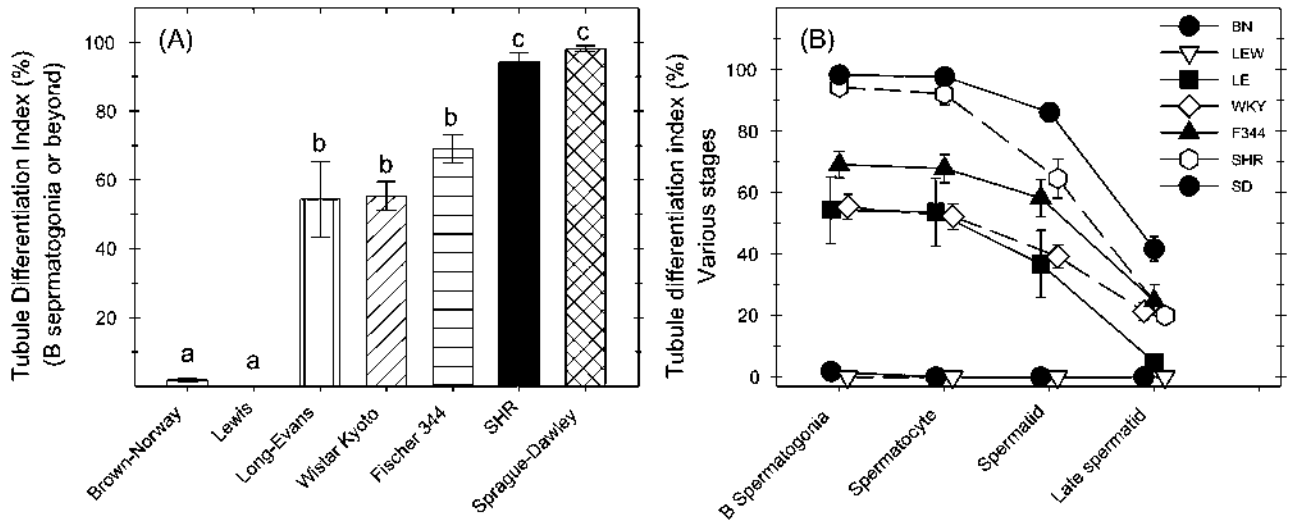

FIG. 3. Recovery of spermatogenesis as measured by the percentage of tubules with morphologically differentiated cells at a specific stage of differentiation or beyond for different strains of rats. (A) TDI defined as differentiation to the B spermatogonial stage or beyond, unless otherwise noted. (B) Percentage of tubules reaching differentiation to specific stages or beyond. The values for groups of irradiated rats with different letters (a, b, and c) are significantly different from each other ($p < 0.05$) and groups with the same letter are not.

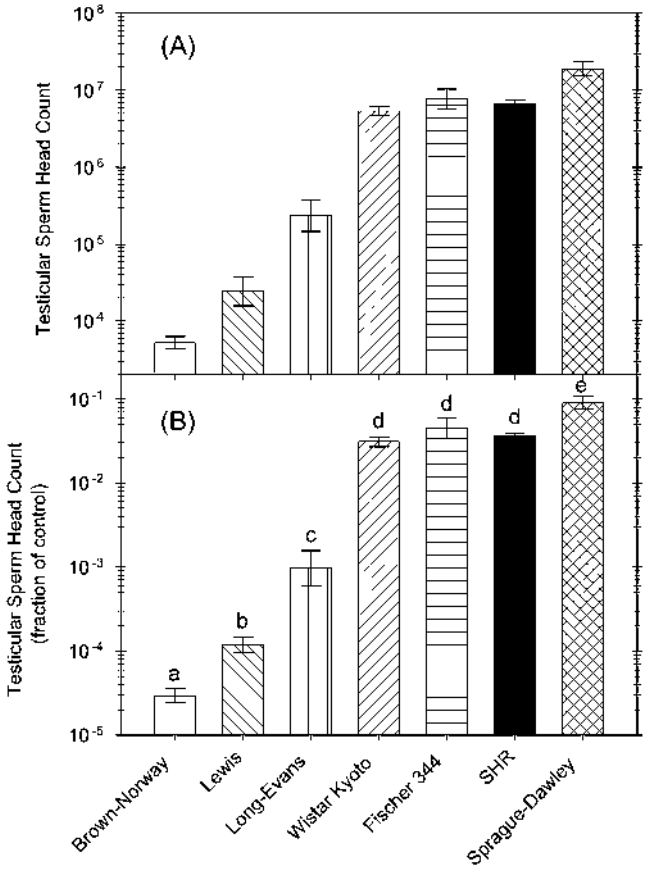

FIG. 4. Testicular sperm production. (A) Numbers of sonication-resistant late spermatids per testis in rats at 10 weeks after irradiation with 5 Gy. (B) Numbers of sonication-resistant late spermatids relative to unirradiated controls of same strain. The values for groups of irradiated rats with different letters (a, b, c, d, and e) are significantly different from each other ($p < 0.05$) and groups with the same letter are not.

stages (Figs. 2C and 3B). We attribute this to damage to the somatic environment, with some tubules being able to support differentiation to only the spermatocyte or early spermatid stage. Although the present data do not rule out the possibility that this heterogeneity reflects variable delays in initiation of differentiation in various tubules, other data (Kangasniemi *et al.*, 1996) (our unpublished results) show that the differentiation in some tubules does not progress beyond a certain stage even at later postirradiation time points.

Because radiation treatment with 5 Gy produced consistent results on the recovery of spermatogenesis within inbred strains of rats but produced differing results between strains, the differences in radiation sensitivity must be attributable to genetic variations between the strains. Consistent with this idea, we found that the standard deviations of the sperm count and tubule differentiation data after irradiation were greater in the outbred Long-Evans rats than in any of the inbred strains, although such a difference was not observed in the outbred Sprague Dawley rats. However, whereas the outbred Sprague Dawley rats were most resistant to radiation effects on spermatogenic recovery, the outbred Long-Evans rats were moderately sensitive, so we cannot conclude that outbred rats are more resistant than inbred ones.

The rat strains were classified according to their differing sensitivities to radiation-induced inhibition of spermatogenic recovery: BN and Lewis were the most sensitive; Long-Evans was intermediate; Wistar Kyoto, Fischer, and SHR were moderately resistant; and Sprague Dawley was most resistant. To investigate a basis for this grouping, we compared the phylogenetic relationships among strains (Saar *et al.*, 2008; Thomas *et al.*, 2003) to resistance levels. The SHR and Wistar Kyoto rats are most closely related and their similar resistance to radiation likely is due to a common set of genes. In contrast, Lewis and Fischer rats, which also are derived from a common ancestor and are relatively closely related, showed a dramatic difference in radiation sensitivity. Lewis are also much more closely related to the more resistant Sprague Dawley rats than they are to the highly sensitive BN strain, which is most genetically distinct of all the rat strains and diverged first in the evolution of strains. Thus, the cause of radiation sensitivity in BN may be different from that in the Lewis rats as it is more likely that two different mutations related to sensitivity would have arisen in the BN and Lewis strains than that mutations to produce resistance arose in all of the five other strains after divergence from the common ancestor with BN. In contrast to the lack of a close relationship between radiation sensitivity and phylogenetic relationship, interstitial fluid accumulation was more closely associated in related strains. The low levels of increase in fluid after irradiation in Lewis, Fischer, Sprague Dawley, and particularly SHR and Wistar Kyoto (Fig. 1D) are consistent with their close phylogenetic relationship; significantly greater increases were observed in Long-Evans and BN, which are more distantly related to the first five strains.

Our results on differential sensitivities of various strains of rats are in general agreement with previous studies using radiation and different toxicant models. The recovery of spermatogenesis after irradiation in Sprague Dawley rats has been shown to be greater than in Wistar rats (Delic *et al.*, 1987). We previously reported that the recovery of spermatogenesis after treatment with the procarbazine was much greater in Sprague Dawley than in Lewis or LBNF1 rats (Parchuri *et al.*, 1993). In addition, Sprague Dawley rats showed greater recovery of spermatogenesis than did Fischer rats after treatment with 2,5-hexanedione, a Sertoli cell toxicant (Blanchard *et al.*, 1996; Boekelheide, 1988; Boekelheide and Hall, 1991). Thus, the interstrain differences appear to be related to the sensitivity to induction of a spermatogonial block after different toxic stresses rather than the sensitivity of the testis to a particular toxicant.

The role of hormones in the strain differences in radiation sensitivity was investigated next. In normal rats, spermatogonial differentiation is qualitatively independent of both testosterone and FSH and occurs even when these hormones are suppressed (Huang and Nieschlag, 1986). However, in

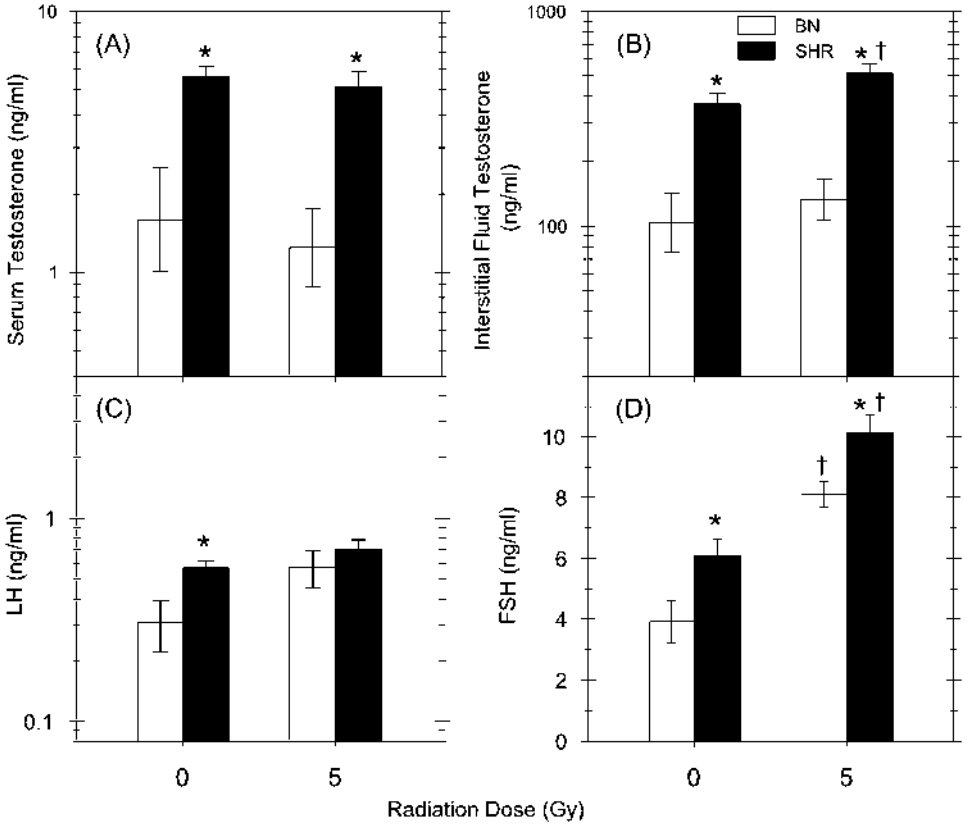

FIG. 5. Testosterone, LH, and FSH levels in BN and SHR rats at 10 week after irradiation with 5 Gy. (A) Serum testosterone. (B) Intratesticular fluid testosterone. (C) Serum LH. (D) Serum FSH. * Indicates values in SHR are significantly different from those in BN, and † indicates value in irradiated is significantly different from that in unirradiated testes ($p < 0.05$, *t*-test).

irradiated rats, we demonstrated that the differentiation of type A spermatogonia can be completely inhibited by moderate levels of testosterone alone, independently of the pituitary hormones, or partially inhibited by high levels of FSH (Shetty *et al.*, 2006). In fact, suppression of testosterone for 10 weeks after irradiation of LBNF1 rats (Meistrich *et al.*, 2001) or BN rats (data not shown) can restore the production of differentiated cells in nearly all tubules, as we observed with the more resistant strains without the need for hormonal suppression (Fig. 3A). We therefore tested whether the block in BN rats but not SHR rats could be due to higher levels of testosterone or FSH. To the contrary, there were lower levels of serum and intratesticular testosterone and FSH in BN rats than in SHR rats both before and after irradiation. An alternative hypothesis, that the high levels of testosterone in SHR are responsible for the greater recovery of spermatogenesis, is not consistent with all of our data, as Sprague Dawley rats, the most resistant strain, had levels of testosterone intermediate between the levels in SHR and BN rats (data not shown).

The possible role of the increase in interstitial fluid levels in the inhibition of spermatogonial differentiation was also evaluated because we previously identified a correlation between the two parameters in irradiated LBNF1 rats (Porter *et al.*, 2006). Radiation induced significant increases in interstitial fluid levels in three of the rat strains, most dramatically in BN, a sensitive strain, and Long-Evans, a strain with intermediate sensitivity. In contrast to BN, the other radiation-sensitive strain, Lewis, showed only a small nonsignificant increase in fluid levels. Examination of the relationship between the increase in interstitial fluid and TDI in the various strains (Fig. 6) failed to indicate any significant correlation between the increase in fluid after irradiation and sensitivity of the different strains to the radiation-induced block in spermatogonial differentiation.

The genetic alterations that are responsible for the differences in the recovery of spermatogenesis after radiation in the various strains are not known. The sensitive and resistant strains identified in this study could be used to determine which specific changes in gene expression that occurred after radiation in LBNF1 rats (Zhou *et al.*, 2010) also occur in a sensitive inbred strain identified in this study but not in a resistant strain. In addition, the regions of the genome (quantitative trait loci, QTL) that contain the candidate genes for the interstrain differences in radiation sensitivity can be determined from genetic crosses between strains. Fortunately the BN and SHR rats, a pair of strains for which recombinant inbred rats already available (Tabakoff *et al.*, 2009) showed

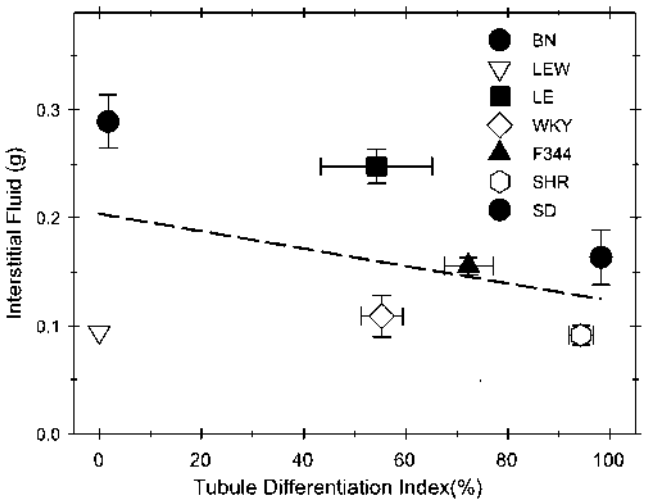

FIG. 6. Radiation-induced increase in testicular interstitial fluid plotted against the recovery of spermatogenesis at 10 weeks after 5 Gy of irradiation. A linear regression was performed on the data points and the correlation was weak ($r^2 = 0.25$) and not statistically significant ($p = 0.25$).

large differences in radiation sensitivity, and we are using these strains to identify QTL related to the radiation sensitivity.

The difference between strains in radiation response highlights the importance of knowledge of this information in choosing an animal species and strain within that species for evaluation of risks to human. Blocks in spermatogonial and later germ cell differentiation were observed in all strains and may correspond to the human situation in which no sperm is produced for a prolonged periods after single doses of 1–6 Gy of irradiation to the testis, despite the presence of surviving stem cells from which there is eventual recovery of spermatogenesis (Clifton and Bremner, 1983). In this study, which used only one dose at one time point (5 Gy, 10 weeks), we found very large differences in recovery of differentiation (0–100% of tubules) and sperm production (100-fold differences). It is not known whether there are qualitative differences between strains or only quantitative differences in the dose at which the complete block occurs or differences in the time course of possible subsequent recovery. These questions are being addressed in further experiments.

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Differentiation of spermatogonial stem cells by soft agar three-dimensional culture system

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ABSTRACT

Since proliferation and differentiation of spermatogonial stem cells (SSCs) in culture system provide successful transplantation in this study, culture of human SSCs was compared to SACS (soft agar culture system), gelatin and control groups. The cells were isolated from seminiferous tubules of non-azoospermia patients (NOA) and cultured in DMEM for 3 weeks. The presence of SSCs in culture system was confirmed by immunocytochemistry of GFR-α1 and ITGα6 antibodies. The proliferated cells were cultured in three mentioned groups in the presence of retinoic acid and Sertoli cells conditioned medium for another 2 weeks. The number of colonies in the SACS group was significantly higher than two other groups. Before 2 weeks of culture, only Oct4 expression was observed in testicular cells (2.32 ± 0.25). After 2 weeks, the expression of Oct4 in the gelatin group was higher than that of the SACS group on day 7. The maximum expression of Stra8 was observed in SACS and gelatin groups after 7 days, but its expression was significantly decreased after 14 days of culture ($p < .05$). The expression of Scp3 and Acrosin genes were higher after 14 days in the SACS group compared to other groups. SACS has positive effects on proliferation and differentiation of hSSCs.

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Introduction

Spermatogenesis occurs in seminal tubules and is regulated by a microenvironment surrounding the spermatogonial stem cells (SSCs), which is called niche [1,2]. Stem cells that leave the niche are more likely to go to an environment that promotes their differentiation to other specific cells [3].

Sertoli cells are the most important somatic cells present in the niche due to the production of growth factors and cytokines and their presence in the blood-testis barrier. During spermatogenesis, Sertoli cells secrete growth factors and cytokines in order to regulate the proper self-renewing and differentiation of SSCs, meiosis of spermatocytes and conversion of round spermatid to spermatozoa [4]. Since the number of Sertoli cells determines the size of the testis, it is assumed that each Sertoli cell supports a predetermined number of germ cells [5]. Many interactions between Sertoli cells and germ cells at each developmental stage have an autocrine/paracrine nature [6–8].

Retinoic acid (RA) is a small polar molecule that spreads throughout the tissues and acts through a link with RA nuclear receptors (RARs). They also undergo heterodimer with nuclear retinoid X receptors. Some RARs and RXRs are expressed by germ cells at an appropriate stage of development. RA as an active derivative of vitamin A controls the entrance of germ cells into meiosis stage. In addition, RA can induce conversion of undifferentiated spermatogonia to differentiating spermatogonia [9].

According to previous studies, the response of cells in three-dimensional culture system is very similar to the behavior of cells *in vivo* compared to two-dimensional culture system. In a 3D culture, the cells communicate with each other, an extracellular matrix (ECM) and the surrounding microenvironment. These interactions in 3D structures affect proliferation, differentiation, cell morphology, gene and protein expression and cell responses to external factors [10]. In 3D culture systems, the cells form assemblages or spheroids between matrixes or in a suspension medium [11–13]. The 3D Soft Agar Culture System (SACS) represents *in vivo* conditions that mimic some of the natural aspects of the 3D environment [14].

Among 2D culture systems, gelatin is a type of extracellular matrix (ECMs) that acts as a substrate for coating the culture dish [15]. The culture of mammalian SSCs has been done in some conventional 2D system using culture dishes or flasks [2]. The physical support for SSCs in a 2D culture is varied from natural microenvironment of the seminiferous tubule.

Since the proliferation and differentiation of spermatogonial cells in culture system provide successful transplantation

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of these cells to testes tissue and also, most studies have investigated the effects of two-dimensional and three-dimensional culture systems on animal's models, so this study proliferation and differentiation of human SSCs was compared in SACS, gelatin-coated plates and control groups. In fact, the new protocol needs to be tested in human species for transfer of results to the clinic, so these techniques should be repeated successfully in humans for treatment of disease. In this study, human spermatogonial cells isolated from testis of non-obstructive azoospermia patients and cultured in DMEM in the presence of 10% fetal bovine serum (FBS) for 3 weeks. The presence of SSCs in culture system was confirmed by immunocytochemistry of GFR-α1 and ITGα6 antibodies. Then, the cells were cultured on SACS, gelatin-coated plates and control groups in 10^{-6} M retinoic acid and Sertoli cells conditioned medium from OA patients for 2 weeks. To investigate the effect of culture system on SSCs differentiation, the expression of pluripotent (Oct4), pre-meiotic (*Stra8*), meiotic (*Scp3)* and post-meiotic *(Acrosin*) germ cells specific genes was investigated by real-time PCR before culture and on days 7 and 14 after culture in 3 groups.

Materials and methods

Isolation of human spermatogonial cells and Sertoli cells

Human testis biopsies were obtained from non-obstructive azoospermia (NOA) men referred to the Infertility Clinic of Khatam Alanbia and Parsian Hospital ($n = 14$, aged 21–41 years, average 32 years) during 2016–2017. Analysis of semen was done according to WHO criteria and biopsy of testis was only prepared in cases where sperm could not be detected in any of the semen samples collected during the year. The experimental protocol and use of human testicular biopsy sample were authorized by the Ethical and National Research Council guidelines of Shahid Beheshti University (Tehran, Iran).

The small pieces of testis weighing approximately 100–200 mg were placed into a medium and transferred to the laboratory within 40 min. After three times washing with PBS (phosphate buffered saline) supplemented with 1% pen/strep, they were placed in Dulbecco's Modified Eagle Medium (DMEM; Thermo fisher scientific, Waltham, MA, USA) which consisted of 0.5 mg/ml collagenase IV, 0.5 mg/ml trypsin, 0.5 mg/ml hyaluronidase and 0.05 mg/ml DNase (with shaking and a little pipetting), at 37 °C for 30 min. During this time, tissues were mechanically dissected using two insulin needles and separated in the enzymatic digestion solution (all enzymes from Sigma (St Louis, MO, USA). Then, the obtained cells and spermatogenic tubules were centrifuged at 112 g for 5 min. After three washes in DMEM and removal of most of the fibroblast, interstitial and endothelial cells, second digestion step (5 min at 37 °C) was done by adding a fresh mixture of enzymes and DMEM to the residual seminiferous tubule fragments. The clumps of cells were gently agitated by repeated pipetting with a pipette tip for a minute. Subsequently, the cells were centrifuged at 542 g for 5 min in order to separate the cells from the residual tubular fragments.

The obtained cells suspension (a mixture of spermatogonial cells and Sertoli cells) were filtered through a 70 μm nylon filter and cultured for three weeks at 37 °C, in the presence of 10% fetal bovine serum (FBS; Thermo Fisher scientific),1% Pen/Strep and 5% CO_2, in a humidified atmosphere [16]. After 3 weeks, the cells were dissociated by (EDTA)–trypsin treatment (0.02% EDTA–0.1% trypsin) at 37° C for 5 min and the presence of spermatogonial stem cells in culture system was confirmed by immunocytochemistry of GFR-α1 and ITGα6 antibodies. Then, the cell suspension was cultured on SACS, gelatin-coated plates and control groups for another two weeks. In this study, the secretions of Sertoli cells were extracted from obstructive azoospermia (OA) patients and used as a conditioned medium for culturing the germ cells of non-obstructive azoospermia (NOA) patients in three groups. The number and diameter of colonies were evaluated on days 3, 7, 10 and 14 after culture using an inverted microscope (Olympus IX71, EXFO 120 Xenon-Hg excitation light). The expression of Oct4, Stra8, Scp3 and Acrosin genes was also evaluated before and after 2 weeks of culture by quantitatively real-time PCR. The Y chromosome microdeletions (including SRY, ZFY, sY84, sY86, sY127, sY134, sY254 and sY255) were investigated in NOA patients by multiplex PCR technique.

The preparation of conditioned medium from OA patients

The small pieces of testis were prepared from obstructive azoospermia (OA) patients ($n = 8$, aged 21–43 years, Tehran, Iran), transferred to the lab and enzymatic digestion method was performed as earlier mentioned for NOA patients. Some culture flasks were coated by 5 μg/ml of Datura stramonium agglutinin (DSA; Sigma, Gimson weed, thorn apple, USA) in PBS at 37 °C for 1 h. The obtained cell suspension was added to the flasks and incubated at 37 °C in the presence of 10% FBS and 5% CO_2, in a humidified atmosphere. To isolate Sertoli cells, the non-adhering germ cells were collected and discarded after leaving overnight. The remaining cells were cultured for 4–5 days in DMEM accompanied by 10% FBS and the medium was changed everyday for increasing the purity of Sertoli cells. Then, the cell was detached by treatment with EDTA-trypsin in PBS (calcium and magnesium free) at 37°C for 5 min, washed with fresh DMEM and centrifuged at 645 *g* for 5 min. Then, the cells were counted and adjusted to desired densities into 3 cm culture dishes and cultured for 10 days and the medium was collected every three days and filtered through a 0.22 μm nylon filter. This medium was considered as conditioned medium and subsequently stored in −80 °C freezer until further use.

Immunocytochemical evaluation for confirmation of Sertoli cells

The Sertoli cells were evaluated with FSH receptors as a marker by immunocytochemistry technique [17]. For this

purpose, Sertoli cells were cultured on the glass slides and fixed for 20 min in 4% paraformaldehyde at room temperature. Following permeabilisation by 0.2% Triton X-100 (MPbiomed Inc, Ottawa, Ontario, Canada) for 1 h, which facilitates antibody penetration, they were incubated overnight at 4°C with the rabbit polyclonal anti-FSH receptor antibody (diluted 1: 200; Abcam, Boston, MA, USA) which has been described as a marker for Sertoli cells [17]. After washing with PBS, the cells were incubated with the secondary antibody (Goat FITC-conjugated anti-rabbit IgG) ((diluted 1:100; Abcam, USA) for 2 h. The control slides were under similar conditions except for the removal of the first antibody.

To culture of human spermatogonial stem cells (hSSCs) on gelatin-coated plates

A total of 24 well plates were coated with 0.1% gelatin. Then, the obtained cells from NOA patients were cultured on them in the presence of 15% FBS accompanied by 50% conditioned medium in addition to 50% low-glucose DMEM supplemented by 1 μM retinoic acid (RA) for two weeks. The medium was changed every three days until 14 days after culture.

Culture of hSSCs on SACS and control groups

To make certain concentrations of agar, at first 0.7% (*w/v*) and 1% (*w/v*) agar were dissolved in distilled water separately. Then, these solutions were mixed with the same volume of low-glucose DMEM to the final concentrations of 0.35 and 0.5% in order to prepare the upper and lower phases, respectively. The gel phase of agar was added on top of the lower layer after solidification. The lower layer (solid agar base) contained low-glucose DMEM, 20% (*v/v*) FBS and 0.5% (*w/v*) agar only [18]. Tubular cells with a final volume of 500 ml were cultured in the upper layer of the soft agar medium (0.35% agar + low glucose DMEM + 20% (*v/v*) FBS in 24-well plates [14].

Cell suspensions and DMEM were mixed prior to adding the agar at 37 °C to preclude premature agar coagulation and cellular stress probably caused by heat. All cell culture plates were incubated for 2 weeks at 37 °C in the presence of 15% FBS, 50% conditioned medium, in addition to 50% low-glucose DMEM supplemented by 1 μM RA. Every three days, the medium was changed until 14 days after culture.

For the control group, the obtained cells from NOA patients were cultured on 24-well non-coated plates in the presence of 15% FBS accompanied by 50% conditioned medium in addition to 50% low-glucose DMEM supplemented by 1 μM retinoic acid (RA) for 2 weeks.

To evaluate the diameter and number of colonies in three different culture system

The diameter and number of colonies were evaluated on days 3, 7, 10 and 14 after culture using an inverted microscope (Olympus IX71, EXFO 120 Xenon-Hg excitation light). Colonies were counted separately in whole fields in each well of three groups. The diameter of the colonies was measured using Image J Software.

The evaluation of some specific genes expression by real-time PCR

The differentiation of spermatogonial cells was evaluated by the expression of Oct4, Stra8, Scp3, and Acrosin genes during different days of culture through real-time PCR. For this purpose, the cultured cells were isolated with trypsin-EDTA and collected by centrifugation in gelatin-coated plates and control groups. Pick up of colonies were performed in SACS group at days 7 and 14 after culture. Total RNA was extracted using Trizol (Life technologies, CA, USA; 15596–026) according to the manufacturer's protocol and DNase treatment was applied by RNase-Free DNase Set "DNase I", Qiagen, Mainz, Germany. cDNA was generated from 500 ng total RNA using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (# 1622). For quantitative PCR, 4 μl 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) was added to each well of the PCR plate (10 μl of water, 1 μl of primers and 5 μl of cDNA), based on the following procedure: 50 cycles at 95 °C for 30 s, 57.4–65 °C for 30 s and 72 °C for 30 s. Based on the ΔΔCt method, the threshold of cycle values was normalized against the threshold value of the β-actin housekeeping gene (as an internal control). Extracted cells from normal testis were considered as a positive control. The primers of genes are: Oct4; Forward: GGGCTCTCCCATGCATTCAAA

Reverse: CACCTTCCCTCCAACCAGTTGC; Stra8 (as a pre-meiotic gene) Forward: CAGCGCTCTTCAACAACCTC and Reverse: ACCTGCCACTTTGAGGCTATG; Scp3 (as a meiotic gene) Forward: GGAAGGAGTTGGAGTTGACAT and Reverse: ATCCC ACTGCTGAAACAAAGTC; Acrosin (as a post-meiotic gene) Forward: ATCACCCCTCCCATTTCGTG Reverse: AGTCCAGGT CGATGAGATCC; β-actin (as a housekeeping gene) Forward; AGGCGGACTATGACTTAGTTGCGTTACACC and Reverse; AAGT CCTCGGCCACATTGTGAACTTTG.

Scanning electron microscopic study of SACS colonies

The ultrastructure images of cell cultured in SACS group was investigated by Scanning electron microscopy (SEM). For this purpose, the cultured cells in soft agar were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, accompanied by 2% paraformaldehyde 14 days after culture. They were further washed with 0.1 M phosphate buffer (pH = 7.4). Specimens were then dehydrated to 100% ethanol using an increased ethanol-water series and then dried. The samples were mounted on aluminum stubs with sticky carbon tabs. A Gatan ion beam coater was used to coat the agar with a layer of Au/Pd. Images were obtained using Philips XL 30 SEM scanning electron microscope (Amsterdam, The Netherlands).

Data analysis

All statistical analysis was performed using SPSS software version 22 (CA, USA) and merged to calculate the

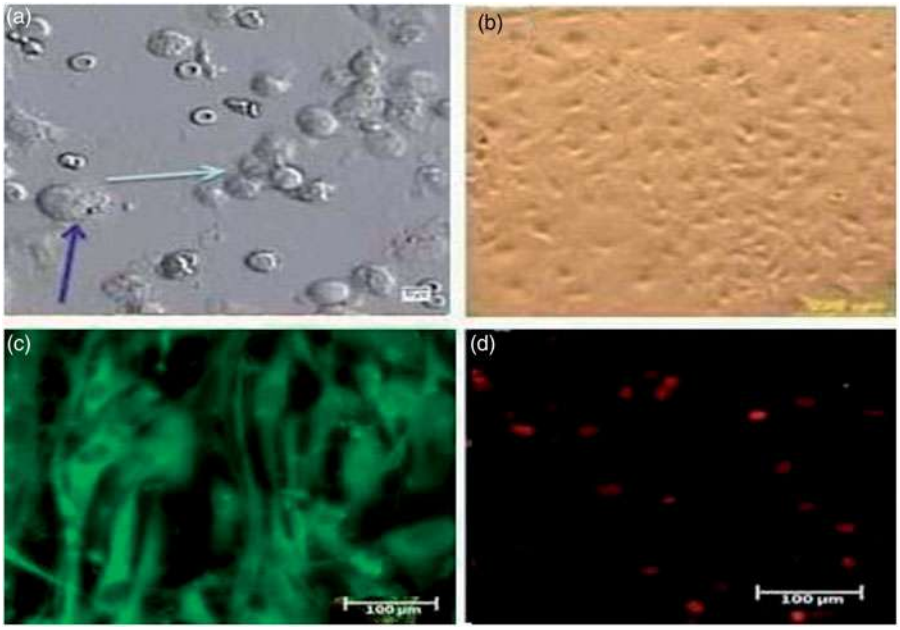

Figure 1. The morphology of isolated cells from NOA testis. (a) Two types of cells are visible. The thick arrow represents the stem cells of the spermatogony. The thin arrow represents the Sertoli cells. (Scale bar: 50 µm). (b) The Sertoli cells attached to the plate and formed spindle-like shape (Scale bar: 200 µm). (c) The present of FSH receptor in Sertoli cells was confirmed by imunocytochemistry technique (Scale bar: 100 µm). (d) The control group without the primary antibody and stained with PI (Scale bar 100 µm).

mean ± standard error of the mean (SEM). The Shapiro–Wilk test was done to determine whether the data were normally distributed. The parametric and non-parametric data were analyzed by independent one-way ANOVA and Kruskal–Wallis test, respectively. Repeated measures test was done for time-frame analysis per group. Probability values $<.05$ were considered statistically significant.

Results

In this study, the human testicular cells were isolated from small biopsies of the testis of NOA patients. Since the size of biopsies was small, following that, the small number of cells was obtained from the digestion process. Therefore, before the culture of cells on SACS, gelatin and control group, the cell suspension was proliferated for three weeks to increase the cell population.

Two groups of cells were identified in the cell suspension based on size and morphology. The first group had a diameter of 8–9 micrometers, with irregular edges and round transparent appearance. These cells were proliferated and formed a layer at the bottom of the plate, which was considered as a feeder layer. The second group of the cells was larger than the first one and had a diameter of 15–17 micrometers and their appearance was spherical and had two or three nucleoli outside the center. These cells were proliferated and several colonies with different numbers and diameters appeared in the culture system, so that the average number of colonies was 13.3 ± 0.7 and the average diameter of colonies was 157.8 ± 16.7. In this study, the presence of FSH receptor as a marker on the surface of Sertoli cells was confirmed by immunocytochemistry method. These cells appeared green color (Figure 1(c,d)).

The nature of Sertoli cells was also proven by flow cytometry technique to test the intermediate filament of vimentin inside the Sertoli cells. Our results indicated a high percentage of positive vimentin cells in the culture system (Figure 2).

In this study, investigation of Y chromosome microdeletions in NOA patients showed that the three NOA patients had microdeletion in AZFc (sY254, sY255) region and were excluded from the study (Figure 3).

Confirmation of SSCs in the culture system

After 3 weeks' culture of testicular cells, an immunocytochemistry reaction was performed on the cells by two antibodies of GFR-α1 and ITGα6 for confirmation of SSCs in culture. The results showed that a large percentage of cells showed a positive reaction for these antibodies. The expression of some genes (Oct4, Stra8, Scp3, Acrosin) was also studied in these cells, after 3 weeks of culture, using real-time PCR technique. The results showed that Oct4 expression was only observed in these cells (2.32 ± 0.25) and the expression of Stra8, SCP3, and Acrosin genes was not observed 3 weeks after culture.

After 3 weeks of proliferation, the cells were isolated and divided between three groups (SACS, gelatin-coated plates and control groups). Since the separation and counting of cells in agar (SACS) culture system was difficult, in order to evaluate the ratio of the cell growth in 3 groups based on constant condition, the number and diameter of colonies appeared in the experimental and control groups was evaluated on days 3, 7, 10 and 14 after culture. The results showed that in the control group, no colony was observed at any time and only small clusters were seen (Figure 4(a)). These clusters were not counted in this study. The colonies

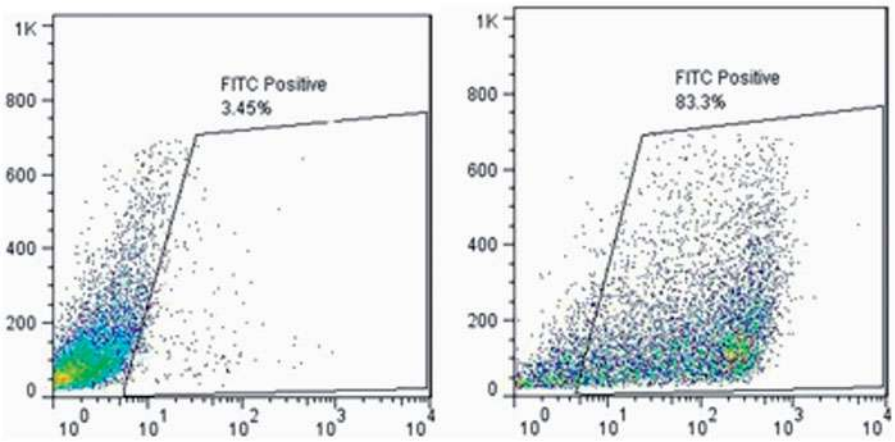

Figure 2. The efficiency of Sertoli cells enrichment was quantitatively assessed by flow cytometry. Attached Sertoli cells were detached by EDTA–trypsin treatment and labeled with mouse monoclonal anti-vimentin antibody overnight at 4 °C. The cells were incubated with FITC-conjugated anti-mouse antibody for 1 h. Negative control was incubated only with FITC-conjugated goat anti-mouse antibody. The results showed Vimentin positive characteristic in attached layer.

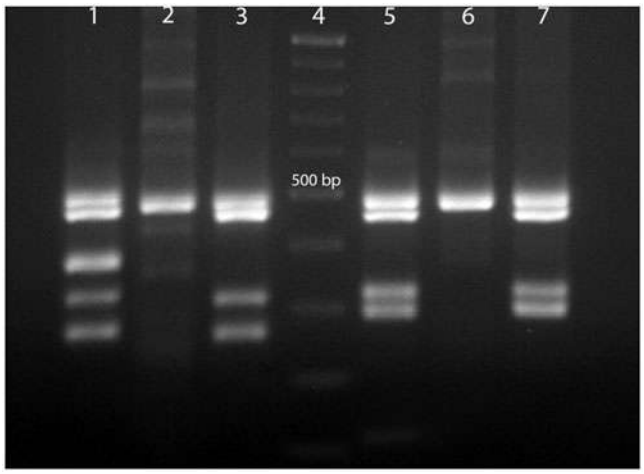

Figure 3. Multiplex PCR from normal man, woman and NOA patients with AZFc microdeletions. Line 1, normal men, multiplex 1; line 2, normal woman, multiplex 1; line 3, NOA patient with AZFc microdeletions in sY254 region, multiplex1; line 4, Gene Ruler™ (DNA Ladder,100 bp, Fermentase # SM0333); line 5, normal men, multiplex 2; line 6, normal woman, multiplex 2; line 7, NOA patient with AZFc microdeletions in sY255 region, multiplex 2. Multiplex 1 included SRY: 427 bp, sY254: 400 bp (AZFc), sY86: 320 bp (AZFa), sY127: 274 bp (AZF b). Multiplex 2 included SRY: 472 bP, sY84: 326 bp (AZFa), sY134: 301 bp (AZFb), sY255: 126 bp (AZFc).

in the gelatin-coated plates group were heavily compacted and exhibited a round shape with sharp edges (Figure 4(b)). The colonies in the SACS group were less dense and showed single cells and small groups of cells in loose contact with the colony (Figure 4(c,d)).

The number of colonies in the 3rd, 7th, 10th and 14th days after culture was higher in the SACS group rather than gelatin group. There was a significant difference between two groups on day 3 ($p < .05$) and on days 7, 10 and 14 after culture ($p < .01$). The mean ± SEM of the SACS and gelatin groups were 31.25 ± 7.06 vs. 1 ± 0.57, 21.5 ± 3.28 vs. 1.3 ± 0.83, 20.16 ± 3.92 vs. 1.33 ± 0.83, 12.37 ± 2.0 vs. 1.5 ± 0.76, respectively (Figure 5(a)).

The maximum diameter of colonies was shown in gelatin-coated plate group on day 14 of culture but no significant difference was noted between this group and SACS on different days of culture. Despite the significant difference between the number of colonies in SACS and gelatin-coated plates on all days, no difference was noted among the two groups for diameter of colonies on days 3, 7, 10 and 14, as far as the diameter of colonies is concerned ($p > .05$) (Figure 5(b)) (112.39 ± 3.79 vs. 121.60 ± 9.76, 118.28 ± 4.68 vs. 125.83 ± 8.62, 118.74 ± 4.99 vs. 128.68 ± 3.64, 132.52 ± 10.60 vs. 144.33 ± 15.93 SACS and gelatin-coated plates on days 3, 7, 10 and 14, respectively) (Figure 5(b))

In this study, the expression rate of some specific genes (Oct4, Stra8, Scp3, Acrosin) was evaluated on days 7 and 14 after culture in all three groups of SACS, gelatin and control by quantitative real-time PCR. The results showed that a significant difference was noted in the expression of the Oct4 gene on day 7 after culture between SACS and gelatin groups ($p < .01$). The expression of this gene on day 7 after culture in gelatin group was higher than that of the SACS group (the mean expression in the gelatin group was 1.06 ± 0.07 versus 0.35 ± 0.1 in the SACS group). There was no significant difference between SACS and control groups and between gelatin and control groups on day 7 after culture ($p > .05$). (Figure 6).

The results showed that the expression of Stra8 in different culture groups was significantly different between days 7 and 14 in SACS and gelatin groups ($p < .01$). The average expression of this gene on day 7 after the culture is higher than that of day 14 in three groups. The mean of Stra8 expression in the control group was higher on day 7 in comparison to day 14 after culture, however, it was not significantly different ($p > .05$) (Figure 6).

In this study, the expression rate of the meiotic gene of Scp3, as a marker of synaptonemal complex formation in the meiosis process, was investigated in three groups. The results showed that on day 7 after culture, the highest expression of this gene was observed in the SACS group, which is significantly higher than the gelatin group ($p < .01$), but there was no significant difference between the SACS and control groups as well as the gelatin and control groups on day 7 after culture. Also, on the 14th day after culture, the expression of this marker was significantly higher in the SACS group rather than gelatin group ($p < .05$). The mean expression of Scp3 gene on day 14 in the SACS group was 3.26 ± 0.16 and in gelatin group was 0.94 ± 0.01 (Figure 6).

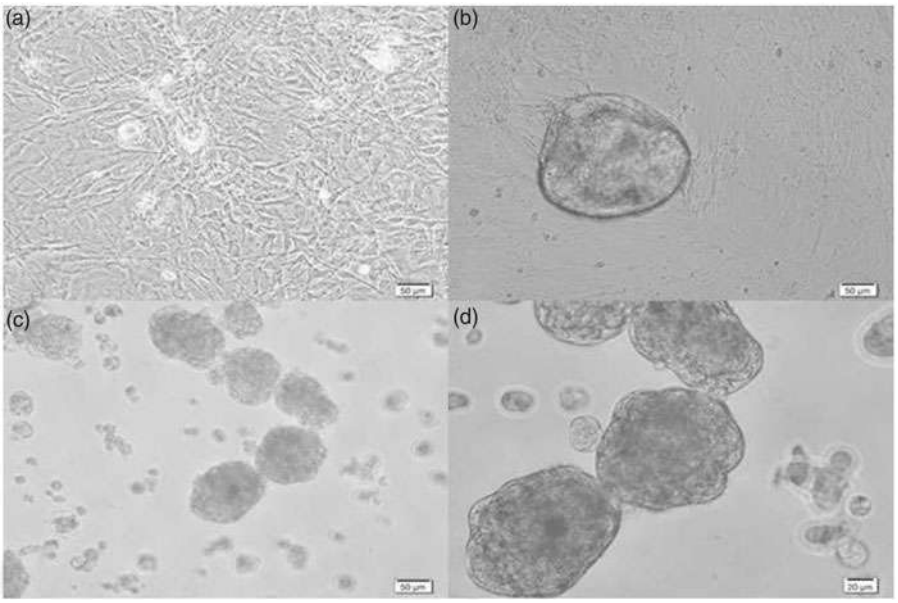

Figure 4. The SSCs colonies in the control, gelatin and SACS groups on day 14 after culture. (a) Control group, (b) gelatin group and (c,d) SACS group. As seen, no colonies were observed in the control group and only single cells and small clusters of SSCs were observed in this group. Magnification: 50 and 20 micrometers.

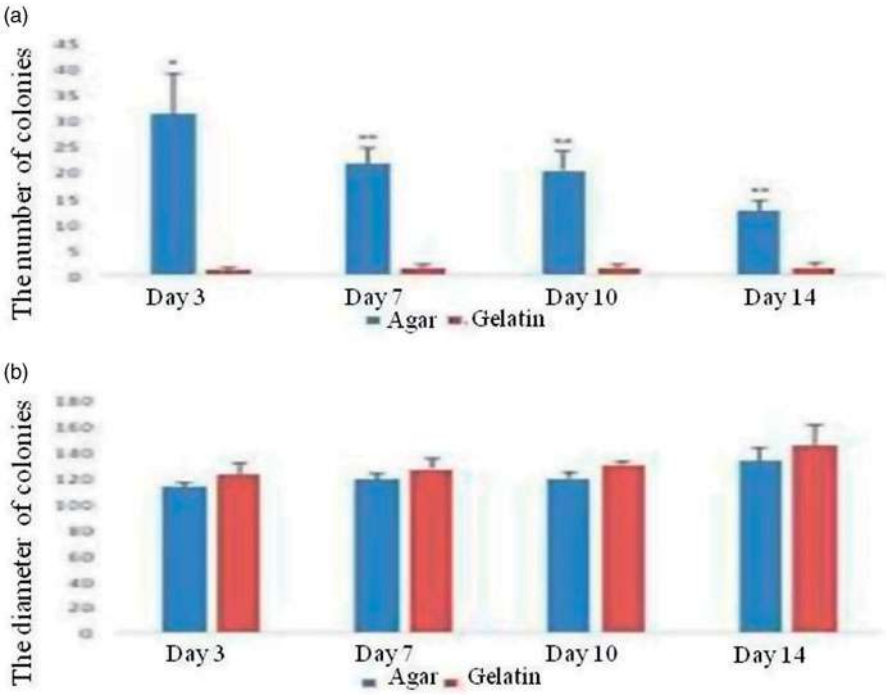

Figure 5. The comparison of the number and diameter of colonies in SACS and gelatin-coated plate groups (*$p < .05$), (**$p < .01$). As it is observed in the figure, there is no significant difference in diameters of colonies between two groups during 14 days.

The results of expression of post-meiotic genes of Acrosin in all three groups showed that, in the SACS group, the expression of this gene increased rather than the other two groups on the 14th day after culture. The expression of this gene in the SACS group increased on day 14 after culture compared to day 7 ($p < .01$). The highest expression of Acrosin gene was observed after 14 days of culture in the SACS group compared to other two groups. However, there is also an increase in the expression of this gene in control and gelatin groups, but the increase of this gene expression in the SACS group is more pronounced after 14 days of culture (Figure 6).

In this study, spermatogonial stem cell colonies on the gel phase of SACS were also examined by SEM. The results showed that spermatogonial cells were located in a three-dimensional arrangement and stem cells connection was clearly visible in the colonies of these cells (Figure 7).

Discussion

As indicated in the study, after isolation of cells from testicular tissue of non-obstructive azoospermia patients, they were cultured in DMEM in the presence of the 10% fetal bovine

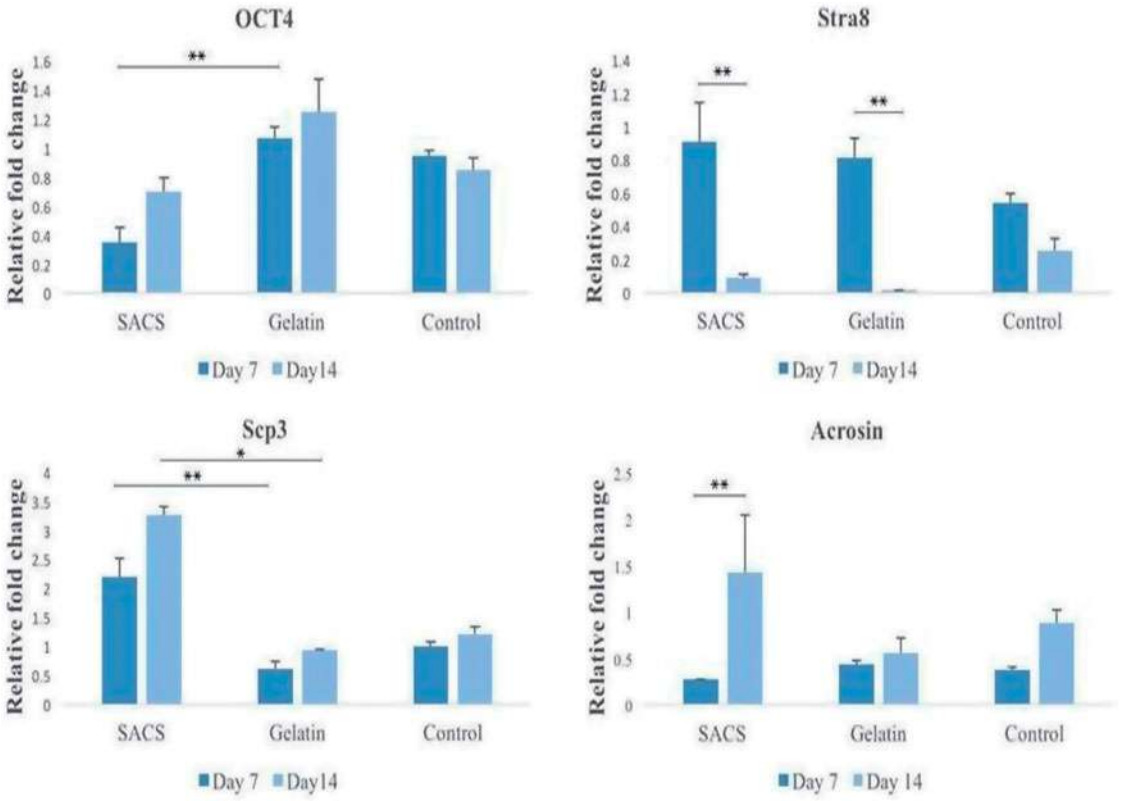

Figure 6. The relative fold change of Oct4, Stra8, Scp3 and Acrosin genes expression in spermatogonial cells that cultured in SACS, gelatin-coated plate and control groups. Notes: Values are mean ± SEM. *indicated statistically significant difference ($p < .05$), **indicated statistically significant difference ($p < .01$).

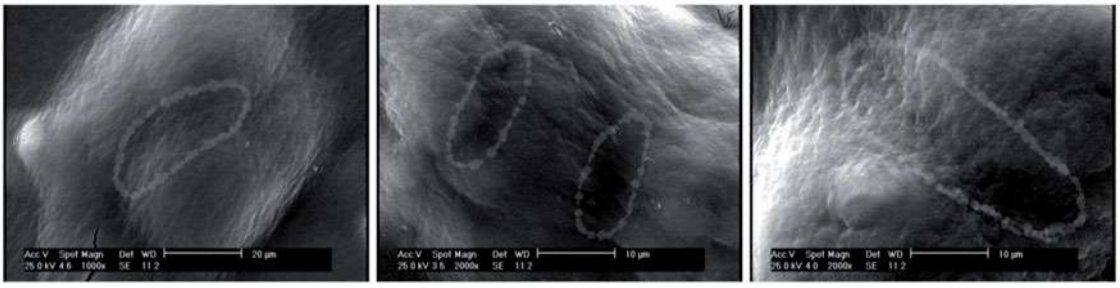

Figure 7. Connection between stem cells in SSCs colonies in SACS group. The dashed lines in the image show the connection of two cells in the colony.

serum,1% Pen/Strep and 5% CO_2 in a humidified atmosphere for 3 weeks to increase the number of cells. In this course, several colonies with different numbers and diameters appeared in the culture system. Positive immunocytochemistry reaction for two antibodies of GFR-α1 and ITGα6 confirmed the presence of SSCs in the culture system.

The expression of Oct4 was only observed in these cells by real-time PCR technique, but the expression of Stra8, SCP3, and Acrosin genes was not observed 3 weeks after culture. This indicates that the cells were not differentiated after three weeks of culture and most of them were spermatogonial stem cells. On the other hand, according to the study of Aponte et al. (2006), during the first week of culture, many, probably differentiating A spermatogonia entered apoptosis while others formed pairs and chains of A spermatogonia. The colonies started to appear that increased in size with time after 1 week of culture. Numbers of single (A_s) and paired (A_{pr}) spermatogonia were significantly higher in culture system on days 15 and 25 and the ratio of A_s to A_{pr} and spermatogonial chains (A_{al}) was also higher indicating enhanced self-renewal of the SSCs [17]. Koruji et al. (2006) also showed that during different passages, most of the differentiated testicular cells that were at different stages of differentiation will be removed by changing the culture medium. So, the remaining cells are undifferentiated spermatogonial stem cells [18].

After three weeks of culture, the cells were isolated and cultured in three different groups (SACS, gelatin and control) in the presence of 15% FBS accompanied by 50% conditioned medium in addition to 50% low-glucose DMEM supplemented by 1 μM retinoic acid (RA) for 2 weeks. Then, the colony formation and differentiation of spermatogonial stem cells of non-obstructive azoospermia patients were investigated in three groups. Various studies have reported the differentiation of spermatogonial cells in mice, but the functional characteristics of these cells in other mammalian species have not yet been determined.

In this study, for the first time, the number and diameter of human SSCs colonies was compared in three groups,

under induction of RA, conditioned medium of OA patients and secretion of Sertoli cells on days 3, 7, 10 and 14 after culture. The nature of Sertoli cells was proven by immunocytochemistry of FSH receptor in the Sertoli cells. In agreement with previous studies, our results indicated that the Sertoli cells were positive for FSH receptor [19]. Vimentin is a cytoskeleton filament which is found in epithelial cells. It is expressed in Sertoli cells from the 14th day of the fetus [20]. Our results indicated a high percentage of positive vimentin cells in Sertoli cells.

Geens et al. (2011) identified that the conditioned medium of Sertoli cells included inductive factors that can represent an origin for *in-vitro* differentiation of germ cells [21]. Based on the study of Ma et al. [4], the mRNA expression of SCF, GDNF and BMP4 was significantly lower in Sertoli cells of NOA patients compared with OA patients.

Microarray analysis also showed that there were lower levels of expression of numerous genes (fold change), including ACAN (aggrecan), AIM1 (absent in melanoma), ANO4 (anoctamin 4), ATRNL1 (attractin-like 1), BDKRB1 (bradykinin receptor B1), CLIC2 (chloride intracellular channel 2), EMB (embigin), EPB41L3 (erythrocyte membrane protein band 4.1-like 3), FGL2 (fibrinogen-like 2), FLJ16171, HSPB8 (heat shock 22 kDa protein 8), IL7 (interleukin 7), MAP2(microtubule-associated protein 2), PAPPA2 (pappalysin 2) and SMC2 (structural maintenance of chromosomes 2) in Sertoli cells of NOA patients compared with OA patients.

In their study, protein expression of SCF, BMP4 and GDNF was also compared in OA and NOA patients. Immunofluorescence revealed that SCF, BMP4 and GDNF proteins were expressed at lower levels in Sertoli cells of NOA patients compared to Sertoli cells of OA patients. Furthermore, Western blots demonstrated that SCF, BMP4 and GDNF proteins were expressed at significantly lower levels in Sertoli cells of NOA patients.

Based on the previous studies, SCF, GDNF and BMP4 regulated the self-renewal and differentiation of SSCs *in vitro* and *in vivo* [22,23]. It has been demonstrated that SCF induces a mouse spermatogonial cell line to differentiate into meiotic spermatocytes and haploid round spermatids, as demonstrated by the formation of a synaptonemal complex and acrosome-like structure, respectively [4].

Generally, the significantly lower or loss of transcripts and proteins of SCF, GDNF and BMP4 may cause abnormal SSC self-renewal and differentiation and eventually contribute to the deficient niche of SSCs in NOA patients. Therefore, in the present study, the conditioned medium of testicular cells belonging to OA patients was used for colony formation and differentiation of NOA SSCs in culture systems.

According to the results of this study, the number of colonies in SACS group was significantly higher than gelatin-coated plates group, which further corroborates the superiority of 3D over 2D culture systems is concerned [24,25]. One possible explanation is that there are many pores in soft agar system that mimic the testis environment and provide a basal, intraepithelial, and adluminal compartment to develop male germ cells. It possibly has better performance in imitating germ-Sertoli cells contact. It stimulates colony formation of SSCs during culture. These results are in agreement with those of Lee et al. that showed the re-aggregation of testicular cells in 3D collagen gel matrix reestablished close contact between germ cells and Sertoli cells [26]. In other words, spermatogenesis requires complicated autocrine and paracrine regulation, as well as direct cell to cell interactions [27].

In this study, a concentration of 10^{-6} M RA was used to stimulate the proliferation and differentiation of spermatogonial cells. According to previous studies, retinoids play an important role in the proliferation of germ cells. Retinoids are dose-dependent materials and a concentration of 10^{-6} M RA is needed to maintain spermatogenesis. RA with higher concentrations of 10^{-6} M inhibits cell proliferation and reduces meiosis process quantitatively [28]. In fact, the expression of Stra8, which is essential for spermatogenesis, is directly related to the presence of RA [29].

In contrast to our study, the study of Eslahi et al. (2013) showed that the number of mouse SSCs colonies significantly decreased in the first and third weeks of culture when cultured on poly-L-lactic acid (PLLA) nanofiber in comparison with the control group [30]. In the study of Navid et al. (2017), the number of mouse spermatogonial colonies significantly increased in SACS culture system compared to two-dimensional culture system. Increasing the number of colonies in our study is in agreement with the study of Navid et al. [31].

This study demonstrated that the diameter of colonies in SACS group was not significantly higher than the gelatin-coated plate group. One possible explanation is that SACS has only an important effect on increasing colony numbers in this timeframe; if agar concentration changes, the size of the pore in soft agar may change and the colonies may have more space to increase their diameter. It also might be that increasing the timeframe has a positive effect on the diameter of the colonies. In contrast, the results of Elhija et al. (2012) confirmed the presence of mouse spermatogonial cell colonies at different sizes on days 14 and 28 after culture on SACS group [24]. In the study of Navid et al., the diameter of colonies significantly increased in SACS culture system compared to two-dimensional culture system [32].

In this study, the expression of some genes was also evaluated after the culture. The results showed that Oct4 gene expression as a nuclear marker of undifferentiated cells was significantly lower in SACS group on day 7 after the culture compared to gelatin group. This decrease was observed on the 14th day after culture in the SACS group compared to the other two groups.

In previous studies, it has been shown that retinoic acid (RA) has a direct or indirect effect on the transcription of Oct4 gene. There is the relationship between induced differentiation of RA and down-regulation of Oct4 in the embryonic cell (EC) and early stem cell (ES) [32–34]. Since, in this study, the retinoic acid as a differentiation factor has been added to all groups, the low-level expression of Oct4 in the SACS group may be explained that RA is more effective in this group and like the *in vivo* condition, the cells responded to this factor faster. Subsequently, the expression of Oct4 decreased in this group 7 days after the culture in comparison to gelatin and control groups. After 14 days of culture, the cells more self-renewed, proliferated and the expression

of Oct4 increased in this group. Increasing of Oct4 in the SACS group is lower than the other two groups. It may be that in this group, the cells are removed from pluripotency and go toward differentiation. Comparison of differentiation genes between three groups can be a clear explanation of this issue.

According to previous studies, Stra8 gene promotes the entrance of spermatogonial cells to prophase I of meiosis during spermatogenesis [34]. Considering the similarity of expression pattern and homology of amino acid sequences between mice and humans, probably human Stra8 also plays similar roles in human spermatogenesis [35,36]. In this study, the highest expression of Stra8 gene was observed in the SACS group 7 days after the culture. This is due to the effect of RA on spermatogonial cells in a 3D culture system, similar to *in vivo* condition, in which many cells start meiosis. This study was in agreement with the study of Snyder et al. (2010) that showed RA increases the expression of known differentiation factors (Stra8, Kit) and reduces the expression of Oct4 that associated with undifferentiated germ cells [37]. The results of this study showed that on the 14th day after culture, the expression of Stra8 significantly decreased in two groups of SACS and gelatin. It may indicate that after 7 days of culture, the cells may start to enter into meiosis phase. Thus, on the 7th day, Stra8 expression increased in both SACS and gelatin groups, but on the 14th day after culture, the number of meiotic cells increased, so stra8 expression in these two groups decreased and the cells can be prepared for the next stage of meiosis. It can conclude that most of the cells are likely to enter the meiosis until the 14th day after culture and the expression of Stra8 decreases. It is also observed in the control group, although it is not significant compared to day 7.

The results of this study showed that the expression rate of Scp3 gene on the 7th and 14th day after culture was higher in the SACS group rather than gelatin and control groups. There is a significant difference in the SACS group in comparison to the gelatin group. This means that the speed of meiosis division is higher in the SACS group and following that, more cells are rapidly entering the next stages of primary spermatocyte divisions (pachytene, dipoltene and diakinesis). The expression of Scp3 gene on day 14 after culture was significantly higher in SACS group rather than gelatin group. It can be argued that the cells in the three-dimensional SACS culture system have more efficiency for starting meiosis division between days 7 and 14.

Acrosin is a serine protease that is used as a marker for spermatogenesis and it is in the acrosome of sperm as an enzyme passive zymogen. Acrosin gene is expressed in both pachytene spermatocyte and round spermatid [38]. In this study, the highest expression of Acrosin gene was observed on day 14 after culture in SACS group, which was significantly higher than that of day 7. Although the expression of Acrosin was higher on day 14 after culture in both gelatin and control groups than day 7, there was no significant difference between two days in two groups. The study of Ventela et al. (2000) showed that low expression of Acrosin in mice was seen in pachytene spermatocytes and after meiosis completion, Acrosin is aggregated in the cytoplasm and lead to strong expression in round spermatid [39]. So, higher expression of Acrosin in SACS group is probably related to a higher number of round spermatid on day 14.

In fact, it can be concluded that the cells in the SACS group started to develop faster during the time of this study. The high expression of Acrosin on this day in the SACS group may be due to the fact that the 3D culture system is more appropriate than other two groups for differentiation of spermatogonial stem cells. The results obtained in this study are in line with the results of Goharbakhsh et al. (2013), which showed that two-dimensional gelatin cannot support spermatogonial stem cell clusters in long-term culture [40]. It can be concluded that, with the support of a medically conditioned medium derived from Sertoli cells of obstructive azoospermic patients, RA and the three-dimensional SACS (due to its greater similarity to the testicular structure in the body), a higher level of SSCs differentiation is observed compared to other studied groups.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Establishing the Minimum Effective Dose and Additive Effects of Depot Progestin in Suppression of Human Spermatogenesis by a Testosterone Depot*

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ABSTRACT

Hormonally induced azoospermia induced by weekly im injections of testosterone enanthate provides effective and reversible male contraception, but more practical regimens are needed. Given our previous findings that six 200-mg pellets implanted subdermally produced more stable, physiological T levels and reduced the delivered T dose by more than 50% while maintaining equally effective suppression of sperm output with fewer metabolic side-effects than weekly 200-mg testosterone enanthate injections, we sought in this study to determine 1) whether further dose-sparing could be achieved by lower testosterone doses while maintaining efficacy and 2) the efficacy of adding a depot progestin to a suboptimally suppressive depot testosterone dose as a model depot progestin/androgen combination male contraceptive. Healthy volunteers were randomized into groups (n = 10) who received either of two lower T doses (two or four 200-mg T pellets) or four 200-mg T pellets plus a single im injection of 300 mg depot medroxyprogesterone acetate (DMPA). Two T pellets (400 mg, 3 mg/day) had a negligible effect on sperm output. Four T pellets (800 mg, 6 mg/day) suppressed sperm output between the second to fourth postimplant months; output returned to normal by the seventh postimplant month, although only 4 of 10 men became azoospermic or severely oligozoospermic (<3 mol/L/mL). The addition of a depot progestin markedly increased the extent, but not the rate, of sperm output suppression, with 9 of 10 becoming azoospermic and 10 of 10 becoming severely oligozoospermic. There were no serious adverse effects during the study. Plasma total and free testosterone levels remained within the eugonadal range at all times with each treatment. Plasma epitestosterone was suppressed by all 3 regimens, consistent with a dose-dependent inhibition of endogenous Leydig cell steroidogenesis. Plasma LH and FSH measured by a two-site immunoassay were suppressed in a dose-dependent fashion by T and further suppressed by the addition of DMPA. Sex hormone-binding globulin levels were decreased by DMPA, but not by either T dose. Prostate-specific antigen and lipids (total, low or high density lipoprotein cholesterol, and triglycerides) were not significantly changed in any group. Thus, a depot testosterone preparation with zero order release must be delivered at between 6–9 mg/day to provide optimal (but not uniform) efficacy at inducing azoospermia. The addition of a single depot dose of a progestin to a suboptimal testosterone dose (6 mg/day) markedly enhances the extent, but not the rate, of spermatogenic suppression, with negligible biochemical androgenic side-effects. These findings provide a basis for the use of a progestin/androgen combination depot for hormonal male contraception. (*J Clin Endocrinol Metab* **81:** 4113–4121, 1996)

HORMONAL contraception for males aims to reduce sperm output reversibly by inhibition of pituitary gonadotropin secretion, which, in turn, depletes intratesticular testosterone and arrests spermatogenesis. Two major multicenter WHO studies that used as the prototype testosterone regimen, weekly im injections of 200 mg testosterone enanthate (TE), have established that hormonally induced azoospermia or severe oligozoospermia (<3 mol/L/mL) provides highly effective, sustained, and reversible contraception with minimal side-effects for 12 months (1, 2). In those studies, a weekly im injection of 200 mg testosterone enanthate was used as a prototype androgen, but the inconvenience, discomfort, and inflexible pharmacodynamics of TE make it necessary to develop better long acting testosterone depot formulations for practical hormonal male contraceptive regimens. For example, TE injections are often uncomfortable (3), and lower doses or frequency of injections have inferior efficacy in suppression of spermatogenesis. The practical requirement for a longer interinjection interval led to development of new long acting depot testosterone preparations (4–6), but their effects on human spermatogenesis have yet to be determined (7). To fill this gap in knowledge, we have studied the effects of an existing depot testosterone formulation, testosterone pellet implants that have near-ideal depot steady state release properties (8, 9), to determine the likely effects of a depot testosterone formulation either alone or in conjunction with a second gonadotropin-suppressing agent (10). In a previous study we established that use of a depot testosterone formulation allowed achievement of major (>50%) reductions in the delivered testosterone dose while maintaining equally effective suppression of spermatogenesis with similar or fewer metabolic side-effects (11). The testosterone dose used in that first study of testosterone implants (1200 mg; testosterone delivery, 9 mg/day) was arbitrarily selected toward the upper range of doses used conventionally for androgen replacement therapy,

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which also correspond with the normal endogenous daily production rate (3–10 mg testosterone/day). Second generation hormonal regimens for male contraception under consideration include androgen alone or in combination with second gonadotropin-suppressing agents, such as progestins or GnRH antagonists (10). As a testosterone depot is the basis of all such hormonal regimens, the properties of long acting depot testosterone formulations alone or in concert with second agents are, therefore, critical to future strategies for the development of hormonal male contraception. This study then aimed to examine lower testosterone doses spanning the range of normal testosterone production rates to determine in healthy men 1) the minimum testosterone dose still consistent with effective spermatogenic suppression and 2) how effectively a depot progestin would be in augmenting the highest suboptimal testosterone dose.

Subjects and Methods

Study design and procedures

The study aimed to 1) undertake a downward dose range to determine the minimum testosterone dose that could maintain effective spermatogenic suppression and 2) to determine the effects of a depot progestin when it was added to the first suboptimally suppressive testosterone dose.

The study was undertaken in two stages. First, 20 men were randomized into two groups to receive either two or four 200-mg testosterone implants (total dose, 400 or 800 mg; daily release rate, 3.0 or 6.0 mg/day) (8, 9). Once the effects of both of these testosterone doses were evident, a third group of 10 men was recruited to be treated with the testosterone dose (eventually identified as four 200-mg implants) that showed partial suppression of spermatogenesis plus a single im injection of 300 mg depot medroxyprogesterone acetate (DMPA; Depo-Provera, Upjohn, Kalamazoo, MI).

Volunteers provided two baseline sets of semen and blood samples at least 2 weeks apart before hormone administration. Pellets of fused crystalline testosterone (Organon, Sydney, Australia) were implanted subdermally in the lower abdominal wall under local anesthesia as previously described (7, 8). The pellets are composed of crystalline testosterone without excipient, thereby being fully biodegradable and not requiring removal. Subsequently, all volunteers provided monthly semen and blood samples for 12 months. Recovery was defined as the point when sperm density reached pretreatment baseline geometric mean or consistently normal levels (>20 million sperm/mL). The study was approved by the Central Sydney Area Health Service Ethics Review Committee.

Subjects

Healthy men, aged 21–50 yr, who were free of chronic medical illness, not taking regular medication, and having normal testicular function, were recruited by advertisement on noticeboards and in the news media. Exclusion criteria were any history of gonadal dysfunction (including infertility), drug abuse, or abnormalities in medical screening tests. Participants were advised to continue reliable contraception throughout this study if they wished to avoid conception. Controls were age-matched men who were screened in an ongoing study (12) of potential sperm donors (n = 509).

Assays

Semen collected by masturbation was analyzed within 60 min according to methods described in the WHO Semen Manual (13) using a Makler chamber (SEFI-Medical Instruments, Haifa, Israel).

Assays of total and free testosterone were performed as described previously (8, 11, 14, 15). LH and FSH were measured by highly sensitive two-site enzyme immunoradiometric assays (IMX/AXSYM, Abbott, North Chicago, IL) with a detection limit of 0.1 U/L for both LH and FSH. In addition, all samples were reassayed by two-site time-resolved fluoroimmunoradiometric LH and FSH assays (Delfia, Pharmacia, Piscataway, NJ). Prostate-specific antigen and sex hormone-binding globulin (SHBG) were measured by solid phase, two-site immunometric assays (Diagnostic Products Corp., Los Angeles, CA). Inhibin was measured by double antibody RIA using the Monash antibody (no. 1989) and recombinant inhibin for standard and iodination material, as described previously (16, 17). Samples were measured within a single assay whenever possible, and between-assay coefficients of variation ranged from 6–12% for all assays.

Epitestosterone (17α-hydroxyandrost-4-en-3-one) was measured by an in-house liquid phase RIA using an antiserum and tritiated epitestosterone tracer (Wien Laboratories, Succasunna, NJ) with a standard dextran-coated charcoal separation. Plasma was extracted by applying 0.4-mL plasma aliquots to a glass Pasteur pipette column filled with ~a 2-mL bed volume of Extrelut (Merck, Darmstadt, Germany). After plasma had soaked in, extracts were eluted with 3 mL hexane-ethyl acetate (3:2; in 0.5-mL aliquots), which were then combined, dried, and reconstituted in assay buffer. Extraction efficiency was 89%, and results were corrected individually for recovery. Cross-reactivity of the antibody with other androgens was low (testosterone, 0.36%; androstenedione, <1.9%; nandrolone, <0.01%; dihydrotestosterone, 0.12%), the detection limit was 2.7 pg/tube (equivalent to 0.09 nmol/L), and the between-assay coefficient of variation was 8.5%. In validation studies, the mean blood epitestosterone concentration was 2.11 ± 0.05 nmol/L (range, 1.4–2.8) in healthy men without known gonadal disorder (n = 42) and 0.76 ± 0.04 nmol/L (range, 0.44–0.96) in castrate men receiving androgen replacement therapy (n = 13).

Hemoglobin, lipids [total, low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol, and triglycerides], renal (urea and creatinine), and liver function tests (bilirubin, albumin, alkaline phosphatase, and transaminases) were assayed by routine autoanalyzer methods.

Data analysis

Results are expressed as the mean ± SEM. Data were analyzed by multiple or repeated measures ANOVA using BMDP software (version 7 for VAX) or exact categorical analysis using StatXact software (version 3 for Windows) as appropriate. Baseline levels for each variable were defined as the arithmetic mean of all pretreatment samples apart from sperm variables, for which the geometric mean was used. Semen data were cube root transformed, and hormonal data were log transformed where required to normalize distribution and stabilize variance. Severe oligozoospermia was defined as a sperm concentration of less than 3 mol/L/mL. The degree of suppression of sperm output was defined on the basis of the lowest recorded monthly sperm density and related to the geometric baseline sperm output. For time-related variables that did not uniformly reach the end point (*e.g.* gonadotropin recovery), life-table estimates of median time to the end point are reported. Results are reported as the mean and SEM or as two-sided 95% confidence intervals unless otherwise stated.

Results

Subjects

The men entering this study did not differ between groups (n = 10) in age, height, weight, body surface area, body mass index, or testis size (Table 1) and were similar to our ongoing control group (12) of healthy men screened as potential sperm donors (n = 509; data not shown).

Implantation of testosterone pellets was well tolerated. There were 2 extrusion episodes among the 30 procedures in this study, both involving a single pellet extruding from men in the combined treatment group and occurring at weeks 11 and 14 after implantation. There were no discontinuations or serious adverse effects reported by participants or any changes in mood or behavior observed by study personnel. Mild acne was reported by 3 of 10 men receiving 800 mg testosterone and 1 of 10 receiving 800 mg testosterone plus

TABLE 1. Baseline variables

Variables	Testosterone Progestin	400 mg –	800 mg –	800 mg 300 mg	*P*
No.		10	10	10	
Age (yr)		27 ± 2	33 ± 3	31 ± 2	0.261
Ht (cm)		177 ± 2	176 ± 2	179 ± 2	0.776
Wt (kg)		75.5 ± 4.0	74.8 ± 4.0	78.2 ± 3.2	0.796
SBW (% ideal)		105 ± 4	106 ± 5	109 ± 4	0.874
BSA (m^2)		1.92 ± 0.06	1.91 ± 0.05	1.96 ± 0.04	0.737
BMI (kg/m^2)		23.9 ± 1.0	24.0 ± 1.1	24.6 ± 1.0	0.884
Mean testis vol (mL)		23 ± 1	24 ± 1	24 ± 1	0.65
Total testosterone (nmol/L)		20.6 ± 0.9	20.6 ± 1.2	18.2 ± 1.2	0.229
Free testosterone (pmol/L)		369 ± 20	352 ± 27	312 ± 23	0.242
Epitestosterone (nmol/L)		1.42 ± 0.08	1.39 ± 0.08	1.26 ± 0.07	0.317
SHBG (nmol/L)		34 ± 2	35 ± 4	44 ± 4	0.123
LH (IU/L)		4.2 ± 0.4	4.9 ± 0.6	5.0 ± 0.6	0.531
FSH (IU/L)		3.7 ± 0.7	2.5 ± 0.5	4.5 ± 0.7	0.105
Inhibin (pg/mL)		302 ± 54	206 ± 27	176 ± 26	0.074
PSA (ng/mL)		0.83 ± 0.07	0.85 ± 0.14	0.90 ± 0.13	0.920
Urea (mmol/L)		5.6 ± 0.5	5.3 ± 0.4	5.7 ± 0.5	0.850
Creatinine (nmol/L)		96 ± 3	85 ± 3	91 ± 3	0.065
Hemoglobin (g/L)		145 ± 2	146 ± 3	150 ± 2	0.411
Total cholesterol (mmol/L)		4.8 ± 0.3	4.6 ± 0.3	5.2 ± 0.5	0.500
LDL cholesterol (mmol/L)		2.8 ± 0.3	2.9 ± 0.3	3.2 ± 0.4	0.693
HDL cholesterol (mmol/L)		1.27 ± 0.10	1.31 ± 0.06	1.36 ± 0.09	0.760
Triglycerides (mmol/L)		1.56 ± 0.23	1.30 ± 0.24	1.49 ± 0.49	0.857

Results are expressed as the mean ± SEM.

DMPA. None required any specific treatment for acne. Increased libido at the start of the study was reported by 5 of 10 men in the 800 mg testosterone plus DMPA group and by 1 of 10 men after 800 mg testosterone alone, but only 1 regarded this as troublesome. One man receiving 800 mg testosterone alone felt that he was transiently more aggressive. There were no adverse effects reported in men receiving 400 mg testosterone. All subjects completed the study, and 412 of 420 (98%) semen samples required for primary endpoint evaluation were obtained.

Sperm output

There were no differences in baseline sperm output (overall median, 80 mol/L·mL) among men entering the three groups (Table 2). The lowest testosterone dose (two implants, 400 mg) had a minimal effect on sperm output, and none became azoospermic (Fig. 1).

The higher testosterone dose (four implants, 800 mg) alone significantly suppressed sperm output, but significant between-subject heterogeneity was evident, with four men ren-

TABLE 2. Baseline, suppression, and recovery of sperm output

Variables	Testosterone Progestin	400 mg –	800 mg –	800 mg 300 mg	*P*
No.		10	10	10	
Baseline					
Abstinence (days)		2.5 ± 0.4	2.1 ± 0.1	5.0 ± 2.0	0.175
Semen volume (mL)		3.6 ± 0.4	3.1 ± 0.4	3.4 ± 0.3	0.738
Sperm density (mmol/L · mL)		89 ± 13	153 ± 37	80 ± 11	0.074
Total sperm (mmol/L · ejaculate)		329 ± 78	469 ± 126	260 ± 41	0.255
Sperm motility (%)		56 ± 3	60 ± 5	59 ± 5	0.429
Rate of suppression					
Decrease at 1st month (% baseline)		77 ± 12	87 ± 20	16 ± 9	<0.001
Decrease at 2nd month (% baseline)		99 ± 14	37 ± 14	1 ± 0	<0.001
Nadir					
Sperm density (mmol/L · mL)		38 ± 7	21 ± 8	0.1 ± 0.1	<0.001
Sperm density (% baseline)		43 ± 6	23 ± 10	0 ± 0	<0.001
Time (months)		5.1 ± 0.9	2.6 ± 0.2	2.5 ± 0.4	0.006
Azoospermia		0 (0)	4 (40)	9 (90)	<0.001
Oligozoospermia (<3 mmol/L · mL)		0 (0)	4 (40)	10 (100)	<0.001
Recovery					
Time to >50% baseline (months)		2.0 ± 0	5.2 ± 1.1	7.6 ± 0.5	<0.001
Time to >20 mmol/L · mL (months)		2.0 ± 0	3.6 ± 0.5	7.3 ± 0.4	<0.001

Results are expressed as the mean ± SEM. Baseline levels are defined as the arithmetic mean of two pretreatment baseline concentrations for all except the sperm variable, in which the geometric mean was used. Percentages are in *parentheses*.

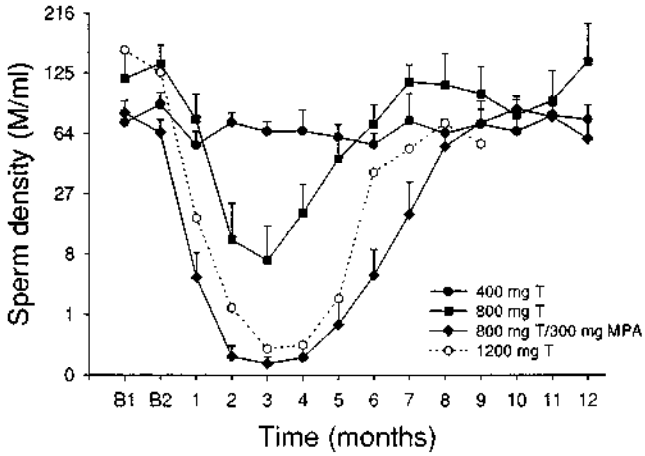

FIG. 1. Time course of sperm output (expressed as sperm density in millions of sperm per mL) before and after implantation of two 200-mg testosterone pellets (400 mg total; *closed circles*), four 200-mg testosterone pellets (800 mg total; *closed squares*), or four testosterone pellets plus depot progestin (800 mg total testosterone plus 300 mg DMPA; *closed diamonds*) in groups (n = 10) of healthy fertile men. For comparison, data from the previous study using six 200 mg (1200 mg total; *open hexagons*) pellets is included. The time of implantation at the start of the study is indicated by the *triangular symbol*. Results expressed as the mean and SEM. Note the cube root transformed scale on the *y*-axis.

dered azoospermic but the other six exhibiting only modest suppression of spermatogenesis. Among men receiving 800 mg testosterone alone, the only significant difference between those who did (n = 4) and those who did not (n = 6) become azoospermic was a lower baseline urea concentration (4.3 ± 0.5 *vs.* 6.0 ± 0.5; $P = 0.046$), but not in any other baseline anthropometric, seminal, hormonal, or biochemical variables. Men who became azoospermic had significantly lower overall total and free testosterone concentrations, but there was no difference in epitestosterone concentrations or time course of total or free testosterone, epitestosterone, LH, or FSH concentrations according to for men became azoospermic *vs.* those who did not (azoospermia × time interaction, $P > 0.05$).

The combination of 800 mg testosterone with 300 mg DMPA caused a striking fall in sperm output, with 9 of 10 reaching azoospermia and all reaching severe oligozoospermia (<3 mol/L/mL). In the two groups receiving 800 mg testosterone, the nadir of sperm output was reached at 2–3 months, with marked suppression lasting for ~3 months followed after the 4th month by a gradual return in sperm output toward normal and reaching baseline levels in the 10th month but without overshoot. Essentially identical patterns were observed whether expressed as concentrations or total output of motile or all sperm. The study provided a power of more than 90% to reject each of the following hypotheses that 1) 800 mg testosterone alone would induce azoospermia uniformly (100%), and 2) the addition of DMPA had no effect on induction of azoospermia.

Reproductive hormones

After all treatments, blood testosterone concentrations remained within the eugonadal ranges for total (10–35 nmol/L) and free (170–510 pmol/L) testosterone throughout the study (Fig. 2). There were, however, significant differences between treatments in the time course of blood total and free testosterone (treatment × time interactions, $P < 0.001$). Total and free testosterone increased modestly after 800 mg testosterone alone, but both decreased after 800 mg testosterone plus 300 mg DMPA, whereas 400 mg testosterone had no consistent effect on testosterone concentrations over time.

Epitestosterone concentrations were significantly reduced by all three treatments in a dose-dependent fashion (Fig. 2). Only in men receiving 800 mg T plus 300 mg DMPA were epitestosterone concentrations consistently suppressed to levels comparable to those in castrate men. Testosterone dose as well as DMPA administration significantly decreased epitestosterone concentrations. The median time to recovery to baseline epitestosterone levels did not differ significantly between groups (overall median 7.4 months, $P = 0.87$).

Plasma LH concentrations were significantly reduced by the first month and remained suppressed for 3 months with all three treatments (Fig. 3). Both the extent and duration of inhibition as well as rate of recovery were dose dependent (Table 3). Undetectable LH levels were observed in 0 (400 mg

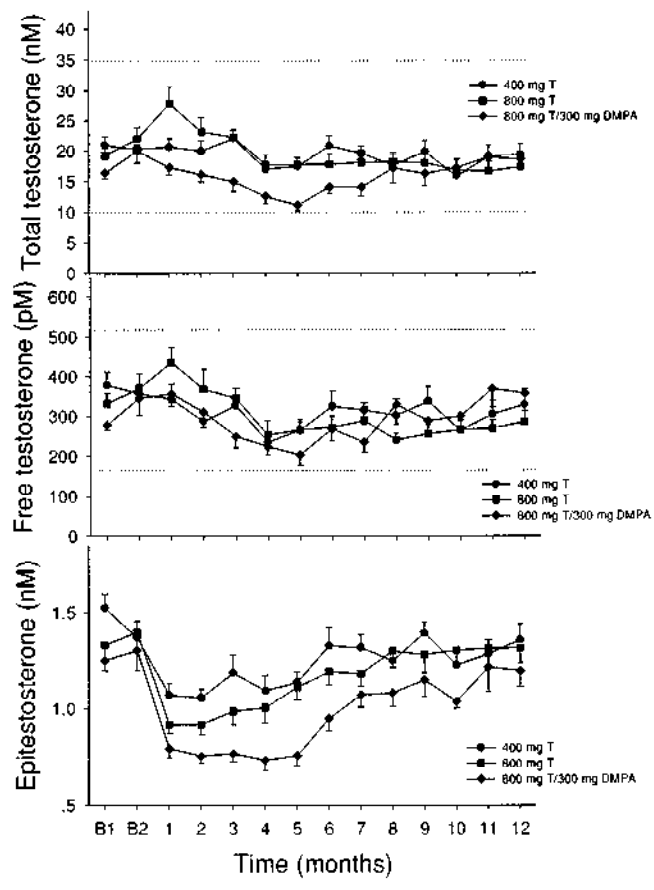

FIG. 2. Plasma total (*upper panel*) and free (*middle panel*) testosterone and epitestosterone (*lower panel*) before and after implantation of two 200-mg testosterone pellets (400 mg total; *closed circles*), four 200-mg testosterone pellets (800 mg total; *closed squares*), or four testosterone pellets plus depot progestin (800 mg total testosterone plus 300 mg DMPA; *closed diamonds*) in groups (n = 10) of healthy fertile men. The eugonadal range is indicated by the *horizontal dashed lines*. Results are expressed as the mean and SEM.

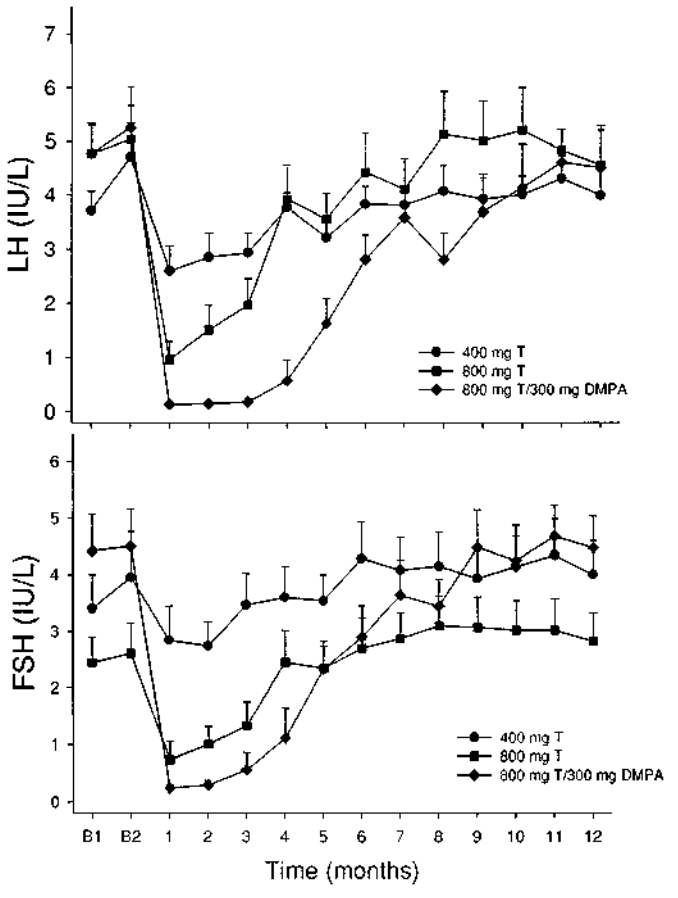

FIG. 3. Plasma LH (*upper panel*) and FSH (*lower panel*) before and after implantation of two 200 mg testosterone pellets (400 mg total; *closed circles*), four 200-mg testosterone pellets (800 mg total; *closed squares*), or four testosterone pellets plus depot progestin (800 mg total testosterone plus 300 mg DMPA; *closed diamonds*) in groups (n = 10) of healthy fertile men. Results are expressed as the mean and SEM.

T), 3 (800 mg T), and 20 (800 mg T plus 300 mg DMPA) of the blood samples taken at weeks 4 (5 samples), 8 (7 samples), 12 (6 samples), 16 (4 samples), and 20 (1 sample). There was no evidence of LH rebound during recovery. Similar findings were confirmed using the Delfia LH assay (data not shown).

Plasma FSH concentrations were significantly reduced in the first month in both groups receiving 800 mg testosterone, but not in the 400 mg testosterone group (Fig. 3). Both the extent and duration of inhibition as well as the rate of recovery were dose dependent, remaining suppressed for 3 months by testosterone alone and for 4 months with the addition of DMPA treatment (Table 3). Undetectable levels were observed in 0 (400 mg testosterone), 3 (800 mg testosterone), and 2 (800 mg testosterone plus 300 mg DMPA) of the blood samples taken at weeks 8 (one sample) and 12 (four samples). There was no evidence of FSH rebound during recovery. Similar findings were confirmed using the Delfia FSH assay (data not shown).

Inhibin concentrations were decreased in a dose-dependent manner (73 ± 10%, 51 ± 9%, and 27 ± 4% of baseline inhibin levels), with a nadir at 3 months and subsequent recovery (Fig. 4). SHBG concentrations were significantly reduced by DMPA administration, but not by either testosterone dose (Fig. 4).

To determine whether the effects of DMPA on testosterone could be explained by the reduced SHBG levels, the greater inhibition of LH levels, or other effects, we examined the effects of DMPA on total testosterone concentrations using either concurrent SHBG or LH levels as covariates. Adjustment for either covariate, however, had little influence on the DMPA effect on the time course of testosterone, which remained highly significant (treatment × time interactions, $P < 0.0001$).

Metabolic effects of testosterone

There were no significant effects of either testosterone dose or DMPA on prostate-specific antigen (Fig. 4), cholesterol fractions (total, LDL, and HDL), or triglycerides (Fig. 5). There were no significant effects of testosterone treatment on any routine biochemical variable, including electrolytes, glucose, phosphate, liver (bilirubin, alkaline phosphatase, and transaminases) or renal (creatinine, uric acid) function tests, or hematological variables (hemoglobin, leukocytes, or platelets). There was no evidence of hepatotoxicity observed.

Discussion

Testosterone implants provide the first opportunity to systematically test the effects of steady state administration of exogenous testosterone on normal human spermatogenesis. These implants provide near zero order release kinetics, ensuring stable dose-dependent testosterone levels within the physiological range for up to 6 months after a single subdermal implantation (8). The characteristics of the spermatogenic suppression with this true testosterone depot should reliably predict the suppression achievable with other depot testosterone formulations, such as testosterone microcapsules (6) or testosterone buciclate (5), both of which have significantly shorter durations of action. We previously showed that the implantation of six 200-mg testosterone pellets suppressed sperm output to the same extent as weekly 200-mg TE injections, whereas daily testosterone exposure was lowered by more than 50%, blood testosterone levels were reduced to remain within the physiological range, and some, but not all, metabolic effects of testosterone were reduced (11). The testosterone dose used in that first study of testosterone implants (1200 mg) was arbitrarily selected toward the upper range of doses used conventionally for androgen replacement therapy (9), and its daily delivery rate of testosterone (9 mg/day) also corresponds with the upper limits of normal endogenous testosterone daily production (3–10 mg/day). These results prompted the present downward dose-ranging study to determine the minimum testosterone dose that could maintain optimal spermatogenic suppression.

This study now identifies the limits of a testosterone depot in the suppression of human spermatogenesis when used alone. We found that a testosterone implant dose of 800 mg (four 200-mg implants), releasing 6 mg testosterone/day, when administered alone achieves inadequate suppression of spermatogenesis for a hormonal male contraceptive. A still lower dose (2 200-mg implants, 3 mg testosterone/day) has

TABLE 3. Suppression and recovery of gonadotropins

Variables	Testosterone Progestin	400 mg –	800 mg –	800 mg 300 mg	*P*
No.		10	10	10	
LH					
Baseline (IU/L)		4.2 ± 0.4	4.9 ± 0.6	5.0 ± 0.6	0.531
Nadir (IU/L)		1.8 ± 0.2	1.0 ± 0.3	0.1 ± 0.02	<0.001
Nadir (% of baseline)		44 ± 4	17 ± 5	2 ± 1	<0.001
Time of nadir (months)		2.5 ± 0.8	1.1 ± 0.1	1.8 ± 0.4	0.158
Recovery (IU/L)		4.0 ± 0.4	4.9 ± 1.8	4.7 ± 0.8	0.626
Recovery (% of baseline)		104 ± 12	101 ± 7	92 ± 8	0.679
Median time to recovery (months)		3.9 ± 1.0	9.0 ± 1.6	10.8 ± 0.6	<0.001
FSH					
Baseline (IU/L)		3.7 ± 0.7	2.5 ± 0.5	4.5 ± 0.7	0.105
Nadir (IU/L)		2.4 ± 0.5	0.7 ± 0.3	0.2 ± 0.1	<0.001
Nadir (% of baseline)		69 ± 4	26 ± 8	4 ± 1	<0.001
Time of nadir (months)		2.3 ± 0.7	1.4 ± 0.2	2.1 ± 0.3	0.351
Recovery (IU/L)		4.6 ± 0.6	3.2 ± 0.5	5.0 ± 0.6	0.096
Recovery (% of baseline)		131 ± 7	136 ± 8	121 ± 17	0.627
Median time to recovery (months)		3.4 ± 0.7	5.1 ± 0.5	9.5 ± 0.8	<0.001

Results are expressed as the mean ± SEM. Baseline levels are defined as the arithmetic mean of two pretreatment baseline concentrations. Recovery levels are defined as the mean of observations at the last three time points (posttreatment months 10–12).

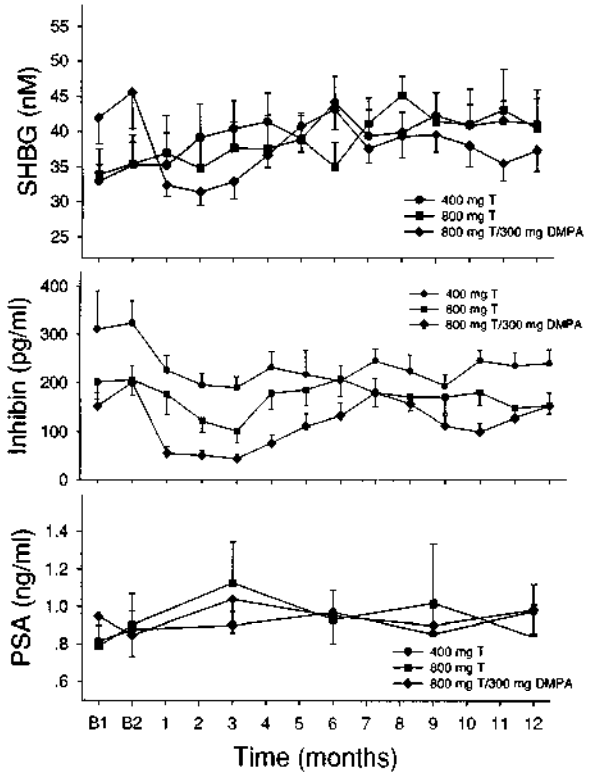

FIG. 4. Plasma SHBG (*upper panel*), inhibin (*middle panel*), and prostate-specific antigen (*lower panel*) before and after implantation of two 200-mg testosterone pellets (400 mg total; *closed circles*), four 200-mg testosterone pellets (800 mg total; *closed squares*), or four testosterone pellets plus depot progestin (800 mg total testosterone plus 300 mg DMPA; *closed diamonds*) in groups (n = 10) of healthy fertile men. Results are expressed as the mean and SEM.

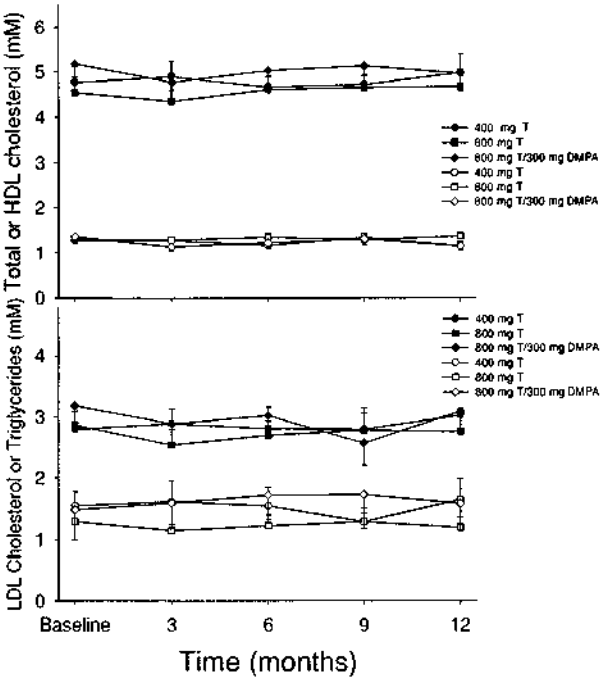

FIG. 5. Plasma total (*filled symbols*), HDL cholesterol (*open symbols*; *upper panel*), LDL cholesterol (*filled symbols*), and triglycerides (*open symbols*; *lower panel*) before and after implantation of two 200-mg testosterone pellets (400 mg total; *circles*), four 200-mg testosterone pellets (800 mg total; *squares*), or four testosterone pellets plus depot progestin (800 mg total testosterone plus 300 mg DMPA; *diamonds*) in groups (n = 10) of healthy fertile men. Results are expressed as the mean and SEM.

negligible effects on sperm output, but produces significant, although submaximal, suppression of gonadotropins and epitestosterone. Our present findings with the 800-mg testosterone dose (four 200-mg implants) alone are strikingly similar to those reported using a single im injection of 1200 mg testosterone buciclate, a novel testosterone ester containing 760 mg testosterone, which produced azoospermia in 3 of 8 healthy men, but minimal spermatogenic suppression in the remaining volunteers (7). Given the prolonged zero order testosterone release by testosterone buciclate injection (5, 7) and extrapolating our previous findings, it can be expected that higher testosterone buciclate doses would improve spermatogenic suppression, but still not provide uniform

azoospermia. Although our findings may be reliably extrapolated to other true testosterone depots (such as testosterone microspheres), they may not apply to synthetic androgens, particularly those metabolically different from testosterone by virtue of restricted activation by aromatization and/or 5α-reduction (18). Extrusions of a single implant were observed in only 2 men among 30 participants in this study, a rate consistent with that of pellet extrusions among hypogonadal men (5–7%) (Handelsman, D. J., unpublished observations). As these 2 men became azoospermic and had no evidence of androgen deficiency, it is unlikely that these extrusions materially affected our findings.

This study is the first demonstration of synergism between a depot progestin and a depot androgen in suppressing human spermatogenesis. We observed markedly greater suppression of sperm output by the addition of a depot progestin to a depot androgen. Although many previous studies have examined various combinations of oral or parenteral progestins with androgens (19), none combined depot formulations of each agent or, until recently, involved controlled prospective comparisons (20, 21). Two recent prospective controlled studies have shown that daily ingestion of an oral progestin augments the spermatogenic suppression produced by weekly injections of 100 mg TE (20, 21) consistent with our findings. One study claimed an acceleration of spermatogenic suppression (20) that we did not observe. The apparent acceleration observed by Bebb *et al.*, however, was due to the use of a suboptimal TE dose (100 mg weekly) shown previously to provide slower, less durable, and less reliable spermatogenic suppression compared with the conventional TE dose of 200 mg weekly (1, 2, 22, 23). Furthermore, a difference of a few weeks in time to adequate spermatogenic suppression have minimal practical importance if a waiting period of months is still required. As with vasectomy (24), any contraceptive method that relies on clearance of sperm from the male reproductive tract will feature a delayed onset and offset of action. This may still be well suited to elective use of hormonal male contraception in circumstances such as the postpartum period, delaying vasectomy, and intolerance of female methods. The inconsistent dose-dependent findings with TE (20, 21, 25), contrasting with those of a true testosterone depot, reinforce the relegation of TE to obsolete status for further path-finding studies for hormonal male contraception. Future studies should use more practical and effective depot testosterone formulations.

Although blood testosterone concentrations are useful to monitor Leydig cell activity, during the administration of exogenous testosterone, the mixture of endogenous and exogenous testosterone negates such interpretation. To resolve this difficulty we monitored blood concentrations of epitestosterone, the natural 17-epimer of testosterone, to indicate Leydig cell secretion. Epitestosterone is a Leydig cell product cosecreted with testosterone, thereby constituting a useful indicator of endogenous testosterone production. This is the basis for the use of urinary epitestosterone to detect the administration of exogenous testosterone among athletes. To avoid the inconvenience of 24-h urine collection and the more complex quantitative gas chromatograph/mass spectrometry (GC/MS) assay, we established a RIA for epitestosterone in blood. Epitestosterone concentrations were decreased in a time- and dose-dependent fashion, such that only the combined treatment depressed epitestosterone concentrations to castrate levels, although both testosterone alone doses decreased epitestosterone concentrations proportionally to dose. Furthermore, recovery toward baseline epitestosterone coincided well with the recovery of testicular function. Our findings support the use of blood epitestosterone concentrations as a valid and sensitive marker of Leydig cell steroidogenesis during the administration of exogenous testosterone.

This study again demonstrates the between-subject heterogeneity in suppression of human spermatogenesis by sex steroids. We previously postulated that a minority of healthy men had become severely oligozoospermic, but not azoospermic, after the administration of 1200 mg testosterone (six 200-mg implants) because blood testosterone concentrations may have been high enough to support spermatogenesis, particularly as we recently demonstrated that spermatogenesis may be induced by physiological levels of testosterone in the gonadotropin-deficient *hpg* mouse (26). The present study, however, refutes this hypothesis, as lower testosterone doses were even less (rather than more) effective, although a subgroup of men (4 of 10) still became azoospermic with the lower (800-mg) testosterone dose. This between-subject heterogeneity was not associated with differences in pretreatment SHBG levels (7) or any other measured variable. Blood testosterone concentrations were consistently, but marginally, higher among those who remained oligozoospermic but never azoospermic, although blood epitestosterone, LH, and FSH concentrations did not differ. Whether this is related to the suggested testosterone-induced increase in 5α-reductase activity (27), although men in our study had much lower, more physiological doses of testosterone, remains to be elucidated. The recent identification of an activating mutation of the human FSH receptor leading to persistence of testosterone-independent (and presumably refractory to testosterone-induced suppression) spermatogenesis (28) raises the possibility of a widely distributed genetic polymorphism as a possible mechanism worthy of exploration. This between-subject heterogeneity within as well as between populations (29) remains unexplained, but clarification of its mechanism might explain how uniform azoospermia may be achieved with hormonal regimens for male contraception.

The mechanism of the additive suppression of spermatogenesis by injection of 300 mg DMPA appears to be multifactorial. DMPA decreased SHBG concentrations and augmented inhibition of blood gonadotropin and testosterone concentrations, presumably reflecting direct hepatic and negative feedback hypothalamic effects, respectively. The fall in blood testosterone concentrations, however, was greater than could be accounted for by the DMPA effects on LH and SHBG in the covariance analysis, suggesting the possible importance of a direct inhibitory effect of DMPA on Leydig cell steroidogenesis. Whether these effects are all due to medroxyprogesterone acetate (MPA) action via progestin receptors or also involve MPA (or metabolite) cross-reactivity with androgen receptors remains unclear. The acute lowering of SHBG levels reflects the pharmacokinetic limitations of DMPA as this older progestin depot formulation has non-

zero order release, and the biochemical effects may reflect excessive early peak blood MPA concentrations. Such transient metabolic changes might be obviated by newer depot progestins with more steady state release kinetics, such as levonorgestrel esters or depot formulations. If the DMPA effects are primarily attributable to effects mediated via progestin receptors, other depot progestins should have similar effects, whereas if effects are partly due to androgen or estrogen receptor cross-reactivity, they may not be replicated exactly by other depot progestins. These observations reinforce the view that changes in SHBG or HDL cholesterol, sometimes considered androgenic effects, are actually toxic or excessive hepatic effects of some sex steroids, notably oral 17α-alkylated androgens or progestins, or a high dose of any parenteral sex steroid. Our findings suggest that optimized depot formulations with effective spermatogenic suppression can be developed with minimal or no biochemical effects on lipids, SHBG, or other nonhormonal biochemical end points. In this respect, SHBG is an easily measured indicator suitable for routine monitoring in path-finding studies as a convenient marker of excessive hepatic steroidal effects.

The absence of significant clinical or biochemical adverse effects or discontinuations during this study confirm and extend our previous observations with a higher dose (1200 mg, 9 mg/day) of testosterone implants. These findings illustrate the advantage of using the minimum testosterone doses that still maintain adequate androgen replacement. Based on our experience with hypogonadal men, this would be 800 mg testosterone (6 mg/day), which closely replicates the normal endogenous testosterone production rate. Although such doses provide inadequate suppression of spermatogenesis when used alone, they would provide adequate androgen replacement if another gonadotropin-suppressing agent, such as a progestin or GnRH antagonist, was used concurrently. Further lowering of testosterone doses would provide inadequate androgen replacement, with likely adverse consequences for structure and function of bone, muscle, and other androgen-dependent tissues, including loss of libido. In deciding the relative advantages of androgen alone *vs.* androgen combinations with a second agent, the key issue is the relative safety of reducing testosterone exposure from 9 to 6 mg/day *vs.* the addition of a second gonadotropin-suppressing agent, and the optimal approach remains to be determined (see discussion in Ref 10).

This path-finding study was not designed to resolve the issue of the long term risks and benefits of androgen usage. These considerations will require evaluation of the risks of cardiovascular or prostate disease balanced against the noncontraceptive benefits on bone, muscle, and general anabolic effects during prolonged surveillance over decades, as has been required for female hormonal contraception. Nevertheless, the short term findings in this study are reassuring. The only relevant established cardiovascular risk association in men is that lowered blood testosterone levels are associated with excess cardiovascular risk (30). The absence of lipid changes together with the dose-sparing effect of a steady state depot formulation and maintenance of completely physiological testosterone concentrations throughout the study indicate that testosterone-based male contraceptive regimens with minimal metabolic impact on biochemical variables can be developed. Further study of the influence of physiological doses of androgens and progestins on nonlipid cardiovascular risk factors, such as vascular reactivity (31, 32), also need evaluation. Similarly, the unchanging prostate-specific antigen concentrations are evidence against any change in total prostate size under the conditions pertaining to this study. This supports the strategy that maintaining adequate physiological testosterone concentrations and avoiding excessive or underreplacement dosages may minimize long term cardiovascular or prostate risk from androgen-based hormonal regimens for male contraception.

The present study demonstrates the feasibility and advantages of using a depot progestin/androgen combination for hormonal male contraception. The ongoing public interest and enthusiastic participation in such contraceptive studies signal the motivation and willingness of men to continue to share the burdens as well as the benefits of reliable contraception. If more convenient depot formulations can be made available, the promise of hormonal contraception for men indicated by the WHO studies and the clear community niche for hormonal male methods can be brought into fruitful conjunction.

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Reproductive Biology and Endocrinology

REVIEW

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Follicle-stimulating hormone signaling in Sertoli cells: a licence to the early stages of spermatogenesis

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Abstract

Follicle-stimulating hormone signaling is essential for the initiation and early stages of spermatogenesis. Follicle-stimulating hormone receptor is exclusively expressed in Sertoli cells. As the only type of somatic cell in the seminiferous tubule, Sertoli cells regulate spermatogenesis not only by controlling their own number and function but also through paracrine actions to nourish germ cells surrounded by Sertoli cells. After follicle-stimulating hormone binds to its receptor and activates the follicle-stimulating hormone signaling pathway, follicle-stimulating hormone signaling will establish a normal Sertoli cell number and promote their differentiation. Spermatogonia pool maintenance, spermatogonia differentiation and their entry into meiosis are also positively regulated by follicle-stimulating hormone signaling. In addition, follicle-stimulating hormone signaling regulates germ cell survival and limits their apoptosis. Our review summarizes the aforementioned functions of follicle-stimulating hormone signaling in Sertoli cells. We also describe the clinical potential of follicle-stimulating hormone treatment in male patients with infertility. Furthermore, our review may be helpful for developing better therapies for treating patients with dysfunctional follicle-stimulating hormone signaling in Sertoli cells.

Keywords: Follicle-stimulating hormone, Sertoli cell, Signaling pathway, Spermatogenesis, FSH treatment

Background

Spermatogenesis is a process that is under complex regulation to achieve successive germ cell proliferation and differentiation [1]. Starting from spermatogonia stem cells (SSCs) producing differentiated spermatogonia, differentiated spermatogonia transform into spermatocytes. Spermatocytes undergo meiosis to produce round spermatids in which the chromosome number is reduced from diploid to haploid [2]. Round spermatids then undergo transformation to form the final spermatozoa which are released into the lumen. In mammals, only Sertoli cells and undifferentiated spermatogonia are detected during prepubertal and juvenile periods while spermatogenesis is initiated at puberty when undifferentiated spermatogonia begin to differentiate and enter meiosis [3–5].

As the only type of somatic cell in seminiferous tubule, Sertoli cell (SC) functions as a 'nurse' to care for spermatogenesis via paracrine actions to provide necessary nutrition and factors as well as forming necessary structures such as the blood-testis barrier (BTB) and Sertoli cell-Germ cell adhesion complex [6–9]. In higher vertebrates, spermatogenesis requires hormonal regulation by the hypothalamic-pituitary gonadal axis [10, 11]. Gonadotropin-releasing hormone (GnRH) is synthesized in the hypothalamus and is released into the pituitary gland where it stimulates the secretion of two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing

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hormone (LH) [12]. FSH and LH then enter the circulation system to act on testis. Only undifferentiated spermatogonia and Sertoli cells are present in seminiferous tubule in the absence of FSH and LH [13]. Hormonal regulation of spermatogenesis is important and is mediated indirectly by SCs. Here, we will focus on the function of FSH signaling in spermatogenesis.

FSH is a glycoprotein that plays an essential role in prepubertal preparation for spermatogenesis and pubertal spermatogenesis regulation [14]. Its receptor FSH receptor (FSHR) is exclusively expressed on the cellular membrane of Sertoli cells [15]. In early life of both primates and rodents, physiological role of FSH signaling in spermatogenesis is to stimulate the transcription of genes related to DNA replication and cell cycle progression [16, 17]. Decades of study using the hypogonadal (*hpg*) model [18], FSHβ subunit knockout model [19], GnRH-immunized model [20] and FSHR knockout model [21–23] have revealed pivotal roles for FSH in regulating Sertoli cell function, increasing spermatogonia number, promoting entry into meiosis and limiting overall germ cell apoptosis. Since adult FSHR knockout mice are fertile but exhibit a reduced sperm output and completion of meiosis mainly depends on testosterone action, FSH is suggested to play a dominant role in establishing the most important parameter for testicular development and spermatogenesis prior to puberty in rodents [21, 24–27]. In men, FSH is essential to maintain fertility. Subfertility with quantitatively reduced spermatogenesis will occur in the absence of FSHR function [28] while a mutation in FSHβ subunit leads to azoospermia and infertility [29]. Although studies have provided a better understanding of the spermatogenesis processes that are regulated by FSH signaling, few molecules participating in these regulating activities have been precisely identified. We are also unable to elucidate the exact role of FSH in human spermatogenesis [30]. As a result, a review of the present work about FSH signaling in SCs is necessary and suggestions for future studies should be proposed. Moreover, FSH treatment has the potential to improve sperm number and motility in patients with hypogonadotropic hypogonadism or normogonadotropic patients with idiopathic impairment of spermatogenesis, highlighting the importance of obtaining a better understanding of FSH signaling in humans [31–34]. All of our hard work aims to achieve the 'bench to bedside' translation and cure more patients with FSH signaling dysfunction.

We surveyed articles in PubMed using the following keywords: 'Sertoli cell', 'FSH', 'spermatogenesis', 'Sertoli cell proliferation', 'Sertoli cell differentiation', 'Spermatogonia stem cell self-renewal', 'meiosis', 'Spermatogonia proliferation', 'apoptosis', 'hypogonadotropic hypogonadism', 'normogonadotropic', 'FSH treatment'. We will present this review at the cellular and molecular levels, covering four parts: 1) Sertoli cell proliferation, differentiation and apoptosis; 2) Spermatogonia pool maintenance, differentiation and spermatogonia survival; 3) Entry into meiosis and spermatocyte survival; 4) Potential use of follicle-stimulating hormone in treating male infertility. Experimental species include rats, mice, zebrafish, sheep, bovines, goats, newts, trout and men. Our review is focused on the function of FSH signaling in SCs during the early stages of spermatogenesis.

Overview of follicle-stimulating hormone signaling in Sertoli cell

FSH is a glycoprotein composed of α and β subunits. FSHα is a subunit shared with other glycoproteins, while FSHβ subunit is unique to FSH [35]. FSH exerts its function through the interaction between FSHβ and FSHR [14, 36]. According to a recent analysis of crystal structure, FSHR, which is a heterotrimeric guanine nucleotide–binding proteins (G proteins)-coupled receptor, is composed of a hormone binding domain, hinge region, hairpin loop, seven-transmembrane α helical domains and an intracellular domain [37–39]. The binding of FSH to the FSHR hormone-binding domain leads to a conformational change in FSHR, which facilitates the interaction between residues of FSHα and FSHβ subunit with the residues of the hinge region of FSHR. This interaction will further alter the conformation of seven-transmembrane α helical domains, resulting in the transmission of the signal to the intracellular domain, where coupling to effectors, recruiting adaptor proteins and transmitting FSH signaling downstream happen [39–41]. For more crystal structures, please see a review [39].

FSHR is present in the testis before a significant concentration of hormone appears in the foetal circulation [42]. In both rodents and primates, FSHR expression begins in the second half of gestation [43]. The interaction between FSH and FSHR is important for the function of FSH signaling. The variation of FSH/FSHR interaction at different age depends on the amount of SCs expressing FSHR with respect to those not expressing FSHR. In mice, FSH binding peaks between Days 7 and 21 but decreases significantly between Days 20 and 37 [44]. In rats, the *Fshr* mRNA level increases until Day 7, remains constant for 10 days and then decreases sharply on Day 40 [14]. The initial increase correlates with the proliferation of SCs and the increase of FSHR density per SC. While the sharp decrease correlates with the wide appearance of spermatocytes and spermatids so that the ratio of SCs to germ cells drops per seminiferous tubule. During one cycle of spermatogenesis in rats, FSH binding and *Fshr* mRNA level peak in stages XIII, XIV and I during the early development of germ cells

but reach their lowest levels in stages VII and VIII when germ cells have developed to mature state [45–47]. Based on these observations, it can be concluded that FSH signaling mainly participates in the initiation and early development phases of spermatogenesis.

To date, at least five FSH signaling pathways have been identified in SCs: cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway, extracellular-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, phosphoinositide 3-kinase (PI3K) pathway, calcium pathway and phospholipase A2 pathway. Here we mainly review the first three types of these five types pathways as well as the newly found retinoid acid pathway. Only these four types of pathways are included in Fig. 1. The cAMP/ PKA signaling pathway was the first to be identified. Upon FSH binding to FSHR on the plasma membrane of SCs, FSHR couples to the Gαs subunit to activate adenylate cyclase (AC). Activated AC recruits ATP and transforms it into cAMP [48]. cAMP then binds to the regulatory subunits of PKA to release catalytic subunits of PKA [49, 50]. Catalytic subunits translocate into the nucleus and phosphorylate cyclic AMP response-element binding protein (CREB) at Ser133 or some cAMP-responsive elemental modulators [14]. These factors bind to the cAMP-response element of target genes to regulate their transcriptional activity during spermatogenesis [51]. Additionally, FSH activates ERK/MAPK pathway by coupling to both the Gαi and Gαs subunits in vitro [52]. The interaction of FSHR with the Gαs subunit leads to ERK activation via a cAMP/PKA dependent pathway while the exact pathway mediated by FSHR coupling to Gαi remains to be determined. Moreover, coupling of FSHR to Gβγ also activates PI3K [53, 54]. Activated PI3K triggers the transition from phosphatidylinositol bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3) [55]. Accumulation of PIP3 leads to the phosphorylation of protein kinase B (Akt) and mammalian/mechanistic target of rapamycin (mTOR) [53, 56–59]. Activated mTOR phosphorylates 70-kDa ribosomal S6 kinase (p70S6K) to promote protein translation and gene expression [53, 60, 61]. Beta-arrestin promotes the internalization of FSHR to sustain the prolonged activation of signaling. This internalization is mediated by clathrin proteins [31, 62]. Interestingly, the levels and biochemical characteristics of signaling messengers are stage-specific upon FSH stimulation. The FSH-induced cAMP production level increases from birth to puberty while the FSH-induced PIP3 production level decreases from birth to puberty. Additionally, the p70S6K phosphorylation sites differ between different developmental periods. p70S6K is phosphorylated at T389, T421 and S424 via the cAMP/PKA pathway and PI3K/Akt pathway during proliferating stage but is only phosphorylated at T389 by the cAMP/PKA pathway during differentiating state [53]. The signaling pathway adopted is also stage-specific. For example, FSH mediated ERK activation in vitro was only detected in 5 and 11 day old rats, not in 19 day-old rats. Recently, FSH signaling was linked to retinoic acid (RA) signaling in SCs. FSH stimulates RA synthesis during the postnatal period via cAMP-dependent upregulation of retinol dehydrogenase 10 (RDH10) and aldehyde dehydrogenase 1A1 (ALDH1A1) [63–65]. During the pubertal period, FSH facilitates the translocation of retinoic acid receptor α (RARα) into the nucleus. With the help of cytoplasmic RA-binding protein 2 (CRABP2), RA interacts with the RAR/retinoid X receptor (RXR) heterodimer and binds to RA response elements (RAREs) to regulate gene transcription [66, 67]. Other signaling pathways, including the calcium pathway and phospholipase A2 pathway have been reviewed by other researchers (For reviews, please see [68–70]).

Follicle-stimulating hormone signaling in Sertoli cell regulates early stages of spermatogenesis

Roles of FSH mediated signaling in Sertoli cell proliferation, differentiation and apoptosis

SCs create a microenvironment and provide necessary nutrition for germ cells to complete spermatogenesis. The final number of SCs in adulthood is determined by the proliferation activity during the prepubertal period. SC differentiation during puberty endows SCs with their functions in spermatogenesis. Additionally, SC apoptosis maintains a healthy SC pool. All three processes are regulated by FSH signaling (Fig. 2).

Sertoli cell proliferation

The final number of SCs determines the quality and quantity of spermatogenesis. SC proliferation occurs in the foetal or neonatal period and in the peripubertal period in all species [16]. Decades of studies using decapitation model, FSH antagonist model, transgenic model and FSHR knockdown model have revealed that FSH signaling in SCs is essential for SC proliferation.

FSH regulates SC proliferation only during foetal and early postnatal life. Pioneering works using the [^{3}H] -thymidine assay, which indicates mitosis activation, found that low levels of endogenous FSH decrease [^{3}H]-thymidine incorporation and the final SC number both in vivo and in vitro [71–73]. Injection of human FSH into immature rats with FSH withdrawal restored the mitotic activation and the final SC number [74–77]. In addition, treating *hpg* mice with recombinant FSH or expressing an FSH transgene in *hpg* mice also counteracted the negative effect of FSH deprivation on SC

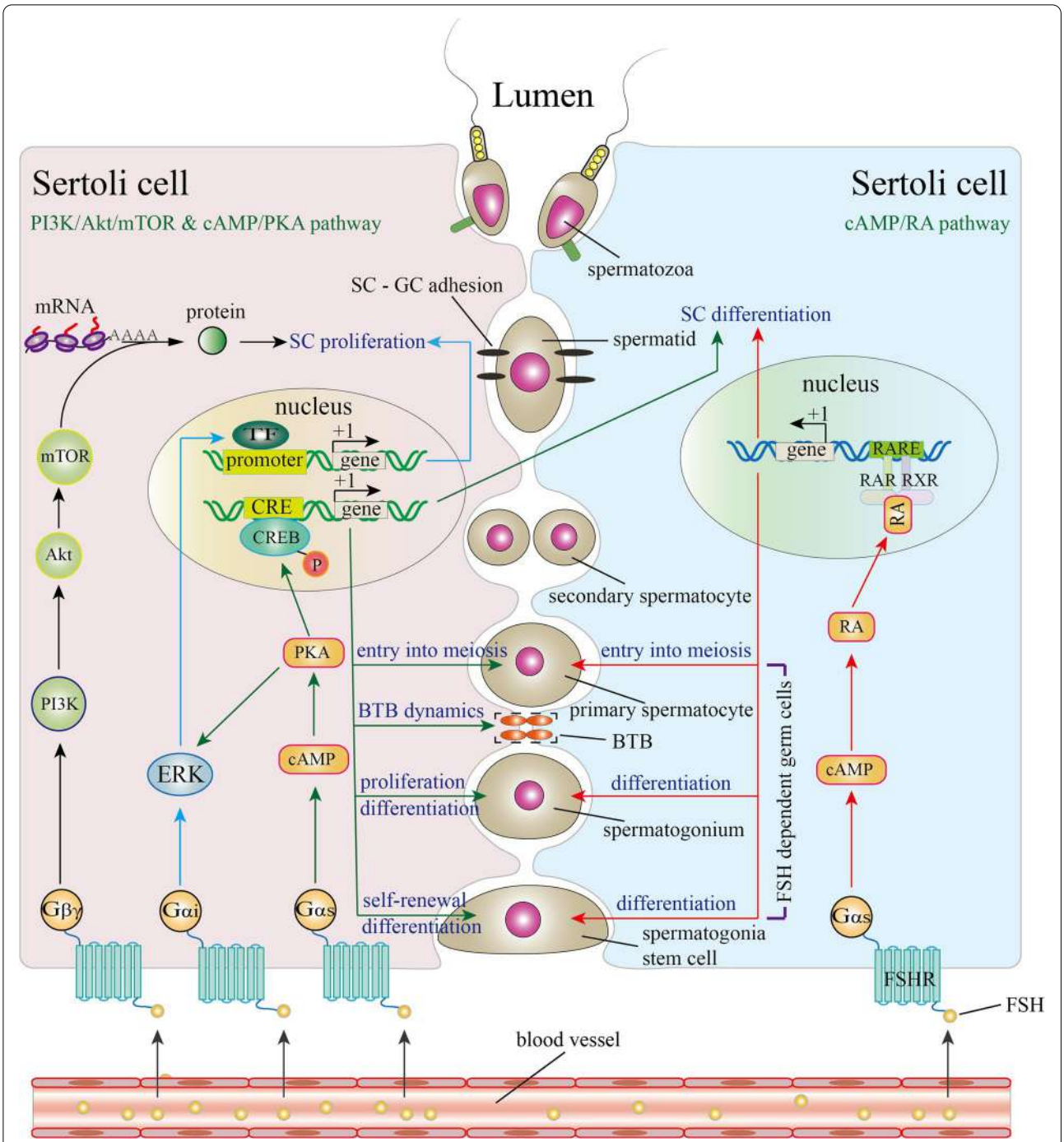

Fig. 1 FSH signaling pathway in SCs. After FSH binds to FSHR on the membrane of SCs, FSH signaling is activated. FSHR recruits different types of G proteins to mediate different signaling pathways. Recruitment of Gβγ subunits activates the PI3K/Akt/mTOR signaling pathway, promoting the translation of mRNAs. Recruitment of the Gα subunit activates the cAMP/PKA signaling pathway. Activated PKA directly phosphorylates the CREB protein in the nucleus. Phosphorylated CREB binds to CREs of target genes to regulate transcriptional activity. In addition, PKA activates ERK during Sertoli cell proliferation. ERK activation is also mediated by recruiting the Gαi subunit. Recently, FSH-induced RA signaling was reported. FSH promotes RA biosynthesis through a cAMP-dependent pathway. RA translocates into the nucleus and binds to RAR/RXR to regulate target gene transcription. The cAMP/PKA signaling pathway participates in Sertoli cell differentiation, SSC self-renewal and differentiation, spermatogonia proliferation and their entry into meiosis, as well as BTB dynamics. The cAMP/PKA/ERK signaling pathway and PI3K/Akt/mTOR signaling pathway induce Sertoli cell proliferation. The cAMP/RA signaling pathway has been shown to participate in SSC differentiation, spermatogonia differentiation and their entry into meiosis. TF: transcription factor

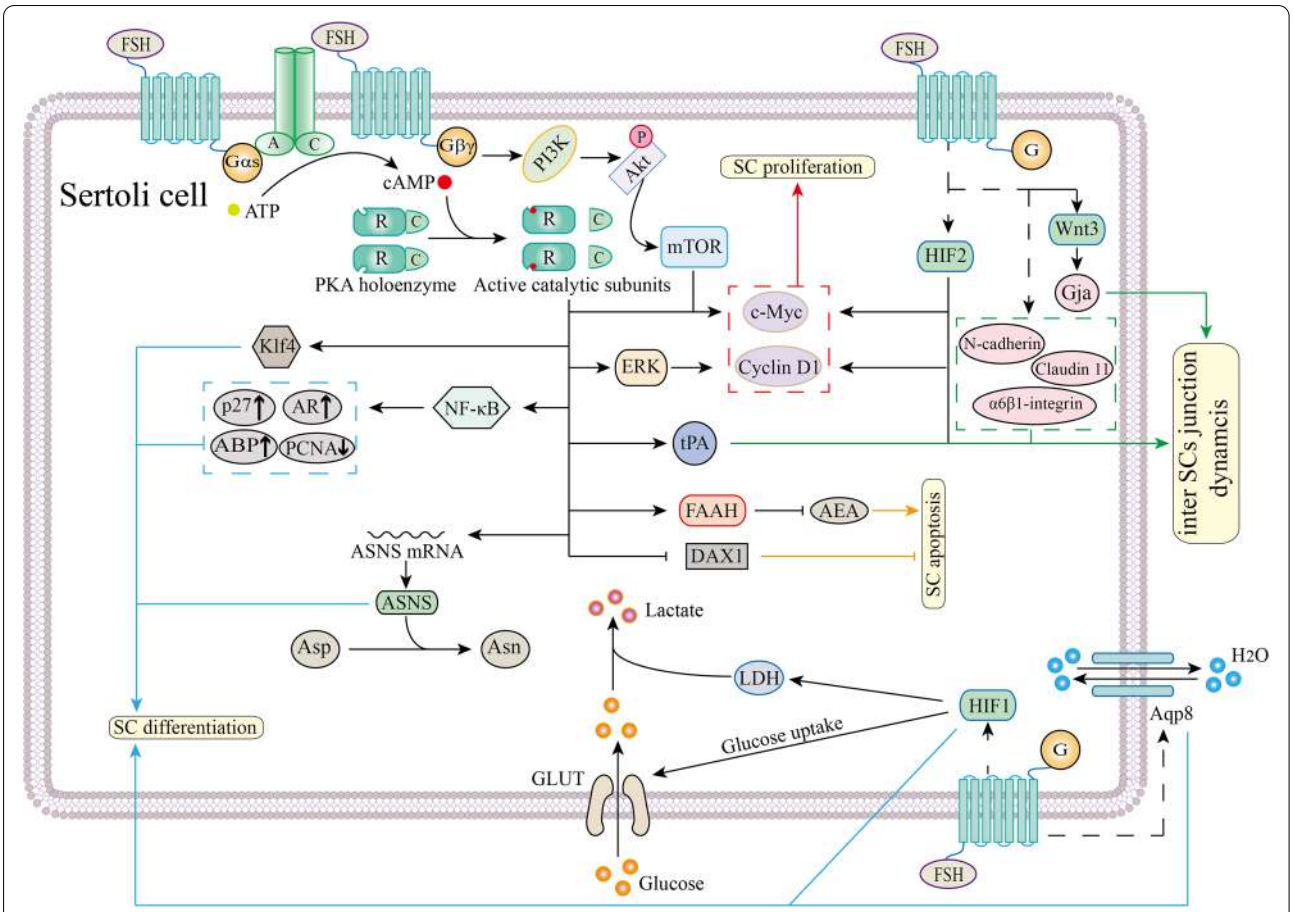

Fig. 2 Functions of FSH signaling in SC proliferation, differentiation and apoptosis. Binding of FSH to FSHR recruits Gαs to FSHR, activating AC. AC transforms ATP into cAMP, and cAMP binds to regulatory subunits of PKA to release catalytic subunits of PKA. Activated PKA induces the expression of c-Myc and Cyclin D1 to promote SC proliferation (red). c-Myc expression is also upregulated by the PI3K/Akt/mTOR pathway. The cAMP/PKA/ERK pathway leads to the expression of Cyclin D1 only during SC proliferation. Through an unknown pathway, FSH induces the expression of HIF2 to promote c-Myc and Cyclin D1 expression. Moreover, inter-SC junction dynamics are also mediated by FSH signaling (green). FSH induces tPA expression via a cAMP/PKA-dependent pathway and three components of the BTB, N-cadherin, α6β1-integrin and claudin 11, through an unknown pathway. Combined with testosterone, FSH stimulates Gja expression via the Wnt3 pathway. Additionally, FSH participates in SC differentiation (blue). Klf4, NF-κB and ASNS expression are mediated by the cAMP/PKA signaling pathway. HIF1 and Aqp8 expression are mediated by an unknown pathway. HIF1 mainly participates in the glycolytic pathway, and Aqp8 is important for water balance. Finally, the cAMP/PKA-mediated balance of FAAH and DAX1 maintains normal SC apoptosis (orange). The dotted line represents an unknown mechanism. For more information, please see the main text

proliferation. FSHR mutation also decreased the SC number in mice, supporting these results [21, 22, 78].

Molecular mechanism underlying this stimulatory effect has been elucidated (Table 1). The main pathway that is included in this period is the PI3K/Akt signaling pathway [69, 79]. PI3K/Akt pathway phosphorylates p70S6K at T389, T421 and S424 [53]. Furthermore, Riera et al. reported that FSH also regulates SC proliferation via the PI3K/Akt/mammalian target of rapamycin complex 1(mTORC1) pathway. Supporting these results, phosphorylated Akt, phosphorylated proline-rich Akt substrate of 40 kDa (PRAS40), phosphorylated mTOR and phosphorylated p70S6K were detected after FSH stimulation in vitro [80]. Moreover, Crépieux et al. showed that FSH supports cAMP/PKA dependent extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) activation and subsequent activation of the MAPK cascade in vitro [52].

Furthermore, genes regulated by FSH signaling that promote SC proliferation have been identified. Most genes are related to DNA replication, the cell cycle, cytoskeletal rearrangement and stem cell factors. Among them, cell-derived Myc (c-Myc) and type D1 cyclin (Cyclin D1) have been linked to the FSH signaling pathway. The proto-oncogene *c-myc* encodes the transcription factor c-Myc, which is important for promoting cell growth and maintaining vitality [81]. In prepubertal rats, the

Table 1 Factors that are under FSH signaling regulation during early stages of spermatogenesis

Process	Molecules	Signaling pathway	References
Sertoli cell proliferation	c-Myc	cAMP/PKA & PI3K/Akt/mTORC1	[80–82]
	Cyclin D1	cAMP/PKA/ERK	[83]
	HIF2	unknown	[84–86]
Sertoli cell differentiation	Klf4	cAMP/PKA	[87, 88]
	NF-κB	unknown	[89]
	HIF1/2	unknown	[90]
	c-jun, jun-B	unknown	[91]
	tPA	cAMP/PKA	[92, 93]
	ASNS	cAMP/PKA	[94]
	Aqp8	unknown	[95, 96]
	Gja	FSH/Wnt3	[97]
	N-cadherin, α6β1-integrin	unknown	[98–100]
	PFKFB1/3	unknown	[101]
	PDK3	unknown	[101]
Sertoli cell apoptosis	FAAH	cAMP/PKA	[102]
	DAX1	cAMP/PKA	[103]
Spermatogonia maintenance	GDNF	cAMP/PKA	[104, 105]
	FGF2	cAMP/PKA	[106, 107]
	PGE2	unknown	[108]
Spermatogonia stem cell differentiation	BMP4	cAMP/PKA	[109, 110]
	SCF, SLF	cAMP/PKA	[111, 112]
	Igf3	cAMP/PKA	[113, 114]
	transferrin	unknown	[115, 116]
Spermatogonia survival	Bok	cAMP/PKA	[117, 118]
Entry into meiosis	Activin A, Inhibin B	cAMP/PKA	[119, 120]
	IL-6	cAMP/PKA & cAMP/PKC	[121]
	nociceptin	cAMP/PKA	[122, 123]
	Nrg1, Nrg3	unknown	[124, 125]
Spermatocyte survival	Gal-3	cAMP/PKA	[126, 127]
	AP-1	unknown	[128]

expression of *c-myc* mRNA was elevated by FSH stimulation via a cAMP-dependent pathway [82]. Further study using rat SCs found that PI3K/Akt/mTOR signaling participate in FSH stimulation of c-myc expression [80]. Cyclin D1, a member of cyclin, binds to cyclin-dependent kinase 4 and 6 to form a complex that promotes cell cycle progression from G1 to S phase [83]. By activating the cAMP-dependent ERK pathway in rat SCs, FSH stimulates Cyclin D1 expression in neonatal rat testes to promote SC proliferation [52]. Hypoxia inducible factor (HIF) is a transcription factor that regulates cell metabolism [84–86]. HIF1 regulates the expression of genes in the glycolytic pathway, while HIF2 regulates the expression of genes related to cell cycle progression [129–132]. During rat SC proliferation, FSH only upregulates HIF2 expression to increase c-Myc and Cyclin D1 expression both in vivo and in vitro through an unknown pathway [130]. Other genes that are regulated by FSH include hairy/enhancer of split gene 1, max-interacting protein repressor and Nur-related protein 1 in murine SCs [133]. Moreover, FSH signaling also cross-talks with insulin growth factor signaling to promote mouse SC proliferation. It is reported that FSH amplifies insulin growth factor signaling mediated Akt phosphorylation [134]. Interestingly, in female mice, FSH can stimulate granulosa cells proliferation via inducing Octamer-binding transcription factor 4 (OCT4) expression [135]. OCT4 is also found to be expressed in human SCs [136]. Whether FSH signaling can promote human SCs proliferation via OCT4 is proposed to be investigated. The precise signaling pathway regulating target gene expression after FSH binds to FSHR remains to be determined.

Sertoli cell differentiation

SC differentiation begins after SC proliferation cessation during puberty in all species [16]. During SC

differentiation, SCs form the BTB to separate the adluminal area and basalarea. Also, SCs undergo metabolism to provide nutrition for germ cells between them. FSH is maintained at a relatively high level during this stage and promotes SC differentiation via an absolutely different signaling pathway compared with SC proliferation [1].

The main pathway by which FSH regulates SC differentiation is the cAMP/PKA signaling pathway. Although debates exist regarding whether FSH promotes SC differentiation, some evidences support our hypothesis. Firstly, FSH deprivation or FSHR knockout in mature mouse SCs led to low sperm counts and the SC transition from differentiation to proliferation [137]. Secondly, FSH activates ERK in immature rat SCs but inhibits its activation in mature SCs via cAMP/PKA signaling [52]. Thirdly, p70S6K is only phosphorylated at T389 in mature rat SCs, while p70S6K is phosphorylated at T389, T421 and S424 in proliferating cells [53]. Fourthly, cAMP and stimulatory Ga production in pubertal rat SCs are greater than those in neonatal rat SCs and FSH mediated cAMP signaling increases stimulatory Ga production in pubertal rat SCs [138]. Further support is obtained from evidence that FSH inhibits Yes-associated protein (YAP) expression to inhibit the Ste20-like protein kinase Hippo (Hippo) signaling pathway in pubertal rat SCs [139]. Hippo signaling pathway is known to promote cell proliferation [140]. Additionally, an increase in FSH level during puberty promotes RARa to translocate into the nucleus, which is important for SC differentiation [67]. In summary, FSH mainly regulates the cAMP/PKA signaling pathway to promote SC differentiation.

FSH regulates SC differentiation directly and indirectly via targeting direct functional factors and transcription factors respectively (Table 1). First class is transcription factors. Krüppel-like factor 4 (Klf4) is a pleiotropic zinc finger transcription factor that induces the expression of genes involved in SC differentiation. Klf4 expression is induced via cAMP/PKA signaling pathway in the TM4 Sertoli cell line [87, 88]. A recent in vivo study using mice demonstrates that FSH is able to induce expression of Klf4 via suppressing microRNA-92a-3p [141]. Nuclear factor (NF)-κB, a transcription factor that induces expression of genes related to SC function such as androgen binding protein, androgen receptor, is activated during SC differentiation following FSH stimulation in rats [89]. In rat mature SCs, both HIF1 and HIF2 expression are induced under FSH regulation. HIF1 increases glucose transporter 1 (*Glut1*) mRNA level to augment glucose uptake while HIF2 promotes the expression of tight junction protein ZO-1, ZO-2 and Occludin levels to establish the BTB [90]. Also, in vitro study using rat SCs indicated that FSH inhibits the expression of cell-derived jun proto-oncogene (*c-jun*) and increases *jun-B* mRNA level to regulate the transcription factor activator protein-1 (AP-1). AP-1 participates in the transcription response to hormones and growth factors which are necessary for SC differentiation [91].

In addition to transcription factors, direct functional factors involved in structural establishment, biochemical reactions and cell morphology were identified. Asp synthetase (ASNS), which promotes Asp accumulation in SCs, is regulated by FSH to induce its transcription in rat SCs. FSH activates the cAMP/PKA signaling pathway to regulate ASNS expression and its function in SC metabolism [94]. Besides Asp accumulation, FSH also positively regulates lactate production via glycolysis process in SCs [142]. Through interaction with PI3K, FSH promotes translocation of Glut1 to plasma membrane to absorb more glucose in rat SCs [143]. Also, FSH induces the transcription level of bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB) isoform 1 (PFKFB1) and 3 (PFKFB3) in rat SCs to regulate synthesis and degradation of fructose 2,6-biphosphate [101]. What's more, FSH can inhibit the transition of pyruvate to acetyl-coA through increasing the expression of pyruvate dehydrogenasekinase 3 (PDK3) in rat SCs [101]. All the efforts are to produce more lactate to nourish germ cells. Aquaporin 8 (Aqp8), which is involved in the water balance of rat and mouse SCs, is also stimulated by FSH [95, 96]. FSH is shown to be permissive for the formation of BTB and Sertoli cell – Germ cell junction such as ectoplasmic specialization and adherent junctions [133, 144]. Neural-cadherin (N- cadherin) and alpha-6beta1-integrin (a6β1 integrin), two molecules known to make up of ectoplasmic specialization, are upregulated during the rat Sertoli cell differentiation process. FSH participates in this promotion in vitro, along with claudin-11, which belongs to tight junction protein [98–100]. Combination of FSH and testosterone also regulates the expression of gap junction protein Gja via wingless-type MMTV integration site family, member 3 (Wnt3) pathway in mice [97]. Gap junctions are pivotal for germ cell development [145]. Tissue plasminogen activator (tPA) was found to be induced by cAMP/PKA signaling pathway in rat and bovine SCs [92]. As a protease, tPA degrades tight junction proteins to regulate BTB dynamics [93]. Further studies could focus on the linkage between the transcription factor and molecules directly related to SC differentiation, which is helpful to elucidate the complete signaling network downstream FSHR.

Sertoli cell apoptosis

Apoptosis is important for maintaining a healthy microenvironment and cell number. SC apoptosis is also regulated by FSH, through the cAMP/PKA signaling pathway. FSH activates the cAMP/PKA signaling pathway to stimulate the expression of N-arachidonoylethanolamine hydrolase (FAAH) and inhibits SC apoptosis that caused by N-arachidonoylethanolamine (AEA) in mice [102]. AEA initiates apoptosis by inducing DNA fragmentation [146]. Additionally, activation of the cAMP/PKA signaling pathway downregulates nuclear receptor subfamily 0 Group B member 1 (DAX1) in maturing rat SCs [147]. Downregulated DAX1 is associated with a higher number of apoptotic cells [103]. The mechanism by which FSH achieves a balance between apoptosis and survival requires further investigation, but is probably mediated by regulating different transcription factors.

In summary, FSH promotes SC proliferation via the cAMP/PKA/ERK and PI3K/Akt/mTORC1 pathways while regulating SC differentiation and apoptosis via the cAMP/PKA pathway. Through sequential and corporative regulation of these three processes, FSH provides a healthy and functional microenvironment for spermatogenesis. However, the pathways that are activated in different processes and molecule function vary between different experiments. One explanation is that no precise boundaries exist between different stages. At the time of SC differentiation, SC proliferation can also occur. Besides, different species have different developmental stages. The two stages may overlap in some species such as rats [16]. Different cell culture conditions might also explain the difference between the results. In addition, it is suggested to consider whether the autocrine action is involved in SC proliferation and differentiation, as well as whether the autocrine action is regulated by FSH.

Role of FSH mediated signaling in Spermatogonia pool maintenance, differentiation and survival

Residing in the basement area of the seminiferous tubule, spermatogonia stem cells function as the original sources for the final spermatozoa [148]. In rodents, undifferentiated type A spermatogonia are classified as SSCs and subsequent progenitors [149]. Differentiating spermatogonia are classified into A_1, A_2, A_3, A_4, intermediate and type B spermatogonia [150, 151]. In humans, undifferentiated spermatogonia are categorized into A_{pale} and A_{dark} spermatogonia. Type B spermatogonia are the differentiating cells [152]. Decades of studies have provided insights into the function of FSH signaling in the spermatogonia pool (Fig. 3).

Spermatogonia pool maintenance

Maintaining the spermatogonia pool ensures normal spermatogonia stem cell self-renewal and proliferation of undifferentiated progenitors. FSH has been shown to positively regulate spermatogonia pool maintenance in vivo and in vitro [153–155]. Impaired FSH signaling in immature SCs or mature SCs decreases the colonization of SSCs [156].

Molecular mechanism behind FSH regulation has been elucidated (Table 1). Glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2) are two factors that are secreted by SCs and positively regulate SSC self-renewal and undifferentiated spermatogonia proliferation [157]. Among them, GDNF activates Akt and MAP kinse-ERK kinase (MEK) signaling pathway, resulting in the production of reactive oxygen species in SSCs. Reactive oxygen species stimulate SSCs self-renewal via the 38 kDa protein (p38) pathway [104, 105]. FSH inhibits autophagy of GDNF in goat SCs via activating the PI3K/Akt/mTOR pathway, which inhibits the translocation of transcription factor EB (TFEB) into the nucleus. Otherwise TFEB induces the expression of lysosomal biogenesis-related genes to degrade GDNF in goat SCs [158]. Recent study reported that GDNF receptor, GDNF family receptor α1, which is expressed by undifferentiated spermatogonia, is also positively regulated by FSH signaling in prepubertal trout testis, though the mechanism is unknown [159]. FGF2 is considered to be a bifunctional factor. For one thing, FGF2 promotes SSC self-renewal along with GDNF [106]. For another thing, FGF2 creates a more suitable environment for SSC differentiation by suppressing GDNF and cytochrome P450 family 26 subfamily B member 1 (Cyp26b1) expression [107]. Possible explanation may be that the combination of GDNF and FGF2 prepares the environment for the formation of progenitors that are ready to differentiate under FSH stimulation. In vivo and in vitro studies using bovine testis and rat testis demonstrates that FSH activates a cAMP-dependent signaling pathway to increase the mRNA levels and protein levels of these two factors [160, 161]. In zebrafish, FSH negatively regulates prostaglandin E2 (PGE2) in SCs by inhibiting Anti Mullerian Hormone (AMH). Otherwise PGE2 promotes SSC self-renewal and inhibits SSC differentiation [108].

Spermatogonia differentiation

Undifferentiated type A spermatogonia are under regulation of signaling network to differentiate into differentiated type B spermatogonia and then preleptotene spermatocytes [162]. FSH seems to initiate type A spermatogonia differentiation and induce differentiating spermatogonia proliferation.

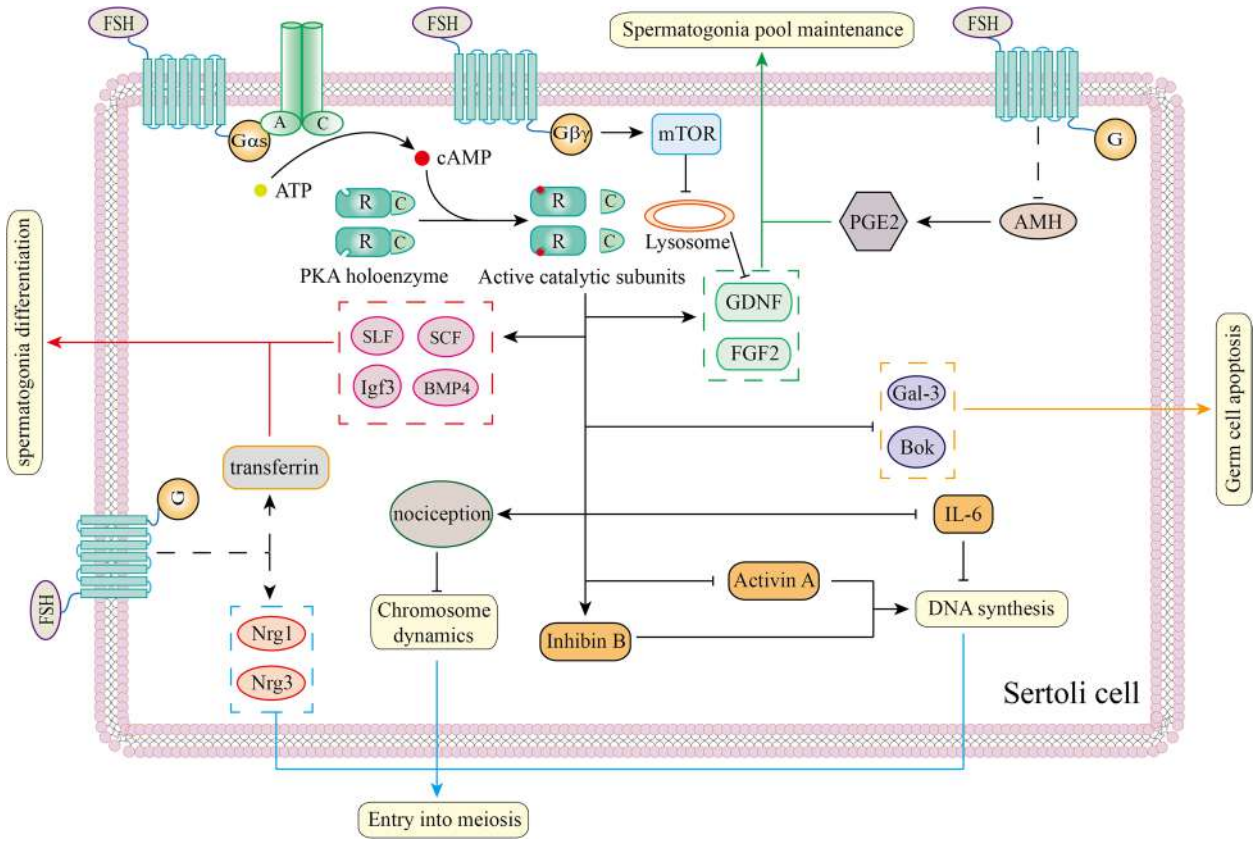

Fig. 3 Functions of FSH signaling in early stages of spermatogenesis. Activating cAMP/PKA signaling pathway induces expression of GDNF and FGF2 in SC which will be secreted to SSC to maintain spermatogonia pool. Notably, FSH can inhibit degradation of GDNF via suppressing lysosomal biogenesis. This process is validated by activating PI3K/Akt/mTOR pathway. Additionally, FSH negatively regulates PGE2 through inhibiting AMH. PGE2 is also important for spermatogonia pool maintenance. SLF, SCF, Igf3 and BMP4 that is activated via cAMP/PKA pathway, along with transferrin through unknown pathway, contribute to spermatogonia differentiation. FSH ensures entry into meiosis through regulating DNA synthesis and chromosome dynamics. FSH regulates DNA synthesis via temporally inhibiting IL-6, Activin A and inducing Inhibin B. Chromosome dynamics is positively regulated by nociception via cAMP/PKA signaling pathway. Also, FSH upregulates Nrg1 and Nrg3 to induce Stra8 expression in spermatocytes. Besides, limiting gem cell apoptosis by inhibiting Gal3 and Bok is another task of FSH signaling in SCs. Dotted line represents unknown mechanism

Molecular mechanism behind FSH regulation has been elucidated (Table 1). FSH activates RA signaling by increasing RDH10, ALDH1A1, CRABP2 levels in primate SCs and this will provide an environment for induction of spermatogonia differentiation [1]. Stem cell factor (SCF) and steel factor (SLF) are two factors secreted by Sertoli cells during postnatal stages that are essential for the expansion of differentiating spermatogonia [111, 160]. FSH signaling induces transcription of SCF and SLF in prepubertal mouse testis via the cAMP-dependent signaling pathway [112]. The same phenomenon was also observed in adult rat testes [111, 160]. Both SCF and SLF are v-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog (kit) ligands (pleiotropic growth factor) and bind to kit (kit ligands receptor, CD117 is the cluster number for KIT receptor tyrosine kinase) on the surface of differentiating spermatogonia [163]. Owning to alternative splicing, kit ligand has transmembrane form or soluble form at different developmental stages and soluble form is favored for SSC differentiation [112]. Supporting this, transmembrane form of kit ligand is detected immunohistochemically in stages VII-VIII of the mouse seminiferous epithelium, during which SCs are less-sensitive to FSH signaling in mice [111, 164, 165]. Bone morphogenetic protein-4 (BMP4), secreted by SC during early postnatal stage, is proposed to promote SSC differentiation after binding to its receptor on spermatogonia [109, 110]. Its expression is under FSH/cAMP regulation. cAMP analogues downregulate BMP4 expression in prepubertal and pubertal mouse SCs while RA upregulates BMP4 expression level in prepubertal and pubertal mouse SCs [166, 167]. In zebrafish, FSH activates insulin growth factor 3 (Igf3) production via cAMP/PKA pathway [113]. Igf3 promotes SSC

differentiation via beta-catenin (β-catenin) pathway in SSCs [114]. Other factors that are regulated by FSH and promote SSC differentiation include transferrin and Doublesex (sex determination and differentiation gene) and mab-3 (sex determination and differentiation gene) related transcription factor [161]. Transferrin functions as an ion transport to provide necessary ions for differentiating spermatogonia [115, 116].

Spermatogonia survival

FSH has been shown to protect spermatogonia from apoptosis which is important for steadiness of spermatogonia pool. Bcl-2-related ovarian killer (Bok) is proapoptotic member of the Bcl-2 gene family. FSH downregulates Bok mRNA level in rat testes to inhibit apoptosis [117]. Supporting this result, FSH suppression in immature rat SCs activates the caspase 9 mediated intrinsic apoptotic pathway [118]. Activation of the intrinsic apoptotic pathway is partially attributed to the activation of Bcl-2 gene family [168].

To sum up, FSH signaling in SCs induces paracrine action to maximize the capacity of spermatogonia ecology by maintaining the undifferentiated spermatogonia pool, promoting spermatogonia differentiation and spermatogonia survival. How to achieve a balance between molecules promoting self-renewal and molecules promoting differentiation in response to FSH stimulation remains to be further investigated. The answer may reside in spermatogonia themselves because it was reported that germ cells can control the local balance of GDNF, BMP4 and kit ligand levels [161].

Entry into meiosis and spermatocyte survival

Transition of type B spermatogonia into spermatocytes facilitates meiosis while surviving spermatocytes are essential for quantitative spermatogenesis. FSH is shown to be indispensable for entry into meiosis and positively regulates spermatocyte survival (Fig. 3).

Entry into meiosis

In vitro study using coculture system containing SCs and spermatogonia showed that FSH initiates the differentiation of secondary spermatogonia into primary spermatocytes in newts [169]. Injection of *hpg* mice with exogeneous FSH or transgenic expression of FSH restores the number of spermatogonia and spermatocytes [170, 171]. This observation is supported by hypophysectomised or GnRH-immunized adult rat models which lack normal circulating FSH levels [26, 172]. Furthermore, knocking out of FSHR and FSHβ in mice resulted in decreased numbers of spermatogonia and spermatocytes, let alone spermatids [25, 173]. These results indicate that FSH is necessary for spermatogonia to differentiate into primary spermatocytes, promoting entry into meiosis.

Detailed mechanisms that FSH adopts to guarantee entry into meiosis are as follows (Table 1). Activin alpha (Activin A, growth and differentiation gene) and Inhibin beta (Inhibin B, growth and differentiation gene) are two structurally-related factors that belong to the transforming growth factor β family. Activin A promotes DNA synthesis in spermatocytes while Inhibin B inhibits this biological process [119]. Through the cAMP/PKA signaling pathway, FSH can activate the production of Inhibin B while inhibit the production of Activin A near the beginning of meiosis [120]. Thus, FSH functions as a monitor for the end of DNA synthesis. This result is further supported by a study of another factor interleukin 6 (IL-6). IL-6 is reported to negatively regulate DNA synthesis. IL-6 expression is downregulated by FSH via the cAMP dependent pathway during stages VII-VIII and upregulated by FSH via the PKC dependent pathway during stages IX-XI in rats [121]. Stages VII-VIII corelate with the initiation of meiotic DNA synthesis while stages IX-XI correlate with DNA synthesis termination. Recently, Eto et al. reported that FSH can promote nociception expression via cAMP/PKA signaling in murine Sertoli cells [122]. Nociceptin (17-residue neuropeptide) is secreted by Sertoli cells and binds to nociception receptor opioid related nociceptin receptor 1 (OPRL1) on the surface of spermatocytes [123]. Binding of nociception to its receptor leads to REC8 meiotic recombination protein (Rec8) phosphorylation in spermatocytes which promotes meiotic chromosome dynamics to prepare for the subsequent meiosis [174]. Similarly, FSH, combined with retinoic acid, stimulates Neuregulin 1 (Nrg1) and Neuregulin 3 (Nrg3) expression in mouse SCs [124, 125]. Nrg1 and Nrg3 are secreted from SCs and bind to their receptor EGFR – Mouse Genome Informatics 4 (ERBB4) on the surface of pre-spermatocytes which will trigger stimulated retinoic acid gene 8 (Stra8) expression [125]. Upregulated Stra8 expression promotes the early stage of meiotic prophase [175]. However, the exact signaling pathway that is adopted by FSH remains to be elucidated. In summary, these results demonstrates that FSH positively regulates entry into meiosis by temporally ensuring the initiation of DNA synthesis and termination of DNA synthesis, as well as monitoring meiotic chromosome dynamics.

Spermatocyte survival

FSH is also pivotal for spermatocyte survival. In FSH-suppressed adult rats and gonadotropin-suppressed adult men, the spermatocyte apoptosis rate showed a significant increase [176, 177].When the androgen level is normal, the suppression of FSH reduced pachytene

spermatocytes numbers in rats [178]. In human SCs, Sá et al. found that the combination of FSH and testosterone maximally maintains spermatocytes because FSH alone was not enough to limit spermatocytes apoptosis during the second week of vero cell conditioned medium [179, 180]. Previous study demonstrated that spermatocyte apoptosis is related to both extrinsic (Caspase 8) and intrinsic (Caspase 9) apoptotic pathways [181, 182]. Supporting this finding, FSH signaling in rats has been shown to inhibit both the extrinsic and intrinsic apoptotic pathways during the first wave of spermatogenesis to promote spermatocyte survival [118].

Another factor, Galectin-3 (Gal-3) is reported to inhibit both the intrinsic and extrinsic apoptotic pathways by blocking cytochrome c release and Fas (death receptor)/Fas-ligand (member of the tumor necrosis factor family of death-inducing ligands)cross linking respectively [126]. FSH induces expression of Gal-3 in porcine and rat SCs at the initiation stage of meiosis and protects spermatocytes from apoptosis, probably via cAMP/PKA dependent pathway [127].

Additionally, FSH can inhibit early meiotic spermatocyte apoptosis via inhibition of transcription factor AP-1 in human SCs. Activation of AP-1 occurs before the activation of effector caspase such as caspase 3 [128]. Caspase 3 was shown to be expressed in human SCs and germ cells [183]. This indicates that FSH signaling in human SCs may control germ cell death via paracrine action.

In summary, FSH exerts its effect at the beginning of meiosis by promoting entry into meiosis and the survival of spermatocytes. This effect may be mainly due to the sufficient number of Sertoli cells and spermatogonia. Further studies are recommended to focus on the effect of FSH on the transition from spermatogonia to spermatocytes as well as whether FSH has effect on the transition from primary spermatocytes to the secondary spermatocytes.

Potential use of follicle-stimulating hormone in treating male infertility

In humans, FSH induces SC proliferation and spermatogonia proliferation and differentiation, while FSH alone is not essential to complete meiosis and spermiogenesis [184, 185]. Testosterone is more important from the beginning of spermatocyte development [186]. The differences in the functions of FSH signaling in spermatogenesis between humans and other experimental animals suggest that further studies should be conducted to understand the FSH regulation in human or we should develop more appropriate experimental animal models.

Currently, FSH treatment is mainly administrated to two types of patients: patients with hypogonadotropic hypogonadism (HH) and normogonadotropic patients with idiopathic impairment of spermatogenesis [187]. The findings described above indicate that FSH promotes the final sperm production by positively regulating Sertoli cell biology. As expected, questions about whether excess FSH is harmful for spermatogenesis arise before FSH treatment. In rodents, high serum FSH levels result in better testis development [78, 188]. Men with pituitary adenoma secreting excess FSH also show normal spermatogenesis and normal testicular development [189]. Supporting this result, enhanced receptor activity resulted from gain of function mutations in FSHR, such as FSHR-D567G and FSHR-N431I, also appears to have little effect on normal spermatogenesis [190, 191]. These results provide a theoretical support for FSH treatment.

In patients with hypogonadotropic hypogonadism, the lack of gonadotropin FSH stimulation or defects in gonadotropin-releasing hormone synthesis and secretion lead to azoospermia or severe oligozoospermia [187, 192]. One method to treat HH is pulsatile GnRH administration which may lead to the secretion of gonadotropin from the pituitary gland [29, 193]. The secreted FSH can stimulate Sertoli cell growth to support normal spermatogenesis. However, this method is costly and troublesome since external GnRH must be pumped subcutaneously [194]. Another method to treat HH is the administration of exogenous gonadotropins. This method, which involves treating patients with human chorionic gonadotropin alone or in combination with FSH, is more direct and may be more successful in most cases [195, 196]. Human chorionic gonadotropin (hCG) functions similarly to luteinizing hormone, but with different bioactivities [197]. hCG is observed to restore sperm production in men. This effect may be enhanced when hCG is administered in combination with FSH [198, 199]. However, the precise dosage and timing of FSH treatment in this method remains controversial. Though this method seems useful at present, limitations have also been noted. For example, exogenous gonadotropin administration is not the same as the gonadotropin secretion stimulated by GnRH. The intrinsic regulatory network cannot be simulated using this method.

FSH treatment appears to be beneficial for normogonadotropic patients with idiopathic impairment of spermatogenesis. Meta-analyses revealed that FSH therapy in these patients increases the rate of clinical pregnancies in female partners [200]. However, since the number of participants taking part in the experiment was relatively low, studies are still needed to determine whether FSH therapy truly affects normogonadotropic patients. As a result, the selection of appropriate normogonadotropic

patients to receive FSH therapy is necessary. First, no identifiable and generally accepted cause for male infertility should be detected [201]. Second, FSH pharmacogenetics is promising in this evaluation. Testing for the single nucleic polymorphism (SNP) p.N680S in patients receiving FSH therapy is important. Male patients with the p.N680S homozygous N polymorphism exhibit a significantly decreased DNA fragmentation index of sperm in the ejaculate after FSH treatment [202–204]. Also, SNP of the FSHβ is another marker to select the normogonadotropic patients to receive FSH therapy [205, 206]. More well-organized and sufficient randomized studies are needed to determine whether FSH therapy is truly helpful for normogonadotropic patients as well as the dosage and timing needed to carry out therapy. In summary, Precision Medicine matters a lot!

Let's return to the exogeneous gonadotropin administration method. How to expand the half-life of gonadotropin is important for treatment efficiency. One way is to conjugate the gonadotropin to polyethylene glycol (PEG). PEGylated FSH not only retains FSH activity but also results in improved bioavailability [207]. Another way is to develop a single-chain recombinant analogue of gonadotropin [208]. These molecules were engineered with the β-subunits oriented at the N-terminus of the α subunit and used the hCGβ carboxy-terminal peptide (CTP) sequence as a linker [209, 210]. These analogues have an increased serum half-life and increased biopotency. Using this method, we obtain dual FSH and LH analogues, such as FSHβ-CTP-LHβ-CTP-α [211]. Recently, fusion analogues of FSH consisting FSHα, FSHβ and immunoglobulin constant fragments were constructed [212]. This type of analogue can improve pharmacokinetics. All the aforementioned analogues have great beneficial to female infertility treatment and ovarian development of experimental animals such as sheep and monkeys. It remains to be determined if present analogues can treat male infertility. Analogs that can be used in clinical trials are being researched [31].

Recently, the relationship between diabetes mellitus and male infertility attracts attention [213–215]. As the metabolic modulator in seminiferous tubule, SC metabolism dysfunction is thought to be one link between diabetes mellitus and male infertility [216, 217]. In human, Glut1 and Glut3 transport glucose into SCs. With the help of lactate dehydrogenase (LDH), glucose can be converted into lactate which will be transported out of SCs and supplied to germ cells via monocarboxylate transporter (MCT) [218–220]. Diabetes mellitus patients displayed low level of FSH, low mRNA levels of Glut1, Glut3 and low protein level of LDH in SCs [221]. Insulin-deprived human SCs, which was similar to diabetes mellitus, presented decreased transcript level of LDH, MCT4, Glut3 and increased transcript level of Glut1 [222]. Moreover, decreased level of sirtuin 1 and increased level of ghrelin in diabetes mellitus patients impair the hypothalamus-pituitary–gonadal axis which leads to low level of FSH [86, 223, 224]. In Klinefelter syndrome male patients which is prone to suffer from diabetes mellitus, high level of FSH along with increased mRNA expression of Glut3 and decreased mRNA expression of Glut1 in SCs may be a try to rescue spermatogenesis [225]. Based on this, drugs can be developed to rescue FSH in diabetes mellitus patients so that normal SC metabolism can occur and sufficient energy can be provided to germ cells.

Hope still exists. Conversation in spermatogenic processes between human and mouse are revealed in previous studies [226]. Since the phenotypes of *Fshr*-knockout mice and men carrying *Fshr* mutations are similar, the *Fshr*-knockout mouse model still has great clinical potential [29]. The identification of additional genes that are regulated by FSH in mice and developing targeted medicines are feasible. Last, the combination of FSH and testosterone treatment is more efficient than a single hormone treatment, since testosterone can augment FSH signaling in SCs [227].

Conclusion

FSH signaling in SCs establishes the appropriate microenvironment for spermatogenesis. FSH signaling plays a dominant role in determining the number and function of SCs. FSH signaling also maintains the spermatogonia pool and induces spermatogonia differentiation through paracrine actions. In addition, FSH signaling promotes entry into meiosis and the survival of germ cells. However, few molecules involved in these paracrine actions have been found. We could detect the changes within protein and mRNA expression level of receptors on the surface of gem cells that are associated with different spermatogenic processes, and then determine whether the levels of their ligands changed after the administration of FSH signaling in SCs. Mass spectrometry and single-cell transcriptomics will be helpful. Moreover, combining the transgenic mouse model with human infertility is necessary to develop therapies for diseases related to dysfunctional FSH signaling.

Abbreviations

α6β1 integrin: Alpha6beta1-integrin; β-catenin: Beta-catenin; AC: Adenylate cyclase; Activin alpha: Activin A; AEA: N-arachidonoylethanolamine; Akt: Protein kinase B; ALDH1A1: Aldehyde dehydrogenase 1A1; AMH: Anti Mullerian Hormone; AP-1: Activator protein-1; Aqp8: Aquaporin 8; ASNS: Asp synthetase; BMP4: Bone morphogenetic protein-4; Bok: Bcl-2-related ovarian killer; BTB: Blood-testis barrier; cAMP: Cyclic adenosine monophosphate; c-Myc: Cell-derived Myc; c-jun: Cell-derived jun proto-oncogene; CRABP2: Cytoplasmic RA-binding protein 2; CREB: Cyclic AMP response-element binding protein; CTP: Carboxy-terminal peptide; Cyclin D1: Type D1 cyclin; Cyp26b1: Cytochrome P450 family 26 subfamily B member 1; DAX1: Nuclear receptor

subfamily 0 group B member 1; ERBB4: EGFR – Mouse Genome Informatics 4; ERK: Extracellular-regulated kinase; ERK1/2: Extracellular signal-regulated protein kinases 1 and 2; FAAH: N-arachidonoylethanolamine hydrolase; FGF2: Fibroblast growth factor 2; FSH: Follicle-stimulating hormone; FSHR: Follicle-stimulating hormone receptor; G proteins: Heterotrimeric guanine nucleotide–binding proteins; Gal-3: Galectin-3; GDNF: Glial cell line-derived neurotrophic factor; GnRH: Gonadotropin-releasing hormone; hCG: Human chorionic gonadotropin; HH: Hypogonadotropic hypogonadism; HIF: Hypoxia inducible factor; hpg: Hypogonadal; Hippo: Ste20-like protein kinase Hippo; Igf3: Insulin growth factor 3; IL-6: Interleukin 6; Inhibin B: Inhibin beta; Kit: V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog; Klf4: Krüppel-like factor 4; LDH: Lactate dehydrogenase; LH: Luteinizing hormone; MAPK: Mitogen-activated protein kinase; MCT: Monocarboxylate transporter; MEK: MAP kinase-ERK kinase; mTOR: Mammalian/mechanistic target of rapamycin; mTORC1: Mammalian target of rapamycin complex 1; N-cadherin: Neural-cadherin; NF: Nuclear factor; Nrg1: Neuregulin 1; Nrg3: Neuregulin 3; OCT4: Octamer-binding transcription factor 4; p38: 38 KDa protein; OPRL1: Opioid related nociceptin receptor 1; p70S6K: Phosphorylated 70-kDa ribosomal S6 kinase; PDK3: Pyruvate dehydrogenasekinase 3; PEG: Polyethylene glycol; PFKFB: 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase; PFKFB1: 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase isoform 1; PFKFB3: 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase isoform 3; PGE2: Prostaglandin E2; PI3K: Phosphoinositide 3-kinase; PIP2: Phosphatidylinositol bisphosphate; PIP3: Phosphatidylinositol 3,4,5-trisphosphate; PKA: Protein kinase A; PRAS40: Proline-rich Akt substrate of 40 kDa; RA: Retinoic acid; RARE: RA response element; RAR**α**: Retinoic acid receptor **α**; RDH10: Retinol dehydrogenase 10; Rec8: REC8 meiotic recombination protein; RXR: Retinoid X receptor; SC: Sertoli cell; SCF: Stem cell factor; SLF: Steel factor; SNP: Single nucleic polymorphism; SSC: Spermatogonia stem cell; Stra8: Stimulated retinoic acid gene 8; TFEB: Transcription factor EB; tPA: Tissue plasminogen activator; Wnt3: Wingless-type MMTV integration site family, member 3; YAP: Yes-associated protein.

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Authors' contributions
J.-M. Wang conceived, wrote, revised the manuscript, and prepared all the figures; Z.-F. Li wrote part of the manuscript and participated in discussion; W.-X. Yang was involved in constructing the frame of the manuscript, participated in the revision and supplied the fund for this research; F.-Q. Tan participated in discussion and revision, and provides the funding of the publication of this work. The author(s) read and approved the final manuscript.

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Declarations

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Review

Genetics of Azoospermia

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Abstract: Azoospermia affects 1% of men, and it can be due to: (i) hypothalamic-pituitary dysfunction, (ii) primary quantitative spermatogenic disturbances, (iii) urogenital duct obstruction. Known genetic factors contribute to all these categories, and genetic testing is part of the routine diagnostic workup of azoospermic men. The diagnostic yield of genetic tests in azoospermia is different in the different etiological categories, with the highest in Congenital Bilateral Absence of Vas Deferens (90%) and the lowest in Non-Obstructive Azoospermia (NOA) due to primary testicular failure (~30%). Whole-Exome Sequencing allowed the discovery of an increasing number of monogenic defects of NOA with a current list of 38 candidate genes. These genes are of potential clinical relevance for future gene panel-based screening. We classified these genes according to the associated-testicular histology underlying the NOA phenotype. The validation and the discovery of novel NOA genes will radically improve patient management. Interestingly, approximately 37% of candidate genes are shared in human male and female gonadal failure, implying that genetic counselling should be extended also to female family members of NOA patients.

Keywords: azoospermia; infertility; genetics; exome; NGS; NOA; Klinefelter syndrome; Y chromosome microdeletions; CBAVD; congenital hypogonadotropic hypogonadism

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1. Introduction

Male infertility is a heterogeneous, multifactorial and complex disorder of the reproductive system, affecting 7–12% of men from the general population [1,2]. Azoospermia (absence of spermatozoa in the ejaculate) can be observed in about 1% of men and the etiology of this condition can be divided into three major categories: (i) hypothalamic–pituitary axis dysfunction, (ii) primary quantitative spermatogenic disturbances, and (iii) urogenital duct obstruction [3]. All of these subgroups can be related to congenital and acquired factors. In case of bilateral distal or proximal obstruction of the ejaculatory ducts, the spermatogenic process is unaffected, and this pathologic condition is termed as Obstructive Azoospermia (OA). On the other hand, primary or secondary testicular failure leads to Non-Obstructive Azoospermia (NOA). NOA is a phenotypic manifestation for which at least three different types of testis histology can be present: (i) Sertoli-Cell-Only Syndrome (SCOS), (ii) Maturation Arrest (MA) at different stages of germ cell maturation (such as Spermatogonial and Spermatocyte Arrest [SGA, SCA]), (iii) hypospermatogenesis.

A number of acquired conditions (such as orchitis, cytotoxic treatment, ejaculatory duct obstruction, CNS tumors, systemic diseases etc.) may lead to azoospermia and may account for approximately 35–40% of cases [3]. Concerning the congenital forms, genetic factors play a role in all the above-mentioned etiological categories and some of them are tested as part of the routine diagnostic workup of infertile men [4]. Genetic screening is relevant for its diagnostic value, clinical decision making, and appropriate genetic counselling [5]. In the clinical practice, karyotype abnormalities and Azoospermia Factor (AZF) microdeletions are routinely screened in azoospermic patients [6] due to primary testicular failure. Gene mutation screening based on targeted-gene panels should be recommended when either Congenital Bilateral Absence of Vas Deferens (CBAVD) or

Congenital Hypogonadotropic Hypogonadism (CHH) is suspected. The diagnostic yield of the above tests is different in the different etiological categories, the highest is in CBAVD and the lowest in NOA due to primary testicular failure (Figure 1). Next Generation Sequencing (NGS)-based Whole Exome Sequencing (WES) or gene panel sequencing have allowed the identification of a growing number of novel monogenic causes. This review is aimed at providing an overview on genetic factors involved in azoospermia.

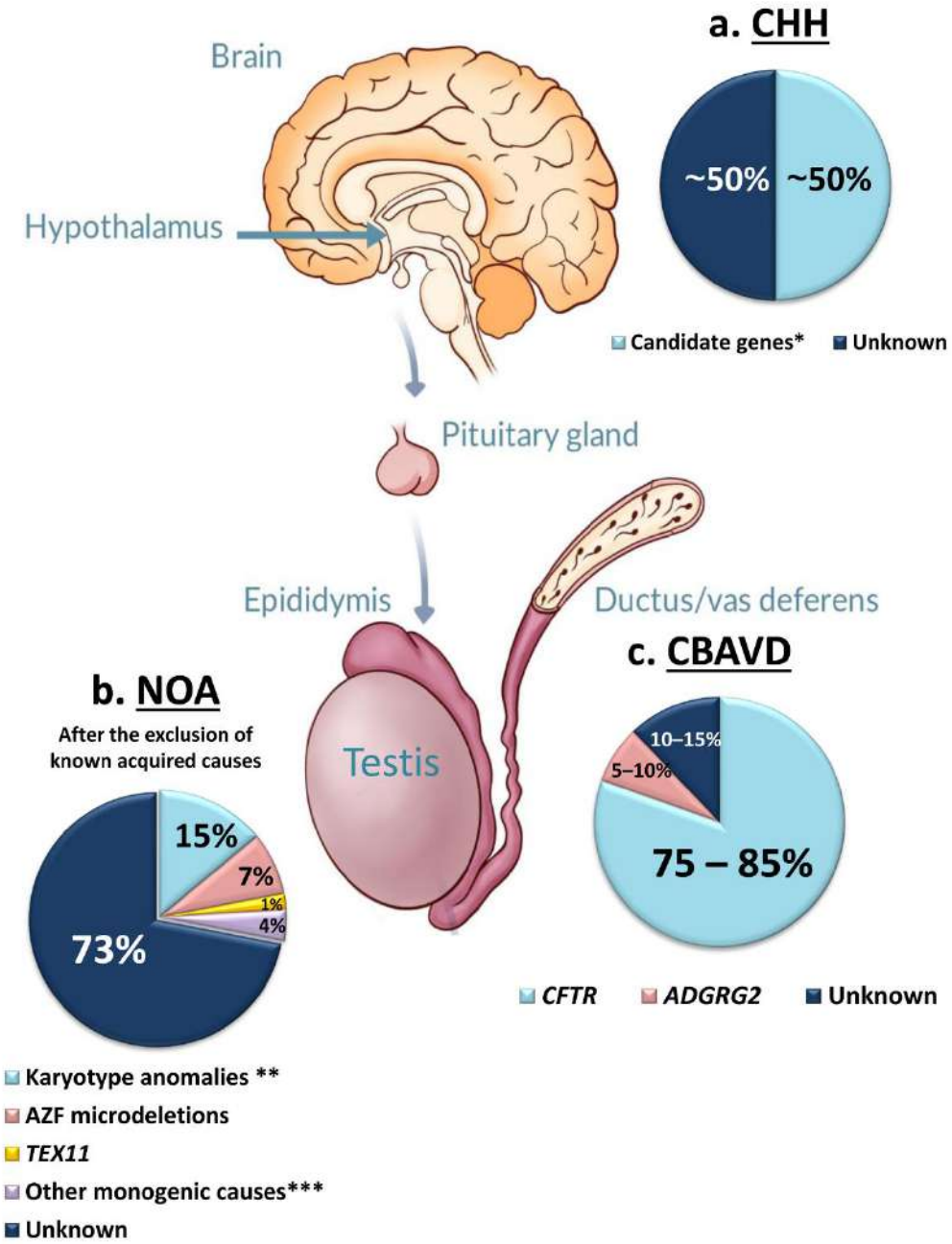

Figure 1. Diagnostic yield of genetic testing in azoospermia with different etiology: (**a**) Congenital Hypogonadotropic Hypogonadism; (**b**) Non-Obstructive Azoospermia due to primary testicular failure, after the exclusion of all know acquired causes; (**c**) Congenital Bilateral Absence of Vas Deferens. Abbreviations: AZF—Azoospermia Factor Region; CBAVD—Congenital Bilateral Absence of Vas Deferens; CHH—Congenital Hypogonadotropic Hypogonadism; NOA—Non-Obstructive Azoospermia; * See Reviews [7,8]; ** 47,XXY Klinefelter syndrome, 46,XX male syndrome, Yq'-'; *** See articles [9–11].

2. Chromosomal Anomalies Causing Azoospermia

2.1. Karyotype Anomalies

2.1.1. Klinefelter Syndrome (47,XXY)

The most common genetic disorder causing NOA is the Klinefelter syndrome (KS), which is characterized by the presence of an extra X chromosome. Although the first description of this pathology was almost 80 years ago [12], due to the extreme heterogeneity in its genetic and clinical presentation, KS continues to pose substantial diagnostic challenges. Its prevalence is 1 out of 600 (0.1–0.2%) in newborn male infants, which rises up to 3–4% among infertile males and 10–12% in azoospermic subjects [13,14]. However, the vast majority of patients (approximately 64%) are still misdiagnosed or remain undiagnosed throughout life [15,16], due to the occurrence of mild forms characterized by paucisymptomatic manifestations [13].

Regarding the karyotype, in the 80–90% of the cases, the pure form with 47,XXY is defined, whereas in the remaining 10–20% of cases higher-grade aneuploidies (48,XXXY or 48,XXYY), structurally abnormal X chromosome (47,iXq,Y) or mosaicisms (47,XXY/46,XY) can be detected [16]. The supernumerary X chromosome could derive from paternal or maternal non-disjunction of the sex chromosomes. This event usually happens either during oogenesis or spermatogenesis, or less frequently (around 3%) during early division of the fertilized oocyte. The failure of chromosome separation at anaphase can occur during meiosis I, meiosis II, or mitosis. Paternal or maternal meiotic aneuploidy appears equally distributed in KS subjects [17], with the difference that paternal non-disjunction can happen only during the first meiotic division, since error in the second meiotic division would result in XX or YY gametes, leading to XXX or XYY zygotes. On the other hand, the age of the mother seems to influence the rate of KS due to post-zygotic origin. In fact, the incidence of KS was 4-fold higher when mothers aged above 40 years were compared to those aged less than 24 years [16]. The reason behind this increase could be that the first three mitotic divisions are controlled by maternal proteins and RNAs, hence, with the advanced maternal age, the chance of mitotic errors increases correspondingly, as well as the possibility of KS of post-zygotic origin [16].

KS has a wide spectrum of clinical manifestations, which includes classical features, such as eunuchoid habitus, hypergonadotropic hypogonadism, gynecomastia, small (volume < 4 mL) firm testes, azoospermia and pervasive neurocognitive deficits.

First of all, mosaic forms are less severe compared to non-mosaic, pure forms of KS. In classic non-mosaic KS patients, the clinical manifestation derives from testis dysfunction (hyalinization), from X–linked gene dosage effect and from the modulatory effect of common polymorphisms [18].

Among typical features are tall stature and eunuchoid habitus. Concerning the former, Short-stature Homeobox-containing gene on chromosome X (SHOX), situated in the pseudoautosomal region 1 (PAR1) on the short arm of the X chromosome (Xp) is involved. Since this gene is implicated in height regulation the presence of three SHOX copies explains the excessive tallness [19].

Another potential genetic modulator is the CAG repeat polymorphism of the Androgen Receptor (AR) gene. CAG repeat length correlates negatively with the function of the Androgen Receptor. It has been investigated in relationship with the broad scale of clinical manifestations seen in KS. The existing literature shows that CAG repeat length has an impact on the androgen-dependent features in KS [13]. Moreover, a positive correlation can be seen with anthropometric parameters such as height, arm span and length, or leg length [20–22]. Comorbidities may also manifest as the consequence of gene dosage increase of non-PAR genes, since a certain proportion of them escape X inactivation. It has been suggested that the increased X chromosome gene dosage may alter protein interactome activity with the consequent alteration of cell function [23]. Moreover, there is raising evidence for a role of epigenetic modifications in the clinical manifestations of KS. A globally changed DNA methylation profile, with both hyper- and hypomethylated areas,

in the genome of KS patients has been reported [24–26]. These epimutations may have regulatory impact on gene transcription with consequent functional effect.

Concerning the fertility potential of KS, it is still not clearly understood how the extra X chromosome affects spermatogenesis. It has been described that extensive fibrosis and hyalinization of the seminiferous tubules occur, with progressive apoptosis of 47,XXY spermatogonia. In the large majority of cases this process results in azoospermia before reaching adulthood [27]. However, spermatogenesis can take place in some seminiferous tubules, which could be explained by two hypotheses: (i) a low level gonosomal mosaicism originated during embryogenesis; (ii) testicular mosaicism due to the loss of the extra X chromosome during mitosis occurring in 47,XXY spermatogonia. Experimental studies based on Fluorescence In Situ Hybridization (FISH), demonstrated that those spermatogonia belonging to tubules with active spermatogenesis were euploid, (46,XY) [28,29]. Hence, these normal diploid germ cells are able to complete the spermatogenic process leading to normal, haploid gametes in the majority of cases [28].

Spermatozoa can be harvested in about 34–44% of KS patients through conventional or micro-Testicular Sperm Extraction (m-TESE) [14,30]. The retrieved spermatozoa can be used for Intra-Cytoplasmic Sperm Injection (ICSI) with an average live birth rate per cycle of 29–43% [14,30]. Since with ageing a progressive loss of germ cells occurs, Rohayem and colleagues [31] investigated the optimal timing of TESE/preventive cryopreservation [32], and predictive factors influencing sperm retrieval rate. The window of opportunity for a higher spermatozoa retrieval success rate was between late adolescence and early adulthood ($\geq$ 15–19 years), with LH $\leq$ 17.5 U/L and testosterone level $\geq$ 7.5 nmol/L. No other positive association have been demonstrated with other parameters, such as testicular volumes, serum levels of FSH, Inhibin B, AMH, estradiol. A history of cryptorchidism was found as a negative predictor. On the other hand, Corona and colleagues [30] by performing a meta-analysis did not find an age-related effect on sperm retrieval rate, therefore this issue remains still debated.

With the combination of m-TESE and ICSI, KS men are not considered as infertile anymore, and are able to have their own biological child. Since spermatozoa from KS subjects originates from euploid germ cells, there is no increased risk of having a KS child compared to infertile men with normal karyotype [33]. In fact, more than 200 healthy offspring were born worldwide from KS fathers and only a few cases of KS fetus/newborns were reported [34–36].

Given the encouraging data that KS offspring seems not to be affected by the genetic disease of the father, it remains still an open question whether Preimplantation Genetic Diagnosis (PGD) or pre-natal genetic analyses should be recommended [13].

2.1.2. 46,XX Testicular/Ovo-Testicular Disorder of Sex Development

Another karyotype anomaly causing azoospermia is the 46,XX testicular/ovo-testicular Disorder of Sex Development (DSD), also known as 46,XX male syndrome. It was firstly described by De la Chapelle and colleagues in 1964 [37], referring to a rare, heterogeneous clinical condition with an incidence of about 1:20,000–25,000 male newborns [38,39]. The phenotype is largely dependent on the presence or absence of the SRY gene. The SRY gene located on the short arm of the Y chromosome (Yp) is the master gene of male sex determination. The majority of 46,XX testicular–DSD cases are SRY positive (SRY+) (90%) [38,40], thanks to the translocation of SRY-containing segments of the Y chromosome onto the X chromosome during paternal meiosis. These patients usually present with completely differentiated male external and internal genitalia, but decreased testis volume. The minority of 46,XX DSD cases are SRY negative (SRY–). These patients have ambiguous genitalia, poor virilization and ovo-testicular –DSD. The pathogenesis behind this condition could be autosomal gene mutations/over-expression, a gain-of-function in key testicular pathway genes, causing testis differentiation. The most common mechanism is the duplication of SRY-Box 9 (SOX9) gene. Seldomly, duplications of SRY-Box 3 (SOX3) and SRY-Box 10 (SOX10) genes have also been described in 46,XX male cases [41]. Both

genes are highly homologous with SOX9, hence their increased expression could mimic SOX9 gene's function, leading to testis development. Moreover, extremely rare genetic defects, such as large duplication of the Fibroblast growth factor 9 (FGF9) gene [42], or, on the other hand, Loss-of-Function (LoF) mutations in the female pathway genes i.e., in R-spondin 1 (RSPO1) and Wnt Family Member 4 (WNT4) genes have also been associated with 46,XX SRY– DSD cases [43].

A common clinical finding among this group of patients is azoospermia, due to the lack of Y chromosome linked AZF regions, which are essential for physiological spermatogenesis. Hence, in these patients the chance to find spermatozoa in their testicles with sperm harvesting methods is zero. If the couple desires to have children, sperm donation is the only viable option, or adoption. Another frequent remark is the decreased height, corresponding to the absence of growth-regulation genes on the Y chromosome and testosterone level ranging from normal to low with increased FSH and LH levels.

2.1.3. Yq–

The absence of the long arm of the Y chromosome (Yq−) is inevitably linked with azoospermia since it contains crucial genes for spermatogenesis mapping to the AZF regions. These patients present with small testes due to SCOS which recapitulates the complete AZFa deletion phenotype.

2.2. Microdeletions of the Y Chromosome: AZF Deletions

The male sex chromosome is particular for its size, genomic structure, content, and evolutionary trajectory [44,45]. It is haploid, and it precludes recombination with the X chromosome for most of its length (except the two pseudoautosomal regions, PAR1 and PAR2). Moreover, it contains segmental duplications arranged in direct or inverted repeats, and palindromes [44]. The existence of these duplicated sequences allows a mechanism called Non-Allelic Homologous Recombination (NAHR), which might lead to recurrent deletions/duplications affecting the gene dosage on the Y chromosome. The first de novo deletions on the long arm of the Y chromosome (Yq) were described by Tiepolo and Zuffardi in 1976 [46], predicting the presence of Azoospermia Factor(s) (AZF). Further research defined three deletion patterns in proximal, middle and distal Yq11, designated as AZFa, AZFb and AZFc [47]. AZF microdeletions are generally de novo and their origin most likely derives in the testis of the patient's father. In fact, during meiosis NAHR between sister chromatids may take place leading to AZF deletions [48]. Five Yq fragile sites exist, resulting in the recurrent removal of DNA segments ranging from 0.8 to 7.7 Mb. The most frequently deleted subregion is the AZFc (around 80%), followed by AZFa (around 0.5–4%), AZFb (around 1–5%), and AZFbc, with two different breakpoints (around 1–3%) [49]. The AZF microdeletions are well-known causes of male infertility since AZF regions comprise important spermatogenesis associated genes and gene families [49]. The frequency of complete AZF deletions is 1:4000 in the general population, but it rises up to 5–10% of patients affected by idiopathic NOA [49,50]. The deletion phenotypes for each region are reported in Figure 2. Due to the presence of several genes and their multicopy nature in these regions, it is difficult to understand which genes cause the associated phenotype.

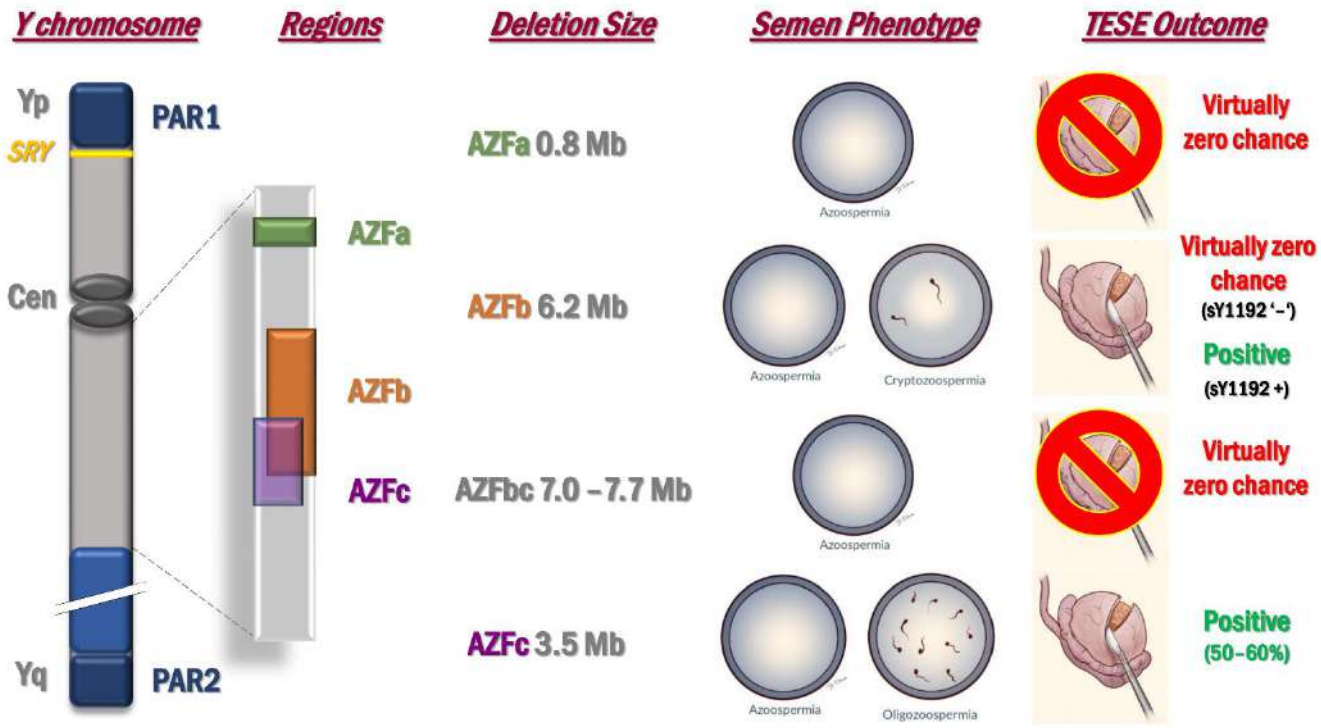

Figure 2. Semen phenotype and TESE outcomes of the different types of AZF microdeletion. Abbreviations: AZF—Azoospermia Factor Region; Cen—centromere; PAR1—Pseudoautosomal Region 1; PAR2—Pseudoautosomal Region 2; *SRY*—Sex-determining Region Y gene; TESE—Testicular Sperm Extraction.

The AZFa region is 792 kb long and contains two ubiquitously expressed, single-copy genes, USP9Y and DDX3Y (former DBY). USP9Y encodes a protein with ubiquitin C-terminal hydrolase activity, which may play an important regulatory role at the level of protein turnover [51]. The isolated absence of this gene is associated with a large spectrum of testis phenotypes, ranging from azoospermia with hypospermatogenesis to normozoospermia [52]. Based on this, USP9Y is more likely to be a fine tuner that improves efficiency, than a gene with an essential function. On the other hand, DDX3Y encodes an ATP-dependent RNA helicase that is a member of the well-conserved DDX3 DEAD Box Helicase family [53]. DDX3Y protein was found predominantly in spermatogonia, whereas its X chromosome homologue, DDX3X, was found to be expressed after meiosis in spermatids. Although isolated mutation or removal of the DDX3Y gene has not been reported, it is likely that the removal of DDX3Y gene is responsible for the AZFa deletion phenotype, which is SCOS. SCOS is characterized by the total absence of germ cells in the testis, low testis volume and high FSH.

Complete AZFb deletion removes a 6.2 Mb DNA segment, including 32 copies of genes and transcription units. These genes are likely to be involved in germ cell maturation since their removal causes spermatogenic arrest. MA is a cessation of germ cell formation mainly at the spermatocyte stage, resulting in azoospermia. MA is typically associated with normal testicular volume and normal gonadotropin levels (LH, FSH), which might mimic obstructive-azoospermia.

The AZFc region involves 12 genes and transcription units, each present in a variable number of copies resulting a total of 32 copies. The clinical manifestation in complete AZFc deletion carriers is largely variable. Spermatozoa may be detected in the ejaculate but typically less than 2 million/mL [50]. Since a progressive decline in sperm count has been observed among these subjects, sperm cryopreservation should be offered to prevent future testis surgery. In those patients who present azoospermia the testis phenotype ranges from SCOS to hypospermatogenesis.

Y chromosome deletion screening is performed in a standardized way, according to the EAA/EMQN guidelines [6]. Two markers are used for each AZF regions besides the control markers of *SRY* and *ZFX/ZFY*. At this regard, it is worth to note that one of the two AZFa markers proposed in the guidelines, contains a polymorphic site in sY84 primer sequence [54]. This SNP (rs72609647) is relatively frequent in the Chinese population while it is yet undetected in the Caucasian population. It is therefore mandatory to use alternative STSs in case of failed amplification of the sY84 marker. For neighboring markers, the "MSY breakpoint mapper" can be consulted (http://breakpointmapper.wi.mit.edu/) [6].

The AZF deletion screening does not only useful for diagnostic purposes but it is also important for TESE prognosis (Figure 2). Carriers of complete AZFa and AZFb deletions have virtually zero chance to find spermatozoa in their testes. In fact, the correct distinction between complete and partial AZFa or AZFb deletions is mandatory through the second step, extension analysis of the AZF deletions. Concerning the AZFb region, Stouffs and colleagues [55] reported two patients with suspected complete AZFb deletion based on the routine first step analysis but with residual sperm production. By performing the extension analysis of the deletion in these patients, the authors were able to amplify a specific distal STS (sY1192) of the AZFb region. This finding indicates that the retention of some copies of multicopy genes such as *PRY*, *RBBMY*, *BPY2*, *DAZ* and some transcription unites may allow residual spermatogenesis and complete maturation. From a clinical point of view, this report called the attention to the importance of testing for sY1192, which is now considered as a decision-making marker: if it is present, TESE might be attempted (Figure 2).

Concerning AZFc microdeletion carriers, spermatozoa can be retrieved through m-TESE from the testicles with a success rate of 50–60% [56]. Data in the literature indicate that a significant proportion of spermatozoa from AZFc microdeletion carriers are nullisomic for sex chromosomes [57,58]. Yq microdeletions could be associated with an overall Y chromosomal instability, which can lead to the formation of 45,X0 cell lines. Karyotype analysis with the search of 45,X0/46,XY mosaicism should be performed on peripheral blood lymphocytes, since mosaicism is considered as a negative predictor of sperm retrieval success [57,59–61].

3. Monogenic Forms of Azoospermia

All the etiological categories of azoospermia include monogenic causes. Some of them are routinely tested in specific pathological conditions, such as CHH and CBAVD [7,8]. The discovery of monogenic defects of quantitative alterations of spermatogenesis due to primary testicular failure is increasing constantly, but their screening has not been introduced into the diagnostic workup of NOA men, so far. In about 70% of NOA without known acquired causes, the etiology remains unknown, and we refer to it as "idiopathic" NOA (iNOA) (Figure 1). The recent application of NGS (especially in familial cases of NOA) has rapidly increased the number of novel NOA candidate genes. Currently 17 of them have been validated by more than one independent study.

3.1. Congenital Hypogonadotropic Hypogonadism (CHH)

CHH is a rare endocrine disease (1:8000 males), caused by the deficient production, secretion or action of the Gonadotropin-Releasing Hormone (GnRH), in the absence of anatomical or functional abnormalities of the hypothalamic-pituitary axis. CHH is a clinically heterogeneous condition covering a wide spectrum of symptoms, where the typical clinical features are delayed puberty and azoospermia. CHH can manifest itself with anosmia/hyposmia (Kallmann Syndrome) or as a normosmic form (nCHH). In addition, non-reproductive features can be recognized in CHH patients, i.e., midline facial defects (cleft lip or palate), unilateral renal agenesis (URA), hearing loss, synkinesia, dental agenesis and short metacarpals [62]. Furthermore, CHH can occur as a part of complex genetic syndromes, such as CHARGE syndrome, Gordon Holmes syndrome and Waardenburg syndrome. CHH is heterogeneous not only clinically but also genetically. To date, more

than 40 genes with variable expressivity, penetrance and inheritance have been identified as the genetic cause of the disease and reviewed in two recent articles [7,8]. Currently, through the sequencing of a targeted-gene panel using NGS, a genetic diagnosis is possible in about 50% of cases, and it is expected that novel CHH-associated genes will be discovered by WES analysis in the near future. To note, the main challenge in NGS –based methods is the interpretation of variants of unknown significance (VUSs), hence appropriate tools and expertise are needed for correct interpretation of these findings in the clinical practice.

CHH presents three peculiar features: (1) distinguishing between KS and nCHH is often difficult from a genetic point of view, because mutations in genes involved in GnRH-mediated neuronal migration might result in both forms of CHH [63]. With the exception of certain genes purely associated with Kallmann syndrome or with nCHH, some others (for instance *FGFR1* and *PROKR2*) can be involved in both clinical manifestations, even within the same kindred [7]. (2) CHH is no longer viewed as a Mendelian monogenic disease, since rare variants in two (digenicity) or more (oligogenicity) candidate genes have been found in the same patient, supporting a digenic/oligogenic inheritance in about 20% of cases [63]. Although the oligogenic basis of CHH makes the genotype-phenotype correlation even more complex, it may explain the variable penetrance of the same pathogenic variant within the same family members [7,8]. (3) The traditional view of CHH as a lifelong disease has been changed following the observation of spontaneous remission in patients affected by Kallmann syndrome or nCHH, regardless of type of identified genetic defect [64]. Thus, a periodic suspension of the substitutive testosterone therapy is advised in order to verify the "reversibility".

In about 80% of CHH patients, spermatogenesis is induced after 9–18 months of gonadotropin treatment [64], and mutations can be transmitted either through spontaneous pregnancy or through Assisted Reproductive Techniques (ART). Overall, the complexity of this disease makes predicting the exact health consequences for the offspring difficult; however, PGD or prenatal diagnosis should be offered to couples, only for syndromic cases and after taking into consideration national legislations.

3.2. Congenital Bilateral Absence of Vas Deferens (CBAVD)

CBAVD is a congenital developmental disease (1:1000 males) characterized by the lack of both vas deferens. It may manifest with various clinical features depending on the association or not with other abnormalities of the male urogenital tract, mainly of the seminal vesicles (50% of cases) and the kidneys (renal agenesis occurring in 5–10% of CBAVD patients) [65]. The prevalence of CBAVD in azoospermic men is estimated to be 4–17% and raises up to 25% in case of OA [3,66]. CBAVD associated with agenesis of the seminal vesicles is characterized by a typical semen phenotype, consisting of azoospermia, low semen volume (<1 mL), acid pH (<7). In contrast to CHH, which is a genetically heterogeneous condition (see above), the genetic contribution of CBAVD without kidney anomalies is limited to two genes: (1) Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene for the 75–80% of cases and (2) Adhesion G Protein-Coupled Receptor G2 (*ADGRG2*) for the 5–10% of cases.

CFTR gene spans 250 kb on the long arm of chromosome 7 (7q31.2), including 27 exons, and encodes for a functional protein of 1480 amino acids. The CFTR protein is involved in the regulation of several ions transporters, including sodium channel, chloride/bicarbonate exchangers, proton exchangers and water channels, and its biallelic dysfunction is responsible for the Cystic Fibrosis (CF) phenotype. Up to now, more than 2000 mutations have been reported in this gene (http://www.genet.sickkids.on.ca/). Disease causing mutations in *CFTR* may affect not only coding regions but also the promoter and deep intronic regions [67,68]. *CFTR* disease-causing alleles can be classified into two main types: (i) "severe" or CF-causing mutations, which are always associated with CF in a homozygous state; (ii) "mild" or non-CF-causing mutations, which have never been observed in CF patients. The presence of two "mild" mutations or one "severe" plus one "mild" allele is causative for CBAVD. Up to 33% of CBAVD subjects of European descendent are compound heterozy-

gous carrying the CF-causing mutation F508del (c.1521_1523delCTT; p.Phe508del) and the non-CF-causing intronic 5T allele (IVS8-5T allele), whose frequency is 4-to-5 times higher in CBAVD patients [69]. However, it is worth noting that some *CFTR* variants are associated with variable expressivity, also called as "variants of varying clinical consequence", for instance R117H (c.350G > A; p.Arg117His) and IVS8-5T allele. It remains difficult to predict the associated phenotypes in those cases in which one of these variants is in trans with another one of the same type or when it is in trans with a known pathogenic variant [70]. Thus, a genotype involving these variants should be interpreted carefully and reported thoughtfully. For instance, the penetrance of 5T allele is dependent on the status of the adjacent poly TG tract, which usually contains 11, 12, or 13 repeats (c.1210–34TG [11], c.1210–34TG [12], c.1210–34TG [13]). When paired with a known CF-causing variant, 5T and 11TG variants in cis rarely predispose to CBAVD, whereas 5T in cis with 12TG or 13TG confers a risk for CBAVD. Given that 1:10 individuals carry the 5T variant, interpretation of its clinical value should always be performed in the context of the number of associated TG repeats [70].

ADGRG2 is an X-*linked* gene encoding an adhesion-class G protein-coupled receptor and is highly expressed in the efferent ducts [71]. In 2016, Patat and colleagues identified 3 hemizygous truncating mutations of the *ADGRG2* gene in 4 out of 26 selected CBAVD subjects with normal kidneys and wild-type *CFTR* gene [72]. Interestingly, *Adgrg2*-mutant mice develop fluid accumulation in the deferent ducts, leading to an obstructive infertility phenotype, which resembles that observed in men with *ADGRG2* mutation [73]. Three other studies have subsequently reported the identification of five new rare variants of *ADGRG2* in 6 CBAVD patients of Asian origin with no pathogenic *CFTR* mutation [74–76]. Recently, six novel truncating mutations of *ADGRG2* have been reported in a cohort of 53 French CAVD men carrying none or only one *CFTR* mutation and not presenting with URA [77]. The authors suggested that hemizygous *ADGRG2* mutations are responsible for CBAVD phenotype with normal kidneys, accounting for approximately 20% of unexplained CBAVD cases after a comprehensive analysis of *CFTR* gene [77].

Despite this later discovery, 10–15% of CBAVDs remains without a genetic diagnosis. A portion of these unexplained CBAVD cases coexists with URA, suggesting an early organogenesis disorder. On the contrary, CBAVD related to *CFTR* or *ADGRG2* mutations might be the result of a progressive degeneration that begins later in fetal life and probably continues after birth [65]. The small percentage of CBAVD cases with normal kidneys lacking of genetic diagnosis could be explained by defects in additional genes. For instance, the *SLC9A3* gene has been proposed to be involved in some idiopathic CBAVD cases of Taiwanese origin [78,79]. In addition, epigenetic or environmental factors could contribute to the development of this disease [65].

The clinical management of men affected by CBAVD without renal agenesis must include *CFTR* analysis, which should be based on two steps: (1) a targeted panel of the most commonly known CF-causing mutations in the Caucasian ethnic group, including R117H and 5T allele; (2) a comprehensive screening of *CFTR* for rare point mutations and large rearrangements, when none or only one single mutation has been identified by the first step. If the CBAVD man has no biallelic mutations after comprehensive *CFTR* testing, the analysis of *ADGRG2* is suggested, especially if there is a family history of male infertility. With the high accessibility to NGS-based testing, it could be more cost-effective to perform directly a comprehensive scanning of both genes [65]. Again, the main issue concerns the assignment of a clinical value to VUSs identified by NGS-based methods.

Since the testicular function of CBAVD patients is usually normal, conception of a biological child is possible through TESE combined with ICSI. Given that the carrier frequency of *CFTR* mutations in the Caucasian ethnicity is high (1:25), the *CFTR* panel screening in the female partners is mandatory. If mutations are detected in both partners, the risk of an offspring with CF (classical CF or non-classical CF) is very high and PGD should be advised to the couple.

3.3. Primary Testicular Failure

NOA (1:100 males) represent >70% of total azoospermia cases and the majority of them shows primary testicular failure due to an intrinsic defect in the onset or progression of spermatogenesis. As reported in the introduction, NOA can manifest with various testicular histologies, including SCOS, MA at different stages and hypospermatogenesis. SCOS and complete MA are characterized by the absence of haploid cells in the testes, hence the TESE procedure to recover spermatozoa for subsequent ICSI is unsuccessful. In case of incomplete MA, some tubules containing round or later stage spermatids may be found. For this purpose, the development of a pre-TESE diagnostic gene panel would be of high clinical benefit to prevent unnecessary surgery in patients with pure SCOS/MA.

To date, the knowledge of monogenic causes of NOA due to primary testicular failure is limited and none of the current clinical guidelines includes mutational analysis of any NOA genes [9,11]. Given that the spermatogenic process is inherently complex and >2000 genes participate in it [80,81], a high genetic heterogeneity seems to be plausible. So far, 38 candidate genes for iNOA were reported based on the resembling reproductive phenotype in the mouse or on the family segregation (Table 1). Currently, only 17 of them have reached "moderate" or higher grade of clinical evidence, according to the classification criteria proposed by Oud and colleagues [9].

These genes are responsible either for isolated form of NOA or for complex phenotypes where azoospermia may represent one of the clinical symptoms underlying a certain syndrome. In this latter case, we can refer to them as "phenocopy" genes. It is worth noting that, for certain congenital diseases, syndromic features may be non-penetrant in the patient, as well as in the family, or may become evident only with age. For instance, in *FANCA*-mutated patients affected by iNOA, the complex Fanconi Anemia (FA) phenotype may be subtle and need to be specifically assessed to reach a diagnosis of "occult" FA in adulthood (see below; [82]). Thus, in case of pathogenic variants in "phenocopy" genes, a clinical re-evaluation of the patients and relatives should always be part of the diagnostic workup of NOA men.

Very recently, by using STRING analysis of physical and functional protein-protein interactions, Kasak and Laan demonstrated that candidate NOA genes belong to a dense network of "predicted functional partners" [11]. In particular, two distinct clusters of proteic interactions were observed, one for isolated NOA and the other one for syndromic conditions, emphasizing the different etiology underlying the two forms of NOA.

With the exception of *TEX11* and *WNK3* which are X-linked, the remaining candidate genes are mapping to autosomes. Apart from *DMRT1, NR5A1, PLK4* and *WT1* which follow the autosomal dominant inheritance pattern, only recessive mutations lead to quantitative alterations of spermatogenesis (Table 1).

In this paragraph, we will discuss the candidate genes for iNOA (Table 1) according to the different associated-testicular histologies such as SCOS, MA, different types of testis phenotype and undefined testis phenotype.

Table 1. List of candidate genes involved in monogenic causes of Human NOA, divided according to the associated-testis histology: (**a**) SCOS phenotype, (**b**) maturation arrest phenotype, (**c**) different types of testicular phenotype (SCOS/MA/hypospermatogenesis) and (**d**) undefined testicular phenotype. Detailed genomic and clinical information, the female and male mouse reproductive phenotypes and references are reported.

(a)

Gene ^	OMIM	Locus °	Function +	Inheritance	Other Phenotypes	POI	Mouse Reproductive Phenotypes #	Segregation in Family	More than One Unrelated Carrier	Independent Cohorts	Refs.
FANCA	607139	16q24.3	Interstrand crosslink repair	AR	Fanconi Anemia	Yes	Abnormal male meiosis, decreased germ cell number, decreased mature ovarian follicle number, absent ovarian follicles	Yes	Yes	No	[82]
PLK4	605031	4q28.1	Centriole duplication during the cell cycle	AD	Microcephaly and chorioretinopathy	No	Decreased male germ cell number	No	No	No	[83]
WNK3	300358	Xp11.22	Regulation of electrolyte homeostasis, cell signaling, survival and proliferation	XLR	n.r.	No	Normal *	Yes	No	No	[84]

(b)

Gene ^	OMIM	Locus °	Function +	Inheritance	Other Phenotypes	POI	Mouse Reproductive Phenotypes #	Segregation in Family	More than One Unrelated Carrier	Independent Cohorts	Refs.
ADAD2	n.a.	16q24.1	dsRNA-binding protein, RNA editing	AR	n.r.	No	Male and female infertility	Yes	Yes	Yes	[10]
C14orf39	617307	14q23.1	Chromosome synapsis during meiotic recombination	AR	n.r	Yes	Arrest of male meiosis, abnormal chiasmata formation, abnormal chromosomal synapsis, abnormal X-Y chromosome synapsis during male meiosis, absent oocytes	Yes **	Yes	No	[85]
DMC1	602721	22q13.1	Meiotic recombination, DNA DSB repair	AR	n.r.	Yes	Arrest of male meiosis decreased oocyte number, absent oocytes, absent ovarian follicles, abnormal female meiosis	Yes **	No	No	[86]
KASH5	618125	19q13.33	Meiotic telomere attachment to nuclear envelope in the prophase of meiosis, homolog pairing during meiotic prophase	AR	n.r.	No	Arrest of male meiosis, female infertility	Yes	No	No	[84]
MAJIN	617130	11q13.1	Meiotic telomere attachment to the nucleus inner membrane during homologous pairing and synapsis	AR	n.r.	No	Meiotic arrest, male and female infertility	No	No	No	[87]
MEI1	608797	22q13.2	Meiotic chromosome synapsis, DBS formation	AR	Hydatidiform mole	Yes	Arrest of male meiosis, female infertility	Yes	Yes	Yes	[10,88,89]

Table 1. *Cont.*

(b)										
Gene ^	**OMIM**	**Locus °**	**Function +**	**Inheritance**	**Other Phenotypes**	**POI**	**Mouse Reproductive Phenotypes #**	**Segregation in Family**	**More than One Unrelated Carrier**	**Independent Cohorts**	**Refs.**
MEIOB	617670	16p13.3	DNA DSB repair, crossover formation and promotion to complete synapsis	AR	n.r.	Yes	Arrest of spermatogenesis, decreased oocyte number, absent oocytes	Yes	Yes	Yes	[10,90,91]
MSH4	602105	1p31.1	Homologous chromosomes recombination and segregation at meiosis I	AR	n.r.	Yes	Azoospermia, abnormal male and female meiosis	No	Yes	Yes	[10]
RAD21L1	n.a.	20p13	Meiosis-specific component of some cohesin complex	AR	n.r.	No	Arrest of male meiosis, absent oocytes, decreased mature ovarian follicle number, absent primordial ovarian follicles	Yes	No	No	[10]
RNF212	612041	4p16.3	Regulator of crossing-over during meiosis	AR	n.r.	No	Arrest of male meiosis, female infertility	Yes	No	No	[92]
SETX	608465	9q34.13	DNA and RNA processing	AR	Amyotrophic lateral sclerosis; ataxia with oculomotor apraxia type 2	Yes	Arrest of male meiosis, globozoospermia, reduced female fertility	No	Yes	Yes	[93,94]
SHOC1	618038	9q31.3	Binds to single-stranded DNA and DNA branched structures; formation of crossover recombination intermediates	AR	n.r.	No	Arrest of male meiosis	Yes	Yes	Yes	[10,95]
SPINK2	605753	4q12	Inhibitor of acrosin	AR	n.r.	No	Kinked sperm flagellum, oligozoospermia, teratozoospermia, abnormal male germ cell apoptosis	Yes	No	No	[1,96]
SPO11	605114	20q13.31	Initiation of DSBs	AR	n.r.	No	Arrest of male meiosis, decreased oocyte number, oocyte degeneration, abnormal female meiosis	Yes	No	No	[84]
STAG3	608489	7q22.1	Cohesion of sister chromatids, DNA DSB repair	AR	n.r.	Yes	Azoospermia, absent oocytes	Yes **	Yes	Yes	[10,92,97,98]
STX2	132350	12q24.33	Sulfoglycolipid transporter	AR	n.r.	No	Arrest of male meiosis	No	No	No	[99]
SYCE1	611486	10q26.3	Chromosome synapsis in meiosis	AR	n.r.	Yes	Arrest of male meiosis, decreased mature ovarian follicle number	Yes	Yes	Yes	[10,100,101]
TDRD7	611258	9q22.33	RNA processing	AR	Congenital cataract	No	Arrest of spermatogenesis, abnormal male germ cell apoptosis	Yes	Yes	No	[2,102]

Table 1. *Cont.*

(b)

Gene ^	OMIM	Locus °	Function +	Inheritance	Other Phenotypes	POI	Mouse Reproductive Phenotypes #	Segregation in Family	More than One Unrelated Carrier	Independent Cohorts	Refs.
TERB1	617332	16q22.1	Meiotic telomere attachment to the nucleus inner membrane during homologous pairing and synapsis	AR	n.r.	No	Arrest of male meiosis, absent oocytes, absent ovarian follicles, abnormal female meiosis I arrest	Yes	Yes	Yes	[10,87]
TERB2	617131	15q21.1	Meiotic telomere attachment to the nucleus inner membrane during homologous pairing and synapsis	AR	n.r.	No	Arrest of male meiosis, absent ovarian follicles, abnormal female meiosis	Yes	No	No	[87]
TEX11	300311	Xq13.1	Chromosome synapsis and formation of crossovers	XLR	n.r.	No	Arrest of male meiosis, meiotic non-disjunction during M1 phase	Yes	Yes	Yes	[10,103–105]
XRCC2	600375	7q36.1	Interstrand crosslink repair, DNA DSB repair	AR	Fanconi Anemia	Yes	Meiotic arrest, POI	Yes **	No	No	[106]
ZMYND15	614312	17p13.2	Transcriptional repressor	AR	n.r.	No	Azoospermia	Yes	No	No	[107]

(c)

Gene ^	OMIM	Locus °	Function +	Inheritance	Other Phenotypes	POI	Mouse Reproductive Phenotypes #	Segregation in Family	More than One Unrelated Carrier	Independent Cohorts	Refs.
DMRT1	602424	9p24.3	Transcription factor involved in male sex determination and differentiation	AD	Ambiguous genitalia and sex reversal	No	Abnormal male meiosis, male infertility	Yes	Yes	Yes	[10,108]
FANCM	609644	14q21.2	DNA DSB repair, interstrand cross-link removal	AR	n.r.	Yes	Azoospermia, decreased mature ovarian follicle number	Yes	Yes	Yes	[109,110]
M1AP	619098	2p13.1	Meiosis I progression	AR	n.r.	No	From arrest of male meiosis to severe oligozoospermia/globozoospermia	Yes	Yes	Yes	[111]
NANOS2	608228	19q13.32	Spermatogonial stem cell maintenance	AR	n.r.	No	Azoospermia, abnormal female meiosis	Yes	Yes	No	[84]
NR5A1	184757	9q33.3	transcriptional activator for sex determination	AD	46, XY and 46, XX sex reversal; adrenocortical insufficiency	Yes	From oligozoospermia to arrest of spermatogenesis, decreased mature ovarian follicle number, absent mature ovarian follicles	Yes **	Yes	Yes	[112–116]
TAF4B	601689	18q11.2	Transcriptional coactivator	AR	n.r.	No	Oligozoospermia, decreased male germ cell number, asthenozoospermia, absent mature ovarian follicles, impaired ovarian folliculogenesis	Yes	No	No	[107]

Table 1. *Cont.*

(c)										
Gene ^	**OMIM**	**Locus °**	**Function +**	**Inheritance**	**Other Phenotypes**	**POI**	**Mouse Reproductive Phenotypes #**	**Segregation in Family**	**More than One Unrelated Carrier**	**Independent Cohorts**	**Refs.**
TDRD9	617963	14q32.33	Repression of transposable elements during meiosis	AR	n.r.	No	Arrest of male meiosis	Yes	No	No	[117]
TEX14	605792	17q22	Formation of meiotic intercellular bridges	AR	n.r.	No	Arrest of male meiosis	Yes	Yes	Yes	[10,84,90]
TEX15	605795	8p12	Chromosome, synapsis, DNA DSB repair	AR	n.r.	No	Arrest of male meiosis	Yes	Yes	Yes	[118,119]
WT1	607102	11p13	Transcription factor	AD	Wilms tumor type 1; Nephrotic sdr type 4; Denys-Drash sdr; Frasier sdr; Meacham sdr; Mesothelioma	Yes	Azoospermia	No	Yes	Yes	[120–122]
(d)										
Gene ^	**OMIM**	**Locus °**	**Function +**	**Inheritance**	**Other Phenotypes**	**POI**	**Mouse Reproductive Phenotypes #**	**Segregation in Family**	**More than One Unrelated Carrier**	**Independent Cohorts**	**Refs.**
MCM8	608187	20p12.3	DNA DSB repair, interstrand crosslink removal	AR	n.r.	Yes	Arrest of male meiosis, decreased oocyte number, decreased mature ovarian follicle number, increased ovary tumor incidence, increased ovary adenoma incidence	Yes **	No	No	[123]
PSMC3IP	608665	17q21.2	Stimulating DMC1-mediated strand exchange required for pairing homologous chromosomes during meiosis.	AR	Ovarian dysgenesis	Yes	Arrest of male meiosis, absent ovarian follicles, abnormal ovary development	Yes **	No	No	[124]

^ based on HGNC; ° according to Human GRCh38/hg38; + based on GeneCards database; # male and female mouse reproductive phenotypes based on MGI database; * *Wnk3* −/− females from *Wnk3* Y/− fathers were reported [125], although the mouse male reproductive phenotype was not investigated (subtle spermatogenic impairment cannot be excluded); ** cosegregating in family with NOA and POI phenotypes. n.r. = not reported; n.a. = not available; POI = Primary Ovarian Insufficiency; DBS = double-strand break; sdr = syndrome; XLR = X-linked recessive.

3.3.1. Candidate Genes for the SCOS Phenotype

FANCA: plays an important role in adult-onset syndromic NOA cases. It belongs to the FA pathway [126], taking part in the interstrand crosslink repair and DNA double-strand break (DBS) repair. The *FANCA* gene is the most commonly mutated gene in the genetically heterogeneous FA disorder with variable age of onset [127]. Starting from WES analysis followed by targeted gene sequencing, Krausz and colleagues identified homozygous *FANCA* pathogenic variants in 3/29 selected iNOA patients with SCOS and with slightly altered/borderline hematological parameters [82]. This study underlies the fact that WES may lead to important incidental findings. In this study, undiagnosed FA cases (late-onset FA) before the appearance of other severe clinical manifestations of the disease, including hematological and solid cancers, have been identified. This finding indicates that andrological evaluation, especially in SCOS patients, should not only include hormone measurement but also blood count in order to diagnose "occult" FA cases [82].

PLK4: encodes for a protein, which plays a crucial role in the centriole duplication, which is a critical process to be completed before primary spermatocytes can undergo meiosis [128]. It is well-known that homozygous LoF variants in *PLK4* cause microcephaly and chorioretinopathy [129]. However, one man affected by iNOA due to SCOS has been reported as a carrier of a heterozygous 13 bp deletion in the Ser/Thr kinase domain of PLK4 [83]. It is noteworthy that a heterozygous *Plk4*-mutation in mice caused patchy germ cell loss in the testes [130], similar to human, strengthening the potential role of *PLK4* as a dominant cause of SCOS.

WKN3: *X-linked* gene with high testis-specific expression, encoding an activator of NKCC1, which has been proposed to cosegregate with the NOA phenotype in a family from Oman [84]. The proband presented with SCOS, but no histological data was available for the affected brothers, thus a phenotypic concordance cannot be established [84]. *Knockout* (KO) *Wnk3* male mice is fertile [125], suggesting that the link between this gene and infertility may not be identical across mouse and men [84]. However, the disruption of the Wnk3-actived *Nkcc1* gene in mice leads to spermatogenic defects resulting in the complete absence of spermatocytes [131].

3.3.2. Candidate Genes for the MA Phenotype

So far, the histological category with the largest number of monogenic defects is represented by the MA phenotype, comprising 22 genes implicated in different stages of spermatogenesis (Table 1b). Six genes involved in meiosis (*MEI1, MEIOB, TEX11, SYCE1, STAG3*, and *SETX*) have been reported to be strictly causative of spermatocyte arrest.

MEI1: is implicated in DBS formation, and, along with other NOA causing genes such as *MEIOB, TEX11, TEX15* and *SYCE1*, contributes to the formation and maintenance of the synaptonemal complex and crossovers between homologous chromosomes [11]. Recessive mutations in *MEI1* have been reported in NOA patients exhibiting complete SCA [10,88,89].

MEIOB: besides crossover formation and promotion of synapsis during meiosis, it is especially required for DBS repair [132,133]. Men carrying biallelic LoF mutations in this gene also present complete SCA [10,90,91], resulting in an arrest at metaphase I [10]. Interestingly enough, *MEIOB* LoF variants cluster in exon 12, suggesting a hotspot variant region, at least in the Arab/Pakistani population [10,91,134].

TEX11: is an X-linked gene belonging to the family of Testis Expressed genes, with the strongest evidence for NOA due to MA [103–105]. By using high-resolution array-Comparative Genomic Hybridization (CGH) to screen men with NOA, a recurring deletion of three exons of *TEX11* in two patients has been identified [105]. Furthermore, by sequencing *TEX11* in larger groups of azoospermic men, disease-causing mutations were detected, accounting for more than 1% of NOA cases [104,105,135]. Very recently, Krausz and colleagues demonstrated that defects in the human gene showed a complete metaphase arrest, suggested by a residual spermatocytic development together with the dramatic increase in the number of apoptotic metaphases [10].

SYCE1: is a member of the synaptonemal complex, which links homologous chromosomes during prophase I of meiosis. Homozygous mutations in this gene are associated with NOA due to complete SCA [10,100,101]. Meiotic analysis on testicular tissue of *SYCE1* mutation carrier revealed a pachytene arrest (no XY body formation), consistent with the mouse phenotype, with features of unrepaired meiotic DNA DBS in spermatocytes [10].

STAG3: is involved not only in DBS repair, but also in the formation of chromosomal axis and cohesion of sister chromatids after DNA replication. Riera-Escamilla and colleagues have demonstrated that biallelic LoF mutations in this gene lead to the persistence of meiotic DBS and to a failure to complete chromosome pairing [92]. Further reports on *STAG3* mutation carriers allowed to classify this gene among those presenting definitive/strong clinical evidence for complete SCA [10,97,98].

SETX: is involved in both DNA and RNA processing, and its functional disruption causes syndromic conditions, i.e., Ataxia with Oculomotor Apraxia Type 2 (AOA2) [136] and amyotrophic lateral sclerosis [137]. In the two articles in which testis histology has been described, AOA2 male patients exhibited MA at primary spermatocyte stage [93,94]. This gene represents a typical example of a "phenocopy" gene causing a syndromic disorder in which male and female primary gonadal failure may be one of the clinical signs of the disease. Hence, particular attention must be given to the clinical evaluation of azoospemic patients carrying recessive *SETX* mutations.

In addition to the above reported MA genes, very recently, four novel recessive genes (***ADAD2***, ***TERB1***, ***SHOC1***, and ***MSH4***) have been identified as responsible for MA [10,87,95]. For all these genes the KO mice's phenotype recapitulates the human phenotype and all of them were validated in independent cohorts of iNOA patients. ADAD2 is a double-stranded RNA binding protein, and it was found to be associated with incomplete SGA in two patients belonging to two independent cohorts [10]. Concerning the other three new genes (*TERB1, SHOC1* and *MSH4*), a complete SCA phenotype was observed in mutated patients. TERB1 is a testis specific telomere-associated protein, which is essential in the regulation of chromosome movement to promote homologous pairing during meiotic prophase I [138]. Meiotic studies of the two *TERB1* variant carriers detected a pachytene arrest [10], which recapitulates the mouse phenotype [138]. Carriers of biallelic defects in *SHOC1* and *MSH4* genes showed a metaphase arrest in the testis that was somewhat less severe compared with the mutant mouse models [10,95].

Besides these recurrently mutated candidate genes, the following 13 genes have been reported only in single studies as causative for MA at different stages with the support of in vitro or in vivo experimental data.

C14orf39: encodes a component of the synaptonemal complex and it interacts with SYCE1 via its alfa-helical domain [139]. Very recently, a homozygous frameshift variant of this gene has been identified in two NOA brothers and in their sister affected by Primary Ovarian Insufficiency (POI) [85]. In addition, two different LoF variants have been reported in a homozygous state in two unrelated Chinese NOA-affected patients [85]. All the male *C14orf39* mutation carriers displayed complete SCA [85]. Meiotic analysis on their testicular tissue revealed severe synaptic defects and no XY body formation, indicating that meiosis was arrested at the pachytene-like stage [85]. These observations were strongly supported by the *Six6os1* (the murine orthologue of human *C14orf39*) mutant mouse model, which recapitulated the phenotypes of the NOA and POI individuals [85,139].

DMC1: is a meiosis-specific recombinase interacting with several DNA repair proteins in the FA pathway [140], thus it is essential for meiotic homologous recombination and DBS repair [141–143]. The lack of this protein results in a block at the leptotene or zygotene stage of meiotic prophase I due to the inability to form synaptonemal complexes [86,142]. Recently, a novel homozygous *DMC1* missense mutation has been identified as the genetic cause of both MA at spermatocyte stage and POI in two siblings from a consanguineous Chinese family [86].

SPO11: is essential to initiate meiotic recombination and formation of the synaptonemal complex between homologous chromosomes [144]. To date, a *SPO11* homozygous missense variant has been identified in two brothers with MA [84].

KASH5 and **RNF212**: are involved in the in the synaptonemal complex and have been recently reported as a potential cause of SCA [84,92]. Importantly, the respective mutant mouse models are supportive to the human genetic data [145–147].

STX2: is a sulfoglycolipid transporter. In mice, *Stx2* nullizygosity is known to cause spermatogenic failure [148]. In human, a homozygous frameshift mutation has been reported in one Japanese patient presenting with maturation arrest and multinucleated spermatocytes, which have been also observed in mice lacking *Stx2* [99,148].

XRCC2: belongs to the FA pathway [126], taking part in the interstrand crosslink repair and DNA DBS repair. Concerning the *XRCC2* gene, a meiosis-specific mutation (p.Leu14Pro) has been proposed as a cause of MA in males and POI in females of a consanguineous Chinese family that not showed other major phenotypesuch as FA [106,149]. The male mouse model with the Xrcc2L14P mutation replicated the human MA phenotype.

RAD21L1: is a testis-specific component of the cohesion complex involved in meiotic chromosome pairing and separation [150]. A homozygous stop gain mutation in this gene has been identified in a patient presenting with complete SCA characterized by XY body formation in more than 70% of the tubules indicating completion of synapsis [10]. The authors suggest that *RAD21L1* may be essential for progression beyond meiotic metaphase, but possible not for homologous chromosome synapsis.

TERB2 and **MAJIN**: TERB2 interacts with TERB1 and MAJIN to form the tripartite meiotic telomere complex (MTC), which has been shown in mouse models to be necessary for the completion of meiosis and both male and female fertility [151,152]. Compound heterozygous frameshift variants in *TERB2* gene have been found to cosegregate with MA phenotype in a non-consanguineous family in which 3 sons were affected [87]. Concerning the *MAJIN* gene, a rare homozygous missense variant has been identified in one sporadic case affected by germ cell maturation arrest with occasional post-meiotic round spermatids in 2–4% of tubules [87].

SPINK2: encodes a member of the family of serine protease inhibitors of the Kazal type, which is necessary to neutralize the action of acrosomal proteases shortly after their synthesis and before they can be safely stored in the acrosome [96,153]. A homozygous truncating mutation in the *SPINK2* gene has been reported cosegregating with NOA phenotype due to the arrest of spermatid differentiation at the round stage [96]. Homozygous KO animals also suffered from azoospermia, thus confirming the potential implication of *SPINK2* in NOA [96].

TDRD7: is a component of chromatoid bodies contributing to the post-transcriptional regulation of specific mRNAs and it plays a role in the development of haploid spermatids in adulthood [154]. Recently, biallelic LoF variants in this gene were reported in two consanguineous Chinese families to cause a rare syndrome combining congenital cataract and NOA due to MA [102].

ZMYND15: is a testis-specific transcriptional repressor that controls normal temporal expression of haploid genes during spermiogenesis [155]. Ahyan and colleagues have proposed this gene as a cause of recessive azoospermia in two consanguineous Turkish families [107]. Its functional disruption results in maturation arrest at the spermatid stage [107], suggesting that NOA can also be induced by post-meiotic defects.

3.3.3. Candidate Genes Associated with Different Types of Testicular Phenotype

Pathogenic mutations in some candidate NOA genes are not associated with a clear-cut testicular phenotype and different testis histology, ranging from SCOS to hypospermatogenesis, can be observed in different mutation carriers (Table 1c).

DMRT1: encodes a transcription factor that plays a key role in testis differentiation. Its monoallelic disruption is well-known to be associated with syndromic and non-syndromic forms of XY gonadal dysgenesis [156,157]. By performing genome-wide array-CGH, four

deletions spanning the *DMRT1* gene were reported in a total of five men from independent cohorts affected by isolated NOA [108]. Both SCOS and SCA testicular phenotypes have been found to cosegregate with the deletion of this gene [108]. Very recently, a heterozygous deletion of exons 1 and 2, resulting in the removal of the entire DM/DNA-binding domain, has been observed in a case of incomplete SGA [10]. These findings suggest that besides XY gonadal dysgenesis, *DMRT1* deletions may play a role in the occurrence of NOA.

FANCM: this testis-enhanced gene belongs to the FA pathway. This is one of the few genes in the pathway that does not cause the FA phenotype [158,159]. It plays a crucial role in major cellular functions, including DNA replication/repair and anti-crossover to maintain genomic stability [160]. Kasak and colleagues reported that biallelic LoF variants in this gene are the most likely cause of the SCOS phenotype diagnosed in four patients [110]. Recently, a homozygous frameshift mutation in *FANCM* was found cosegregating with male infertility in a consanguineous Pakistani family, in which three brothers presented with either oligoasthenozoospermia or azoospermia [109]. Hence, the spectrum of the seminal phenotype in patients with biallelic truncating *FANCM* variants seems to be widespread, implying that some mutations may lead to milder phenotypes. Interestingly, the *Fancm*-mutant mice displayed SCO tubules and a progressive loss of germ cells [109,161,162], which may derive from the defective repair of interstrand crosslink occurring during DNA replication of the germ cells. These features strengthen the link between *FANCM* mutations and SCOS phenotype in humans.

NANOS2: encodes an RNA-binding protein that contribute to the maintenance of the spermatogonial stem cell population and suppression of meiotic entry [163]. *Nanos2* KO models lead to male-specific complete germ cell loss in both Drosophila and mouse [164]. After the first study on the role of *NANOS2* as a potential cause of SCOS phenotype [165], a homozygous mutation in this gene was recently reported to cosegregate with SCOS [84] in two brothers from a consanguineous family. However, one additional sporadic patient carrying a homozygous start loss variant presented with MA [84], questioning the clear-cut relation with the SCOS phenotype.

TEX14 and ***TEX15*** belong to the family of Testis Expressed genes. TEX14 is required for the formation/maintaining of intercellular bridges (IC) in vertebrate germ cells, which are essential for meiosis during spermatogenesis [166]. This gene appears to be exclusively expressed in the human and mouse testis and it is conserved among mammals [90]. Severe spermatocyte depletion was observed in *Tex14* KO mice [166]. In humans, recessive mutations in *TEX14* were found to be strong candidates for NOA with testis histology ranging from SCOS to early MA phenotype with negative TESE outcome [10,84,90]. TEX15 plays a key role in the recruitment of DNA repair proteins into DBS locations. It is worth noting that, unlike *TEX14* which is a negative predictor of sperm retrieval in testis, *TEX15* has been found mutated both in patients with NOA and crypto/oligozoospermia [103,118,119,167].

NR5A1 and ***WT1***: encode the Steroidogenic factor 1 (SF1) and the Wilms' tumor protein, respectively, which are well-known and functionally interacting transcription factors implicated in gonadal development in both sexes. In fact, WT1 modulates SF1 in a sex-specific manner [168]. Mutations in *NR5A1* and *WT1* primarily cause AD syndromic phenotypes (reviewed in [169]) associated with NOA due to SCOS or MA [112,115]. Mutations in *NR5A1* are well-known to cause AD primary adrenal insufficiency and 46, XY disorders of sexual development (DSD) [170], besides hypospadias, bilateral anorchia and micropenis in addition to women with POI [171]. Some pathogenic *NR5A1* variants are responsible only for NOA phenotype, without any clearly identifiable developmental defects in the testis [112,113,115]. Mutated or deleted *WT1* leads to a spectrum of congenital defects in kidneys and genitalia (such asDenys-Drash syndrome, Wilms' tumor, nephropathy), including DSD disorder [172]. To note, pathogenic *WT1* missense variants have also been reported in patients with the solely diagnosis of NOA without malformations in the genitourinary tract [120–122]. We can conclude that defects in both genes are characterized by variable expressivity and incomplete penetrance, including asymptomatic family members [173,174], thus the clinical management of these patients needs careful

evaluation. In particular, defects in these genes could be suspected in patients presenting hypospadias, cryptorchidism, and other signs of a congenital testicular damage [170,175].

TAF4B: is a ubiquitous transcription factor acting as a gene-selective coactivator, whose homozygous truncating variants lead to the NOA/oligozoospermia phenotype in a Turkish consanguineous family [107]. Interestingly, *Taf4b* KO mice are subfertile with extensive pre-meiotic germ cell loss due to altered differentiation and self-renewal of the spermatogonial stem cell pool [107].

TDRD9: is essential for silencing of Line-1 (L1) retrotransposon in the male germ line, both in mouse and in human, for enabling fertility [117,176]. A homozygous frameshift mutation in the *TDRD9* gene has been identified in five men of a large consanguineous Bedouin family, diagnosed as having cryptozoospermia/azoospermia due to incomplete MA [117]. The authors conclude that the mutation is the cause of the spermatogenic impairment, resembling that observed in the *Tdrd9* knockout mice, without any involvement in female infertility [117,176].

M1AP: encodes a protein that is likely to function in meiotic progression. Recently, *M1AP* has been found to be mutated in patients affected by NOA due to MA [111]. Data from four independent cohorts revealed that biallelic LoF mutations of *M1AP* are associated with a variable spectrum of severely impaired spermatogenesis, mostly meiotic arrest resulting in azoospermia, but occasionally spermatids and rarely a few spermatozoa in the semen were observed. A similar phenotype has been described for mice with disruption of *M1ap* [111].

3.3.4. Candidate Genes for iNOA with Undefined Testicular Phenotype

Novel promising candidate genes for NOA have been recently reported, without providing the clinical histological data of the carriers, precluding their classification into the above-mentioned testis phenotype categories (Table 1d).

MCM8: has been recently suggested to interact with members of FA pathway in crosslink repair during replication [177]. This gene proved to be crucial for gonadal development and maintenance in humans, both males and females. In fact, homozygous *MCM8* mutations resulting in genetic instability due to meiotic DNA repair defect have been demonstrated to be the cause of NOA in males and POI in females [123].

PSMC3IP: encodes a critical coactivator of DMC1 and RAD51 proteins [178–180] and it is implicated in meiotic recombination. In a consanguineous Yemeni family, a homozygous *PSMC3IP* stop gain mutation deleting the C-terminal portion of the protein has been found to cosegregate with POI and NOA phenotypes [124]. It was found that PSMC3IP protein deprived of C-terminal domain fails to associate with the DMC1 and RAD51 proteins required for homologous recombination [179]. In mice, the absence of Psmc3ip protein leads to the arrest at the primary spermatocyte stage, indicating a block at meiosis I [181].

4. Common Monogenic Defects in Male and Female Primary Gonadal Failure

An emerging issue in the field of human reproduction concerns common genetic factors between male and female infertility. Several genes causing NOA in males are also considered to be involved in female reproduction, leading to the POI phenotype (Table 1). POI is a heterogeneous disorder characterized by primary or secondary amenorrhea in women younger than 40 years of age [182]. The three main mechanisms leading to POI can be: (i) an impaired formation of primordial follicles leading to a reduced number of their pool; (ii) an impaired recruitment and/or an altered maturation of the follicles; (iii) an increased follicular atresia [183]. Isolated or non-syndromic POI is recognized in ~1–2% of women and it has a heterogeneous genetic basis [182,184], which accounts for approximately 20–25% of POI patients [185]. Given both the similar incidence and the identification of shared genetic factors, POI can be considered as the corresponding female phenotype of oligo/azoospermia. In support of this, similarly to NOA, POI is associated with a significantly higher morbidity in respect to females with physiological

menopause [182]. In addition, an increased risk of osteoporosis, cardiovascular diseases, type 2 diabetes has also been reported [182,186], making this condition a public health problem [186]. Candidate genes involved in both POI and NOA pedigrees are mainly related to DNA damage repair (*FANCM*, *FANCA*, *XRCC2*, *MCM8*), homologous recombination and meiosis (*STAG3*, *SYCE1*, *C14orf39*, *MSH4*, *PSMC3IP*, *DMC1*, and *MEIOB*), along with the transcriptional activator involved in sex determination, such as *NR5A1*. In addition, candidate genes of syndromic male and female infertility are *SETX* and *WT1*. It is noteworthy that the inheritance pattern of POI is complex, since at least two mutations in distinct candidate genes have been recognized in 42% of patients, arguing in favor of a oligogenic nature [187].

FANCM: has been first reported in two Finnish sisters affected by non-syndromic POI [188]. The homozygous stop gain *FANCM* mutation identified in this family may provoke meiotic defects leading to a depleted follicular stock, as in *Fancm* −/− mice [188]. Interestingly, the parents and the 20-years-old brother carrying the mutation in a heterozygous state were healthy, confirming the recessive inheritance mode of *FANCM*. Notably, the same homozygous nonsense variant in *FANCM* was identified in an Estonian NOA-affected case [110].

FANCA: two rare heterozygous missense variants have been recently identified by WES in two unrelated females, one with primary amenorrhea and the other one with non-syndromic POI [189]. In order to verify the potential pathogenic effect of heterozygous mutations, the authors performed in vitro studies showing that the mutations in a heterozygous state partial affect FANCA expression levels and its signaling pathways [189]. Heterozygous mutated female mice (*Fanca*+/−) showed reduced fertility and progressive decline of follicles with aging when compared with the wild-type female mice, suggesting a possible contribution of *FANCA* haploinsufficiency to POI [189]. However, given that the mode of transmission is autosomal recessive for most of the meiosis or DNA repair genes, especially for genes of the Fanconi Anemia pathway, it is still debated whether a causal link between heterozygous *FANCA* variants and POI may exist [190].

XRCC2: as mentioned in the previous paragraph, a homozygous missense variant (p.Leu14Pro) of the gene has been proposed as a meiosis-specific mutation causing both NOA and POI [106,149]. Homozygous female mice for the *Xrcc2-L14P* allele exhibited reproductive disorders that were consistent with POI [106].

MCM8: similarly to the above gene, mutations in *MCM8* have been reported as a recessive cause of both isolated and syndromic POI [123,191–196] and isolated NOA [123]. *Mcm8*-deficient mice have small gonads and are infertile (female and male) [197], as observed both in women and in men carrying homozygous *MCM8* inactivating variants [123,191–196]. KO mice models suggested that in both sexes the gonadal function impairs with aging [197].

STAG3 was first described as a POI gene in 2014 [198], and since than recessive high-impact variants have been described as a rare but recurrent cause of non-syndromic POI [198–204]. Very recently, a homozygous *STAG3* missense variant cosegregated with the infertility phenotype in a consanguineous family including a proband with POI and her brother with NOA [97]. These findings are consistent with *Stag3* KO mice, showing an early prophase I arrest and apoptosis in both male and female germ cells [205].

SYCE1: the first report identified a homozygous point mutation in a 13-member-family in which two sisters born to consanguineous parents suffered from POI [206]. Hernandez Lopez and colleagues [207] have demonstrated that the homozygous state of the previously described point mutation severely affects homologous chromosome synapsis, which would most probably account for the observed gametogenesis failure both in male and in female mice. As stated in the previous paragraph, a similar observation was made in male carriers [10]. In addition, the female mutant mice with the absence of recognizable oocytes and follicles in the ovary resemble the clinical description of the sisters who were homozygous for the mutation [206]. A recent case report provided further support for the involvement of this gene in POI: a homozygous gross deletion affecting

4000 bp of *SYCE1* in two POI sisters have been identified in a highly consanguineous Chinese family [208].

MSH4: was found to be mutated in two Colombian sisters presenting with secondary amenorrhea [209]. Segregation analysis of the *MSH4* splicing variant in the family was consistent with a recessive mode of inheritance [209]. Its KO in mice leads to both female and male infertility secondary to defective chromosome synapsis during meiosis [210,211].

Interestingly, both NOA patients described by Krausz and colleagues [10] with variants in *SYCE1* and *MSH4* had at least one infertile sister, further supporting a common genetic origin for NOA and POI.

C14orf39: a homozygous frameshift mutation has been recently identified in a POI-affected patient from a consanguineous Pakistani family, in which two male siblings carrying the same variant presented with meiotic arrest [85]. This mutation is located in the N terminus of the protein, which contains two SYCE1 binding regions [85]. The mutant protein can still interact with SYCE1, but its ability to form aggregates with SYCE1 is diminished [85]. Importantly, *Six6os1* mutant female mice mimicked the POI phenotype of the affected sister, further confirming the pathogenic role of *C14orf39* both in male and female infertility [85,139].

PSMC3IP and ***DMC1***: homozygous variants were reported in consanguineous families, in which the affected females presented with POI while the male proband had NOA [86,124].

MEIOB: a homozygous truncating mutation has been recently reported as the cause of POI in two sisters of a consanguineous family where the parents are double first cousins [134]. This *MEIOB* variant is expected to provoke meiotic defects and a depleted follicular stock, consistent with the phenotype of the *Meiob* −/− mouse that displays infertility in both sexes due to meiotic arrest [132,133].

NR5A1: in rare cases, sequence variants of the gene may result in POI [174,212], or in various disorders of gonadal development (DGD) or adrenal insufficiency. Notably, no genotype-phenotype correlation was observed with *NR5A1* variations. For instance, p.Gly146Ala, the most frequently described NR5A1 sequence variant, was detected in three 46, XY-DGD cases [213–215], in four POI [174,216,217], and in two infertile men [112,114]. Safari and colleagues [116] reported a case of two Iranian siblings affected by azoospermia and POI, due to the same heterozygous *NR5A1* mutation segregating in the family. Very recently, *NR5A1* variants have been reported in two families including individuals with 46, XY DGD and POI [218], further complicating the clinical significance of pathogenic *NR5A1* variants in a context of highly variable expressivity.

SETX: as described in males, homozygous mutations of the gene lead to a syndromic phenotype, including progressive ataxia and ovarian failure [219].

WT1: heterozygous missense and splicing variants have been associated with Frasier syndrome, a rare disease characterized by male pseudo-hermaphroditism and progressive glomerulopathy [220,221]. Mutated patients presented normal female external genitalia, streak gonads, XY karyotype and frequently developed gonadoblastoma. Glomerular symptoms arise during childhood and consist of proteinuria and nephrotic syndrome, progressing to end-stage renal failure in adolescence or early adulthood [220,221]. Apart from the syndromic manifestation, this gene has been reported both in male and in female as isolated cause of NOA and POI [121,122,222,223].

Interestingly, knockout of *Adad2*, *Terb1* and *Rad21l1* in mice leads to infertility in both sexes [138,210,224], suggesting a potential common role of these genes in NOA and POI phenotypes.

All these findings imply that a special attention has to be paid to female relatives of male patients with primary testicular failure, as approximately 37% (14/38) of NOA candidate genes are also implicated in either POI, female genital anomalies or complex phenotypes. In contrast to gonadal ambiguities and primary amenorrhea usually documented at birth and during puberty, respectively, POI is usually not recognizable until amenorrhea occurs. Thus, genetic counseling for NOA is of great relevance not only to the male family

members but also to the female ones, in whom oocyte vitrification would allow fertility preservation before ovarian failure occurs.

5. Conclusions

For the last 40 years the diagnostic armamentarium for the detection of genetic factors involved in azoospermia has been restricted to a few routine tests such as Karyotype analysis, Y-chromosome microdeletions and the search for a few monogenic causes in selected cases of pre- and post-testicular azoospermia. Thanks to the widespread diffusion of WES, an increasing number of novel candidate genes of azoospermia have been identified, especially in CHH. In this disease, the yield of genetic testing is already over 50% (Figure 1). Large-scale exome sequencing in the frame of international networks, such as COST Action (BM1105) (https://www.chuv.ch/en/hhn/hhn-home/research/our-basic-scientists) and European reference network on rare endocrine conditions (Endo-ERN) (https://endo-ern.eu/), will further improve the molecular diagnosis of CHH. The clinical impact of discovering novel disease-causing genes in this condition is especially relevant for genetic counselling since the majority of these patients can generate their own biological child with the risk of transmission of the identified mutation(s).

Regarding the primary testicular failure, the missing genetic diagnosis is still high, accounting for about 70% of cases after the exclusion of all known acquired and genetic causes (Figure 1). Given the complexity of the spermatogenesis and the highly heterogeneous testicular phenotypes, only large exome and genome studies involving thousands of well-characterized patients have the potential to unravel recurrent genetic causes of NOA. Moreover, for this type of azoospermia, a major breakthrough is expected from ongoing consortia-based efforts. In fact, a growing number of novel candidate genes of MA were found and validated, thanks to the data sharing between different laboratories belonging to the International Male Infertility Genomics Consortium (IMIGC) (http://www.imigc.org) and to the Genetics of Male Infertility Initiative (GEMINI) consortium (http://www.gemini.conradlab.org) (see [225]). Similar to other medical fields, the major challenge in the monogenic diagnosis of NOA is represented by the attribution of a pathogenic role to the identified variants, especially if they are classified as VUSs. A possible solution of the issue seems to lie in the high-resolution phenotyping of candidate male infertility mouse mutants [226], thanks to the CRISPR-Cas9 technologies. This approach will indeed allow to overcome the difficulty in interpreting missense variants, demonstrating a cause-effect relationship between a given genotype and NOA phenotype. Very recently, genome sequencing in combination with single-cell RNA sequencing (scRNAseq) allowed to connect potential pathogenic mutations directly to the testicular cell type where the effect is likely to be exerted [81]. This technology could clarify the effect of potential disease-causing variants on the complex cellular structure of spermatogenesis.

Besides the Mendelian inheritance, other mode of transmission may underlie the NOA phenotype. As for CHH, which can also be explained by a digenic/oligogenic inheritance, the combined effect of two or more rare mutations in different candidate genes of NOA should be taken into consideration. Kasak and Laan showed a highly significant enrichment of active connections and complementary functions among loci implicated in NOA [11]. In this context, a possible scenario of digenicity/oligogenicity underlying the etiology of this complex and heterogeneous condition could be considered in the near future.

In this review we provided a brief description of those potential candidate genes which may be part of a gene panel-based diagnostic testing in the future. We classified these genes according to the associated testicular histology underlying the NOA phenotype. For some gene defects, the testis phenotype consistently shows pure SCOS/MA phenotypes, providing a pre-TESE prognostic value for the identified NOA-causing gene. Currently, the sole prognostic pre-TESE genetic test is based on the AZF deletion screening but, if these monogenic causes will be validated in large cohorts, the gene panel will complement AZF screening also as prognostic test for testicular sperm retrieval. The emerging data on

shared genetic factors between NOA and POI will have a clinical impact both on family history taking and genetic counselling.

In the past three years we witnessed to a continuous increase of novel genetic factors causing NOA [11,225,227,228]. Although we expect to uncover many more candidate genes, we anticipate that besides mutations in protein coding genes, other genetic and epigenetic alterations may contribute to the NOA phenotype. Concerning the former, it is possible that some unanalyzed genetic alterations, such as synonymous single nucleotide variants and variants located in the regulatory regions (UTR), could be responsible for the loss of function of a NOA-associated gene. Concerning the epigenetic aspects, small and long non-coding RNAs are reported to have a regulatory role in spermatogenesis, potentially resulting in NOA [229,230]. In addition, environmental exposures and lifestyle factors could have an influence on the expression of genes involved in spermatogenesis [231]. Recently, significant changes in DNA methylation of spermatogenic cells have been observed in NOA patients, although further studies are needed to determine the impact of the epigenetic regulations on development of male infertility [232]. From a diagnostic point of view, genetic factors remain clinically the most relevant and we expect that a male infertility diagnostic gene panel will be available in the near future.

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Hormone Suppression with GnRH Antagonist Promotes Spermatogenic Recovery from Transplanted Spermatogonial Stem Cells in Irradiated Cynomolgus Monkeys

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SUMMARY

Hormone suppression given before or after cytotoxic treatment stimulates recovery of spermatogenesis from endogenous and transplanted spermatogonial stem cells (SSC) and restores fertility in rodents. To test whether the combination of hormone suppression and transplantation could enhance the recovery of spermatogenesis in primates, we irradiated (7 Gy) the testes of 12 adult cynomolgus monkeys and treated 6 of them with GnRH-antagonist (GnRH-ant) for 8 weeks. At the end of this treatment, we transfected cryopreserved testicular cells with GFP-lentivirus and autologously transplanted them back into one of the testes. The only significant effect of GnRH-ant treatment on endogenous spermatogenesis was an increase in the percentage of tubules containing differentiated germ cells (tubule differentiation index; TDI) in the sham-transplanted testes of GnRH-ant-treated monkeys compared to radiation-only monkeys at 24 weeks after irradiation. Although transplantation alone after irradiation did not significantly increase the TDI, detection of lentiviral DNA in the sperm of one radiation-only monkey indicated that some transplanted cells colonized the testis. However, the combination of transplantation and GnRH-ant clearly stimulated spermatogenic recovery as evidenced by several observations in the GnRH-ant-treated monkeys receiving transplantation: (a) significant increases (~20%) in the volume and weight of the testes compared to the contralateral sham-transplanted testes and/or to the transplanted testes of the radiation-only monkeys; (b) increases in TDI compared to the

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DISCLOSURES

The authors have no conflicting financial interests.

AUTHOR CONTRIBUTIONS

GS, KEO & MLM, conception and design; GS, collection of data; RKU, study design, treatment and surgeries; WZ, PCR analysis of semen and testicular samples; SHS, cell preparation and immunostaining; CCW, xenotransplantation and hormone assays; RCT, dosimetry for the testicular radiation of the monkeys. BPH, study design, transplantation of testicular cells into the monkey testes; KEO, transplantation of testicular cells into the monkey testes; MLM & GS analysis of results and preparation of the manuscript.

transplanted testes of radiation-only monkeys at 24 weeks (9.6% *vs*. 2.9%; *P*=0.05) and 44 weeks (16.5% *vs*. 6.1%, *P*=0.055); (c) detection of lentiviral sequences in the sperm or testes of five of the GnRH-ant–treated monkeys; and (d) significantly higher sperm counts than in the radiation-only monkeys. Thus hormone suppression enhances spermatogenic recovery from transplanted SSC in primates and may be a useful tool in conjunction with spermatogonial transplantation to restore fertility in men after cancer treatment.

Keywords

Radiation; spermatogenesis; infertility; transplantation; GnRH-antagonist

INTRODUCTION

As many as 30% of male survivors of cancer in childhood and young adulthood are at risk of sterility due to treatment with high-dose chemotherapy, total-body irradiation, or irradiation with scatter to the genital region (Thomson *et al*., 2002; Meistrich *et al*., 2005). Whereas adults have the option of cryopreserving semen before therapy to ensure that they can produce offspring, prepubertal or peripubertal patients cannot supply appropriate semen sample either due to sperm insufficiency or sociological reasons. Thus they do not currently have any fertility preservation choices that have proven effective. Development of new methods of fertility preservation to prevent these effects or restore normal reproductive function after cytotoxic treatment are of great importance to these young male cancer survivors.

If spermatogonial stem cells (SSC) survive after cancer therapy, there is the possibility for endogenous spermatogenic recovery either by spontaneous or stimulated differentiation of these cells. Suppression of gonadotropins and testosterone stimulated endogenous recovery of spermatogenesis from surviving stem cells in rats after exposure to cytotoxic agents, which was surprising since testosterone and follicle-stimulating hormone (FSH) are the hormones responsible for completion of the process of spermatogenesis (Meistrich & Kangasniemi, 1997; Shetty *et al*., 2000; Shetty *et al*., 2006). Transient suppression of these hormones after radiation stimulated recovery of spermatogenesis and fertility in both rats and in mice (Meistrich *et al*., 2001; Wang *et al*., 2010). Furthermore, hormone suppression in rats during or after exposure to the cancer chemotherapy agents procarbazine or busulfan also stimulated spermatogenic recovery and restored fertility (Velez de la Calle & Jegou, 1990; Meistrich *et al*., 1999; Udagawa *et al*., 2001) . Of the several clinical studies attempting to use hormonal suppression to preserve human spermatogenesis after radiation or chemotherapy (reviewed in (Shetty & Meistrich, 2005), only one was successful (Masala *et al*., 1997). The one study using hormonal suppression after prepubertal radiation or chemotherapy to stimulate recovery (Thomson *et al*., 2002) was unsuccessful, probably because the high-dose treatment killed all stem cells (Shetty & Meistrich, 2005).

If SSC are completely lost after gonadotoxic therapy, harvesting and cryopreservation of tissue or a cell suspension containing SSC prior to therapy and a method to produce sperm from those cells is the only way to preserve fertility in prepubertal and peripubertal males. Several techniques are being tested for potential future production of sperm, including SSC transplantation, testicular tissue grafting, and *in vitro* development of sperm (Brinster, 2007; Rodriguez-Sosa & Dobrinski, 2009; Sato *et al*., 2011). Only SSC transplantation has the potential to restore spermatogenesis from an individual's own testis *in vivo*, enabling the recipient male to father his own genetic children, possibly through normal coitus. Hence, autologous transplantation of SSC, such as those collected and cryopreserved before therapy, is an important potential option for fertility preservation (Orwig & Schlatt, 2005;

Brinster, 2007). Intratesticular transplantation of cryopreserved testicular cell populations has been well documented to restore fertility in rodent models and some farm animals (Honaramooz & Yang, 2011). However, there are only two reports of modest spermatogenic recovery after transplantation of cryopreserved germ cell suspensions into irradiated monkey testes (Schlatt *et al*., 2002; Jahnukainen *et al*., 2011), but the progeny of the donor cells could not be distinguished from endogenous-derived cells. In a recent study, however, spermatogenesis could be restored from either autologously or allogeneically transplanted genetically marked germ cells in rhesus monkeys exposed to busulfan (Hermann *et al*., 2012).

Experiments in rats showed that spermatogonial differentiation is blocked after radiation because of damage to the somatic compartment but not to the spermatogonia (Zhang *et al*., 2007) and that the block could be ameliorated by hormone suppression. These findings suggest that hormone suppression should also enhance differentiation and recovery from transplanted germ cells by improving the niche and somatic environment. The enhancement of colonization and differentiation of transplanted spermatogonia via suppression of gonadotropins and intratesticular testosterone has been demonstrated in busulfan-treated and in irradiated recipient rats (Ogawa *et al*., 1999; Zhang *et al*., 2007) and mice (Ogawa *et al*., 1998; Dobrinski *et al*., 2001; Ohmura *et al*., 2003), resulting in donor-derived fertility in two of these studies (Zhang *et al*., 2003; Wang *et al*., 2010). Comparison of stimulation of recovery of endogenous and donor spermatogenic recovery by hormone suppression in irradiated mice showed a greater stimulation of the recovery from transplanted cells. This result indicates that, besides stimulating proliferation or differentiation of both endogenous and transplanted spermatogonial stem cells, hormone suppression also has a positive effect on homing of transplanted cells (Wang *et al*., 2010).

To test whether these concepts of stimulation of spermatogenic recovery by hormonal suppression could be applied to primates, we treated irradiated cynomolgus monkeys with a gonadotropin-releasing hormone antagonist (GnRH-ant) in conjunction with spermatogonial stem cell transplantation. Our hypothesis was that GnRH-ant treatment enhances spermatogenic recovery from surviving endogenous and from autologously transplanted SSC in irradiated monkeys.

MATERIALS AND METHODS

Animals

A total of 16 adult (6- to 10-year-old) male cynomolgus monkeys (*Macaca fascicularis*) were purchased from Charles River Laboratories from their facility in Houston, Texas. The animals were individually housed in steel cages in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care at The University of Texas MD Anderson Cancer Center. They were fed Harlan TEKLAD Primate diet #7195 with daily enrichment foods, such as seeds, peanuts, fruits, and vegetables. Their environment was maintained at a constant temperature (75°F–80°F) and humidity (40%–55%) with a 12-hour light/12-hour dark cycle.

For xenotransplantation of monkey testicular cells, adult nude (Swiss nu-nu/Ncr) mice bred at The University of Texas MD Anderson Cancer Center were used as recipients. The animals were maintained on a 12-hour light/12-hour dark cycle and were allowed food and water ad libitum.

All animal care and treatment protocols were approved by the Institutional Animal Care and Use Committee of MD Anderson Cancer Center.

Experimental design

A preliminary experiment was performed involving four monkeys: one untreated control, one receiving GnRH-ant only, one receiving testicular radiation only, and one receiving both radiation and GnRH-ant (Fig. S1). No transplantations were performed.

In the main experiment, twelve other monkeys were divided into two treatment groups of six each, such that the age and weight distributions were similar (Table S1). All monkeys underwent irradiation followed by autologous germ cell transplantation into one testis (Fig. 1). One group received GnRH-ant treatment and the other group received no hormone-suppressive treatment.

General surgical and post-surgical procedures

Monkeys undergoing testicular biopsy and spermatogonial transplantation were first sedated with IM injection of telazol (2.2–4.4 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and then anesthetized with 1–3% isoflurane (Butler Schein Animal Health, Dublin, OH) in oxygen. Before all surgical manipulations, 2% lidocaine (Hospira, Inc., Lake Forest, IL) (IM) was instilled into the surgical site to provide local anesthesia. All surgical procedures were performed under aseptic conditions. Postsurgically, all animals received, at the discretion of the Clinical Veterinarian, one daily IM injection of Baytril antibiotics (5mg/kg) for a week post-surgery, and an analgesic (buprenorphine; 0.01–0.03 mg/kg; Bedford Laboratories, Bedford, OH) prior to and at the end of the day of surgery, and 2 times per day for up to 3 days as needed by the appearance of the animal under constant monitoring.

Irradiation

The monkeys were anesthetized with telazol and were maintained with isoflurane. Each monkey was irradiated to the testes, using a cobalt-60 gamma-irradiator, based on dosimetry performed in a simulated phantom made from 1.5% agarose. Tissue-equivalent bolus material (5-mm thick) was placed over the scrotum to provide a build-up layer. For dose uniformity, both postero-anterior and antero-posterior positions were used, and half of the dose was given in each position. The monkeys were irradiated at a total calculated dose of 7 Gy at a rate of 73–78 cGy/minute, maintaining a field size of ~10 × 10-cm and a source-to-skin distance of 76.5 cm measured to the bolus.

Mouse recipients underwent irradiation to eliminate endogenous spermatogenesis prior to transplantation of monkey testicular cells. The mice were restrained in a plastic chamber and then placed into a metal shield module with a 3-cm diameter hole, so that only the lower abdominal and scrotal area of the animal was irradiated. Radiation was delivered by a cesium-137 gamma-ray unit (Zhang *et al*., 2006) as an initial 1.5-Gy dose followed by a second dose of 12 Gy.

GnRH antagonist treatment

The GnRH-ant Acyline was obtained from the Contraceptive Discovery and Development Branch (formerly Contraception and Reproductive Health Branch) of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (Bioqual; Rockville, MD). A stock solution of Acyline (2 mg/ml) in 5% aqueous mannitol was prepared as needed and stored at 4°C for a maximum of 1 week. Various GnRH-ant treatment regimens were used in the preliminary experiment to determine the most effective dose regimen for suppressing serum testosterone (Fig. S2). One unirradiated monkey was initially given daily subcutaneous injections of Acyline at 50 μg/kg/day for 2 weeks, followed by twice-weekly injections, at doses of 200 μg/kg (Monday) and 300 μg/kg (Thursday) during weeks 3 and 4 and 300 and 450 μg/kg during weeks 5 through 8. One irradiated monkey was initially given

a bolus injection of 600 μg/kg and then twice-weekly injections at doses of 200 μg/kg and 300 μg/kg from weeks 3 through 8. On the basis of those results, the monkeys in the main experiment were given twice-weekly subcutaneous injections of Acyline on Mondays and Thursdays at doses of 200 μg/kg and 300 μg/kg, respectively. The hormone-suppressive treatment was started immediately after irradiation, since in irradiated rats this efficiently stimulated recovery of spermatogenesis from surviving stem cells (Meistrich & Kangasniemi, 1997). Hormone suppression was continued for 8 weeks and at the end of the eighth week, transplantation was performed.

Semen and blood collection

Semen was obtained from anesthetized monkeys by electro-ejaculation using a rectal probe (Beltron Instruments, Longmont, CO). The probe was inserted gently into the rectum with the electrodes adjacent to the prostate. Stimulation was applied for 1 second every 3–5 seconds, initially at 10 volts and gradually increased to 15 volts until an ejaculate was obtained. The sample was allowed to liquefy at 37°C for an hour before sperm were counted in the exudate using a hemacytometer. Sperm counts were expressed per total ejaculate (volume of exudate plus remaining coagulum). The exudate was stored at −80°C for later polymerase chain reaction (PCR) analysis of lentiviral DNA.

Blood (5–10 ml) was drawn from each monkey by venipuncture of the saphenous vein with the animal under ketamine (Fort Dodge Animal Health, Fort Dodge, IA) sedation. Serum was prepared and stored at −20°C.

Testicular measurements and sampling

Testis volume was determined by measuring the length and width of each testis within the scrotum of anesthetized monkeys with calipers and modeling the testis as a prolate ellipsoid, applying the following formula: testis volume = $\pi \times \text{width}^2 \times \text{length}/6$. Since the pretreatment volume of all testes were measured, testis volumes could be presented as a fraction of the pretreatment volume, providing a correction for interanimal variability.

Testicular biopsy specimens were collected from anesthetized animals by making an incision in the scrotal skin and then in the tunica albuginea to expose the testicle. Biopsy samples of up to1 g, depending on the size of the testis, to obtain cells for transplantation or of 100 mg for histological and hormone studies, were collected from a region midway between the poles avoiding the major blood vessels and the rete testis. At the end of the study, the remaining testes were harvested intact, weighed, and prepared for histology. Absolute testis weights are given since pretreatment testis weights were not known; thus there is more interanimal variability than in testis volume, which is normalized to the pretreatment value.

In 15 of the 16 monkeys studied, we did not observe any adverse effects of multiple testicular biopsies or the transplantation procedure on the testes. No focal or generalized damage to somatic structures or inflammation was observed. Only in one monkey (main experiment, #5, radiation-only) the sham-transplanted testis became almost completely necrotic after the 24-week biopsy and was excluded from the analysis at subsequent time points. Thus, biopsy by itself does not seem to be deleterious to the remaining testicular tissue, and occasional necrosis may be a result of damage to a major blood vessel.

Preparation of testis cells for transplantation

The testis cells were prepared with slight modification of previously published procedures (Hermann *et al*., 2007). Biopsy samples were digested with collagenase type IV (1 mg/ml; Worthington Biochemical Corporation, Columbus, OH) and DNase I (100 μg/ml; Sigma-

Aldrich, , St. Louis, MO) in Hanks' balanced salt solution (HBSS; Gibco/Life Technologies, Grand Island, NY) for 5–10 minutes at 37°C with vigorous shaking. Dispersed seminiferous tubules were sedimented and washed in HBSS to remove interstitial cells. Isolated seminiferous tubules were further digested with trypsin (2.5 mg/ml; Gibco) containing 1 mM EGTA, 1 mM $MgCl_2$, and DNase I (0.4 mg/ml) in HBSS for 10–15 minutes at 37°C with pipetting. The cell suspension was filtered through a 70-μm nylon mesh, pelleted, and resuspended at 40×10^6 per ml in minimum essential medium α (MEMα; Gibco) containing 10% fetal bovine serum (FBS).

Cells were aliquoted into cryovials, and an equal volume of freezing medium (MEMα + 20% FBS + 20% dimethyl sulfoxide [DMSO]) was added drop-wise. Vials were frozen at −1°C/minute in controlled-rate freezing containers (Nalge Nunc International, Penfield, NY) to −80°C and stored in liquid nitrogen.

Lentiviral Transfection of Testicular Cells

Prior to use, the frozen vials with testicular cells were thawed rapidly at 37°C, excess MEMα + 10% FBS was added to the cell mixture drop-wise, and cells were washed three times. Cells were transfected with a lentiviral vector modified from the FUGW construct (Lois *et al*., 2002) and containing EF1α (promoter)–EGFP (Hermann *et al*., 2012) which was obtained from the Transgenic and Molecular Research Core at Magee-Womens Research Institute. Cells were incubated overnight with the lentivirus particles in MEMα containing 10% FBS and polybrene (6 μg/ml; Sigma-Aldrich) at a total multiplicity of infection (MOI) of 60 (three additions at MOI 20, at 3-hour intervals). Lentivirus-treated cells were washed several times with fresh medium to remove excess lentivirus. The labeling of SSC by EGFP-lentivirus by this method was demonstrated previously although the labeling efficiency was apparently low (Hermann *et al*., 2012).

Autologous transplantation

Each monkey underwent autologous transplantation of cells into one testis 8 weeks after irradiation essentially as described (Hermann *et al*., 2012). Briefly, cells prepared for transplantation were suspended at approximately 1.3×10^8 cells/ml in MEMα containing 10% FBS, trypan blue (Sigma-Aldrich; 0.4 mg/ml), 20% (v/v) Optison ultrasound contrast agent (GE Healthcare, Waukesha, WI), 1% antibiotic-antimycotic (a combination of penicillin, streptomycin, and amphotericin B; Gibco), and DNase I (0.1 mg/ml) in a total volume of as much as 1 ml, depending on recipient testis size and number of available cells. The cells were transplanted via ultrasound-guided injections into the rete testis. A 13MHz linear superficial probe and a MicroMaxx ultrasound machine (Sonosite, Bothell, WA) were used to visualize the rete testis space and to guide a 25-gauge, 2" spinal needle into the space. Cells were injected under slow constant pressure and chased with saline solution. The average total numbers of viable cells injected into the radiation-only monkeys and the irradiated and GnRH-ant–treated monkeys were 56×10^6 and 81×10^6, respectively (Table S1). The contralateral testes were sham transplanted at the same time by injection of the suspension medium with all constituents except the cells.

Xenotransplantation to mice

Seminiferous tubules of adult nude mice were injected via the efferent ducts with 7–10 μl of donor testis cell suspension containing about 40×10^6 cells/ml at 3 weeks after testicular irradiation as described previously (Zhang *et al*., 2006). One to three recipient testes per monkey cell suspension was successfully transplanted for this study. At 10 weeks after transplantation, intact seminiferous tubules were recovered, dispersed, fixed, and stained in whole-mount with an anti-rhesus testis-cell antibody (Hermann *et al*., 2007). Samples were dehydrated stepwise in methanol and then incubated in MeOH:DMSO:H_2O_2 (4:1:1) for 2–3

hours. The rhesus testis-cell antibody was used at a 1:800 dilution and detected with goat anti-rabbit IgG conjugated to AlexaFluor 488 (1:300 dilution; Invitrogen, Carlsbad, CA). Samples were mounted with Vectashield medium containing DAPI (Vector Laboratories, Burlingame, CA) on slides with raised coverslips and visualized by fluorescence microscopy. The DAPI staining was used to determine the position of the donor rhesus cells within the seminiferous epithelium. Donor stem cell–derived colonies with at least four cells exhibiting spermatogonial morphology located on the basement membrane of the recipient seminiferous tubule (<100 μm between cells) were counted (Hermann *et al*., 2009).

Detection of lentiviral vector DNA in sperm and testis

Attempts to detect green fluorescent protein (GFP)–positive sperm or cells using direct fluorescence or immunofluorescent staining of the testicular sections, as had been used with GFP-transfected rat SSC (Ryu *et al*., 2007), were unsuccessful, in accordance with other studies with monkey testis cells (Hermann *et al*., 2012). Thus PCR was used to screen for the presence of lentiviral genetic material. DNA was extracted from as many as 1.5×10^7 monkey sperm from each sample (Hermann *et al*., 2012). To eliminate somatic cells, sperm were suspended in 700 μl phosphate-buffered saline solution (PBS) with 0.2% sodium dodecyl sulfate and pelleted (Zheng *et al*., 2000). The pellets were resuspended in 300 μl Cell Lysis Solution (Puregene, Cat#158906; Qiagen, Valencia, CA) and then mixed with 33 μl of 100 mM dithiothreitol and 30 μl of proteinase K (20 mg/ml). Samples were then incubated at 55°C overnight. Each sample was supplemented with 100 μl Protein Precipitation Solution (Cat#158910; Qiagen) and vortexed. Samples were subjected to centrifugation, and supernatants were collected. For samples that contained fewer than 1.5×10^7 sperm, 2 μl of glycogen (20 mg/ml) was added to enhance DNA precipitation. Then 1 ml of ice-cold 100% ethanol was added to each sample, mixed thoroughly and subjected to centrifugation. The resulting pellets were washed with 70% ethanol and air-dried.

For monkeys with spermatogenesis in at least 4% of tubules, DNA was extracted from testis slices using Qiagen AllPrep DNA/RNA Mini Kit (Cat #80204). For each PCR reaction, 6–200 ng DNA template and 0.75 U Platinum Taq High Fidelity (Invitrogen) were diluted in a final 15-μl volume containing 0.1 mM deoxy-NTPs, 2.5 mM $MgSO_4$, 0.2 μM of each primer, and buffer. A touch-down PCR protocol was used: 5 minutes at 94°C, then 28 cycles of 30 seconds at 94°C, 30 seconds initially at 70°C with the annealing temperature decreasing by 0.5°C every cycle, and 45 seconds at 72°C, followed by 20 more cycles at the final annealing temperature (56°C) and a final extension step at 72°C for 10 minutes. The amplified DNA was visualized in ethidium bromide–stained agarose gels.

Primers were designed for amplifying the HIV envelope glycoprotein (*env*) gene and *GFP* gene in the lentiviral vector and the primate-specific gene *BC042682* of rhesus monkeys, which has the same size and sequence in the cynomolgus macaques (Table S2). To confirm that all the sperm and testis DNA samples contained good quality monkey DNA, primer pair BC1 for *BC043682* was used; it showed a strong signal in all samples. To detect lentiviral vector DNA sequences, primer pairs for *env* and *GFP*, designated env1 and GFP1, respectively, were used initially. Samples were then subjected to another round of nested PCR for more sensitive detection using env2 or GFP2 primer pair. Later, the most sensitive primer pair, env2, was used directly for the remaining sperm and all the testis samples. The nested PCR or the env2 primer pair alone detects positive signals from as low as 0.1 ng of sperm DNA from a monkey (M036) previously shown to have transfected donor-derived sperm in the ejaculate (Hermann *et al*., 2012).

Hormone assays

Intratesticular testosterone was measured in tissue (20–67 mg) from each biopsy that was frozen immediately in liquid nitrogen, stored at −20°C, and homogenized at the time of radioimmunoassay (RIA) (Boekelheide *et al*., 2005). Serum testosterone and intratesticular testosterone concentrations were measured using coated-tube RIA kits (TKTT1, Siemens Health Care Diagnostics, Deerfield, IL) according to a method described elsewhere (Shetty *et al*., 2011). The intraassay and interassay coefficients of variation were 10% and 16%, respectively. The sensitivity of testosterone assay was 0.041 ng/ml.

Circulating concentrations of FSH and luteinizing hormone (LH) were determined by using homologous RIA reagents supplied by the National Hormone and Peptide Program as described previously (Ramaswamy *et al*., 2003). The sensitivities of the LH and FSH assays were 0.12 ng/ml and 0.06 ng/ml, respectively, using 100-μl samples. The intraassay and interassay coefficients of variation were 6% and 15%, respectively, for FSH, and 3% and 9%, respectively, for LH.

Histological procedures

The monkey testis tissue was fixed in Bouin solution and embedded in paraffin or methacrylate, and sections were stained with periodic acid Schiff reagent and hematoxylin. The stained sections were quantitatively assessed by scoring seminiferous tubule cross-sections at regular intervals across the whole tissue section for the presence or absence of germ cells and the most advanced germ cell type present. In the single biopsy samples taken at interim time points after irradiation, an average of 309 tubules (range: 144–515) were counted per testis in in the main experiment and 138 tubules (79–159) were counted in the preliminary experiment. In whole testes harvested at the end of the studies, the testes were transversely sliced into 5–6 pieces and every alternate slice was used for analysis. Since the slices from the mid region were large, they were halved into two, one of which was used for histological scoring. An average of 3980 tubules (range: 1985–5143) were scored in these testes in the main experiment and 3617 tubules (range 3539–3695) were scored in the preliminary experiment. It should be noted that the tubule count data for the 24 week time point is from the single biopsy samples, and while it may not be the exact representation of the spermatogenesis in the whole testis, it certainly should be indicative. At 44 weeks large portions of the testes were systematically analyzed and hence likely to be more accurate estimations of spermatogenesis. A tubule differentiation index (TDI) that represents the percentage of seminiferous tubule cross sections containing at least one differentiated germ cell type (B spermatogonia or later stages), was computed. In addition, the extent of the progression of germ cell differentiation was assessed by determining the percentages of tubules with germ cells that contained spermatocytes, round spermatids or elongating/elongated spermatids as the latest germ cell type present; no tubules containing only spermatogonia were observed.

Statistical analysis

The testis weights and TDI were represented as arithmetic means ± SEM. For sperm counts, FSH, LH, and testosterone measurements, the averages and SEM were calculated on log-transformed data. The significance of differences between treatment groups was evaluated by the statistical tests indicated in the figure legends. Since the parameters measured were not normally distributed, nonparametric statistics were used. Comparisons between groups of independent samples were done using the Mann-Whitney test. Wherever possible when samples were related (e.g., same monkeys or testes at different time points, contralateral testes from same monkeys), more powerful paired tests, such as the Wilcoxon signed-rank test, was used. A computer-assisted statistics program (PASW statistics 17, SPSS Inc,

Chicago, IL) was used. A value of $P<0.05$ for the asymptotic significance was considered statistically significant.

RESULTS

Preliminary experiment

A preliminary experiment with four monkeys was performed to find an appropriate dose of the GnRH-ant, Acyline, for hormone suppression and to obtain information on the effect of a 7-Gy dose of radiation on spermatogenesis (Fig. S1).

The bolus dose of 600 μg/kg of Acyline (given to the irradiated monkey) transiently suppressed serum testosterone level to 0.6 ng/ml, but levels returned to normal within 7 days. However, daily injections (to the unirradiated monkey), initially at 50 μg/kg/day, for 2 weeks effectively suppressed serum testosterone levels to about 3 ng/ml (Fig. S2A). Twice a week Acyline injections of 200 μg/kg and 300 μg/kg were enough to keep the serum testosterone levels at about 3 ng/ml in the unirradiated monkey during weeks 3 and 4 and reduced them to <1 ng/ml in the irradiated monkey during weeks 3–8. Giving a slight increase in dose of the twice-weekly injections, to 300 and 450 μg/kg, during weeks 5–8 did not further suppress serum testosterone levels in the unirradiated monkey. The testosterone suppression was rapidly reversible, and testosterone level was restored to normal levels within 1 or 2 weeks of the end of treatment. These treatment regimens suppressed intratesticular testosterone levels to between 10% and 20% of the control levels in both monkeys at the end of the 8-week treatment (Fig. S2B).

The GnRH-ant treatment was biologically effective in suppressing spermatogenesis, as indicated by the reduction in testicular volume and the shrinkage of tubules with sloughed germ cells in the unirradiated monkey at the end the 8-week treatment (Fig. S3). Both effects were reversible: testis volume had recovered with normal histology at the next biopsy 12 weeks later.

In the monkey treated with radiation alone, 0.6%, 0%, and 0.7% of the tubule cross-sections contained germ cells at 8, 20, and 44 weeks, respectively, after irradiation. Although the irradiated monkey treated with GnRH-ant showed no germ cells in the biopsy sampled at the 8-week time point, germ cells were observed in 1.5% and 6.2% of tubule cross-sections at 20 and 44 weeks, respectively.

Main experiment

We used the experimental design shown in Figure 1 to determine the benefits of hormone suppression alone, spermatogonial transplantation alone and the two approaches combined on the recovery of spermatogenesis after radiation. Pre-irradiation testicular biopsies from both testes, amounting to ~5% of the testis and an average of 2.2 g tissue, were collected from each monkey (Table S1). Histologic analysis showed normal spermatogenesis in all testes (data not shown). Cell suspensions prepared from this tissue yielded an average of 277 million cells per monkey with 80% viability (131± 19 cells/g tissue); there was no significant difference in this yield and viability between the monkeys who went on to receive GnRH-ant treatment and the radiation-only group. All suspensions were cryopreserved.

Response to irradiation

The radiation appropriately depleted endogenous spermatogenesis; testis size in the radiation-only monkeys declined to 49% of that of pretreatment controls by 8 weeks (Fig. 2A). At 24 and 44 weeks after irradiation, only 3% and 7%, respectively, of tubule cross-

sections in the sham-transplanted testis contained germ cells, (Figs. 3B, 4A & Fig. S4). This increase in TDI with time was statistically significant (P=0.043). All tubules with germ cells contained cells at the spermatocyte stage or later; no tubule cross-sections containing only spermatogonia were observed. At 24 and 44 weeks, respectively 22% and 67% of the tubules containing germ cells had late spermatids.

Radiation did not induce any changes in serum testosterone or LH levels (Fig. 5). However, as expected, the loss of germ cells after radiation caused large increases in serum FSH levels in the radiation only monkeys within 4 weeks after irradiation and in the irradiated, GnRH-ant–treated monkeys after the hormone suppression was stopped.

Effect of hormone suppression alone

Treatment of the irradiated monkeys with GnRH-ant markedly suppressed serum FSH, LH, and testosterone levels during the treatment period (Fig. 5) and resulted in a more extreme decline in testis volume (Fig. 2A). However, after the treatment period the hormone levels and testis volumes returned within 2 to 8 weeks to the levels observed in radiation-only monkeys

The effect of hormone suppression on endogenous spermatogenic recovery was assessed by comparing the volume, weight, and histology of the sham-transplanted testes of GnRH-ant–treated monkeys with those of the radiation-only monkeys at times ≥24 weeks after irradiation. Testis volumes were slightly but not significantly greater in the GnRH-ant–treated monkeys than in the radiation-only monkeys at all time points from 24 to 44 weeks (Fig. 2A). No differences were observed in the average testis weights when removed (Fig. 2B).

Histologic analysis at 24 weeks after irradiation showed that, whereas only 2.5% of the tubule cross-sections in the sham-transplanted testes of the radiation-only monkeys contained differentiated germ cells, 10.7% of the tubules in the sham-transplanted testes of the GnRH-ant–treated monkeys were recovering spermatogenesis (P=0.037) (Fig. 4A, and Fig. S5). At 44 weeks after irradiation, these TDI values increased to 7.1% (radiation-only) and 12.8% (GnRH-ant), but the difference between the treatment groups was not significant at this time point. Similar to the radiation-only monkeys, 22% and 60% of tubules containing germ cells in the GnRH-ant-treated monkeys had late spermatids at 24 and 44 weeks after irradiation, respectively.

Effect of transplantation alone

At the end of the hormone suppression period, the cryopreserved germ cells from all monkeys were thawed and cultured with EGFP-lentivirus overnight. After incubation, about 113 million cells with 54% viability remained per monkey in the radiation-only group (Table S1). The germ-cell suspensions were injected back into the rete of one of the testes of the monkey from which they were obtained. To validate the presence of stem cells in these preparations, aliquots of the cell suspensions from each monkey were also transplanted to germ cell–depleted nude mice, and donor colonies of monkey spermatogonia were identified by immunostaining (Fig. S6). The cells from the monkeys in the radiation-only group yielded an average of 8.0 ± 2.6 colonies/10^5 viable cells (Table S3). Based on the xenotransplant assay and the numbers of viable cells autologously transplanted into these monkeys (average of supplementary data, Table S1), we calculated that the radiation-only monkeys received 4600 ± 1500 cells with stem cell potential.

The effect of transplantation alone on spermatogenic recovery was assessed by differences in testis volume, weight, and histology between the transplanted and sham-transplanted

testes of radiation-only monkeys and by the presence of lentiviral DNA in their sperm or germ cells. The volumes of the transplanted testes were slightly larger than those of the sham-transplanted testis between 24 and 44 weeks after irradiation, and the difference was significant at two time points (Fig. 2A). However, there was no difference in average testis weight at the end of the study (Fig. 2B). In addition, the average percentage of tubules with differentiated germ cells was not significantly changed by transplantation at either time point (Fig. 4A), and no individual radiation-only monkey showed notably higher percentages of tubules with germ cells in the transplanted testis than in the sham-transplanted testis at the end of the experiment (Fig. 4B).

PCR analysis for lentiviral DNA could only be performed effectively on sperm from three of these monkeys (the other three were azoospermic, Table 1) and on testis tissue from the one monkey that showed germ cells in about 25% of tubules (Fig. 4B) but was azoospermic. Lentiviral genetic material was detected in the sperm of one monkey at several time points after transplantation, indicating that some transplanted SSC did indeed colonize the testis.

Effects of combined hormone suppression and transplantation

For transplantation into the GnRH-ant treated monkeys an average of 134 million cells with 64% viability (Table S1) were used, a small portion of each was also used for xenotransplantation. The xenotransplantation assay indicated that these cells yielded 7.6 ± 2.8 colonies/10^5 viable cells (Table S3). From these numbers, we calculated that the GnRH-ant-treated monkeys received 6900 ± 2800 cells with stem cell potential. Although the radiation-only monkeys received only 4600 ± 1500 potential stem cells, the numbers were not significantly different between the groups.

In contrast to the minimal effects of hormone suppression or transplantation alone on spermatogenic recovery, enhanced spermatogenic recovery from the transplanted cells was clearer in the hormone-suppressed monkeys. The volumes of the transplanted testes in the GnRH-ant–treated monkeys were greater than those of the other groups at all time points starting at 24 weeks after irradiation (Fig. 2A), the difference being significant at nearly all points. The volumes of the GnRH-ant–treated transplanted testes averaged 20% larger than those of the radiation-only transplanted testes. The volumes of the GnRH-ant–treated transplanted testes averaged 17% more than the contralateral sham-transplanted testes, and there also was a significant difference (P=0.043) in testis weights at the end of the study (Fig. 2B).

The transplanted testes of the GnRH-ant–treated monkeys showed the highest percentage of tubules with differentiated germ cells (Figs 3C, 4A & Fig. S7). The TDI value of 9.6% at week 24 was significantly higher than the TDI of 2.9% of the transplanted testes of radiation-only monkeys (P=0.05) and at week 44, the TDI was increased to 16.5%, which was close to being significantly different from the value of 6.1% in the radiation-only monkeys (P=0.055) (Fig. 4A). Although the difference in the average percentages of tubules with germ cells between the transplanted and control testes of the GnRH-ant–treated monkeys was not statistically significant, two of the six monkeys (#s 11 and 12) treated with GnRH-ant showed marked increases (~2-fold) in the percentage of tubules with germ cells in the transplanted *vs*. the sham-transplanted testis (Fig. 4B). As in the other treatment groups, in the transplanted testes of the GnRH-treated monkeys, there were no tubules with spermatogenesis arrested at the spermatogonial stage and 33% and 66% the tubules showing differentiated germ cells contained late spermatids at 24 and 44 weeks, respectively.

PCR analysis of lentiviral DNA could be performed on sperm and testes of five GnRH-ant–treated monkeys; the sixth monkey (# 8) was azoospermic and had very few tubules with germ cells. Four of the five GnRH-ant–treated monkeys with sperm showed lentivirus DNA

in the sperm by PCR analysis (Table 1). The fifth monkey, which produced very scant or sometimes no ejaculate, had lentiviral sequences in DNA extracted from the transplanted testis but not in the DNA from the sham-transplanted testis.

Sperm counts in all the monkeys were assessed. The GnRH-ant–treated monkeys showed higher sperm counts than the radiation-only monkeys at all time points starting 24 weeks after irradiation; the differences at several of the time points were statistically significant (Fig. 6). When data from all these time points were pooled, the statistical significance of the difference between the groups was $P<0.001$. Furthermore, five of the six GnRH-ant–treated monkeys repeatedly showed $>10^4$ sperm per ejaculate (three monkeys ≥1 million), compared to only one of the six radiation-only monkeys (maximum count, 0.26 million).

DISCUSSION

In the current study we investigated the ability to enhance recovery of spermatogenesis in irradiated monkeys by hormone suppression, spermatogonial transplantation, and the two strategies combined. Hormone suppression alone appears to accelerate recovery of endogenous spermatogenesis. Transplantation alone did not have any effect on overall spermatogenic recovery, although sperm production from transplanted cells could be demonstrated. However, hormone suppression clearly enhanced spermatogenic recovery from transplanted spermatogonia in this nonhuman primate model.

Hormone suppression alone induced a significant increase in the TDI from 2.7% to 10.7% at 24 weeks after 7-Gy. Although the TDI values in the irradiated GnRH-ant-treated monkeys increased to 12.8% at 44 weeks, the difference between the two treatment groups at that time point was not significant. Thus we suggest that GnRH-ant-treatment may accelerate the initiation of endogenous spermatogenic recovery but may not produce a sustained enhancement. Two previous studies also failed to show any protection or stimulation of recovery in irradiated cynomolgus (Kamischke *et al*., 2003) or stump-tailed (Boekelheide *et al*., 2005) macaques.

Acyline suppressed serum testosterone to 2% of control values while, in the previous studies, Cetrorelix suppressed testosterone levels only to 21% (Kamischke *et al*., 2003) and 10% (Boekelheide *et al*., 2005) of controls. Moreover, the previous studies employed much higher doses of GnRH-ant (450 μg Cetrorelix/kg/day, compared with 500 μg Acyline/kg/week used here), which was likely the cause of the prolonged suppression of testosterone levels for about 15 weeks after the end of treatment, compared with only 1 to 2 weeks in the present study. Furthermore, in one of those studies (Boekelheide *et al*., 2005), the volume of the testes of the unirradiated monkeys recovered to only 40% of the pre-treatment values after cessation of hormone suppression, and the volumes of the testes of the irradiated, hormone-suppressed monkeys remained permanently below those of the radiation-only monkeys. In the present study, the biological effect of the GnRH-ant was indeed transient, as evidenced by full recovery of testicular volume to that of non–hormone-suppressed controls within 8 weeks after the end of Acyline treatment.

The absence of substantial recovery with transplantation alone was disappointing in view of earlier reports. Although lentivirus signal in sperm indicated that we achieved transplantation, the enhancement of recovery of spermatogenesis (Schlatt *et al*., 2002; Jahnukainen *et al*., 2011) and the incidence of donor marker sequences in sperm (Hermann *et al*., 2012) were lower than reported in previous studies. Two of these studies used unilateral autologous transplantation of testicular cells in adult cynomolgus monkeys after 2 Gy radiation (Schlatt *et al*., 2002) or in prepubertal/pubertal rhesus monkeys after 10 Gy (Jahnukainen *et al*., 2011). In two of five adult monkeys and in one of five immature

monkeys (a prepubertal monkey) in those studies, recovery of spermatogenesis was enhanced in the transplanted testis as compared to the sham-transplanted testis. In one of these cases, however, there could have been selective damage to the sham-transplanted testis by a previous unilateral biopsy (Jahnukainen *et al*., 2011). Following transplantation of SSC in busulfan-treated rhesus monkeys using lentivirus-transfected autologous and allogeneic testicular cells (Hermann *et al*., 2012), ejaculated sperm from donor cells were detected by PCR in nine of twelve recipients of autologous cells (marked by lentivirus) and two of six recipients of allogeneic cells (microsatellite markers). In one of the allogeneic transplanted recipients, about 10% of the sperm were of donor genotype. In our study we are unaware of any technical problems that might have caused reduced colonization, as cell preparation, cryopreservation, and lentiviral transduction were done according to the same procedures and transplantation was performed by the same individuals as in the previous study (Hermann *et al*., 2012). Possible factors include the use of a rather high dose of radiation in adult monkeys and the culturing of cells, which was not done in other irradiation studies. Whatever the cause, the low level of colonization with transplantation alone made the system very sensitive to detection of the increase resulting from hormone suppression.

Most importantly, our results, clearly show augmentation of spermatogenic recovery in the transplanted testes of GnRH-ant–treated monkeys by multiple criteria. These testes: (1) had greater weights than the testes of other treatment groups; (2) had increased percentages of tubule cross-sections showing spermatogenesis, including two monkeys with greatly increased spermatogenesis in the transplanted *vs*. the sham-transplanted testis; (3) had detectable lentivirus-transfected germ cells or sperm in five of six cases; and (4) produced higher sperm counts than those from monkeys not treated with GnRH-ant. Although the quantitative contribution of endogenous vs. transplanted stem cells to this sperm production could not be determined, the presence of lentiviral DNA in most of the samples from hormone suppressed monkeys demonstrates that the increased sperm production must have been derived in part from transplanted cells. Since the stimulation of spermatogenic recovery from donor cells was greater than that from endogenous cells, we conclude that the hormone suppression primarily enhances the homing, colonization, and survival of donor SSC.

It is not clear why the positive signals for the lentiviral DNA in sperm were discontinuous over time. The same phenomenon was also observed after autologous transplantation study of lentivirus-transfected cells to busulfan-treated rhesus monkeys (Hermann *et al*., 2012). This may have been due to the low labeling efficiency and cyclical release of the sperm originating from a limited number of stem spermatogonia transduced by lentivirus as they self-renewed and differentiated in the tubules.

As indicated in the Results, the GnRH-ant treated monkeys received 48% more potential viable stem cells during the transplantation than did the irradiated-only monkeys, although the numbers varied between animals and were not significantly different between the groups. The recovery of spermatogenesis in transplanted testes of the GnRH-ant treated monkeys compared to the radiation-only ones, indicated by multiple endpoints, appeared to be greater than that which could be contributed by a modest increase in cells transplanted, but we cannot rule out some enhancement due to the greater numbers of functional cells transplanted.

It is useful to analyze the hormone suppression regimen selected in this study; although it was effective in enhancing recovery of spermatogenesis from the transplanted stem cells, changes might produce even greater recovery. Acyline suppressed serum testosterone to 2% of control values while, in the previous studies, Cetrorelix suppressed testosterone levels only to 21% (Kamischke *et al*., 2003) and 10% (Boekelheide *et al*., 2005) of controls.

Moreover, the previous studies employed much higher doses of GnRH-ant which caused prolonged suppression of testosterone levels after the end of treatment and incomplete recovery of spermatogenesis in an unirradiated monkey (Boekelheide *et al*., 2005). The initiation of the hormone suppression 8 weeks before transplantation as based on a study in mice indicated that only hormone suppression prior to transplantation induced enhancement of donor-generated spermatogenesis in mice (Dobrinski *et al*., 2001). However, others found that extending the treatment after transplantation gave slightly greater enhancement (Wang *et al*., 2010) or that treatment after transplantation was as effective as treatment before transplantation (Ohmura *et al*., 2003). However, because differentiation of spermatogonia to the B spermatogonial stage in normal monkeys is inhibited when both testosterone and FSH are suppressed by GnRH-ant (Marshall *et al*., 2005), whereas in rodents hormonal suppression has little effect on premeiotic development, we limited the hormone suppression to the period before transplantation.

Suppression of both testosterone and FSH by using GnRH-ant was employed since that was used in most rodent studies. Since the hormone suppression in this study primarily stimulated recovery from transplanted spermatogonia, efficient homing of these cells to the stem cell niche in the basal region of the epithelium, which involves passage though the tight junctions at the Sertoli cell ("blood-testis") barrier (Kanatsu-Shinohara *et al*., 2008), may be a critical step. Because androgen suppression increases the permeability of the Sertoli cell barrier (Meng *et al*., 2005), we suggest that it is androgen suppression that leads to enhanced homing efficiency. However, spermatogenic recovery from transplanted cells involves the processes of stem cell survival, stem-cell proliferation, self-renewal, and differentiation, and it is possible that FSH might actually have a stimulatory role.

The dose of radiation used in the current study is relevant to the human exposures during the radiation therapy. The testicular dose is about 8 Gy is when single-dose total body radiation is given as part of a bone-marrow transplant conditioning regimen for leukemia or Hodgkin’s disease (Anserini *et al*., 2002; Jacob *et al*., 1998). The spermatogenic response of the human testis seems similar to that of the monkey as about 10% of these patients, who also received a temporarily sterilizing dose of cyclophosphamide, eventually recovered their sperm count.

Although the present study demonstrates that hormone suppression significantly enhances spermatogenic recovery from transplanted stem spermatogonia in primates, the efficiency of the process is low and must be improved if it is to be clinically effective. Elucidation of the relative roles and mechanisms of testosterone and FSH in the inhibition or stimulation of spermatogenic recovery from donor stem cells after cytotoxic treatment in monkeys will help to develop a better clinical protocol for spermatogenic recovery, perhaps by suppressing only one of the hormones, optimizing suppression time, and/or directly targeting a downstream effector of the hormone action. Further development of the spermatogonial stem cell preparation and technology for transplantation in a clinically relevant nonhuman primate system, along with optimizing hormone suppression, will facilitate addressing issues of safety and feasibility for human applications in the restoration of male fertility after cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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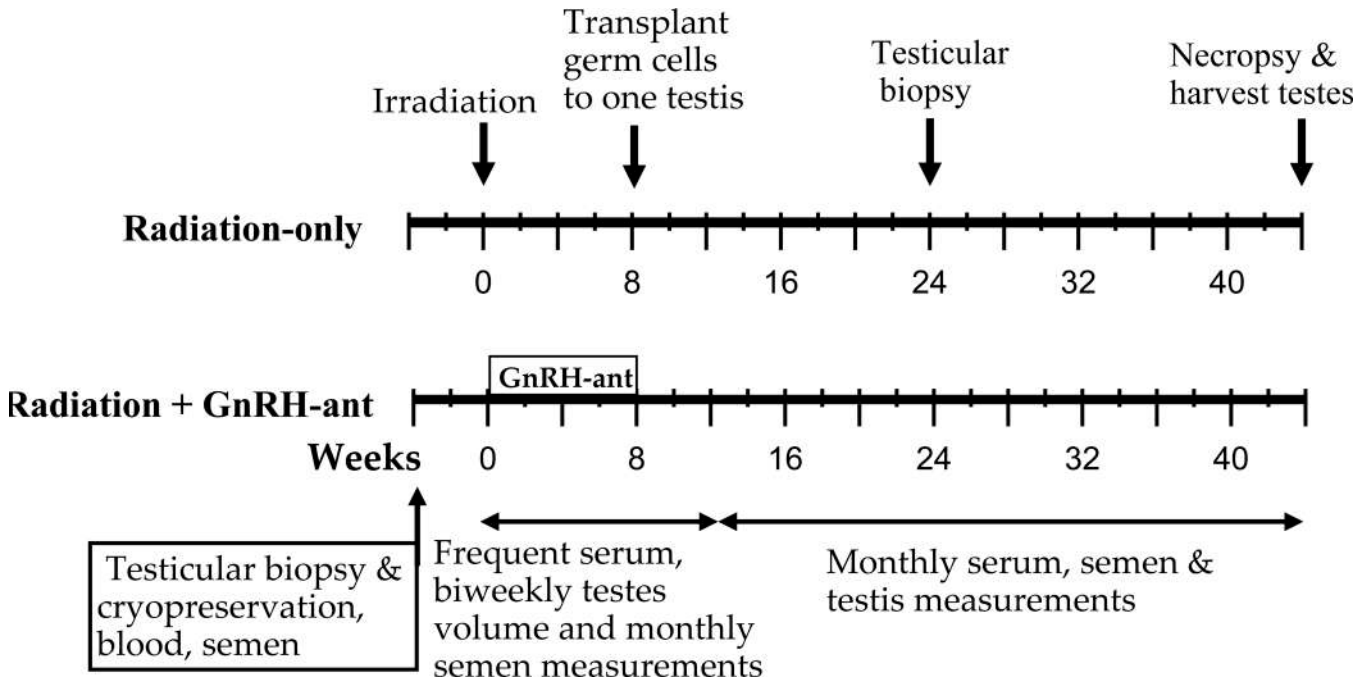

Figure 1.

Overall design of the main study. Monkeys were evaluated before treatment and periodically after exposure to radiation, hormone suppression, and transplantation. Evaluation included sampling of serum and semen, measurements of testis volume and weight, and testis biopsies as indicated. All 12 monkeys were given testicular irradiation; six then underwent GnRH-ant–mediated hormone suppression for 8 weeks, while the other six did not. At the end of the 8-week period, all monkeys received autologous transplantation of GFP-lentivirus–labeled germ cells into one testis.

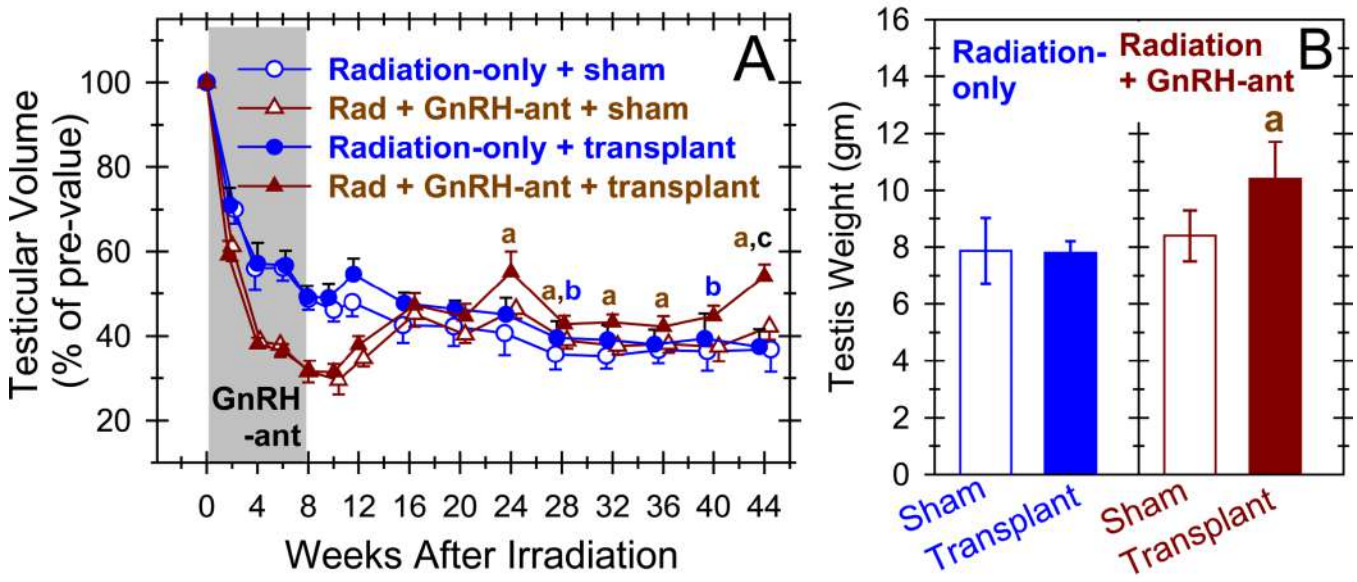

Figure 2.

Increases in testis volume and weight suggest that combined hormone suppression and germ cell transplantation promote spermatogenic recovery. Testicular volume (expressed as a percentage of the pretreatment volume) (A) and testis weight at the end of the study, at 44 weeks after irradiation (B) are shown in radiation-only monkeys and monkeys that received GnRH-ant, for both the testes that did or did not receive transplantation at 8-weeks after irradiation. The shaded area represents the period of hormone suppression. Recovery of testis volume during the period from week 24 to week 44 and the testis weight at week 44 was compared between various groups, and significant (P<0.05) differences are shown by different letters, as follows: a: between the transplanted and sham-transplanted testes of GnRH-ant–treated monkeys (Wilcoxon test); b: between the transplanted and sham-transplanted testes of radiation-only monkeys (Wilcoxon test); c: between the transplanted testes of GnRH-ant–treated and radiation-only monkeys (Mann-Whitney test). The differences between the sham-transplanted testes of GnRH-ant–treated and radiation-only monkeys were not significant.

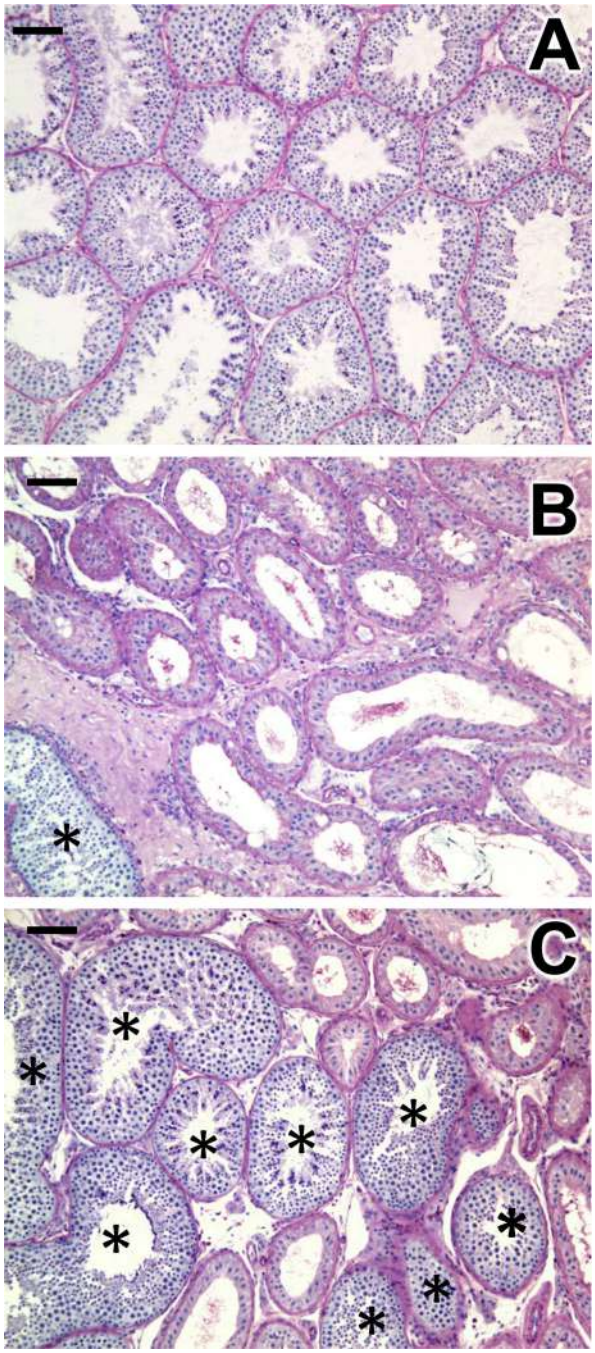

Figure 3.
Combined hormone suppression and germ cell transplantation results in spermatogenic recovery. The three panels show histology of representative monkey testes. (A) Testis from the unirradiated untreated monkey used in the preliminary experiment. (B) Testis of a monkey that received radiation 44 weeks previously and neither GnRH-ant treatment nor transplantation. Very few of the tubules contain germ cells (marked with *) in this radiation-only monkey. (C) Testis of a monkey that received radiation 44 weeks previously and underwent both GnRH-ant treatment and germ cell transplantation. Note the large cluster of tubules containing germ cells. Bar represents 100 μm.

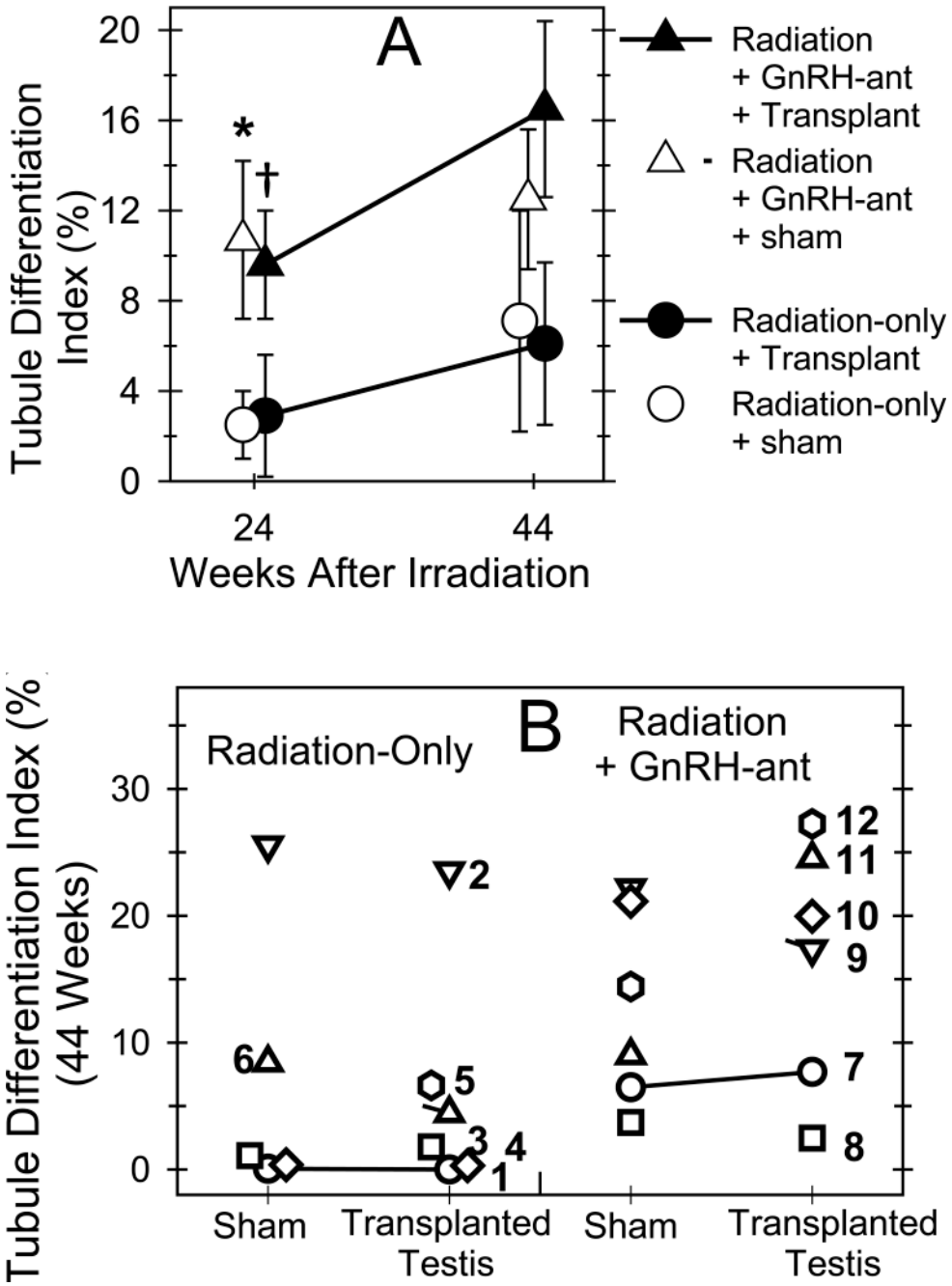

Figure 4.

Combined hormone suppression and germ cell transplantation induced greater development of germ cells in monkey testes than either approach alone. The average (A) and individual (B) percentages of tubules showing differentiated germ cells (tubule differentiation indices) in radiation-only and irradiated GnRH-ant–treated monkeys that received autologous transplantation of testicular cells to one of the testes. Tubule differentiation indices were compared between various groups at week 24 and at week 44, and significant ($P<0.05$) differences are shown as follows: *: between the transplanted testes of GnRH-ant–treated and radiation-only monkeys (Mann-Whitney test); †: between the sham-transplanted testes

of GnRH-ant–treated and radiation-only monkeys (Mann-Whitney test). (B) The tubule differentiation indices for individual monkeys, with the individual animal numbers indicated, were obtained at 44 weeks after irradiation. Only the transplanted testis of monkey #5 is shown, as the sham-transplanted testis became necrotic after week 24.

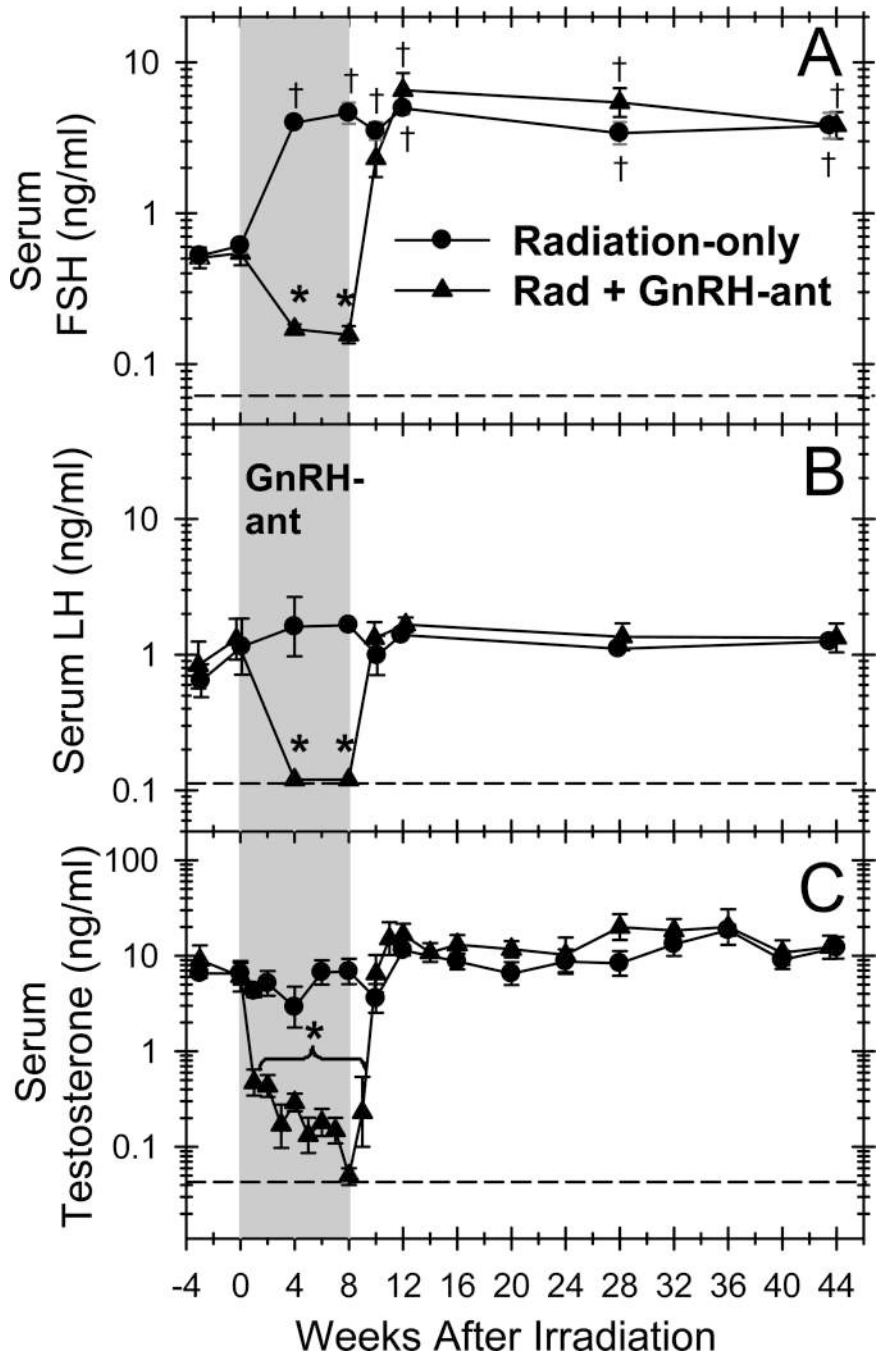

Figure 5.

Serum hormone levels are suppressed by treatment with GnRH-ant and return to normal levels when the treatment is stopped. Serum FSH (A), LH (B), and testosterone (C) levels in radiation-only and irradiated monkeys treated with GnRH-ant for 8 weeks are shown. The shaded area represents the period of hormone suppression. Dashed lines indicate minimum levels of detection. The serum hormone levels after irradiation with or without GnRH-ant treatment were compared to the respective values before irradiation, and significant (P<0.001) differences are shown for the values lower (*) and higher (†) than the pre-irradiation values (Dunnet test).

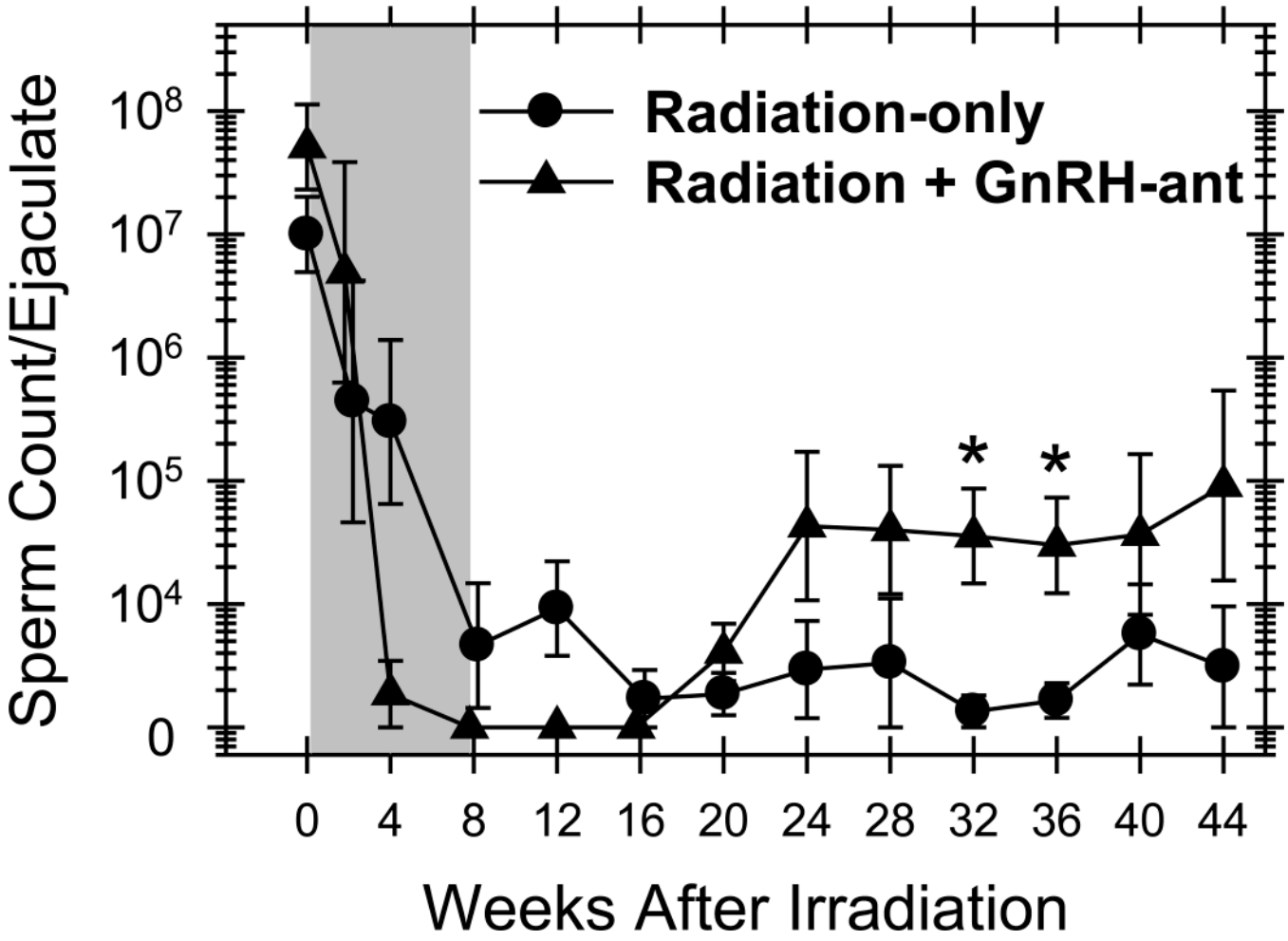

Figure 6.

Treatment with GnRH-ant stimulates recovery of sperm counts in monkeys transplanted with germ cells. Sperm counts in radiation-only and GnRH-ant–treated irradiated monkeys that received autologous transplantation of testicular cells to one of the testes are shown. The shaded area represents the period of hormone suppression. For the purpose of averaging log-transformed value, azoospermic counts were set at 1,000/ml which is the lower limit of detection of sperm with hemocytometer counting. Recovery of sperm counts was compared between the two groups during the period from week 24 to week 44, and significant (P<0.05) differences are shown by the symbol:* (Mann-Whitney test).

Table 1

Presence or absence of lentiviral vector and/or green fluorescent protein (GFP) DNA in the periodic semen samples and the testes samples collected at the end of the study.

Treatment	Monkey Number	Sperm from Semen								Testes	
										Sham	Trans-planted
		Pre-Irrad	Time after Irradiation (weeks)[a]								
			16	20	24	28	32	36	40	44	44
Irradiated	1	−	0	0	0	n	0	n	−	n	nd	nd
	2	nd	0	0	0	0	0	0	0	0	−	−
	3	nd	0	0	0	0	0	0	0	0	nd	nd
	4	−	0	0	nd	n	0	0	0	0	nd	nd
	5	nd	0	nd	nd	0	nd	−	−	0	nd	nd
	6	−	+	−	−	−	−	+	−	+	_[b]	_[b]
Irradiated + GnRH-ant	7	−	0	−	−	−	−	+	−	+	_[b]	_[b]
	8	−	0	0	0	0	0	0	0	0	nd	nd
	9	−	0	+	−	−	+	+	−	ne	−	−
	10	−	0	0	−	−	−	+	−	+	−	−
	11	−	0	−	nd	n	−	n	ne	n	−	+
	12	−	0	ne	n	+	nd	+	n	−	−	−

Abbreviations: n = no ejaculate; ne = not enough ejaculate for analysis; 0 = azoospermic; + = presence of lentiviral vector and/or GFP DNA in the semen sample; − =absence of lentiviral vector and/or GFP DNA in the semen sample; nd = not done.

[a] Note transplantation was done 8 weeks after irradiation

[b] Low numbers of germ cells in testes (see Fig. 4B).

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Hormone Treatment After Irradiation Stimulates Recovery of Rat Spermatogenesis From Surviving Spermatogonia

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ABSTRACT: The possibility of stimulating the recovery of spermatogenesis after irradiation using hormone treatment was tested in LBNF$_1$ rats. At 10 weeks after irradiation with 3.5 Gy, the percentage of tubules showing recovery of spermatogenesis (repopulation index) was 37% in rats that received no hormone treatment. GnRH agonist (GnRH-Ag) treatment with Zoladex or continuous treatment with testosterone markedly stimulated the recovery of spermatogenesis. When GnRH-Ag treatment was started immediately after 3.5-Gy irradiation and maintained for 10 weeks, the repopulation index was 91%. When an additional 6.5 weeks without further treatment was allowed between the 10-week GnRH treatment and killing the rats, the repopulation index recovered to 100% and sperm counts to 83 × 10^6. These sperm counts were more than 100-fold higher than those in rats not given hormone treatment and 50% of normal nonirradiated control levels. GnRH-Ag for 10 weeks also stimulated spermatogenic recovery in rats irradiated with 6 Gy, even when the start of treatment was delayed until 18 weeks after irradiation. Without GnRH-Ag, the repopulation index was 0, but in GnRH-Ag-treated rats it was 14.5%. Since all of the hormone treatments suppress intratesticular testosterone, high levels of testosterone may be inhibiting differentiation and their suppression may stimulate recovery. Even though the exact mechanism is not yet known, this method may still be applicable for clinical use to activate spermatogenesis in patients rendered azoospermic by irradiation or possibly by other cytotoxic treatments.

Key words: Testis, stem cells, GnRH-agonist, LHRH, testosterone.

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Irradiation of the testis with low doses of 1–3 Gy selectively destroys differentiating spermatogonia, resulting in depletion of more advanced spermatogenic cells (Dym and Clermont, 1970; Kangasniemi et al, 1990). Stem spermatogonia that are more radioresistant (Huckins, 1978; Meistrich et al, 1978; van der Meer et al, 1993) are not killed at low doses of irradiation and, in the mouse and certain strains of rat (e.g., Sprague-Dawley), immediately begin repopulating the seminiferous epithelium with differentiating cells (Oakberg, 1959; Dym and Clermont, 1970). In human beings, doses as low as 2 Gy appear to prevent this immediate repopulation from stem cells because azoospermia is induced and sperm do not reappear until after 50 weeks postirradiation (Clifton and Bremner, 1983). However, in some men, spermatogenesis recovers after prolonged periods of azoospermia following irradiation (Rowley et al, 1974; Hahn et al, 1982) or chemotherapy (da Cunha et al, 1984; Marmor et al, 1992; Meistrich et al, 1992). This suggests that there are surviving stem cells that are at first incapable of producing differentiating spermatogenic cells but are eventually activated.

We have recently shown that the LBNF$_1$ rat testis is similar to the human testis in its sensitivity to low doses of irradiation (Kangasniemi et al, 1996). After a dose of 3.5 Gy, there was no recovery of spermatogenesis despite the presence of A spermatogonia in the seminiferous tubules for up to 60 weeks following irradiation.

The recovery of spermatogenesis in these rats can be enhanced by administration, prior to irradiation, of steroid hormones that reduce luteinizing hormone levels and, consequently, testosterone production and the completion of spermatogenesis (Kurdoglu et al, 1994). It has also been shown (Pogach et al, 1988) that there is a greater enhancement of recovery of spermatogenesis in procarbazine-treated rats when hormonal treatments were given both before and after the procarbazine than when the hormonal treatment was only given before procarbazine. This finding suggested that hormonal posttreatment alone might be sufficient to improve recovery from cytotoxic treatment.

In the present study, we tested this possibility primarily using the GnRH-Ag, Zoladex, that has been shown to reduce intratesticular testosterone levels in the LBNF$_1$ rat (Kangasniemi et al, 1995a). To better understand the hormonal basis of stimulation of spermatogenic recovery, we

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also used testosterone capsules (Pogach et al, 1988) as a hormonal posttreatment.

Materials and Methods

Animals and Irradiation

$LBNF_1$ hybrid rats (F_1 generation of Lewis and Brown Norway strain parents) were used. They were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, Indiana) at 10 weeks of age and were acclimatized for 1 week before initiation of experiments. Animals were housed under standard lighting (12 hours light–12 hours dark) and were allowed food and water *ad libitum*. They were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care; all procedures were approved by our Institutional Animal Care and Use Committee.

The lower part of the body of rats was irradiated using a ^{60}Co gamma-ray unit (Eldorado 8, Atomic Energy of Canada Ltd., Ottawa, Canada). Rats anesthetized with 72-mg ketamine/kg and 2.2-mg acepromazine/kg (i.m.) were placed on their backs and 5 mm of tissue-equivalent bolus material (Superflab, Mick Radio-Nuclear Instruments Inc., Bronx, New York) was placed over the scrotum to provide a build-up layer. The field extended distally from a line about 5 cm below the diaphragm or about 6.5 cm above the base of the scrotum. Single doses of 3.5 or 6.0 Gy were administered at a dose rate of 0.96 Gy/minute. There was no toxicity or reduction in body-weight gain from these irradiations.

Fifty rats were irradiated with 3.5 Gy. Twenty were not given any hormone treatments and killed at 10 (8 rats), 16.5 (4 rats), or 20 (8 rats) weeks after irradiation. Starting at the time of irradiation, 16 were given GnRH-Ag treatment for 3 (4 rats), 6 (4 rats), or 10 (8 rats) weeks and killed at 10 weeks after irradiation; four were given GnRH-Ag treatment for 10 weeks and killed at 16.5 weeks after irradiation. Eight rats were given 24-cm testosterone implants and killed either 10 (4 rats) or 20 (4 rats) weeks after irradiation; four rats were given 2-cm testosterone implants and killed 20 weeks after irradiation. One rat from the 24-cm testosterone, 20-week group was excluded because one of the implant capsules was lost. Six rats were irradiated with 6 Gy. Two received no hormone treatment and the other four were treated with GnRH-Ag from weeks 18 through 28. In addition, between five and 14 untreated rats, assayed in the same runs, were used for control serum FSH, LH, and testosterone and intratesticular testosterone measurements. For the hormone measurements, between four and seven additional rats irradiated with 3.5 Gy and killed between 10 and 20 weeks after irradiation or four rats irradiated with 6 Gy and killed at week 30 were also included.

Hormone Treatments

Zoladex (goserelin acetate, D-Ser(Bu^t)^6-Aza-Gly^{10}-GnRH, Zeneca Pharmaceuticals, Wilmington, Delaware) was given to rats anesthetized with ketamine as subcutaneous depot injections of 1.8 mg, as used in our earlier studies (Kangasniemi et al, 1995a); this is one-half of the commercially available 3.6-mg preparation. Since the 3.6-mg preparation releases GnRH-Ag for 28 days (Furr and Hutchinson, 1992), and since the duration of release is related to the ratio of surface area to volume, we estimated that a 1.8-mg depot would release GnRH-Ag for between 3 and 4 weeks. Rats were either given a single GnRH-Ag depot (3-week treatment), two depots 23 days apart (6-week treatment), or three depots 23–24 days apart (10-week treatment). Control animals received anesthesia similar to those injected with GnRH-Ag. The GnRH-Ag treatment was usually started immediately after irradiation; however, in one experiment, it was initiated 18 weeks after irradiation.

Testosterone was administered by implantation of capsules made from Silastic tubing (catalog number 602-305, Dow Corning, Midland, Michigan) (Ewing et al, 1979). Capsules making up 2-cm or 24-cm (three capsules of 8-cm each) total length were implanted immediately after irradiation and maintained for up to 20 weeks after irradiation, when rats were killed. Capsules were used within several days after preparation. Although there is a spike of testosterone release immediately upon implantation, within several days the release rate has been reported to equilibrate at about 30 μg/day·cm (Robaire et al, 1979). The 2-cm capsules suppress spermatogenesis in otherwise untreated $LBNF_1$ rats, whereas 24-cm capsules maintain spermatogenesis (Meistrich et al, 1996).

Evaluation of Spermatogenesis

For histological analysis, the left testis was fixed in Bouin's fluid, embedded in plastic (JB4, Polysciences Inc., Warrington, Pennsylvania), and 4-μm sections were cut and stained with PAS-hematoxylin. To evaluate the recovery of spermatogenesis from irradiation, 400 seminiferous tubules in one section from each animal were scored for the most advanced germ-cell stage present in each tubule. A tubule was scored as repopulating if it contained three or more spermatogonia that had reached type B or later (Meistrich and van Beek, 1993). The repopulation index (RI), which is the percentage of tubules showing repopulation, was computed.

The weight of the right testis was measured after removing the tunica albuginea and the tissue was homogenized and an aliquot sonicated as described previously and sperm heads were counted (Meistrich and van Beek, 1993). The sonication-resistant sperm heads represent nuclei of step 12–19 spermatids.

Testing of Fertility

The effect of GnRH-agonist treatment for the first 10 weeks after irradiation with 3.5 Gy on fertility of male rats was evaluated. One female was caged with each male for 7 days (week 13) and then replaced with a new female (week 14). None of these were pregnant so the first females were recaged with the males (week 15), and then the second group of females was recaged with males for 4 days (week 16). At the start of week 19, females that had not produced litters were killed, the uteri were examined, and the number of embryos were counted.

Hormone Assays

Blood was collected by cardiac puncture at the time the rats were killed, and serum was prepared from it and stored at −80°C. After an aliquot was taken from the homogenate of the right

testis for sperm counts, the remainder was frozen and stored at −80°C.

Serum LH concentrations were measured by a supersensitive immunofluorometric assay developed for the rat (Haavisto et al, 1993). The NIDDK rat LH RP-2 was used as standard. FSH concentrations were determined by double-antibody radioimmunoassay using the NIDDK assay kits and standards (rFSH RP-2) (Clayton et al, 1980). To correct for the interassay variation in LH and FSH measurements, values from each of two runs of the assays were normalized to the average of the two runs based on values for samples run in both assays.

Testosterone was measured using coated-tube RIA kits (catalog number DSL 4000, Diagnostic Systems Laboratories, Webster, Texas); the interassay coefficient of variation was 14%. Serum testosterone was assayed according to manufacturer's instructions. This coated-tube RIA kit was also used to directly measure testosterone in testicular homogenates, the validity of which was demonstrated as follows. Known amounts of testosterone were diluted into serum, water, charcoal-stripped testis-tissue homogenates, and testis-tissue homogenates with known low testosterone levels. Measurement of testosterone concentrations in tissue homogenates was highly correlated with the concentrations in serum ($r^2 = 0.98$) but gave higher apparent values (apparent testosterone concentration in homogenate/concentration in serum = 1.62). In contrast, measurement of testosterone in tissue homogenates gave similar values as in water (ratio = 1.10). Since the standards used in the assay are diluted in serum, it was necessary to correct the measured values in tissue homogenates by the following equation to allow application of the standard curve:

$$\log_{10}(\text{corrected T concentration}) = [\log_{10}(\text{measured T in homogenate}) - 0.233] \times 1.1147.$$

To further test the validity of the assay and check the application of this correction, testosterone concentrations in 9 testis-tissue samples were measured by the coated-tube assay either directly or after extraction with ethyl acetate (Kangasniemi et al, 1995b). The two values were highly correlated ($r^2 = 0.95$) and agreed quite well (apparent testosterone concentration in unextracted homogenate/concentration using extraction = 1.02).

Data and Statistical Analyses

For presentation of sperm counts, the means and standard errors of the means were obtained on log-transformed data. Statistical analyses of comparisons between groups was performed by using first a Kruskal-Wallis one-way ANOVA to determine whether there were differences between all groups ($P < 0.05$) and then a nonparametric Mann-Whitney test to determine the significance of the differences between pairs of groups. SPSS for Windows, version 5.0 (SPSS Inc., Chicago, Illinois) software was used. The fertility data was analyzed by a Chi-square test.

Results

Effects of GnRH-Ag on Recovery of Spermatogenesis After Irradiation

At 10 weeks after irradiation with 3.5 Gy without any subsequent hormone treatment, the majority of seminiferous tubules had only Sertoli cells or Sertoli cells with surviving A spermatogonia, which was not considered to be evidence of repopulation (Fig. 1A). Some tubules were, however, repopulating, yielding a repopulation index of 37% (Fig. 2). Of the repopulating tubules, most had B spermatogonia or pachytene spermatocytes as the most advanced spermatogenic cells; 22% (±6%, SEM) contained round spermatids and 4% (±1%) elongating or condensed spermatids.

Three different treatment times were tested to study the

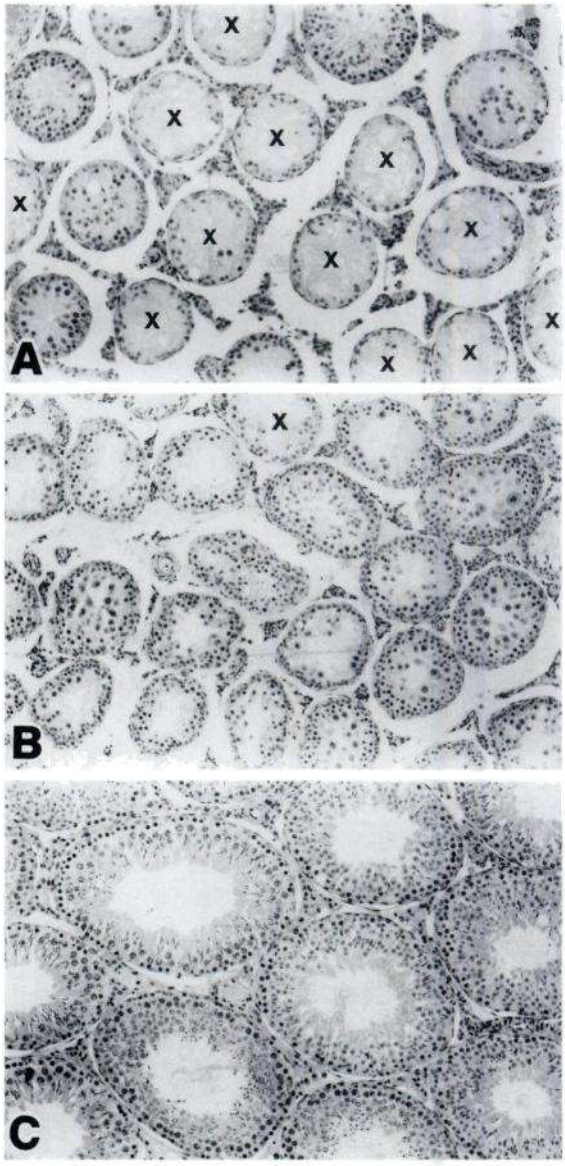

FIG. 1. Photomicrographs (magnification, ×76) of sections of rat testes. (A), 10 weeks after 3.5 Gy of irradiation. Most of the tubules show no recovery of spermatogenesis (x). (B), 10 weeks after 3.5 Gy of irradiation with continuous GnRH-Ag treatment started immediately after irradiation and maintained up to 10 weeks after irradiation. Most tubules show recovery of spermatogenesis; in this field only 1 tubule has not recovered (x). (C), 16.5 weeks after irradiation, GnRH-Ag was given up to 10 weeks after irradiation. All tubules show active spermatogenesis.

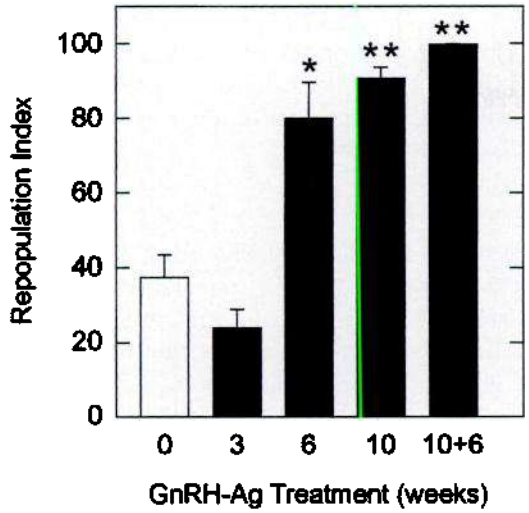

FIG. 2. Effect of GnRH-Ag treatment on repopulation indices in rats irradiated with 3.5 Gy. GnRH-Ag treatment was started immediately after irradiation and maintained for 3, 6, or 10 weeks. Three groups were killed 10 weeks after irradiation. One group of rats was treated for 10 weeks after irradiation but killed 16.5 weeks after irradiation (10 + 6). Mean ± SEM, $n = 4$ rats per group (3, 6, 10 + 6 weeks) or $n = 8$ (control, 10 weeks). Statistical significance of difference compared to rats that received no GnRH-Ag: * $P < 0.02$; ** $P < 0.005$.

enhancement of the recovery of spermatogenesis by GnRH-Ag; all rats were killed 10 weeks after irradiation. Treatment given for 3 weeks starting immediately after irradiation had no effect on the recovery of spermatogenesis (Fig. 2). In rats that received GnRH-Ag for 6 weeks, the repopulation index was enhanced to 80%, and in rats treated with GnRH-Ag for 10 weeks after irradiation, the repopulation index was 91% (Fig. 1B). The stimulatory effect of the 10-week treatment was measured in two independent experiments. Despite the stimulation of repopulation, round spermatids were seen in only 15% (±3%) and elongating or condensed spermatids in only 1% (±1%) of the recovering tubules.

The low number of tubules with late spermatids is likely due to the suppression of androgen levels by GnRH-Ag. To study whether the increase in repopulation indices is maintained and whether the germ cells present continue to mature into sperm after cessation of the hormone treatment, rats were irradiated with 3.5 Gy and treated with GnRH-Ag for 10 weeks thereafter, but not killed until 16.5 weeks after irradiation. At this time, the repopulation index increased to 99.7% (Fig. 2). Furthermore, 92% (±2%) of the recovering tubules contained elongating or condensed spermatids (Fig. 1C). For comparison, in rats that received 3.5 Gy but no GnRH-Ag treatment, and were killed at 16.5 weeks after irradiation, repopulation index was 10% (±3%).

At 16.5 weeks after irradiation, testicular sperm count recovered to 83 × 10^6 in the hormone-treated rats. This was 127-fold higher than in rats not given hormone treatment and 48% of normal nonirradiated control levels (Table 1). One of the four GnRH-treated, irradiated rats produced a pregnancy on week 15 and two did on week 16; none of those given 3.5 Gy but no hormone treatment produced any pregnancies. The fertility of the GnRH-treated rats differed from those not given GnRH at $P = 0.10$.

Table 1. *Testicular weights and sperm-head counts 16.5 weeks after irradiation with 3.5 Gy. GnRH-Ag treatment was started immediately after irradiation and maintained for 10 weeks (n = 4 rats per group)*

Hormone treatment	Irradiation dose (Gy)	Testis weight (g) Mean ± SEM	Sperm head count (×10^6) Mean (−SEM, +SEM)
None	3.5	0.45 ± 0.03	0.65 (0.41, 1.03)
GnRH-Ag	3.5	0.87 ± 0.05*	83 (73, 93)*
None	0	1.69 ± 0.05†	173 (154, 194)†

* Significantly different from values for irradiated rats without GnRH-Ag treatment, $P < 0.03$.

† Untreated control value in age-matched LBNF, rats from Kangasniemi et al (1995b).

To determine, whether GnRH-Ag treatment stimulates recovery of spermatogenesis in testes following regression, GnRH-Ag treatment was started 18 weeks after 6 Gy of irradiation. At this time type A spermatogonia are the only remaining spermatogenic cells. The hormone treatment was continued for 10 weeks, at which time the rats were killed. In rats that received no hormone treatment, the repopulation index was nearly 0. When GnRH-Ag treatment was given for 10 weeks before the rats were killed, spermatogenesis recovered in 14.5% of the tubules (Table 2). This result shows that some stimulation of recovery is also possible after radiation doses as high as 6 Gy. However, the development of germ cells was limited as spermatids were present in only 1% (±1%) of the recovering tubules.

Effects of Testosterone on Recovery of Spermatogenesis After Irradiation

To better understand the mechanism of stimulation of spermatogenesis, effects of testosterone treatment given

Table 2. *Repopulation indices 28 weeks after irradiation with 6.0 Gy. GnRH-Ag treated rats were given the hormone for 10 weeks prior to killing. Mean ± SEM*

Hormone treatment	Number of rats	Repopulation index (%)
None	6*	0.1 ± 0.1
GnRH-Ag	4	14.5 ± 2.9†

* Two rats were irradiated and killed concurrently with the GnRH-Ag treated rats and four were from Kangasniemi et al (1996) and killed at 30 weeks after irradiation.

† Significantly different from values for irradiated rats without GnRH-Ag treatment, $P < 0.01$.

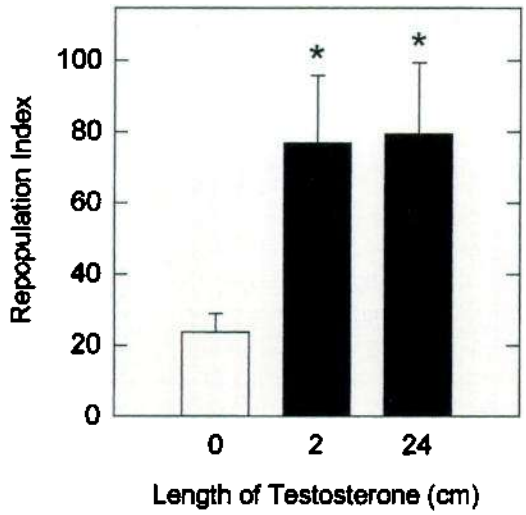

FIG. 3. Effect of testosterone treatment on repopulation indices in rats irradiated with 3.5 Gy and killed 20 weeks after irradiation. Testosterone capsules of 2-cm or 24-cm lengths were implanted after irradiation and left in place until the rats were killed. Mean ± SEM, $n = 3$ (24-cm group), 4 (2-cm group), or 8 (control) rats per group. Statistical significance of difference compared to rats that received no testosterone: * $P < 0.03$.

after irradiation were also investigated. Ten weeks of treatment with 24-cm testosterone capsules after irradiation with 3.5 Gy appeared to enhance the recovery of spermatogenesis; however, the increase in repopulation index from 37% (±6%) to 52% (±13%) was not statistically significant. A longer time period was used to try to further enhance the recovery. When 24-cm testosterone treatment was maintained for 20 weeks after irradiation, repopulation index was significantly increased to 79% from the value of 24% observed with no hormone treatment (Fig. 3). Treatment with 2-cm testosterone capsules also significantly enhanced the repopulation index to 77%. Whereas 77% (±18%) of repopulating tubules contained elongating or condensed spermatids in the rats implanted with 24-cm testosterone capsules, only 34% (±1%) showed development to this stage in the rats implanted with 2-cm capsules.

Hormone Levels

As reported earlier (Kangasniemi et al, 1996), there are 50% and 200% increases in immunoreactive FSH and LH, respectively, in serum following irradiation of $LBNF_1$ rats but no significant changes in serum testosterone (Table 3). There is also a marked 300% increase in the intratesticular concentration of testosterone.

Some of the hormone treatments that stimulate the recovery of spermatogenesis result in lowering of immunoreactive FSH back to control levels, but others do not. Similarly, with immunoreactive LH, only the 24-cm testosterone capsules were effective at significantly reducing the serum levels of this gonadotropin. It was surprising that 2-cm testosterone implants failed to reduce LH levels in irradiated $LBNF_1$ rats, whereas they significantly reduced LH levels in the unirradiated rats (Meistrich et al, 1996). As expected, serum-testosterone levels were reduced by GnRH-Ag treatments, maintained by 2-cm testosterone capsules, and elevated by 24-cm testosterone capsules.

The one consistent feature of the different treatments that stimulated recovery of spermatogenesis was the lowering of ITT concentrations. This was not due to an increase in testis mass, as all of these treatments actually reduced testis weights. It was noted that although GnRH-Ag treatment resulted in the best stimulation of spermatogenesis, 2-cm testosterone capsules appeared most effective at reducing ITT concentrations.

Table 3. *Hormonal levels in $LBNF_1$ rats following irradiation and treatment with GnRH-agonist or testosterone for the indicated time prior to killing. (n = 4–23 samples per group, except for ITT measurement after 6 Gy, no GnRH-Ag, in which there were only two samples)*

Irradiation dose (Gy)	Time after irradiation (weeks)	Hormone treatment	Duration of hormone treatment (weeks)	Serum FSH (ng/ml)*	Serum LH (ng/ml)†	Serum T (ng/lm)†	ITT (ng/g testis)†
0		None		19 ± 1	0.073 (0.057–0.094)	0.59 (0.39–0.89)	30 (26–36)
3.5	10–20	None		26 ± 1[a]	0.18 (0.15–0.20)[a]	0.76 (0.66–0.86)	131 (120–143)[a]
3.5	10	GnRH-Ag	10	17 ± 1[b]	0.18 (0.17–0.20)	0.06 (0.05–0.09)[a,b]	4 (3–6)[a,b]
3.5	20	24-cm T	20	18 ± 1[b]	0.043 (0.034–0.053)[b]	14.8 (13.9–15.7)[a,b]	9 (6–15)[b]
3.5	20	2-cm T	20	25 ± 5	0.18 (0.16–0.21)	1.2 (1.1–1.3)	2.3 (1.9–2.7)[b]
6	30‡	None		26 ± 1[a]	0.14 (0.13–0.14)	1.2 (1.1–1.3)	122 (103–145)
6	28	GnRH-Ag	10	22 ± 5	0.14 (0.13–0.15)	0.05 (0.04–0.06)[a,c]	13 (12–15)[c]

* Mean ± SE.

† Mean calculated from log-transformed data (−1 SEM, +1 SEM).

‡ Rats killed at 30 weeks for serum assays were from Kangasniemi et al (1996). Intratesticular testosterone was measured on rats killed at 6 weeks.

[a] Significantly different from unirradiated control, $P < 0.01$. [b] Significantly different from corresponding irradiated group without hormone treatment, $P < 0.01$. [c] Group size too small to reach the 0.01 level of significance by Mann-Whitney test; significantly different from corresponding irradiated group without hormone treatment by *t*-test, $P < 0.01$.

Discussion

This study is the first to demonstrate stimulation of recovery of rat spermatogenesis from surviving stem cells when hormonal treatment was given only after irradiation or cytotoxic drugs. There have been several studies showing stimulation of recovery of spermatogenesis when hormone treatment with steroids or GnRH antagonists were given before irradiation (Schally et al, 1987; Schlappack et al, 1988; Jegou et al, 1991; Kurdoglu et al, 1994). Our results are similar to those of Pogach et al (1988), who had indicated that recovery of spermatogenesis can be stimulated by testosterone given after procarbazine. However, interpretation of their study is complicated by their use of GnRH-antagonist treatment before and during chemotherapy. They also failed to present any statistical analysis of their data.

In this study, we presented preliminary data indicating that GnRH-Ag treatment restores fertility. In a subsequent experiment, in which rats were given the GnRH-Ag Lupron for 10 weeks after 3.5 Gy of irradiation and mated on weeks 18 and 19, there was also a trend (but not statistically significant) towards increased fertility above that observed in the group receiving irradiation only. Further experiments with larger numbers of animals need to be done to unequivocally determine whether fertility is enhanced. In addition, further studies are needed to optimize the treatment to maximize the recovery with the minimal dose of hormone.

Mechanism of Stimulation

We recently characterized dose- and time-responsive effects of a single dose of irradiation on the testis of the $LBNF_1$ rat (Kangasniemi et al, 1996). Spermatogenesis initially recovered from surviving stem cells during the first 6 weeks after irradiation. However, at doses of 3.5–6.0 Gy, repopulation indices declined after 6 weeks, eventually to between 0% and 2%, and no recovery of spermatogenesis was seen up to 60 weeks after irradiation. Despite the lack of recovery, A spermatogonia were observed in the nonrepopulating seminiferous tubules. The mechanism of this decline is unknown, but may involve loss of paracrine effectors that would otherwise maintain the continued differentiation of spermatogenic cells from surviving stem cells.

Since GnRH-Ag treatment enhanced the recovery of spermatogenesis when given for 6–10 weeks after irradiation, the possibility exists that hormone treatment prevented the secondary decline of spermatogenesis. While that may be involved, it cannot be the only mechanism, since we also observed enhancement of recovery in rats that received GnRH-Ag treatment starting 18 weeks after irradiation. At this time, the repopulation index is 0 and undifferentiated A spermatogonia are the only spermatogenic cell type in the testis (Kangasniemi et al, 1996). We postulate that the enhanced recovery of spermatogenesis after irradiation is due to hormonal modulation of Sertoli cells that in turn alters the paracrine regulation of the differentiation of A spermatogonia. This could prevent the secondary decline or induce a secondary recovery after the decline has occurred.

The possibility was considered that the hormonal treatments were acting by suppressing the completion of spermatogenesis and that was producing a feedback signal to stimulate spermatogonial differentiation. However, since 24-cm testosterone capsules have been shown to maintain spermatogenesis, this mechanism cannot explain the stimulation of recovery seen with this treatment.

The hormonal changes produced by the different stimulatory treatments were analyzed. The slightly elevated levels of FSH after irradiation are not consistently lowered by all of the stimulatory treatments, and hence, FSH levels are unlikely to be involved in the stimulation of spermatogenic recovery. Although there is a large increase in LH after irradiation, it was not reduced by the GnRH-Ag or by 2-cm testosterone capsules in this study. Therefore, reduction of LH alone is not directly responsible for the stimulation, but, rather, it may contribute to stimulation by reduction of testosterone production. The different stimulatory treatments can increase, decrease, or leave serum testosterone unchanged, and hence, serum testosterone levels are not related to stimulation of recovery.

The consistent feature of the hormone treatments used here to stimulate recovery of spermatogenesis is the suppression of ITT. This suggests the possibility that the high ITT levels observed after irradiation, or possibly high levels of a metabolite of testosterone such as estradiol, are actually detrimental to spermatogenesis and that reduction of these levels is required for resumption of normal spermatogenesis. This hypothesis needs to be tested in future experiments. Since hormone receptors have not been localized in A spermatogonia, the hormonal effects are likely mediated through paracrine interactions with Sertoli or other cells.

Several studies have indicated that rat spermatogenic stem cells are protected from irradiation-induced (Schally et al, 1987; Schlappack et al, 1988; Jegou et al, 1991; Kurdoglu et al, 1994) or procarbazine-induced (Delic et al, 1986; Glode et al, 1990; Velez de la Calle and Jegou, 1990; Ward et al, 1990; Parchuri et al, 1993; Kangasniemi et al, 1995a,b) damage by hormone treatments given before cytotoxic treatment. None of those studies have addressed the question of whether hormone treatment actually improves stem-cell survival from cytotoxic treatment or whether it only enhances the recovery of spermatogenesis from the surviving stem cells. The possibility remains that stem cells are not protected from killing by

the cytotoxic agent but that, since hormone levels may still be altered in the period immediately after cytotoxic treatment, the mechanism of enhanced recovery resulting from hormonal pretreatment also involves stimulation of differentiation of surviving spermatogonia.

Potential for Clinical Application

After cancer therapy for Hodgkin's disease, non-Hodgkin's lymphomas, osteosarcoma, nonosteogenic sarcoma, or testicular cancer, some patients suffer from iatrogenic infertility. Some men receiving irradiation or chemotherapy have prolonged azoospermia, but eventually recover to oligo- or normospermia. As mentioned in the Introduction, this implies that there are surviving stem cells. In some men, spermatogenesis never recovers, but even in them we cannot rule out that some may still have stem cells. The present data demonstrating that hormone treatment stimulates recovery from surviving stem cells after cytotoxic treatment might be applied to humans to either shorten the period of azoospermia or induce recovery in some men in whom spermatogenesis would never otherwise recover.

Hormone treatment that controls recovery of spermatogenesis from remaining spermatogonia also expands approaches to male contraceptive development. Some attempts at development of contraceptives have been limited by the apparent irreversibility of the treatment in rats despite the presence of surviving A spermatogonia (Richard Blye, NICHD, personal communication). Hormonal methods stimulating the differentiation of surviving A spermatogonia would have a significant impact in potentially making such contraceptive procedures reversible.

Acknowledgments

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Hormonal Suppression for Fertility Preservation in Males and Females

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Abstract

Methods to restore fertility of men and women sterilized by medical treatments and environmental toxicant exposures are under investigation. Rendering spermatogenesis and ovarian follicular development kinetically quiescent by suppression of gonadotropins has been proposed to protect them from damage by cytotoxic therapy. Although the method fails to protect the fertility of male mice and monkeys, gonadotropin and testosterone suppression in rats before or after cytotoxic therapy does enhance the recovery of spermatogenesis. However the mechanism involves not the induction of quiescence but rather the reversal, by suppression of testosterone, of a block in differentiation of surviving spermatogonia caused by damage to the somatic environment. In men, only one of eight clinical trials was successful in protecting or restoring spermatogenesis after cytotoxic therapy. In females, protection of primordial follicles in several species from damage from cytotoxic agents using GnRH analogues has been claimed; however only two studies in mice appear convincing. The protection cannot involve induction of quiescence in the already dormant primordial follicle but may involve direct effects of GnRH analogues or indirect effects of gonadotropin suppression on the whole ovary. Although numerous studies in female patients undergoing chemotherapy indicate that GnRH analogues might be protective of ovarian function, none of the studies showing protection were prospective randomized clinical trials and thus are inconclusive. Considering interspecies differences and similarities in the gonadal sensitivity to cytotoxic agents and hormones, mechanistic studies are needed to identify the specific beneficial effects of hormonal suppression in select animal models that may be applicable to human.

I. Introduction

Medical treatments required for life-threatening diseases such as cancer or exposure to environmental toxicants may jeopardize the fertility of men and women of reproductive age. In men, such exposures can lead to effects ranging from temporary oligospermia to permanent azoospermia, and occasionally to androgen insufficiency. In women, such exposures can result in a range of effects from temporary amenorrhea to premature menopause and permanent amenorrhea, with the associated estrogen insufficiency. Whereas transient effects do affect the quality of life, the most serious effects of concern are the irreversible, permanent effects.

Methods to prevent these effects on fertility and to restore gonadal function after the toxic treatment are of great importance to men and women of child-bearing age. A variety of biochemical and biological approaches (thiol radioprotectors, prostaglandin analogues, growth factors, blockers of apoptotic pathways, and reduction in blood flow) have been tested to protect the testes in experimental animal model systems against radiation and chemotherapy (reviewed in (Meistrich *et al.*, 2007). However, the greatest research interest and nearly all clinical trials

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have involved hormonal modulation in attempts to prevent or reverse damage to the germline from radiotherapy and chemotherapy. We will discuss the current status of the knowledge on the hormonal suppression as a means to preserve fertility in men and women separately.

II. History and Hypotheses

The use of hormone suppression for protecting gonadal function after cytotoxic exposure is based on the observation that non-cycling cells are generally more resistant to killing by certain toxicants, particularly antineoplastic agents, than are rapidly proliferating cells. The greater sensitivity of cycling as compared to non-cycling cells is the basis for the antitumor action of many of these antineoplastic agents.

The mechanism originally proposed for protection of spermatogenesis was that interruption of the pituitary-gonadal axis would reduce the rate of spermatogenesis and render the resting testis more resistant to the effects of chemotherapy (Glode *et al.*, 1981). Although the Glode study claimed that pretreatment with gonadotropin-releasing hormone (GnRH) protected spermatogenesis in the mouse from damage from cyclophosphamide, attempts to repeat these original observations using more quantitative endpoints revealed that there was no protection (da Cunha *et al.*, 1987). Since suppression of gonadotropins and testosterone only blocks the completion of spermatogenesis but has no effect on the kinetics of the developing cells (Meistrich *et al.*, 1997), the premise on which the mechanism was based was incorrect and the negative outcome should be expected. Furthermore, the stem spermatogonia, which are more important targets than the differentiating germ cells for the long-term effects of cytotoxic damage, did not appear to be affected at all by hormonal suppression.

Despite the failure until now to observe protective effects in mice, it has been convincingly shown that suppression of gonadotropins and intratesticular testosterone levels prior to or during exposure of rats to chemotherapy or radiation enhances the subsequent recovery of spermatogenesis (Delic *et al.*, 1986). Thus other mechanisms must be involved and careful attention must be given to the species used.

Similarly suppression of gonadotropins with GnRH agonists or steroidal oral contraceptives has been proposed to suppress ovarian function, specifically to halt follicular development and follicular cell division, with the goal of protecting these now dormant ovarian follicles from destruction (Ataya *et al.*, 1985, Chapman & Sutcliffe, 1981). However, gonadotropins act primarily on the cyclic recruitment of antral follicles, while the initial recruitment of primordial follicles is not directly controlled by gonadotropins (McGee & Hsueh, 2000). It is these primordial follicles that provide the source for the long-term growing follicle and ova production and thus should be the target for protection. They lack gonadotropin receptors and besides are normally non-proliferating. Furthermore, although ovaries contain GnRH receptors, there is no evidence for the presence of GnRH receptors on primordial follicles (Danforth *et al.*, 2005).

Although several early studies concluded that such suppressive treatments could protect rat ovarian follicles against the damaging effects of chemotherapy (Ataya *et al.*, 1985, Bokser *et al.*, 1990), these results did not show that primordial follicles were protected (Meistrich, 1994). Recent studies using a GnRH agonist (Danforth *et al.*, 2005) or an antagonist (Meirow *et al.*, 2004) do, however, show protection of primordial follicles in mice from the damaging effects of cyclophosphamide. However, the originally proposed mechanism does not appear to be valid.

III. Experimental Studies - Males

The generally accepted model for the male gonadal toxicity from antineoplastic agents is that actively dividing differentiated spermatogonia are most sensitive to these agents, which will lead to a reduction in the sperm count. The reduction should be temporary provided the stem spermatogonia survive. However, stem spermatogonia are killed by some of these agents at varying degrees and recover only gradually, resulting in prolonged reductions of sperm count; in the mouse this reduction is directly related to stem cell killing (Meistrich, 1982). It is rare that surviving stem spermatogonia fail to differentiate in mouse testes (Kangasniemi *et al.*, 1996b). In contrast, after exposure of rats to several antineoplastic agents (Kangasniemi *et al.*, 1996a) and other toxicants (Boekelheide & Hall, 1991), the stem spermatogonia that survive are blocked from differentiating and their progeny undergo apoptosis instead (Meistrich & Shetty, 2003). This block has been shown to be a result of damage to the somatic environment within the testis, not to the spermatogonia (Zhang *et al.*, 2007). There is, however, no evidence of a similar spermatogonial block in monkeys (Boekelheide *et al.*, 2005).

Several studies further support the conclusion that gonadotropin suppression does not protect spermatogenesis in mice from damage (Crawford *et al.*, 1998, Kangasniemi *et al.*, 1996b, Nonomura *et al.*, 1991). In contrast numerous reports suggest that hormone suppression protects rat testes from damage due to irradiation, procarbazine, doxorubicin, an indenopyridine compound, and heating (Delic *et al.*, 1986, Hild *et al.*, 2001, Jégou *et al.*, 1991, Kangasniemi *et al.*, 1995, Manabe *et al.*, 1997, Morris & Shalet, 1990, Parchuri *et al.*, 1993, Setchell *et al.*, 2002, Weissenberg *et al.*, 1995) and enhances future fertility in the face of these toxicants. In addition to direct suppression of gonadotropins with GnRH agonists or antagonists, which also results in both direct and secondary suppression of intratesticular testosterone, these studies also utilized combinations of GnRH analogues with antiandrogens, systemic physiological doses of testosterone (which suppresses gonadotropins and results in reduced intratesticular testosterone levels), progestins (which are very effective at suppressing gonadotropins but have weak androgenic activity), and estrogens (which both suppress gonadotropins and inhibit testosterone synthesis) It should be noted that in all these studies protection was not assessed directly at the time of cytotoxic exposure, but rather by the enhanced ability of spermatogenesis to recover from surviving stem cells (Meistrich *et al.*, 2000), which is actually the most relevant endpoint for future fertility.

Attempts to protect spermatogenesis in other animal species (dog, monkey) have not yielded any reproducibly positive results. Although one group reported that GnRH-agonist shortened the time to recovery of spermatogenesis after treatment of dogs with cyclophosphamide, cisplatin, or radiation (Nseyo *et al.*, 1985), another study reported potentiation of the damage (Goodpasture *et al.*, 1988). Similarly, one preliminary report based on an extremely small number of baboons suggested that GnRH-agonists might decrease the gonadal damage from cyclophosphamide (Lewis *et al.*, 1985), while larger studies showed neither protection nor stimulation of recovery of spermatogenesis in macaques from radiation damage by GnRH-antagonist treatment (Boekelheide *et al.*, 2005, Kamischke *et al.*, 2003).

We proposed that prevention of the pronounced block in differentiation of surviving stem spermatogonia in rat testes after exposure to cytotoxic agents (Fig. 1A,B) is the mechanism by which hormone suppression appears to protect spermatogenesis from toxicant exposure (Meistrich *et al.*, 2000). It is important to note that many of the studies showing protection involved subchronic exposure to the cytotoxic agent, so the hormonal suppression was given after the initial exposures, and in some cases even extended beyond the last exposure (Pogach *et al.*, 1988). Furthermore, when the hormonal suppression was administered to the rats only after the cytotoxic insult, either immediately or after a delay (Fig. 1C), the numbers of differentiated germ cells still dramatically increased (Meistrich & Kangasniemi, 1997).

However, because testosterone, which is required for spermatid differentiation, was suppressed, spermatogenesis proceeded only to the round spermatid stage: no sperm were produced. Nevertheless, when additional time without further suppressive treatment was allowed before the rats were killed, all tubules showed almost complete spermatogenic recovery, sperm counts increased, and the fertility of the rats significantly increased (Meistrich *et al.*, 2001). This phenomenon appears to be quite general: post-treatment with GnRH agonists or antagonists, with or without antiandrogen, low-dose systemic testosterone, estradiol, or hypophysectomy are all effective at stimulating recovery (Shetty *et al.*, 2002, Shetty *et al*., 2006), and recovery has been stimulated following gonadal toxicity from anticancer agents such as radiation, procarbazine (Meistrich *et al.*, 1999), or busulfan (Udagawa *et al.*, 2001), environmental toxicants such as hexanedione (Blanchard *et al.*, 1998) or dibromochloropropane (Meistrich *et al.*, 2003), an indenopyridine compound (Hild *et al.*, 2001), or heat treatment (Setchell *et al.*, 2001). The endogenous hormone primarily responsible for the inhibition of spermatogonial differentiation in toxicant-treated rats was testosterone, although FSH also had a minor inhibitory effect (Shetty *et al.*, 2006), and other exogenously administered androgens were also inhibitory (Shetty *et al*., 2002). We have also observed that hormonal suppression after irradiation of mouse testes modestly but highly significantly increased the percentage of tubules in which differentiation of surviving spermatogonia occurred (G. Wang and M. L. Meistrich, unpublished observations).

Hormonal suppression with GnRH analogues or hypophysectomy has also been shown to promote the survival and differentiation of spermatogonia that are transplanted into testes of animals that were depleted of endogenous stem cells. Although the effects were most dramatic when rat testes depleted by cytotoxic treatments were used as recipients (Ogawa *et al.*, 1999, Zhang *et al.*, 2007), enhanced proliferation and differentiation of transplanted spermatogonia were also observed when mouse testes were used as recipients (Dobrinski *et al.*, 2001, Kanatsu-Shinohara *et al.*, 2004, Ohmura *et al.*, 2003, G. Wang and M. L. Meistrich, unpublished observations).

The mechanism by which somatic cells in rat testis are protected from damage if testosterone is suppressed at the time of cytotoxic exposure is not known. Neither is the mechanism by which testosterone suppression after cytotoxic exposure enhances the ability of the somatic elements of the testis to maintain the differentiation of spermatogonia. We had previously ruled out the possibility that the protective effect of hormonal suppression given before cytotoxic drug exposure was due to reduced delivery to the tissue or altered metabolism of the drug (Meistrich *et al.*, 1994). We have recently shown that the block in spermatogonial differentiation in rats treated with cytotoxic agents is associated with the increased levels of interstitial edema in the testes (Porter *et al.*, 2006). Based on data indicating that more rapid stimulation of recovery of spermatogonial differentiation in irradiated rats was achieved by elimination of Leydig cells with ethane dimethane sulfonate than with total androgen ablation (G. Shetty and M.L. Meistrich, unpublished data), we are analyzing the role of Leydig cells as targets for the testosterone-induced inhibition of spermatogonial differentiation in toxicant-treated rats.

IV. Experimental Studies - Females

Studies have utilized mice, rats, and monkeys to investigate protection against cyclophosphamide and radiation-induced ovarian damage. In these studies, the important target for which protection should be demonstrated was the primordial follicle, since it provides the reserve for production of growing follicles and ova over a prolonged period of time. Protection of developing follicles would result only in a short term enhancement of ovarian function, since these follicles are either recruited in a cyclic manner to undergo further development and ovulation or otherwise undergo atresia (McGee & Hsueh, 2000).

The mouse appears to be a good model to study the protective effects of various agents against cyclophosphamide damage since the primordial follicles in mouse are moderately sensitive to killing by cyclophosphamide (Plowchalk & Mattison, 1991). An initial study claimed protection of mouse ovaries against cyclophosphamide with the synthetic steroid danazol, but that study examined only developing follicles, so the results do not bear on long-term protection of ovarian function (Budel *et al.*, 1988). Protection of mouse primordial follicles from the damaging effects of cyclophosphamide was first reported in a study using a GnRH antagonist (Meirow *et al.*, 2004). Although one study claimed protection using a GnRH agonist based on marginal statistical significance (Yuce *et al.*, 2004), a subsequent study did show significant protection (Danforth *et al.*, 2005). However, that study made the surprising observation that GnRH antagonists, even given without cyclophosphamide, had deleterious effects and depleted primordial follicles, which needs to be further investigated and confirmed (Gupta & Flaws, 2005).

In contrast, studies using rats for assessing the protection of ovarian function from cytotoxic agents such as cyclophosphamide did not yield any information on the long-term effects because rat primordial follicles are not sensitive to killing by cyclophosphamide (Ataya *et al.*, 1985). The ability of GnRH-agonist treatment to maintain the numbers of small follicles and fertility in cyclophosphamide-treated rats (Ataya & Ramahi-Ataya, 1993, Ataya *et al.*, 1985) is therefore most likely a result of inhibition either of initial recruitment or of physiological loss of primordial follicles (Ataya *et al.*, 1989). This observation that GnRH-agonist treatment maintains the number of primordial follicles was surprising, since these follicles should be unaffected by gonadotropins, and other studies have failed to reproduce this observation (Bokser *et al.*, 1990, Jarrell *et al.*, 1987).

The one study in cyclophosphamide-treated monkeys showed that prolonged (1.5 years) GnRH-agonist treatment reduced the rate of loss of primordial follicles (Ataya *et al.*, 1995b). Although the results were interpreted as indicating the GnRH agonist can protect primate ovaries against cyclophosphamide-induced damage, there was no control group treated with GnRH agonist alone, so the result may also represent GnRH-agonist inhibition of recruitment of primordial follicles.

GnRH agonist has also been shown to prevent the doxorubicin-induced inhibition of estradiol production by granulosa cells *in vitro* (Imai *et al.*, 2007). However, this study utilized granulosa cells from mature follicles, whereas the important target, the primordial follicles, may not have GnRH receptors.

Even less success has been reported with respect to hormonal protection of ovarian function from radiation. Radiation kills primordial follicles in all mammals studied, but those of the mouse are exquisitely sensitive and those of the rat are moderately sensitive (Baker, 1978). In mice, gonadotropin reduction due to a hypogonadal mutation or GnRH-antagonist treatment failed to protect primordial follicles from radiation (Gosden *et al.*, 1997). Treatment with a GnRH agonist, but not with medroxyprogesterone acetate, partially protected against radiation-induced loss of primordial follicles in rats (Jarrell *et al.*, 1987, Jarrell *et al.*, 1989). No protection from radiation-induced loss of primordial follicles in monkeys was observed with GnRH-agonist treatment (Ataya *et al.*, 1995a).

In summary, very mixed results have been obtained in experimental studies regarding protection of primordial follicles from damage by cytotoxic agents by hormonal suppression. Some studies that claimed protection examined only growing follicles and thus could not support the notion that hormonal suppression protects the primordial follicles. Other studies showing that extended GnRH agonist treatment reduced the primordial follicle loss in animals treated with cytotoxic agents do not demonstrate that these follicles are protected but may be

interpreted as inhibition of the normal initial recruitment of primordial follicles. Such a mechanism would not benefit women receiving GnRH-agonist treatment during cytotoxic therapy; however, if the mechanism is valid, prolonged GnRH -agonist treatment would preserve surviving primordial follicles for a longer period of time if the women want to delay childbearing. In cases where protective effects have been observed against cyclophosphamide-induced depletion of primordial follicles in mice, the protection may involve direct effects of the GnRH analogues on the ovary or indirect effects of gonadotropin suppression such as reduced drug delivery due to a reduction of ovarian blood flow (Meirow *et al.*, 2004).

V. Clinical Trials - Males

Seven clinical trials have been performed in attempts to demonstrate protection of spermatogenesis in humans by hormone suppression treatment before and during cytotoxic therapy, but six indicated no protection (Table 1). Three of these trials involved patients treated for Hodgkin's lymphoma and three involved testicular cancer patients. Treatment with GnRH agonist resulted in only 20% of patients recovering sperm count after cessation of chemotherapy (Johnson *et al.*, 1985). However, no concurrent control group of patients receiving similar regimens of chemotherapy without GnRH agonist was enrolled in this study. In another study, hormone suppression with testosterone combined with GnRH agonist prior to and during chemotherapy was randomized with no hormonal suppression (Waxman *et al.*, 1987), but none of the patients from the control and treated groups showed evidence of recovery of spermatogenesis at 1 to 3 years after completion of therapy. Suppression of gonadotropins and intratesticular testosterone levels with testosterone injections alone during treatment (Redman & Bajorunas, 1987) also did not provide gonadal protection benefit: 70% of the patients in both the treated and control group showed spermatogenic recovery at 3 years. Suppression of gonadotropins with medroxyprogesterone acetate during chemotherapy combined with radiotherapy did not improve the recovery of sperm count or normalize FSH levels, which was used as a surrogate for sperm count in patients in whom sperm counts were unavailable; indeed, they appeared to be lower in the patients receiving concurrent treatment with hormonal suppression than in controls (Fossa *et al.*, 1988). Two more studies used GnRH agonist (Kreuser *et al.*, 1990) or GnRH agonist plus an antiandrogen (cyproterone acetate) (Brennemann *et al.*, 1994) prior to and for the duration of chemotherapy or radiation therapy, respectively. In these studies the chemotherapeutic regimen was only 2 courses of PVB and the gonadal dose of radiation was 0.2 Gy, which allowed spontaneous recovery of sperm counts in all the control patients within 2 years. The time course of recovery of spermatogenesis after chemotherapy was identical for the groups of patients with or without GnRH-agonist treatment. Although fluctuations in sperm counts made it difficult to determine whether the time course of recovery of spermatogenesis was affected by hormonal treatment, the time course of reduction of elevated FSH levels back to pretreatment values was similar in controls and in the patient groups treated with GnRH agonist and antiandrogen.

The one study that demonstrated hormonal treatment preservation of sperm production in men involved testosterone therapy of men who received cyclophosphamide as an immunosuppressive therapy for nephrotic syndrome (Masala *et al.*, 1997). During the treatment, the testosterone suppressed gonadotropin levels and suppressed the completion of spermatogenesis. All but one of the men who received cyclophosphamide alone remained azoospermic 6 months after the end of immunosuppressive therapy, whereas sperm concentrations returned to normal in all five men who received cyclophosphamide in combination with testosterone therapy.

The one attempt to restore spermatogenesis by steroid hormone suppression after cytotoxic therapy was also unsuccessful (Thomson *et al.*, 2002). Seven men with azoospermia secondary to high-dose chemo- and/or radiation therapy for leukemia or lymphoma in childhood were

treated with medroxyprogesterone acetate combined with testosterone to suppress gonadotropin and likely intratesticular testosterone levels many years after the anticancer treatment. None of the men recovered any sperm production during the 24-week follow-up after the end of hormonal treatment.

Even if the hormonal suppressive treatments that were successful in protecting and stimulating spermatogenic recovery in rats are applicable to human males, there may be many reasons for the unsuccessful outcomes of the aforementioned clinical trials. The use of testosterone or medroxyprogesterone either alone (Fossa *et al.*, 1988, Redman & Bajorunas, 1987, Thomson *et al.*, 2002) or combined with a GnRH analogue (Waxman *et al.*, 1987) is suboptimal given that, in animal studies, both of these steroids act directly on the testis to reduce the stimulatory effects of GnRH analogues on the recovery of spermatogenesis after cytotoxic damage (Shetty *et al.*, 2002, Shetty *et al.*, 2004); however, this might not be as significant in humans, which have higher intratesticular androgen levels (Jarow *et al.*, 2001). The number of patients and controls studied was small (Johnson *et al.*, 1985) and the cancer therapies variable. Some treatment regimens were not sufficiently gonadotoxic to cause sterility (Brennemann *et al.*, 1994, Kreuser *et al.*, 1990); conversely some regimens may have delivered doses well above that needed to ablate all spermatogonial stem cells, since no evidence of spermatogenesis was observed in almost all patients even after many years (Johnson *et al.*, 1985, Thomson *et al.*, 2002, Waxman *et al.*, 1987). Thus the application of these procedures to humans remains uncertain.

VI. Clinical Trials - Females

The greater proportion of prepubertal than of postpubertal women who maintain normal ovarian function after chemotherapy or radiotherapy (Horning *et al.*, 1981) has been used to promote the concept that the ovary that is not stimulated by gonadotropins has a greater tolerance for these cytotoxic treatments. However, the larger store of follicles at the younger ages is an alternative explanation (Faddy *et al.*, 1992). This alternate explanation is supported by the greater resistance of 20-year-old women than of 35-year old women to induction of permanent ovarian failure from chemotherapy or radiation therapy (Meistrich *et al.*, 2005).

Several studies reported by Blumenfeld's group (reviewed in Blumenfeld, 2007, Blumenfeld & Eckman, 2005), including a recent update of patients treated for Hodgkin lymphoma (Blumenfeld *et al.*, 2008), showed that 7% of ~125 women treated with a GnRH agonist during chemotherapy exhibited premature ovarian failure characterized by hypergonadotropic amenorrhea, whereas 53% of ~125 women in the control group treated with chemotherapy but not GnRH agonist (some concurrent with the GnRH-treated group and some historical controls) suffered premature ovarian failure. None of these studies were randomized prospective trials, although attempts were made to demonstrate similar age ranges and chemotherapy and radiation doses in the GnRH-agonist and control groups. In another study, adolescent girls (9 patients) received high-dose chemotherapy for bone marrow transplantation; those receiving GnRH agonist resumed menstrual cycles whereas those who were not treated with GnRH agonist had secondary amenorrhea (Pereyra Pacheco *et al.*, 2001). In a recent study 56 women were treated with a GnRH agonist and a synthetic estrogen; a control group did not take the treatment. The study reported that 90% of the gonadotropin-suppressed women recovered ovulatory and menstrual function, versus 23% of those not treated (Castelo-Branco *et al.*, 2007). The interpretation that protection occurred is, however, weakened by the fact that different treatments were given and age distributions in the control and treated groups were not comparable. Two phase II studies with adult breast cancer patients (100 patients in one and 29 patients in the other) suggested that a GnRH-agonist treatment protected ovarian function, but the claims were based only on historical data in the literature and not a control group (Del Mastro *et al.*, 2006, Recchia *et al.*, 2006). In addition, an observational study of 145 patients

reported that the patients taking oral contraceptives had only a 10% incidence of post-chemotherapy amenorrhea compared to 44% in the group not taking oral contraceptives (Behringer *et al.*, 2005). One study utilized treatment with a GnRH antagonist, in addition to an agonist, during chemotherapy and reported good short-term recovery of menstrual cycles, but the study did not have a control group (Potolog-Nahari *et al.*, 2007).

Although these studies indicate that suppression of gonadotropins and estrogen might be protective of ovarian function, none of them were prospective, randomized clinical trials, and hence must be considered inconclusive (Lee *et al.*, 2006). There was only one randomized study of the effects of GnRH agonist on chemotherapy-induced sterility that involved 18 patients, and there was no protective effect (Waxman *et al.*, 1987). However the more recent suggestive evidence discussed above indicates that randomized clinical trials, several of which are in progress, need to be pursued.

VIII. Relationship of Experimental to Human Studies

The results of studies of protection of long-term gonadal function by hormonal suppression in experimental animals and humans are summarized in Table 2. In males, hormonal suppression reliably stimulated recovery of spermatogenesis from surviving stem cells in rats but did not influence the survival of spermatogonial stem cells. In humans, only one of eight clinical trials showed that hormonal suppression enhanced subsequent gonadal function. In females, hormonal suppression provided mixed results on protection of mouse primordial follicles from damage due to cyclophosphamide and of rat primordial follicles from damage due to radiation. Several human clinical studies showed that GnRH-agonist treatment produced good prolonged maintenance of ovarian function after chemotherapy, but this conclusion must be viewed with caution, as none of these studies were randomized clinical trials.

Experimental studies, particularly in rodents, are of great value in that they may be highly controlled, have larger sample sizes, and can be used to optimize treatments and to elucidate mechanisms. Primate studies have greater variability and uncertainties and sample sizes are limited. The main question is what aspects of the rodent studies are applicable to the human and what aspects are not. Since primates and rodents diverged 66 million years ago (Mya) there will be differences. It is also noteworthy that mouse and rat diverged 41 Mya, whereas humans and macaques diverged 23 Mya. Thus, significant differences are expected among rodents and between rodents and different primates. It is important to understand the mechanism of protection or stimulation of recovery by hormonal suppression in order to determine which individual steps in the process will be similar or different between rodents and primates.

Cytotoxic agents to which rodents and primates have similar sensitivities with respect to their effects on spermatogenesis are appropriate to utilize in experimental studies for extrapolation to men. Mouse and human spermatogenesis are both sensitive to certain alkylating agents (procarbazine, chlorambucil, busulfan) and radiation as measured by stem cell killing and prolonged azoospermia, respectively (Meistrich, 1993). However there are differences as spermatogonial stem cells in mice are sensitive to killing by doxorubicin (Adriamycin) but not cyclophosphamide, whereas cyclophosphamide, but not doxorubicin, strongly induces long-term azoospermia in humans. In rats, both radiation and procarbazine (Meistrich *et al.*, 1999), but not cyclophosphamide (Meistrich *et al.*, 1995), produce a prolonged block to spermatogonial differentiation.

In rats exposed to moderate doses of cytotoxic agents, the induction of a block in spermatogonial differentiation is a much more likely cause of prolonged azoospermia than is spermatogonial stem cell killing. The reversal of this block in spermatogonial differentiation appears to be the mechanism by which hormone suppression protects or restores spermatogenesis in toxicant-exposed rats (Meistrich *et al.*, 2000) and the occurrence of such

a block should indicate whether hormonal suppression might stimulate spermatogenic recovery in another species. Although in many cases the seminiferous tubules in testicular biopsies taken from men with chemotherapy- or radiotherapy-induced azoospermia contain only Sertoli cells and no spermatogonia (Van Thiel *et al.*, 1972), occasionally the presence of isolated spermatogonia have been observed at relatively long times after chemotherapy treatment (Kreuser *et al.*, 1989). In addition, spontaneous recovery of spermatogenesis in some men more than 1 year after radiation (Hahn *et al.*, 1982) or chemotherapy (Drasga *et al.*, 1983) also implies a block in the differentiation of spermatogonia that survive these cytotoxic exposures. These results suggest that after some cytotoxic therapy regimens, there is a potentially reversible block to spermatogonial differentiation in men. It should be noted that there is no evidence of a similar spermatogonial block in monkeys (Boekelheide *et al.*, 2005). The human data emphasize that the cytotoxic therapy regimens need to be carefully selected in these clinical trials from a range of regimens. To have a chance of success, doses of cytotoxic therapies should be chosen at which there is an appreciable block to spermatogonial differentiation but not killing of all stem spermatogonia.

Since many chemotherapeutic and radiotherapeutic regimens may result in complete killing of the stem spermatogonia and the hormonal methods do not protect these cells from cytotoxicity, consideration should be given to the applications of hormonal suppression in combination with spermatogonial transplantation. Cryopreservation of spermatogonia and autologous transplantation is considered a potential method for restoring spermatogenesis and possibly rescuing fertility after chemo- or radiotherapy (Orwig & Schlatt, 2005). Hormonal suppression could restore the somatic environment in human testes sufficiently to promote the ability of transplanted stem spermatogonia to develop, as was the case with rat testes (Zhang *et al.*, 2007).

It is also important to consider whether the molecular and cellular effects of hormonal suppression are similar in humans and in rats. Although the basic processes by which GnRH analogues suppress gonadotropin and testosterone levels and induce a block in the completion of spermatogenesis in normal adult males are similar in rodents and primates, there are quantitative differences. Whereas in rats and humans, GnRH antagonist reduced intratesticular testosterone concentrations to about 2% of that observed in controls (Matthiesson *et al.*, 2005, Shuttlesworth *et al.*, 2000), in macaques it only reduced intratesticular testosterone concentrations to 28% of control (Zhengwei *et al.*, 1998). Despite the less marked reduction in intratesticular testosterone levels, spermatogenesis was blocked at the B spermatogonial stage by GnRH antagonist treatment of the primates (Zhengwei *et al.*, 1998) compared to the round spermatid stage in rats (Kangasniemi *et al.*, 1996b). In humans, the block in spermatogenesis was also largely at the B spermatogonial levels, but later germ cells to the round spermatid stage were still produced at 20% of control levels (Matthiesson *et al.*, 2005). The restimulation of spermatogonial differentiation by hormonal suppression in rats may be dependent upon ability of germ cells to develop to the spermatocyte stage during the testosterone suppression, and hence might occur in men. Further germ cell differentiation in the presence of suppressed testosterone can be induced in human testes by treatment with FSH (Matthiesson *et al.*, 2006).

To extrapolate results of studies of protection of ovarian function from rodent systems to women, it is important that the cytotoxic mechanism of chemotherapy action on the follicles in the experimental model be similar to that in women. The mouse model seems to be appropriate for investigation of the protection against cyclophosphamide, as primordial follicles in the mouse and in women are moderately sensitive and show a dose-responsive loss (Plowchalk & Mattison, 1991) or a dose-dependent increase in sustained amenorrhea (Boumpas *et al.*, 1993), respectively, after exposure to increasing doses of cyclophosphamide. Although primordial follicles in both rodents and humans are killed by radiation, the exquisite

sensitivity of murine primordial follicles to death by apoptosis and the relative radioresistance of human primordial follicles makes this a less comparable model system (Baker, 1978).

It is also important to elucidate the mechanisms by which hormonal suppression protects primordial follicles in female mice from cytotoxic therapy in order to better determine how to apply the results to women and select an appropriate hormonal treatment for maximal protection.

It needs to be determined whether or not suppression of gonadotropins is involved in the protective mechanism. All successful experimental and clinical trials utilized GnRH analogues, and almost exclusively agonists rather that antagonists. GnRH receptors are present in the ovary so that the action of GnRH might be at the local level, rather than in the pituitary. Possible differential effect of GnRH agonists and antagonists on oocytes and granulosa cells also need to be investigated (Yano *et al.*, 1997).

Since currently there is no evidence that the primordial follicle is a direct target for action of gonadotropins or GnRH analogues, effects that are mediated at the level of the whole ovary should be considered. The possibility that the protective effect on mouse primordial follicle against chemotherapy could result in a decline in ovarian blood flow during GnRH-analogue treatment-induced ovarian quiescence (Meirow *et al.*, 2004), as had been observed in human females (Dada *et al.*, 2001), needs further investigation.

Another step that needs further study is the mechanism of death of the follicle after chemotherapy or radiation, which in the mouse is a result of apoptosis (Morita *et al.*, 2000). It is important to determine whether or not GnRH analogue treatment affects the apoptotic machinery in oocytes and/or granulosa cells, particularly the pregranulosa cells in primordial follicles, and whether it renders them less sensitive to the induction of apoptosis, and if it does, that it does not also protect the tumor cells from apoptotic death.

Based on recent reports that the primordial follicle pool may be dynamic and that there is a mechanism for replenishment of primordial follicles in adult mice (Johnson *et al.*, 2004), the replenishment of the primordial follicle pool may have to be considered, in addition to its maintenance. Although this work remains controversial and, if valid, its extension to human is uncertain, this phenomenon should be considered when trying to explain the effects of hormone suppression on the numbers of primordial follicles after exposure to cytotoxic cancer therapies.

In conclusion, the most immediate need is for randomized prospective clinical trials to test whether the promising results of previous non-randomized clinical studies on the use of GnRH-agonist treatment to protect ovarian function during cancer chemotherapy can be supported by a rigorous test. In addition, mechanistic studies in appropriate animal models are needed to determine which aspects of the beneficial effects of hormonal suppression on maintenance of both male and female fertility in select animal models may be applicable to human and to predict hormonal regimens that may offer maximal protection.

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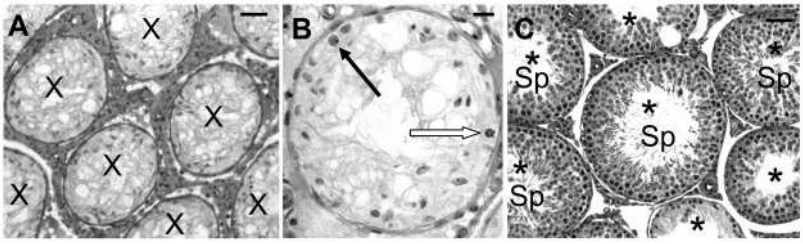

FIG 1.

Photomicrographs of LBNF1 rat testes showing the radiation-induced block in the differentiation of spermatogonia (A & B) and the reversal of this block by GnRH-antagonist treatment. The testes were harvested 13 weeks after 5 Gy irradiation with or without GnRH antagonist treatment during wks 3 to 7 after irradiation. Note that with no GnRH antagonist treatment all tubules are atrophic (X) and contain only Sertoli cells and type A spermatogonia (A), with normal (filled arrow) and dividing (open arrow) spermatogonia shown at higher magnification (B). With GnRH antagonist treatment all tubules are repopulating (*) with mature spermatids in many of them (Sp) (C). Bars = 50 μm (A and C) and 20 μm (B).

Table 1

Results of hormone suppression treatments given before and during cytotoxic therapy on spermatogenic recovery in men.

Study	Disease	Cytotoxic therapy	Hormone treatment	Recovery[b]	
				Hormone-treated	Controls
Johnson et al., 1985	Hodgkin's	MOPP 3–6 cycles	GnRH agonist	1 of 5	No controls
Waxman et al., 1987	Hodgkin's	MVPP, ChlVPP	GnRH agonist + testosterone	0 of 20	0 of 10
Redman & Bajorunas, 1987	Hodgkin's	MOPP ~4 cycles	Testosterone	≈70% of 23[c]	≈70% of 22[c]
Fossa et al., 1988	Testis Ca	PVB, ADR/CY, Radiation	Medroxy-progesterone	0 of 4 (2 of 12)[d]	2 of 3 (7 of 13)[d]
Kreuser et al., 1990	Testis Ca	PVB	GnRH agonist	6 of 6	8 of 8
Brennemann et al., 1994	Testis Ca (Seminoma)	Radiation	GnRH agonist + antiandrogen	12 of 12	8 of 8
Masala et al., 1997	Nephritis	Cyclophosphamide	Testosterone	5 of 5	1 of 5

[a] Chemotherapy regimens are as follows: MOPP, mechlorethamine, vincristine, procarbazine; and prednisone; MVPP, mechlorethamine, vinblastine, procarbazine, and prednisone; ChlVPP, chlorambucil, vinblastine, procarbazine, and prednisone; PVB: cisplatin, vinblastine, bleomycin; ADR/CY: Adriamycin, cyclophosphamide.

[b] Fraction of men recovering testicular function as assessed by restoration of sperm counts to normospermic levels unless otherwise noted.

[c] Actuarial recovery calculated by Kaplan-Meier analysis

[d] Recovery assessed by restoration of FSH levels to within the normal control range.

Table 2

Summary of Effects of Hormonal Suppression on Protection and Stimulation of Gonadal Functions

Species	Effects of Hormonal Suppression in Males	Effects of Hormonal Suppression in Females
Mouse	Pretreatment suppression does not protect endogenous spermatogenesis. Suppression moderately enhances spermatogenesis from transplanted spermatogonia. Posttreatment suppression slightly stimulates recovery from surviving stem cells	Mixed results on protection of primordial follicles from cyclophosphamide. No protection of primordial follicles from radiation.
Rat	Pretreatment and posttreatment suppression markedly stimulate spermatogenic recovery from stem cells. Suppression markedly enhances spermatogenesis from transplanted spermatogonia	Mixed results on maintenance of primordial follicle number during prolong GnRH agonist treatment (independent of cytotoxic exposure). GnRH agonist, but not progestin, partially protects primordial follicles from irradiation damage.
Non-human primate	Neither pretreatment nor posttreatment suppression enhance recovery of spermatogenesis after irradiation.	Prolonged GnRH agonist treatment maintains primordial follicle numbers during cyclophosphamide treatment but no proof of protection against cyclophosphamide-induced damage. Suppression offers no protection from radiation-induced loss of primordial follicles.
Human	Suppression before and during therapy fails to protect spermatogenesis from damage from cancer chemotherapy or radiotherapy (6 studies). Suppression with testosterone before and during therapy protected spermatogenesis from damage from cyclophosphamide (1 study). Delayed posttreatment suppression failed to restore spermatogenesis.	Several non-randomized studies (some with concurrent controls) indicate that suppression markedly protects against premature ovarian failure. One small randomized study showed no protective effect of suppression.

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Postpubertal Spermatogonial Stem Cell Transplantation Restores Functional Sperm Production in Rhesus Monkeys Irradiated Before and After Puberty

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Abstract

Background: Cancer treatment of prepubertal patients impacts future fertility due to the abolition of spermatogonial stem cells (SSCs). In macaques, spermatogenesis could be regenerated by intratesticular transplantation of SSCs, but no studies have involved cytotoxic treatment before puberty and transplantation after puberty, which would be the most likely clinical scenario.

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Authors' roles

G.S. participated in the conceptualization and design of the study, performed the majority of the experiments, analyzed the results, and prepared the manuscript. J.M.Mitchell participated in the study design, treatment of the monkeys, and surgeries. T.N.A.L and T.T.P. prepared the cells for transplantation and performed immunostaining of the tissue sections, testosterone assays and xenotransplantation. J.Z. assisted in the histological preparations of tissues. R.C.T. performed the dosimetry for the testicular radiation of the monkeys. K.A.P. assisted in the transplantation of testicular cells into the monkey testes. C.M.P. performed microsatellite analysis of the samples. C.H. participated in oocyte retrieval, ICSI and embryo culture. A.T.C. contributed to conceptual input and participated in design of the study. K.E.O. provided important conceptual input, participated in design of the study, and performed transplantation of testicular cells into the monkey testes. M.L.M. participated in the conception of the study, design of the experiments, analysis of results, and preparation of the manuscript.

Conflict of interest: The authors have no conflicts of interest to declare.

Objectives: To evaluate donor-derived functional sperm production after SSC transplantation to adult monkeys that had received testicular irradiation during the prepubertal period.

Materials and methods: We obtained prepubertal testis tissue by unilaterally castrating 6 prepubertal monkeys and 2 weeks later irradiated the remaining testes with 6.9 Gy. However, because spermatogenic recovery was observed, we irradiated them again 14 months later with 7 Gy. Three of the monkeys were treated with GnRH-antagonist (GnRH-ant) for 8 weeks. The cryopreserved testis cells from the castrated testes were then allogeneically transplanted into the intact testes of all monkeys. Tissues were harvested 10 months later for analyses.

Results: In three of the six monkeys, 61%, 38% and 11% of the epididymal sperm DNA were of the donor genotype. The ability to recover donor-derived sperm production was not enhanced by the GnRH-ant pretreatment. However, the extent of filling seminiferous tubules during the transplantation procedure was correlated with the eventual production of donor sperm. The donor epididymal sperm from the recipient with 61% donor contribution were capable of fertilizing rhesus eggs and forming embryos. Although the transplantation was done into the rete testis, two GnRH-ant treated monkeys, which did not produce donor-derived epididymal sperm, displayed irregular tubular cords in the interstitium containing testicular sperm derived from the transplanted donor cells.

Discussion and Conclusion: The results further support that sperm production can be restored in non-human primates from tissues cryopreserved prior to prepubertal and postpubertal gonadotoxic treatment by transplantation of these testicular cells after puberty into seminiferous tubules.

Keywords

Transplantation; radiation; spermatogenesis; GnRH-antagonist; ICSI

1. INTRODUCTION

Sustained progress in cancer therapies over the past several decades has led to a rise in pediatric cancer survival rates to approximately 88%.[1] However, the gonadotoxicity and risk of infertility from the treatment remains a major health concern in these survivors as it affects the quality of life. Since prepubertal boys are not producing sperm, there are currently no standard-of-care options to preserve their fertility. We estimate that each year an additional 1,400 young men will become sterile due to cancer therapy and myeloablative conditioning therapy for hematopoietic stem cell transplants for non-malignant conditions.[2] This is a significant human health concern[3,4] and development of new methods of fertility preservation to prevent these effects or restore normal reproductive function after cytotoxic treatment are of great importance to these young male cancer survivors.

If spermatogonial stem cells (SSCs) are completely lost after gonadotoxic therapy, the only way to preserve future fertility of prepubertal males is by harvesting tissue containing SSCs prior to therapy and cryopreservation. With increased awareness and need for fertility preservation, it is the current clinical practice in various centers in the world to cryopreserve the testicular tissues before gonadotoxic therapies in boys,[5–8] hoping that a satisfactory technique will be developed to produce sperm from the SSCs present in this tissue.

Transplantation of a cell suspension containing SSCs into the seminiferous tubules is one of the techniques that have the potential to restore spermatogenesis and sperm production *in vivo*. Sperm can be obtained from the testis, epididymis or the ejaculate and have been successfully used to produce live offspring in rodents[9–11] and goats,[12] and embryos in non-human primates.[2,13]

Previously, we showed that, in macaques, irradiated during adulthood, autologous[14] or allogeneic[2] transplantation of SSCs to the testis produced donor-derived sperm in the recipient. These sperm were also competent to fertilize the eggs to produce embryos by ICSI.[2] In an attempt to model the prepubertal boys undergoing gonadotoxic therapy and requiring fertility restoration when they reach adulthood, we cryopreserved testis tissue from prepubertal monkeys, irradiated them prepubertally, and planned to transplant the stored cells into the testes after puberty and subsequently test the establishment of donor-derived spermatogenesis and the fertilizing potential of the sperm produced. However, a second dose of irradiation, had to be given to these monkeys, since there was spermatogenic recovery at puberty. Furthermore, although autologous transplantation is desired in the clinical scenario, allogeneic transplantation was used in this study so that donor-derived spermatogenesis and the paternity of embryos produced could be reliably quantified using microsatellites that differed between the donor-recipient pairs.

In an attempt to increase the success of the recovery of spermatogenesis from transplanted cells in a non-human primate, we also tested the effect of gonadotropic and gonadal hormone suppression with a GnRH-antagonist, a method that had proved very successful in rodents.[15,16] Although one of our previous studies with macaques[14] had indicated that hormonal suppression just prior transplantation enhanced the recovery of spermatogenesis from the donor, a second study[2] failed to indicate such a beneficial effect.

Furthermore, we previously reported a single case in which transplantation of a suspension of testicular cells from a prepubertal monkey resulted in the development of donor-derived *de novo* tubules containing advanced germ cells in the interstitium.[17] Since this study also involves transplantation of prepubertal monkey cells into the testes, we scrutinized the transplanted testes tissues for such structures.

2. MATERIALS AND METHODS

2.1. Animals

Six male rhesus monkeys (*Macaca mulatta*) were purchased from the Michale E. Keeling Center for Comparative Medicine and Research, MD Anderson Cancer Center, Bastrop, Texas as both donor and recipient monkeys for testicular cell transplantation to the testis. They were prepubertal at the time of purchase and were housed in pairs initially at the M.D. Anderson Cancer Center, Houston, Texas in steel cages with a sliding panel between two adjacent compartments to allow social interaction with another companion of the same study group. The animals were fed Harlan TEKLAD Primate diet #7195 with daily enrichment foods, such as seeds, peanuts, fruits, and vegetables; the environment was maintained at a constant temperature (24°C–27°C) and humidity (40%–55%) with a 12-hour light/12-hour dark cycle. During parts of the study when there were minimal interventions for procedures,

the monkeys were temporarily housed at the MD Anderson Cancer Center facility in Bastrop, Texas with the same conditions as described above.

All animal care and treatment protocols were approved by the Institutional Animal Care and Use Committees of MD Anderson Cancer Center and Magee-Womens Research Institute.

2.2. Experimental design

Prepubertal monkeys, at 40–41 weeks of age, were unilaterally castrated (Figure 1). The castrated testes were weighed and 6–27 mm^3 pieces from the testes were cryopreserved for later allogeneic transplantation. Two weeks later, the remaining testes of the monkeys were given 6.9 Gy of irradiation.

Testis volumes, testosterone levels and sperm counts in the ejaculates were monitored, generally every four weeks, to observe signs of puberty. At about 50 weeks after the first irradiation, the monkeys showed increased testosterone levels, suggesting their entrance to puberty. However, because the testis volumes and sperm counts indicated significant spermatogenic recovery, they were given one more dose of 7-Gy irradiation at 64 weeks from the first dose to deplete endogenous SSCs and the recovering spermatogenesis. The monkeys were then divided into two groups of 3 each; one group was treated with GnRH-antagonist (GnRH-ant) for 8 weeks. At the end of 8 weeks, the testis of each monkey was allogeneically transplanted with cells prepared from the cryopreserved testis pieces of another monkey in the group. To prevent rejection of the transplanted cells, the monkeys were immunosuppressed; the testes and epididymis were harvested 44 weeks after transplantation for analyses as in previous studies.[14]

2.3. General surgical and post-surgical procedures

For all procedures, the monkeys were first sedated with an IM injection of ketamine (10–25 mg/kg) and then anesthetized with 1–3% isoflurane in oxygen. Before castration surgery, 2% lidocaine was instilled into the spermatic cord to provide local anesthesia. All surgical procedures were performed under aseptic conditions. Each animal received an analgesic (buprenorphine, 0.01–0.03 mg/kg body weight) prior to and at the end of the day of surgery, and 2 times per day for up to 3 days as needed by appearance of the animal under constant monitoring. In addition, at the discretion of the Clinical Veterinarian, daily IM injections of Baytril antibiotic (5 mg/kg) were given for a week post-surgery.

2.4. Semen and blood collection

Blood (5–10 ml) was drawn by venipuncture of the saphenous vein of sedated animals. Serum was separated and stored at −20°C. In general, blood sampling was done at monthly intervals, but was drawn more frequently during and immediately after GnRH-ant-treatment, to assess its effects on hormone levels.

Semen was obtained from anaesthetized monkeys by electro-ejaculation using a rectal probe (Beltron Instruments, Longmont, CO, USA), as described previously.[14] The sample was allowed to liquefy at 37°C for an hour before spermatozoa were counted in the exudate using a hemocytometer. Sperm counts were expressed per total ejaculate (volume of

exudate plus remaining coagulum). Semen collection was done only once before the second irradiation to confirm puberty and assess spermatogenic recovery after the first irradiation. Monthly semen collections were then performed starting at 16 weeks after transplantation.

2.5. Testicular measurements

Individual testis volumes were determined by measuring the length and width of each testis within the scrotum of anesthetized monkey with calipers and modeling the testis as a prolate ellipsoid, applying the following formula: testis volume = $\pi \times \text{width}^2 \times \text{length}/6$.

2.6. Hemicastration and tissue cryopreservation

A scalpel incision was made in the scrotum of anesthetized prepubertal monkeys and the dartos and tunica vaginalis were dissected to expose the left testis. The blood supply to the testis was tied off and the testis along with the epididymis was removed by cutting the spermatic cord, and the incision was closed by suturing.

The removed testis tissues were washed in Petri dishes using Hanks' balanced salt solution (HBSS) and, using a single edged blade, were cut into small pieces of about 6–27 mm^3 and cryopreserved.[18] About 5–7 pieces of tissue were placed in a 2 ml cryovial containing 5% DMSO and 5% fetal bovine serum (FBS) in minimal essential medium (MEMα). The vials were placed on ice for 30 min, transferred to −1°C/min containers and placed at −20°C for 90 mins and then these containers were placed overnight at −80°C. Next day, the vials were plunged into liquid nitrogen.

2.7. Irradiation

The testes of anesthetized monkeys were irradiated using a cobalt-60 gamma-irradiator[14,17] with a 5×5 cm field size in an antero-posterior direction. Tissue-equivalent bolus material (5-mm thick) was placed over the scrotum to provide a build-up layer. The remaining right testes of prepubertal monkeys were irradiated at a total calculated dose of 6.9 at a rate of 77– 91 cGy/min, with a source-to-skin distance of 80 cm measured to the bolus. This dose was chosen because 7 Gy was previously shown to provide prolonged depletion of spermatogenesis in adult macaque testes.[2,14] Although we were aware of reports that this dose might not deplete spermatogenesis in immature macaques,[19] higher doses were not given to the prepubertal testis because our preliminary data (not shown) and studies of others[20] have shown that irradiation of prepubertal testes with 10 Gy produced failure of the development of the somatic elements of the testis. Instead, it was necessary to give the monkeys a second dose of 7-Gy irradiation when they reached adulthood to eliminate most of the surviving endogenous SSC and the recovering endogenous spermatogenesis.

2.8. GnRH antagonist treatment

The GnRH-ant, Acyline, was obtained from the Contraceptive Development Program of the NICHD, Rockville, MD, USA. Stock solutions of Acyline (2 mg/ml) in 5% aqueous mannitol were prepared and stored at 4°C for a maximum of 1 week. Based on the pharmacokinetics of Acyline,[21] and our previous data on hormone suppression in macaques, [14] one group of three monkeys was given twice-weekly subcutaneous injections of Acyline

on Mondays and Thursdays at doses of 200 μg/kg and 300 μg/kg, respectively;[14,17] the other group of three was sham-injected with bacteriostatic water.

2.9. Allogeneic transplantation

To prepare the cells for transplantation, the cryovials were thawed in a 37°C water bath and washed with HBSS. Tissue pieces were incubated with collagenase IV and DNase I to digest interstitial tissue and the undigested tissue was then incubated with trypsin-DNase to release tubular cells.[22,23] The recovered cells were washed, counted and prepared for transplantation as in our previous study.[14] The remaining right testes of the unilaterally castrated monkeys were allogeneically transplanted with these cells, choosing the donor-recipient pairs to maximize the unique microsatellite markers between these monkeys.

Transplantation of cells was done essentially as described previously.[13,14] Briefly, cells were suspended at 54 −140 × 10^6 viable cells/ml in MEMα containing 10% FBS, 0.4 mg Trypan blue/ml, 20% (v/v) Optison ultrasound contrast agent (GE Healthcare, Waukesha, WI), 1% antibiotic-antimycotic (a combination of penicillin, streptomycin, and amphotericin B; Gibco), and 0.1 mg DNase I/ml. The cells were transplanted in volumes between 350 and 500 μl via ultrasound-guided injections into the rete testis. A 13 MHz linear superficial probe and a MicroMaxx ultrasound machine (Sonosite, Bothell, WA) were used to visualize the rete testis space and to guide a 25-gauge, 1.5” hypodermic needle into the space. Cells were manually injected under slow constant pressure and chased with saline solution. A transplantation efficiency score was recorded for each transplantation as done previously, based on an ultrasound-visualized estimate of the percentage of the circumference of tubules, going outward from the rete testis, that were filled by donor cell suspension.[2] The scores were as follows: 5 = >80%; 4 = 60–80%; 3 = 40–60%; 2 = 20–40%; and 1= <20%. For example, the filling of the tubules recorded in a previous study,[13] Movie S1, would be a score of 5. To prevent T cell–mediated rejection of the transplanted allogeneic cells, the recipients were immunosuppressed with human/mouse chimeric anti-CD154 IgG 5C8 (NIH Nonhuman Primate Reagent Resource, University of Massachusetts Medical School, Boston, MA) at 20 mg/kg on days (relative to transplant) −1, 0, 3, 10, 18, 28, and monthly thereafter for an additional 8 months. This treatment of rhesus monkeys was shown to functionally protect renal allografts[24] and had been successfully used in allogeneic transplantation of SSCs.[2,13]

2.10. Hormone assays

Testosterone was assayed using radioimmunoassay (RIA) kit KIR1709 (Immuno-Biological Laboratories America, Minneapolis, MN). We used our own testosterone standard for the assay that was diluted in the zero-standard provided in the kit[18]. The detection limit of the assay is 0.05 ng/ml. The intra- and inter-assay coefficients of variation were 5% and 16%, respectively.

Circulating concentrations of FSH and luteinizing hormone (LH) were determined by RIA at the Endocrine Technologies Support Core, Oregon National Primate Research Center, Beaverton. The sensitivities of both the FSH and LH assays were 0.05 ng/ml. The intra-assay coefficients of variation were 12.5 % and 8.2%, respectively, for FSH, and LH.

2.11. Histological and immunohistochemical procedures

After harvest the testes were first weighed and pieces of tissue were fixed in either Bouin solution, 4% PFA or 70% ethanol.

For histology, Bouin-fixed pieces were embedded in paraffin, and sections were stained with periodic-acid-Schiff reagent and hematoxylin. For analysis of spermatogenic recovery at the end of the study, at least three sections chosen from different regions of the testis were assessed by systematic scanning across the entire section and a minimum of 2654 tubules were scored per testis. Sertoli cell-only tubules were categorized into two types: those with normal appearing columnar Sertoli cells with a relatively small empty lumen, and those with flatter Sertoli cells with a large empty lumen. The presence of germ cells was scored by calculating the tubule differentiation index (TDI), which is the percentage of seminiferous tubule cross sections containing at least three differentiated germ cell type (B spermatogonia or later stages). In addition, the extent of the progression of germ cell differentiation was assessed by determining the percentages of tubules with germ cells that contained spermatocytes, round spermatids or elongating/elongated spermatids as the most advanced germ cell type present.

In some sections, areas packed with irregularly shaped tubular cords containing germ cells, often with incomplete basement membranes were observed. These were readily distinguished from normal seminiferous tubules and appeared identical to the donor-derived *de novo* tubules we observed in a previous study.[17]

2.12. Epididymal sperm isolation

The cauda epididymis was minced thoroughly in about 200 μl of pre-warmed modified human tubal fluid (HTF, Cat. No. 90126; Irvine Scientific, Santa Ana, CA) in a 60 mm Petri dish and transferred to a 2 ml microfuge tube. The epididymal mince was incubated thrice with 500 μl of HTF, each time suspending the tissue and allowing it to settle at unit gravity and aspirating the supernatant containing the sperm. The supernatant was filtered through a prewet 100-μm cell strainer basket (BD 352350) into a 50-ml conical tube and the total volume of the filtrate was brought to 2 ml by adding pre-warmed HTF. The number of sperm, their motility and the number of blood cells were counted in the filtrate, which was then divided into two portions: one for genotyping and one for ICSI.

When the level of contaminating somatic cells was <50%, the sperm samples for genotyping were washed in DPBS and pellets were frozen at −80°C. However, when the level of somatic cells was >50%, the sperm were further purified by Percoll gradient separation, reducing the somatic contamination of ~5%, prior to washing and freezing.

The ICSI samples were transferred to 5-ml tubes and equal volumes of pre-warmed Test Yolk Buffer freezing medium were added drop-wise over a 30-second period, mixing thoroughly after each drop of freezing medium was added to avoid osmotic shock to the sperm. The mixture was allowed to equilibrate for 10 minutes at room temperature and then transferred into multiple 2-ml vials. The samples were chilled for 1 hour in the refrigerator (2–5°C), followed by exposure to liquid nitrogen vapor for 30–60 minutes, and then transferred to a liquid nitrogen tank for storage at −196°C.

2.13. Preparation of DNA from the blood, tissue, and sperm

To genotype the monkeys used as donors and recipients, DNA was prepared from non-coagulated blood using the DNeasy Blood & Tissue Kit from Qiagen (Cat No.: 69504).

To extract DNA from sperm, the pellets were suspended in saline sodium citrate buffer and were treated with 0.2% sodium dodecyl sulfate (SDS) to lyse remaining non-sperm cells. In cases in which sperm were not Percoll purified, the sperm were washed one additional time and treated again with SDS; this further eliminated the somatic contaminants and consequently decreased the percentages of recipient DNA in the sperm samples by 1 to 5%. The sperm samples were lysed and digested using proteinase K and dithiothreitol (DTT) at final concentrations of 2 mg/ml and 10 mM, respectively, for 3 hours at 56°C. Then the proteinase K was heat inactivated at 95°C for 15 min, and the extract was directly used for PCR.

For genotyping the suspected *de novo* regions, we first identified regions with irregularly shaped tubules in PAS-hematoxylin stained, 70%-ethanol fixed testicular sections. These slides were used as guides to identify suspected regions of interest in adjacent unstained serial sections. The surrounding unwanted tissues were scraped off using a razor blade, under a dissection microscope. The proteinase K/DTT lysis solution was carefully dropped on the slide containing the required section, the tissue was released into the solution using a pipette tip and aspirated into a microfuge tube, and processed as was done for sperm above.

2.14. DNA microsatellite analysis

Microsatellite repeat fingerprinting was done with a panel of 29 microsatellites as described previously.[17] Microsatellites were amplified and the PCR products were separated by capillary electrophoresis on ABI 3730 DNA Analyzer (Applied Biosystems). Fragment size analysis and genotyping was done with the computer software STRand.

To determine parental origin and sex of ICSI embryos, genotyping was done as above except that before PCR the cells were put through the Whole Genome Amplification (WGA) process using the REPLI-g kit, which contains reagents and primers that will replicate most of the cell genome, producing sufficient DNA for testing: https://www.qiagen.com/us/service-and-support/learning-hub/technologies-and-research-topics/wga/replig-principal-procedure/. In addition to the panel of 29 microsatellites, the primers 5'-CCCTGGGGCTCTGTAAAGAATAGTG-3' and 5'-ATCAGAGCTTAAACTGGGAAGCTG-3' were used to amplify sequences from the amelogenin gene which differs on the X and Y chromosomes, to determine the gender of the embryos.

2.15. Intracytoplasmic Sperm Injection (ICSI)

Controlled ovarian stimulation was performed on six female rhesus macaques as previously described.[25,26] Oocytes were collected and fertilized with sperm by ICSI, and resulting embryos were cultured as described.[27–29] Additional details are provided in the Supplementary Information. Following ICSI and in vitro development, individual embryos

were vitrified and sent from the Oregon National Primate Research Center to the Veterinary Genetics Laboratory, University of California, Davis, for microsatellite analysis.

2.16. Statistical analysis

The serum FSH levels, testis volume, and testis weights are presented as arithmetic mean ± SEM. The serum testosterone and LH levels were represented as means ± SEM calculated from log-transformed values. Comparison of the group treated with GnRH-ant and the control group was done using a *t*-test. When multiple longitudinal measurements were made, the Bonferroni correction for multiple comparisons was applied. Correlations between different endpoints were analyzed using the non-parametric Spearman's rank-order correlation coefficient. Analyses were performed with the IBM SPSS (version 23) statistical package.

3. Results

3.1. Observations during course of the study

We used the experimental design shown in Figure 1. The monkeys were 40–41 months of age with an average testis volume of 1.5 cm^3 and serum testosterone levels of 0.6 ng/ml (Table 1), when the unilateral castration was performed. Histology showed that the castrated testes of all the monkeys were indeed prepubertal containing only spermatogonia, mostly A_{dark} and A_{pale} (Figure S1). The recipient monkeys were monitored to determine when they reached puberty, as indicated by the serum testosterone levels consistently at or above 0.9 ng/ml (Figure 2B, Figure S2; and Table 1) which began at about 40 weeks after the hemicastration and irradiation. The achievement of puberty was confirmed by increases in testis volume resulting from increases in somatic elements and/or development of spermatogenesis (Figure 2A). In 4 of the 6 monkeys, testis volumes increased to at least 10 cm^3 (Figure S3), which is greater than that observed in adult monkeys in which spermatogenesis had been well depleted by irradiation.[2] The volume increase and the presence of sperm in the ejaculates (Table 1) indicated that much of the volume increase was due to regeneration of endogenous spermatogenesis. Because of this, at 64 weeks after the first irradiation, the now postpubertal monkeys were given another dose of 7-Gy testicular irradiation, which resulted in a decrease in testis volume (Figure 2A) as expected due to the depletion of the germ cells.

The monkeys were assigned to two treatment groups so that the distributions of ages, testes sizes, and testosterone levels were similar in the two groups (Table 1). One group of 3 monkeys were treated with GnRH-ant for the 8 weeks between the second irradiation and transplantation, and the other 3 monkeys received only sham injections. All 6 monkeys received allogenic transplantation of testis cells from other monkeys in the group at 72 weeks after the first irradiation dose.

As anticipated, [2,14] serum LH and testosterone levels were markedly suppressed during GnRH-ant treatment and, when the treatment was stopped, they reverted to normal levels for irradiated monkeys (Figure 2B and Figure S4). The reductions in testes volumes after the second irradiation (Figure 2A) were consistent with the loss of germ cells due to irradiation;

the group treated with GnRH-ant had a tendency towards a greater decrease consistent with the loss of Sertoli and Leydig cell volume seen with hormone suppression in other species.[30,31]

The ability to obtain ejaculates was not very successful in these monkeys, even during the breeding season of October-February.[32,33] After the second irradiation and transplantation, only one ejaculate greater than 1 ml was recorded (Table S1). The ejaculates that were obtained during this period were azoospermic ($<6\times10^2$/ml), with one exception that had only a few sperm.

3.2. Results from harvested tissues at the end of the study

At the end of the study, 44 weeks after transplantation, the remaining right testis and the cauda epididymis of all the 6 monkeys were harvested.

The testis weights in these monkeys varied between 3.3 and 7.0 g (Table 2, Figure 3A). Histology, as expected, showed that the majority of the tubule cross-sections contained only Sertoli cells with the complete absence of germ cells (Figure 4A). In 5 out of 6 monkeys, Sertoli cells were mostly columnar with a small empty lumen (asterisks in Figure 4B, C, D), which were considered normal for irradiated macaque testes. However, in one of the monkeys (#092), 99% of the Sertoli-only tubules displayed large empty lumens and lower epithelial height of the Sertoli cells (Table 2; Figure 4E, F). This tubule dilation is likely a consequence of damage from the first prepubertal irradiation, as this monkey showed very little increase in testis size after the first irradiation (Figure S3A). Low numbers of such dilated tubules (< 1% of tubules) were also observed in three monkeys: #094, #122 and #124 (Table 2, Figure 4C, D).

Germ cell differentiation, identified by nuclear morphology and location, was observed in the seminiferous tubules of all monkeys (Figure 5). In monkey #092 with the extensive dilated tubules, only one normal tubule showed spermatogenic cell differentiation. In the other five monkeys, germ cell differentiation, quantified by the tubule differentiation index (TDI), was observed in 2% to 33% of the normal tubules (Figure 3B). Spermatogenesis proceeded to the late spermatid stage in 70% of the differentiating tubules (Figure 5C). In addition, two monkeys (#094 and #122) displayed irregular tubule-like cords, as will be discussed below.

Epididymal sperm counts in five of the monkeys varied between 0.6 and 26×10^6; one monkey (#092) had no sperm in the epididymis (Figure 3C, Table 2). As expected, there was a perfect positive correlation between the cauda epididymal sperm count and the TDI (Spearman coefficient 1.0, $P<0.01$) among the different animals. Microsatellite analysis revealed that, in three of the recipient monkeys, 61%, 38%, and 11% of the epididymal sperm were of the donor genotype, but in the other two of the monkeys with epididymal sperm, there were no donor-derived sperm (Table 3, Figure S5). The monkeys with 61% and 38% donor sperm had high epididymal sperm numbers, with 8 and 10 million donor sperm, respectively, indicating the potential for fertility preservation.

We next assessed the factors that might be responsible for the variability in the success of the transplantation as measured by the numbers of donor sperm in the epididymis (Table 2, Figure 3). The GnRH-ant treatment before transplantation had no significant effect on donor spermatogenic output (*t*-test $P>0.6$). The numbers of donor cells injected and donor cell viability were unrelated to the extent of donor spermatogenesis (Spearman correlation, P>0.9). However, there were trends or significance that the testis volume ($P=0.02$) and serum T ($P=0.08$) measured at the time or second irradiation (8 weeks before transplantation) and the transplantation efficiency score ($P=0.14$) were positively related to the numbers of donor sperm in the epididymis. For instance, the two monkeys with a transplantation score of 5 had the highest numbers of donor sperm in their epididymis. On the other hand, the presence of dilated tubules ($P=0.04$) and the irregular tubule-like cords ($P=0.15$) appeared to be negatively related to the success of the transplantation. Testicular damage from the first irradiation, as evidenced by small testis volumes and low levels of serum T in monkey #092, likely contributed to the dilated tubules and extremely low levels of both endogenous and donor spermatogenic recovery observed in this monkey at tissue harvest.

The irregular-tubule-like cords observed in monkeys #094 and #122 appeared to be identical to the *de novo* tubular cords we have described previously (Figure 6A,B).[17] These abnormal cords filled an estimated 1.5–4.2% of the testis volume. They possessed incomplete basal laminae and contained germ cells up to and including round spermatids and, although rarely, mature spermatids (Figure 6B). Immunostaining for Vasa and acrosin confirmed the identification of germ cells and spermatids, respectively, in these cords (data not shown). In both of these cases, these cords were observed in the interstitium adjacent to the rete testis (Figure 6C). Microsatellite analysis of the DNA extracted from the regions containing these abnormal cord structures showed 65% and 80% of donor genotypes in the two monkey (Table 3), confirming that they indeed originated *de novo* from transplanted donor cells (Figure S6). The remaining percentages were likely contributed mostly by the recipient interstitial cells in addition to any possible minor contaminants from the endogenous tubular area.

3.3. Intracytoplasmic Sperm Injection (ICSI) Results

To test whether the donor-derived sperm obtained after transplantation were functional, we injected the cryopreserved sperm from the recipients into *in vivo* matured rhesus oocytes. A total of 85 ova were injected with sperm from recipients #124 and #114; 14 developed into zygotes (16% of injected ova) and were maintained for 8 days in culture (Table S2). Six reached the compact morula stage (Figure 7A) and one reached early blastocyst. In the first set of injections using the epididymal sperm from recipient #124, which had 61% donor contribution, 4 embryos were successfully genotyped by microsatellite analysis and 2 had the paternal genotype of the transplant donor and 2 had the genotype of the transplant recipient (Table S2, Figure 7B). These results confirmed that the sperm produced from the transplanted SSCs are fertilization competent and can produce embryos. Three embryos from the second set of injections were transferred into timed recipients (Supporting Methods), but no pregnancies were established. In the third set of injections, when the recipient (#114) with 38% donor sperm was used, embryo genotyping was successful in 4

embryos; 2 had developed parthenogenetically and 2 were derived from endogenous sperm produced by the recipient male. It was not known why embryo development was suboptimal and no pregnancies were achieved; it was not specific to possible quality problems with the donor-derived sperm, since ICSI with sperm derived from endogenous SSC did not yield any better results.

4. DISCUSSION

The most important finding in the current study was that, in a model that closely relates to gonadotoxic cancer treatment before puberty and transplantation of prepubertal testis cells back into the testis after puberty, significant donor-derived spermatogenesis was obtained in 3 out of 6 monkeys. In two of these monkeys, 13 and 26 million sperm were recovered from the cauda epididymis and 61% and 38% of these sperm, respectively, were donor derived. The high percentages of donor sperm demonstrate the success of the transplantation. The observation that the tubule differentiation indices in these two monkeys were 28% and 33%, respectively, as compared to 0.03– 20% in the remaining 4 monkeys, supports the conclusion that this was a result of enhanced donor cell colonization. A third monkey had 11% donor representation in the epididymal sperm, but there were only 0.6 million sperm

Comparison with previous studies (Table 4) emphasizes that this is the only study in which some of the gonadotoxic treatment was delivered prepubertally, and the transplantation was done after puberty, which will most likely be the clinically used strategy in humans. Unfortunately, because the 6.9-Gy radiation dose was insufficient for the desired level of SSC depletion in the prepubertal testis, it was also necessary to give another radiation dose after they reached puberty, which deviates from the usual clinical scenario. Nevertheless, the result that 3 of 6 recipient monkeys produced donor sperm is within the range of most of the previous studies.

It is important to determine what specific factors might be associated with good colonization with donor cells. The effect of GnRH-ant treatment on the presence and yield of donor sperm was evaluated. Whereas two of the three control monkeys not treated with GnRH-ant produced donor sperm, only one of the three GnRH-ant treated monkeys produced donor sperm (Table 2), and we concluded that overall the GnRH-ant treatment showed no correlation with spermatogenic recovery from donor SSCs. This result is similar to that observed recently in a study involving allogeneic transplantation in rhesus monkeys,[2] but differs from the stimulation of recovery of donor spermatogenesis by GnRH-ant-treatment observed earlier in a study involving autologous transplantation in cynomolgus macaques (Table 4).[14] The inability to see any favorable effects of GnRH-ant in these allogeneic transplantation studies might be due to a possible enhancement of immune responses when testosterone is suppressed,[34] resulting in the immune suppression being inadequate. This possibly could offset any benefit the hormone and immune suppression might have on colonization and recovery, but could also be a result of the species difference.

The effect of the efficiency of the filling of seminiferous tubules with the donor cell suspension on the production of donor sperm was assessed. The two cases with a transplantation efficiency score of 5 resulted in the highest levels of donor sperm in

the epididymis. Although these data suggested a trend that transplantation efficiency was important for the success of the transplant, the correlation was not statistically significant (P=0.14). A similar trend towards higher donor sperm production with better transplantation efficiency was also observed in our previous study[2], and when we combined the data of both studies, the association was highly statistically significant (Spearman's correlation, ρ=0.58, P=0.006). Thus, efficient transfer of the injected cell suspension to the seminiferous tubules is indeed an important factor in successful transplantation.

The characteristics and functional integrity of the recipient testis may be factors in the ability to colonize, especially after the damaging prepubertal cytotoxic treatment.[35] Both the serum T levels and testis volumes, measured at the time of the second irradiation, 8 weeks before transplantation, seemed to be correlated with the donor sperm production. The serum T level is a measure of pubertal development and testis volume is a measure of both pubertal development and recovery of spermatogenesis after the first irradiation. These results indicate that success from transplantation is dependent on the somatic cells of the testis going through relatively normal pubertal development despite the prepubertal irradiation. Further studies of transplantation are needed with a model of more complete spermatogenic cell depletion, because the transplantation is only needed when there is a failure of endogenous spermatogenic recovery.

Also the presence of dilated tubules, observed in the final histological samples taken 10 months after transplantation, was negatively correlated with the yield of donor sperm and appeared to be a factor limiting the development of donor spermatogenesis. Whereas in previous studies we have never observed such structural damage to the seminiferous tubule from 7 Gy testicular irradiation of adult monkeys,[2,14] four of the six monkeys receiving 6.9 Gy prepubertally had dilated tubules in the final histological sample taken 10 months after transplantation, and in one (#092) of them, nearly all the tubules were dilated. It is likely that this was due to damage incurred from the first dose of 6.9 Gy since this monkey failed to show the increase in testicular volume (Figure S3A) that would be expected from maturation of the somatic elements of the testis during puberty. Dilated tubules in adult rhesus monkeys after prepubertal irradiation had also been observed previously, but no dose-response was reported,[19] and we have also observed such tubule dilation (7%, 24%) in two rhesus monkeys that had received only 1 dose of 6.9 Gy before puberty (data not shown). The immature Sertoli cells in prepubertal testis, which are expected to be still proliferating,[36] are likely one of the targets for such sensitivity of the somatic structure of the testis in these juvenile monkeys. Future studies of molecular markers of Sertoli cell functional status in such cases are important for further development SSC transplantation.

It has been suggested that it might be possible to restore tubular function and the SSC niche by donor Sertoli cells, as was demonstrated after chemical ablation of Sertoli cells in mice. [37] Our previous studies [38] showed that transplanted donor Sertoli cells colonized irradiated rat tubules but did not restore the somatic environment to support differentiation of endogenous spermatogonia, which were otherwise blocked from differentiation. Although transplantation of Sertoli cells as a niche replacement strategy may be beneficial to enhance recovery from transplanted cells in prepubertally irradiated monkeys, we as yet have no data

as to whether or not the rhesus Sertoli cells in the transplantation suspension colonize the tubules of these irradiated monkeys.

However, in two of the six recipients, we observed the formation of *de novo* tubular cords containing somatic and germ cells, derived from the donor, in the interstitial space (Figure 6). This result extends our previous observation on a single irradiated adult monkey transplanted with prepubertal testis cells in which we confirmed that both the germ and somatic components of the *de novo* tubules were of donor-origin.[17] As was the case in the previous study, the ultrasound visualization of the transplantation demonstrated that the cells were indeed injected into the rete testis and entered the tubules (transplant efficiency score ≥4) (Table 1). However, various studies have shown that even when injection is done into the rete, there is significant leakage of the cells to the interstitium.[39,40] The location of *de novo* cords adjacent to the rete testis suggest that the rete itself may be the source of the leakage. Based on the observation that all 3 monkeys, in which we observed *de novo* cords in the interstitium, were treated with GnRH-ant, we suggest that the hormone suppression might increase the leakage of transplanted cells into the interstitium and/or create an environment favorable to the development of these *de novo* cords. It was noteworthy that none of these three monkeys showed any evidence of donor spermatozoa in the epididymis, indicating the there was no intratubular development of transplanted cells. Although the leakage and formation of *de novo* cords in the interstitium appears negatively correlated with intratubular donor spermatogenesis, the production of donor spermatozoa in these cords potentially can be used for fertilization. Thus, if the seminiferous tubules do not support donor spermatogenesis from the cryopreserved SSCs due to endogenous Sertoli cells rendered defective by gonadotoxic therapies, spermatogenesis from such *de novo* derived cords may be an alternative strategy for fertility preservation.

Since there have been no reports as to whether chemotherapy treatment also results in similar damage to the Sertoli cells, we reanalyzed the testicular tissues from a previous study [13], in which 5 prepubertal monkeys were treated with 8–12 mg/kg busulfan when they were prepubertal and then given autologous transplantation of lentivirus transduced testicular cells. There were no dilated tubules nor any morphological abnormalities in these testes that were harvested about 3 years after busulfan exposure. Even in the few Sertoli-only tubules, the Sertoli cells had a regular columnar appearance. However it should be noted that even at these high doses of busulfan, 97% of tubules showed recovering spermatogenesis, 83% of which progressed to the spermatid stage. Most of this recovery must be from endogenous surviving SSC since two of the monkeys were negative for production of lentivirus marked sperm. This is in contrast to our results with two prepubertal monkeys (not shown) irradiated with 6.9 Gy that showed spermatogenic recovery in only 33% of tubules at 2 years after irradiation. Thus although busulfan does not produce the damage to the somatic testis tissue that irradiation does, busulfan in not as effective at producing prolonged loss of spermatogenesis in prepubertal animals. Since even 6.9 Gy did not fully eliminate endogenous spermatogenesis, there is a need for a better model that would kill SSCs with minimal somatic testicular tissue damage.

In conclusion, we have demonstrated that SSC transplantation after puberty can restore spermatogenesis and fertilization-competent sperm production after prepubertal and

postpubertal irradiation and have characterized the factors that may be related to the success of the technique. Particularly the precise delivery of cells and filling of tubules at the injection appears to be an important factor. However, complete depletion of germ cells without causing somatic damage was not possible with single doses of radiation during the prepubertal period, and improvements in the treatment paradigm are necessary. Since the spermatogenic function of the human testis is more sensitive to fractionated doses of radiation [41] or combining radiation with alkylating agents, such as busulfan [42], than to single doses of radiation, these may be better models to deplete the germ cells. It is hoped that these procedures will more closely model the cohort of patients treated prepubertally with gonadotoxic cancer therapies, who have normal tubular somatic cells but with spermatogenic depletion, and are in need of fertility preservation procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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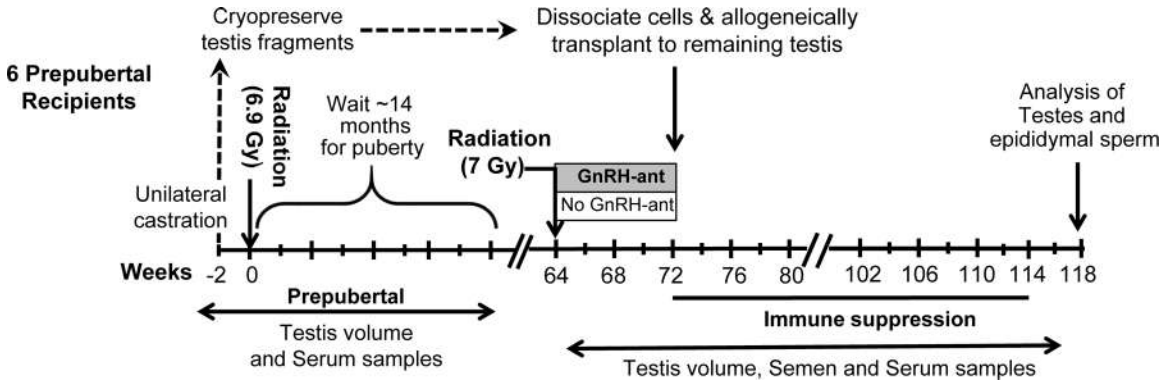

FIGURE 1.

Study design. The monkeys were evaluated before unilateral castration and periodically after exposure to two doses of radiation, hormone suppression, and transplantation. Evaluation included sampling of serum and measurements of testis volume. In addition, periodic semen analysis was performed after the animals reached puberty. Starting immediately after second exposure to testicular irradiation, three monkeys underwent GnRH-ant-mediated hormone suppression for 8 weeks; the other 3 received only sham injections. At the end of the 8-week period, they received allogeneic transplantation of cryopreserved testis tubular cells into one testis, followed by 9 months of immune suppression.

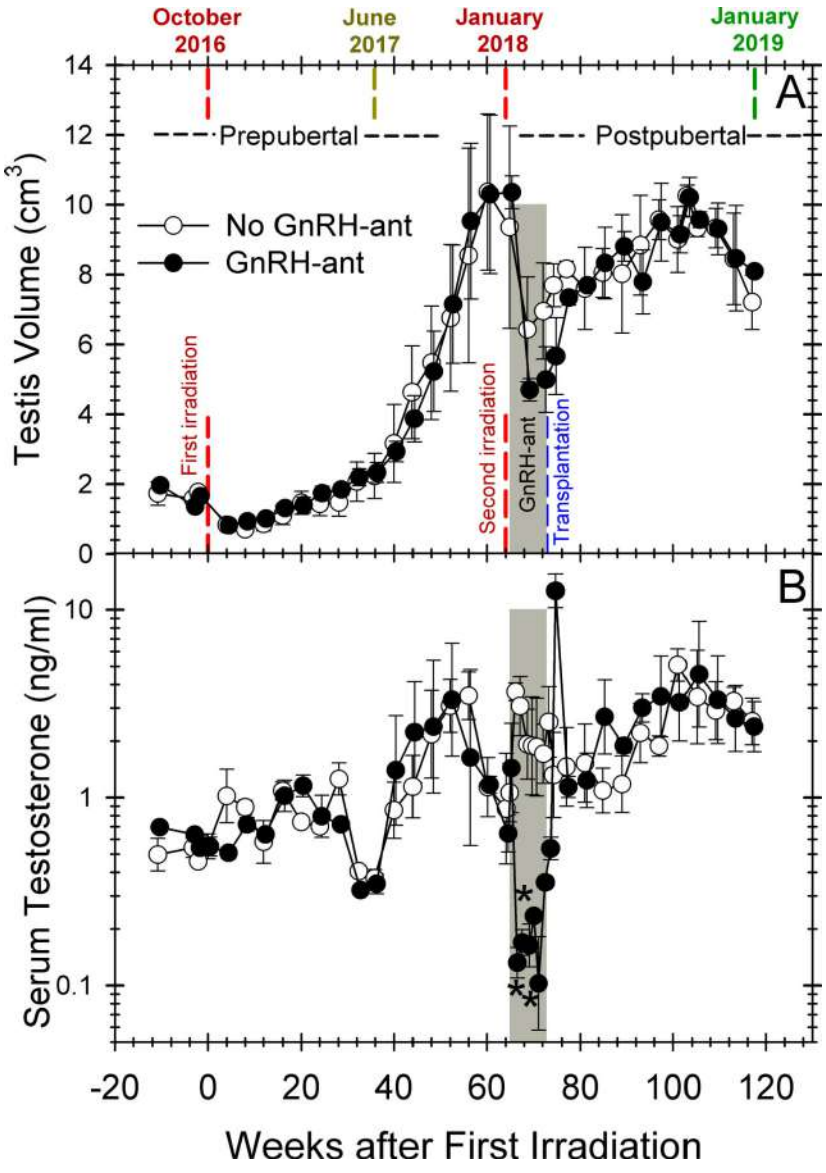

FIGURE 2.

Changes in testis volumes **(A)** and serum testosterone levels **(B)** in monkeys during the study. The vertical red and blue dashed lines represent the times of the two doses of irradiation and of transplantation, respectively. The average values for the 3 monkeys receiving GnRH-ant treatment (filled cicle) (n=3) or sham injections (open circle) (n=3) before transplantation are plotted. The grey shaded area represents the duration of the GnRH-ant treatment. For statistical analysis the axis was divided into three time segments, after initial irradiation, after the second irradiation during GnRH-ant treatment, and after transplantation, during which there were 15, 6 and 13 comparisons, respectively. The only statistical difference between the two treatments groups (marked with asterisks) was decreased serum testosterone during the GnRH-ant treatment.

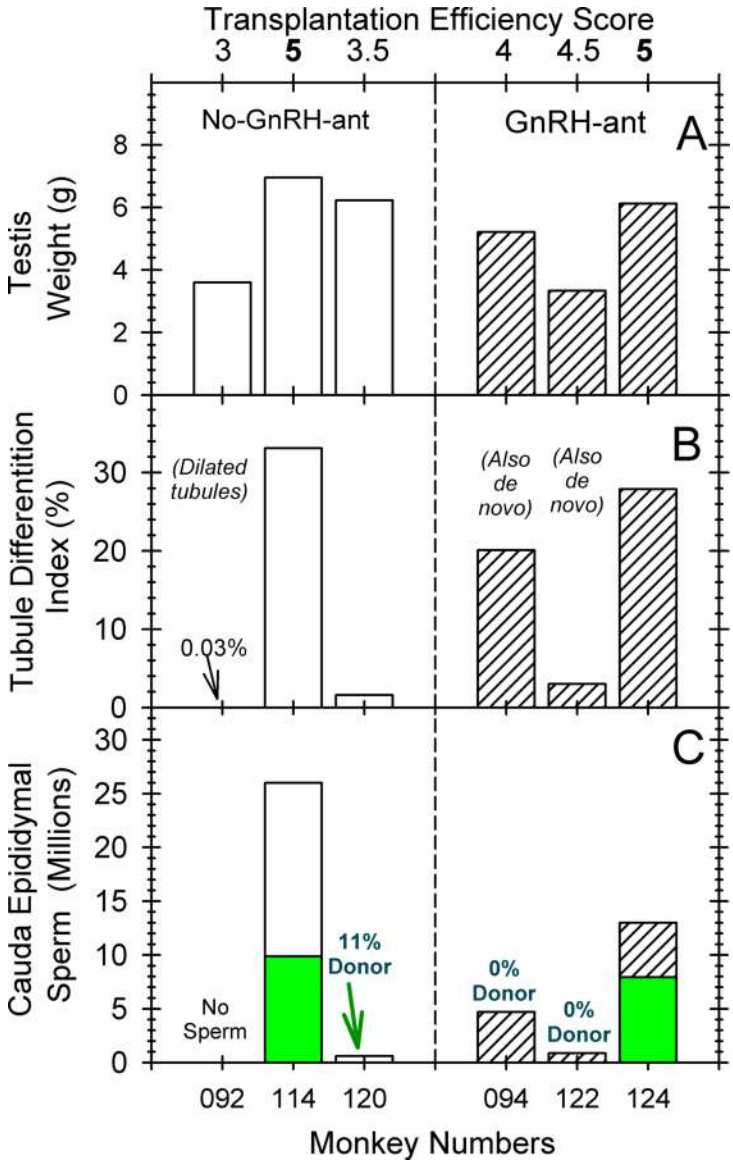

FIGURE 3.

Spermatogenic endpoints in individual monkeys. Testis weights **(A)**, tubule differentiation indices **(B)** and yield of sperm from the cauda epididymis **(C)** are shown for the monkeys treated with GnRH-ant (hatched bars) and those receiving only sham injections. Testes with abnormal tubules (dilated or *de novo*) are indicated in **(B)**. The portion of the columns filled with green in **(C)** shows the numbers of spermatozoa that were donor-derived.

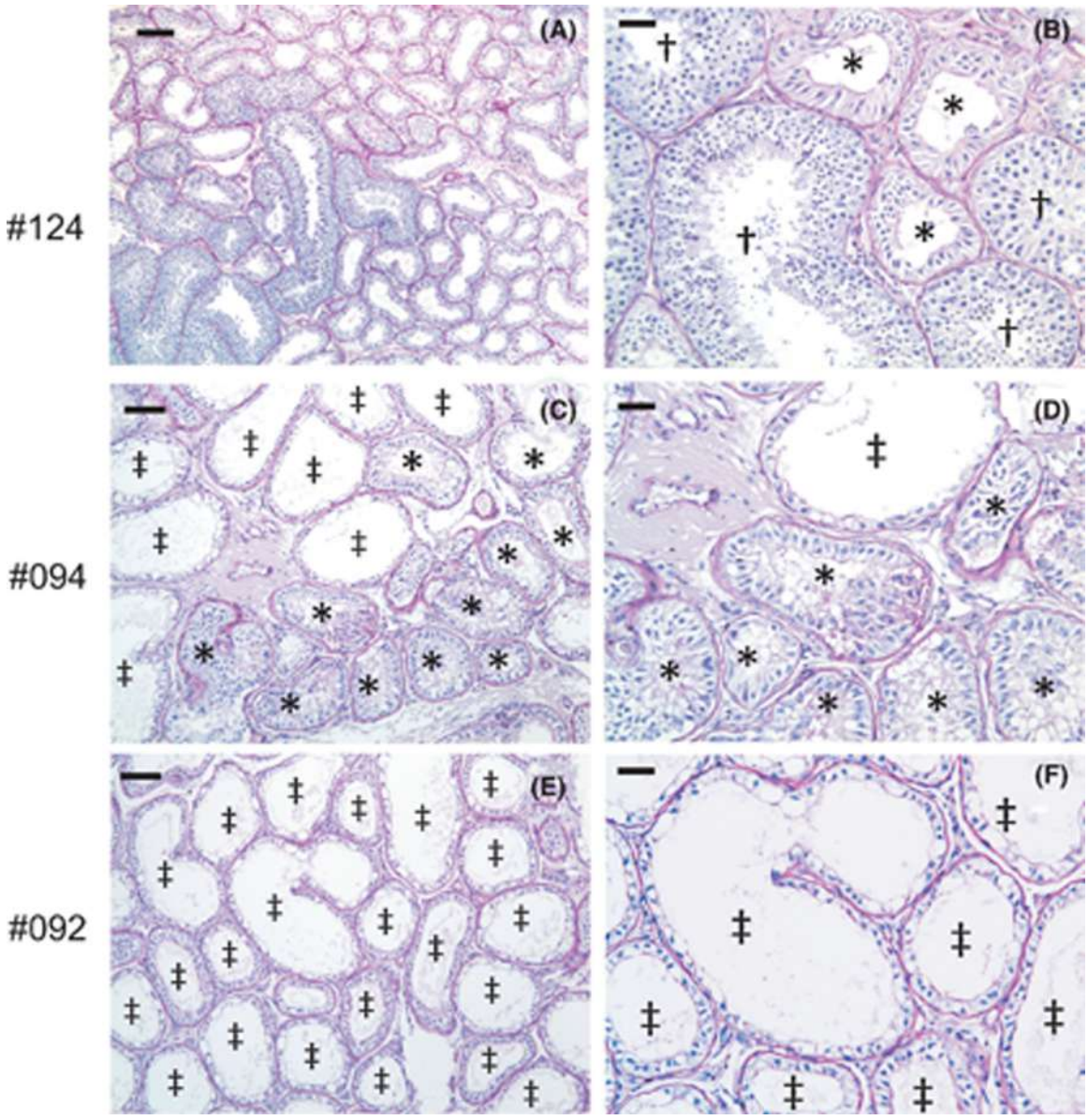

FIGURE 4.

Testis histology at tissue harvest, 44 weeks after transplantation. (**A**) Most tubules only contain Sertoli cells (**B**) Normal Sertoli-only tubules (*) and tubules showing regeneration of spermatogenesis (†). Note that the Sertoli cells in normal tubules have columnar appearance with a small lumen often with the presence of cytoplasmic processes. **(C-F)** Abnormal dilated Sertoli-only tubules (‡) with low epithelial heights and large empty lumens and some adjacent normal Sertoli-only tubules (*). Monkey numbers are indicated beside panels. Scale bars: A: 200 μm; C, E: 100 μm; B,D,F: 50 μm.

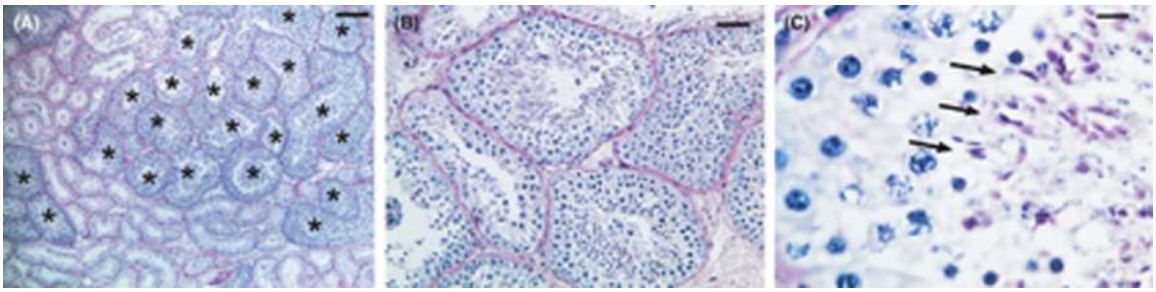

FIGURE 5.

Histology of the testis of a monkey that showed donor-derived sperm in the epididymis. Representative PAS-Hematoxylin stained testis sections at the end of the study from monkey #114. Tubules showing differentiating germ cells in (A) are indicated by asterisks. Note the presence mature spermatids (arrows in **C**) indicating complete spermatogenesis. Scale bars: A: 200 μm; B: 50 μm; C:10 μm.

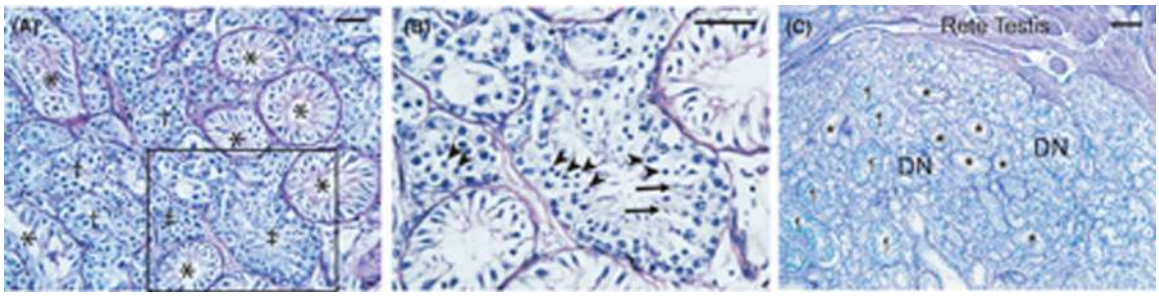

FIGURE 6.

(**A)** Region of irregular *de novo* tubular cords with interspersed endogenous Sertoli-cell only tubules (*). *De novo* cords with spermatogenic development to the spermatocyte (†) and spermatid (‡) stages are indicated. (**B**) Higher magnification of region from **A** showing round spermatids (arrowheads) and elongated spermatids (arrows). (**C**) Region of *de novo* cords (DN) showing that it is adjacent to the rete testis area. Interspersed normal tubules that are Sertoli-cell-only (*) and with recovery spermatogenesis (¶) are indicated. Scale bars: A &B: 50 μm; C: 200 μm.

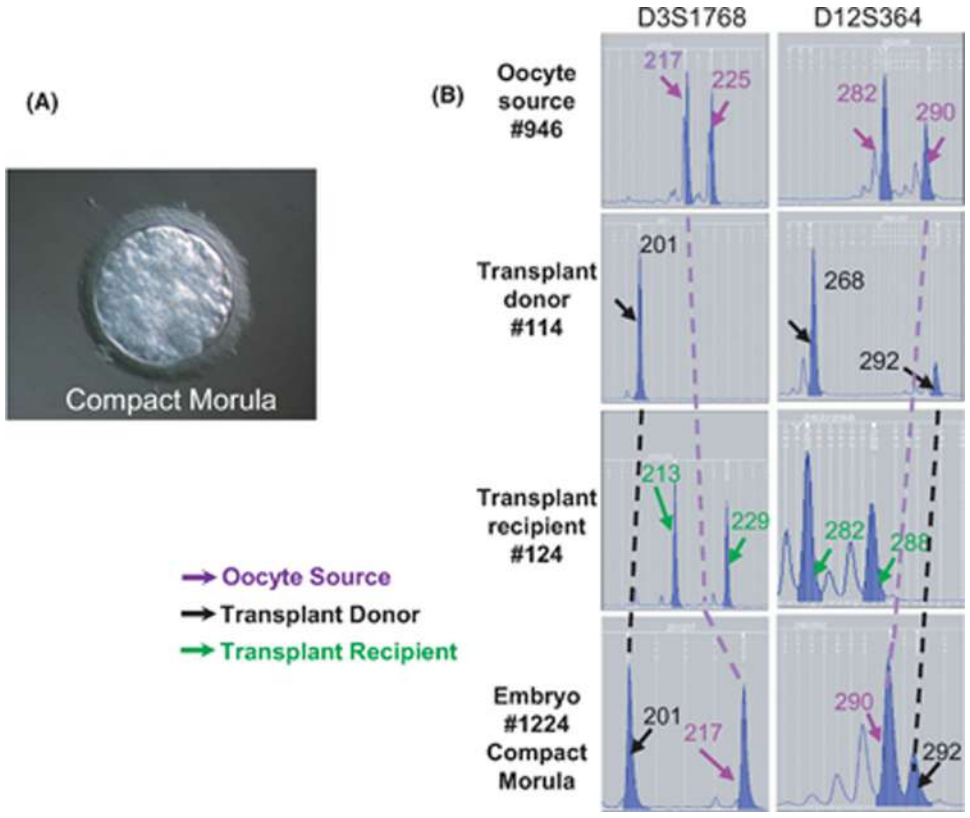

FIGURE 7.

Embryo produced by ICSI with epididymal sperm from a monkey (#124) with high percentage of donor-derived sperm. **(A)** Compact morula resulting from in vitro culture of the fertilized oocyte. **(B)** Microsatellite DNA analysis of one donor-derived embryo and comparison with the oocyte, SSC donor and recipient male profiles. Alleles specific for the oocyte donor (represented by purple font and arrow), transplant donor (represented by black font and arrow), and transplant recipient (represented by green font and arrow) are indicated on the electropherogram panels. The presence of the alleles at 201 and 292 nucleotide pairs in the embryo demonstrates the paternal origin as being from the donor.

Table 1.

Baseline recipient and donor monkey characteristics, treatments, and cells for transplantation.

Treatment groups	Parameters of monkeys at time of unilateral orchiectomy and first irradiation (6.9 Gy)				Parameters of monkeys at time of second irradiation (7 Gy)				Donor cells and transplantation				
	Recipient Monkey Number	Age (months)‡	Serum T (ng/ml)†	Average testis volume (cm^3)†	Age (months)	Serum T (ng/ml)**	Testis volume (cm^3)	Sperm count/per ejaculate ($\times 10^6$) §	Donor Monkey Number	Testis weight (g)	Total cells injected (millions)	Viability (%)	Transplant efficiency score
No-GnRH-ant control *	**092**	40.0	0.60	1.7	55.2	1.21	5.8	0	**120**	1.02	40	81%	3
	114	41.0	0.45	1.5	56.1	2.57	14.0	11.24	**094**	0.93	41	92%	5
	120	40.7	0.46	1.8	55.8	3.89	13.6	1.06	**124**	1.46	42	84%	3.5
GnRH-ant *	**094**	40.3	0.64	1.4	55.5	2.51	9.9	6.51	**122**	1.12	62	95%	4
	122	40.9	0.48	1.5	56.0	0.90	6.8	NE	**092**	0.93	17	89%	4.5
	124	40.4	0.66	1.6	55.5	5.81	15.1	0.08	**114**	1.21	32	81%	5

‡Measured at the time of irradiation.

†Average of last two measurements made on the day of (but just before) unilateral orchiectomy, and a week before. First testicular irradiation of the remaining testis was performed 2 weeks after unilateral orchiectomy.

**Average of last 5 measurements.

§Collected on 12/7/17; NE indicates no ejaculate was obtained.

*No differences were observed between GnRH-ant treatment groups in any parameters (*t*-test, P>0.05).

Table 2.

Parameters of spermatogenic recovery in the recipient testis/epididymis.

Treatment groups	Recipient Monkey Number	Testis volume (cm^3)†	Testis weight (g)*	TDI (%)*	Percent of differentiating tubules with late spermatids*	Percentage of dilated tubules *	Presence of *de novo* cords in testis	Cauda epididymal sperm ($\times 10^6$)*	Percentage of donor sperm in cauda epididymis*	Donor sperm in cauda epididymis ($\times 10^6$)*
No GnRH-ant control	092	5.7	3.6	0.03%	0%	98.7%	No	0	NA	0
	114	7.9	7.0	33.1%	76%	0%	No	26	38%	9.9
	120	8.1	6.2	1.6%	56%	0.0%	No	0.6	11%	0.07
GnRH-ant	094	8.3	5.2	20.1%	82%	0.9%	Yes	4.7	0%	0
	122	7.8	3.3	3.0%	64%	0.4%	Yes	0.9	0%	0
	124	8.1	6.1	27.9%	66%	0.1%	No	13	61%	7.9

*No differences were observed between hormone treatment groups (t-test, $P>0.05$).

†Measurements include scrotal wall thickness

Table 3.

Microsatellite analysis of the cauda epididymal spermatozoa, or testicular cells (retrieved from regions of the section suspected to contain *de novo* cords), from testicular irradiated monkeys transplanted with testicular cells containing SSCs. Wherever chimerism was observed, the percent donor DNA was calculated from the heights of donor and recipient peaks, and the results are averages and SEM of analyses of at least 4 microsatellite loci.

Treatment groups	Recipient monkey #	Donor monkey #	Percentage of donor genotype transplanted		Unique microsatellite loci analyzed
			Epididymal sperm	Testicular cells from suspected *de novo* region	
No GnRH-ant control	092	120	No sperm	No *de novo*	NA
	114	094	37.7±1.2	No *de novo*	D2S1333, D3S1768, D4S2365, D6S501
	120	124	10.7± 0.3	No *de novo*	D2S1333, D3S1768, D6S501, D11S2002
GnRH-ant	094	122	0	65.1± 0.8%	D2S1333, D3S1768, D6S501, D7S794
	122	092	0	80.4± 1.6%	D2S1333, D3S1768, D6S501, D8S1106
	124	114	61.1± 2.0%	No *de novo*	D3S1768, D4S2365, D11S2002, D12S364

NA: Not applicable

Table 4.

Comparison with Previous Studies of Spermatogonial Stem Cell Transplantation in Non-human Primates.

Species of Macaque	Recipient Prep	Age Cytotoxic Treatment	Age Transplant	Donor SSCs	Donor Marking	Enhanced recovery in transplant testis [a]	Donor sperm [a]	Percent Donor (%)	Reference
Cyno-molgus	Irradiation (2 Gy)	Adult	Adult	Autologous	None	2/5	ND [b]	– –	43
Rhesus	Irradiation (10 Gy)	Prepubertal	Prepubertal	Autologous	None	1/4	ND [b]	– –	20
Rhesus	Irradiation (10 Gy)	Pubertal	Pubertal	Autologous	None	0/2	ND [b]	– –	20
Rhesus	Busulfan (8–12 mg/kg)	Prepubertal	Prepubertal [c]	Autologous	Lentivirus	NA [d]	3/5	– –	13
Rhesus	Busulfan (8–12 mg/kg)	Adult	Adult	Autologous	Lentivirus	NA [d]	9/12	– –	13
Rhesus	Busulfan (8–11 mg/kg)	Adult	Adult	Allogeneic	Microsatellites	NR [e]	2/6	10, 1.1	13
Cyno-molgus	Irradiation (7 Gy)	Adult	Adult	Autologous	Lentivirus	2/11 [g]	6/12 [h]	– –	14
Rhesus	Irradiation (7 Gy)	Adult	Adult	Allogeneic	Microsatellites	2/15 [i]	5/15 [j]	93, 84, 1.7, 0.4, 1.0	2
Rhesus	Irradiation (6.9+7 Gy)	Prepubertal & Adult [f]	Adult	Allogeneic	Microsatellites	NA	3/6 [k]	61, 38, 11	Present Study

[a] Number positive results/number transplanted.

[b] ND: Not detectable because autologous transplantation was done with unmarked cells.

[c] Transplantation was done 9–15 weeks after busulfan treatment. The pubertal status at this time was not reported.

[d] NA: Not applicable because all or nearly all of the recipients were hemicastrated.

[e] NR: Not reported.

[f] 6.9 Gy was given prepubertally and an additional 7 Gy was given after puberty because of recovery of spermatogenesis.

[g] 2/6 for GnRH-ant-treated, 0/5 for no GnRH-ant

[h] 5/6 for GnRH-ant-treated, 1/6 for no GnRH-ant

[i] 1/10 for GnRH-ant-treated, 1/5 for no GnRH-ant

[j] 3/10 for GnRH-ant-treated, 2/5 for no GnRH-ant

$k_{1/3}$ for GnRH-ant-treated, 2/3 for no GnRH-ant.

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STEM CELL BIOLOGY

Proliferation of small number of human spermatogonial stem cells obtained from azoospermic patients

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Abstract
Purpose This study aims to proliferate spermatogonial stem cells (SSCs) and compare the in-vitro effects of laminin and growth factors on the proliferation of adult human SSC.

Capsule Proliferation of SSCs obtained from NOA patients are increased by GDNF, bFGF, EGF and LIF in the presence or absence of laminin.

Methods Isolated testicular cells were cultured in DMEM supplemented with 5 % fetal calf serum (FCS). During the culture, enriched spermatogonial cells were treated with a combination of glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and mouse leukemia inhibitory factor (LIF) in the presence or absence of human placental laminin-coated dishes. Cluster assay was performed during culture. Presence of spermatogonia was determined by an ultrastructural study of the cell clusters, reverse transcription polymerase chain reaction (RT-PCR) for spermatogonial markers and xenotransplantation to the testes of busulfan-treated recipient mice. Statistical significance between mean values was determined using statistical ANOVA tests.
Results The findings indicated that the addition of GDNF, bFGF, EGF and LIF on laminin-coated dishes significantly increased in-vitro spermatogonial cell cluster formation in comparison with the control group ($p \leq 0.001$). The expression of spermatogonial markers was maintained throughout the culture period. Furthermore, a transplantation experiment showed the presence of SSC among the cultured cells. In addition, a transmission electron microscopy (TEM) study suggested the presence of spermatogonial cells of typical morphology among the cluster cells.
Conclusions It can be concluded that human SSCs obtained from non-obstructive azoospermic (NOA) patients had the ability to self-renew in the culture system. This system can be used for the propagation of a small number of these cells from small biopsies.

Keywords Spermatogonial stem cells · Non- obstructive azoospermia · Testicular biopsy · Culture

Abbreviations

SSCs	spermatogonial stem cells
GDNF	glial cell line-derived neurotrophic factor

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bFGF	basic fibroblast growth factor
EGF	epidermal growth factor
LIF	leukemia inhibitory factor
NOA	non-obstructive azoospermic
RT-PCR	reverse transcription polymerase chain reaction
TESE	Testis biopsies
PBS	phosphate buffered saline solution
FCS	fetal calf serum
PLZF	promyelocytic leukaemia zinc-finger
DAZL	deleted in azoospermia-like
Oct4	Octamer-binding transcription factor 4
ITGB1	β_1-integrin
ITGA6	α_6-integrin
TEM	transmission electron microscopy
BrdU	5-Bromo-2-deoxyuridine
ES-like	embryonic stem cell-like
GSC	germ-line stem cells

Introduction

In 1994, with the successful transplantation of spermatogonial stem cells (SSCs) in the buslfan-treated mouse, a great evolution occurred in the treatment of male infertility [1]. Since then, researchers have proposed the idea that human testicular tissue could be harvested and cryopreserved in children with testicular cancer prior to start of chemotherapy or radiotherapy. Such cells could subsequently be transplanted back into the testis to resume spermatogenesis and sperm production [2, 3]. Until now, autotransplantation has been carried out in a number of animal models such as bovine, goats and monkeys [4–6], but autologous transplantation was only able to successfully resume complete spermatogenesis in bovine. As yet, no evidence has been found in human studies.

SSCs similar to other stem cells are generally rare [7]. It has been demonstrated that the approximate number of SSCs in mice and rats is 0.03% of all germ cells [8]; therefore, we predict that human SSCs may be rare and similar to rodent SSCs. The success rate of transplants depends on the enrichment and concentration of transplanted SSCs in vitro [9–11]. Proliferation of SSCs in vitro enhances SSC numbers [2, 12, 13] and probability of successful transplantation [14]. In addition, it provides large numbers of stem cells for biochemical or molecular analysis [15]. On the other hand, the usual testicular biopsy does not have an adequate number of SSCs for transplantation therapy. Indeed, obtaining a whole testis from a patient is impossible. Therefore, access to sufficient numbers of SSCs is essential for study of their regulations and further biomanipulation [9]. So, in vitro proliferation of a few SSCs to obtain appropriate cell numbers is essential.

Recent studies have shown that soluble growth factors such as glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and mouse leukemia inhibitory factor (LIF) along with serial passaging of clusters result in long-term SSC maintenance and stimulate SSC division in animals [16–19] as well as in humans [13]. Also, long term SSC maintenance can be achieved when cultured on laminin coated plates in animals [17, 20] and humans [13, 21].

Controversies present with respect to the use of somatic cells as a feeder layer. Somatic cells are able to differentiate [22] or support mice [23–26] and human SSCs in cultures [13, 27, 28]. Recently, in vitro propagation of human SSCs has been reported with small pieces of normal human testis [13] and small testicular biopsies from azoospermic patients [7, 28] with different culture systems. Mirzapour et al. [28] cultured SSCs from human adult azoospermic testes in co-cultured with Sertoli cells or co-cultured with Sertoli cells with adding LIF and FGF. As a result, SSCs co-cultured with Sertoli cells proliferated with the largest number of colonies [28]. Lim et al. [7] propagated SSCs derived from obstructive azoospermic and non-obstructive azoospermic (NOA) patients for a long-time. The testicular cells were treated with a combination of GDNF, FGF and EGF during culture. They didn't perform transplantation of obtained germ cell colonies into recipient mice for functional assessment of SSCs [7].

The aim of this study was to compare the in vitro effects of laminin and growth factors on the proliferation of adult human SSC obtained from patients suffered from NOA. To accomplish this objective, isolated testicular cells were treated with a combination of different growth factors in the presence or absence of human placental laminin-coated dishes during culture. Cluster assay was performed during culture. Presence of spermatogonia was determined by ultrastructural study of cell clusters, reverse transcription polymerase chain reaction (RT-PCR) for spermatogonial markers. The presence of functional SSC in culture was confirmed through xenotransplantation into busulfan-treated recipient mouse testes.

Materials and methods

Experimental samples

Testis biopsies (TESE) obtained for the diagnosis of male fertility through the Clinical Urology and Embryology Department of Royan Institute (Tehran, Iran) following informed consent. The use of human testicular biopsies and the experimental protocol were approved by the Ethical and National Research Council guidelines of Royan Institute (Tehran, Iran).

All 20 samples used for this study were obtained from individuals diagnosed with azoospermia due to incomplete

or complete maturation arrest (age 32–50 years, during 2008–2009). Each patient in this research had a complete medical history in Royan institute. Semen analysis was performed according to WHO criteria and testicular biopsy was only performed in cases where sperm could not be detected in any of the semen samples collected during 1 year.

Isolation of human spermatogonial stem cells

Testicular cells were isolated using the method described previously, with some modifications [12, 13]. Briefly, after using TESE samples in the andrology and embryology Laboratories, the remainder of the testes tissues (≈50± 10 mg) were placed into DMEM medium (DMEM; Gibco, Paisley, UK) supplemented with 13.5 g/L, $NaHCO_3$ (Sigma, St Louis, MO), single-strength non-essential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin, 40 μg/ml gentamycin (all from Gibco, Paisley, Uk) and 5 % FBS. Samples were carried to the stem cell laboratory for isolation. Tissues were initially washed two or three times with phosphate buffered saline solution (PBS) supplemented with 1 % pen/strep before being placed in DMEM medium for a second time. Only two or three samples per isolation were pooled and used. Then, tissues were mechanically dissected using two insulin needles and dissociated in PBS. After two washes, tissues were suspended in a DMEM medium containing 1 mg/ml collagenase type I 1 mg/ml hyaluronidase, 1 mg/ml trypsin and 0.05 mg/ml DNase for 30 min with some shaking and pipetting at a temperature of 37 °C. All enzymes were purchased from Sigma Company (Sigma, St Louis, MO). Fragmented tubules, tissues and cells were centrifuged for 2 min at 1100 rpm and washed two–three times in DMEM medium.

For the second digestion step, fragmented tubules was resuspended in DMEM by addition of fresh enzymes and incubated for 30–45 min at 37 °C (with shaking and pipetting). After filtration through a 40 μm nylon filter, collected cells were washed three times with DMEM medium that contained 10 % fetal calf serum (FCS) before using for culturing. After overnight incubation, floating cells were collected and cultured. Prior to culturing, the cell numbers were determined with a hematocytometer. Cell viability was evaluated by the dye exclusion test (0.04 % trypan blue solution).

Spermatogonial stem cell cultures

The obtained cells were incubated at 32 °C and 5 % CO_2, in a humidified atmosphere in the presence of 5 % FCS. The culture groups included: (1) control: SSCs cultured on plastic dishes without growth factors and laminin (2) growth factor: SSCs cultured on dishes treated with different growth factors and (3) growth factors plus laminin (Sigma, St Louis, MO): SSCs-cultured on laminin-coated dishes (at a concentration of 20 μg/ml) supplemented with different growth factors. In the treatment group, cells were grown for 2 months in the presence or absence of laminin and different growth factors including recombinant human GDNF 20 ng/ml, recombinant human bFGF 10 ng/ml, mouse EGF 20 ng/ml (all from Sigma, St Louis, MO) and recombinant human LIF 10 ng/ml (Chemicon, Temecula, CA). The cells were cultured in uncoated 4-well culture plates (Cole-Parmer, Vernon Hills, Illinois). Two days post-plating, most testicular cells were attached to the growing surface and the media was changed. Depending on the culture groups, several small clusters were observed on top of the monolayer of testicular cells, approximately after 4 weeks. In order to proliferate these clusters and prevent SSCs from differentiating, every 5–7 days until confluency, cells were passaged with trypsin- EDTA (0.25 %) (Invitrogen) and re-cultured or sub-cultured. Differential plating was performed by considering the ratio of somatic vs. germ cells.

Cluster assay

The cells were cultured for 2 months; the number of clusters which appeared in these cultures as well as the diameter of each cluster was evaluated. An inverted microscope (Zeiss, Jena, Germany) was used to determine the number of the clusters. Furthermore, the diameter of each cluster was measured using Image J software.

Identity confirmation of the spermatogonial cells

RNA extraction and reverse transcription

The presence of spermatogonial cells during culture was determined by the expression of spermatogonial genes based upon previous animal and human studies. Total RNA from the testis samples (positive control), testicular cells obtained before cultivation and cultured testicular cells were extracted using RNX kit standard (Cinnagen, Iran) according to the manufacturer's instructions. The purity and integrity of RNA was checked by a 260/280 nm ratio measurement. Total RNA was treated with DNase I to remove genomic DNA contamination from samples. First, strand cDNA was performed using oligodT primers and superscript II reverse transcriptase system. All reverse transcription reagents were purchased from Fermentas Corporation (Germany).

Polymerase chain reaction (PCR)

The primers specific for *PLZF(promyelocyticleukaemia zinc-finger), DAZL(deleted in azoospermia-like), Oct4*

(Octamer-binding transcription factor 4), VASA, ITGB1(β1-integrin), ITGA6(α_6-integrin), and *β -actin* genes were designed using previously described human sequences (GenBank) and Gene runner software (version 3.02; Hastings Software) as shown in Table 1. *β –actin*, a housekeeping gene, was included as an internal control to normalize the PCR reaction. RT-PCR was performed using the prepared cDNA, the primers, and PCR Supermix (Cinnagen) under the following conditions: 35 cycle at 95 ° C for 30 s, specific annealing temperature for each primer (*PLZF*, 55 °C; *DAZL*, 62 °C; *Oct4*, 60 ° C; *VASA*, 62 °C; *ITGA6*, 52 °C; *ITGB1* 55 °C; and *β-actin*, 60 °C) for 45 s, and finally at 72 °C for 45 s. To separate PCR products, 1 μl of each sample was resolved on a 1.7 % agarose gel, then electrophoresis was performed with 1x TAE Loading buffer and a voltage of 95 for 45.

The bands were visualized by using Gell logic, and images were obtained. The amplified PCR products were sequenced to confirm the identity of the amplified products.

Ultrastructural study of cell clusters

Both clusters of GSCs (small and big) were removed and fixed in 2.5 % glutaraldehyde in PBS (pH 7.4) for 2 h, next post-fixed with 1 % osmium tetroxide in the same buffer for 2 h. After dehydration in an ascending series of ethanol, specimens were placed in propylene oxide and embedded in Epon 812 (TAAB, UK). Semithin sections (0.5 mm) were stained with toluidine blue for a light microscopy. Ultrathin sections (60–80 nm) were contrasted with uranyl acetate and lead citrate before being examined by a transmission electron microscopy (TEM; Zeiss EM 900, Germany).

Cell labeling, recipient mice and transplantation

At passage 7, more than 34 SSC clusters and underlying somatic cells were trypsinized followed by adding 5-Bromo-2-deoxyuridine (BrdU) to the medium for cell labeling and tracing in the recipient mice 72 h before transplantation. Then, spermatogonial cells were transplanted into the seminiferous tubules of recipient mice,age 10 weeks, via the rete testis that was treated with 35 mg/kg busulfan prior to the transplantation. The treated recipient mice were devoid of endogenous spermatogenesis at the time of transplantation (6 weeks after treatment) [29]. Adult recipient mice were anesthetized with 10 % ketamine and 2 % xylazine (Alfasan, Woerden, Netherlands). Approximately, 10^5 of the cultured cells in 10 μl DMEM were injected into the seminiferous tubules in one testis of each recipient mouse (n=3). The other testis served as an internal control. Transplantation was performed by retrograde injection through the efferent ducts.

Recipient testes assessment

Transplanted testes of the recipient mice were examined two months after transplantation. The testes were fixed in 4 % paraformaldehyde, dehydrated and embedded in paraffin. Presence of transplanted cells in 5 μm sections was assayed by immunohistochemical detection of BrdU incorporated with a kit according to manufacturer's instructions (Sigma). For immunohistochemistry, following deparaffination, sections were treated in 25 % formamid in SSC2X for 2h at 60 °C, then washed in SSC2X for 10 min. Antigen retrieval were performed in $CaCl_2$ solution for 20 min at 37 °C and blocked with 10 % goat serum for 1 h at 37 °C (Vector, Burlingame, CA). The slides were incubated for overnight at 4 °C with mouse monoclonal anti BrdU (dilution, 1:300; Sigma). After being extensively washed with PBS, the

Table 1 Sequences of the designed primers used for RT-PCR

Genes	Primer sequences (5′-3′)	Annealing temperature (°C)	Size (bp)
PLZF	F: 5′ GGTCGAGCTTCCTGATAACG 3′ R: 5′ CCTGTATGTGAGCGCAGGT 3′	55 °C	396
DAZL	F: 5′ GCC CTT CTTTCAGTGACTTC 3′ R: 5′ TGCTTCACTCCAACAAAGAC 3′	62 °C	381
Oct4	F: 5 ′GTT CTTCATTCACTAAGGAAGG 3′ R : 5′CAAGAGCATCATTGAACTTCAC 3′	60 °C	100
VASA	F: 5′ TACTTGCTGGACGAGATTCTG 3′ R: 5′ ATCCATCAGTCTTCCAGGAG 3′	62 °C	320
ITGA6	F: 5′ TCATGGATCTGCAAATGGAA 3′ R: 5′ GCGGGGTTAGCAGTATATTCA 3′	52 °C	300
ITGB1	F: 5′ GTGGGTGGTGCACAAATTC 3′ R: 5′ GGTCAATGGGATAGTCTTCAGC 3′	55 °C	300
β-actin	F: 5′ CAAGATCATTGCTCCTCCTG 3′ R: 5′ ATCCACATCTGCTGGAAGG 3′	60 °C	90

secondary antibody (goat anti-mouse labeled with fluorescein isothiocyanate (FITC); dilution, 1:100; Sigma) was applied for 45 min. The control slides were under similar conditions except for the removal of the first antibody.

Statistical analysis

Results were expressed as mean ± SD. Data were analyzed using ANOVA and results were assumed significant at $p \leq 0.05$.

Results

Owing to the presence of a variety of germinal cells in the testes (Fig. 1 a), purification of spermatogonia was difficult after isolation, thus differential plating was used. In addition, the cell population obtained was of different sizes and morphologies. Sertoli cells, as specified by their nuclear morphology, were 32–33 μm in diameter with an irregular outline and a granular appearance (Fig. 1 b). During the first week of culture, they proliferated and created a monolayer of cells. SSC had a diameter of 24–25 μm and a spherical outline with prominent nucleus centrally located (Fig. 1 b). They created embryonic stem cell-like (ES-like) colonies (Fig. 1 c) or big and small germ-line stem cells (GSC) clusters (Fig. 1 d–f) after proliferation over several weeks of culture. Testicular cells could be proliferated for up to 2 months and 7 passages. Of these, only the clusters of GSC were assayed.

Assessment of spermatogonial stem cell clusters

Cell viability was assessed after isolation of testicular cells by the dye exclusion test (0.04 % trypan blue solution). The

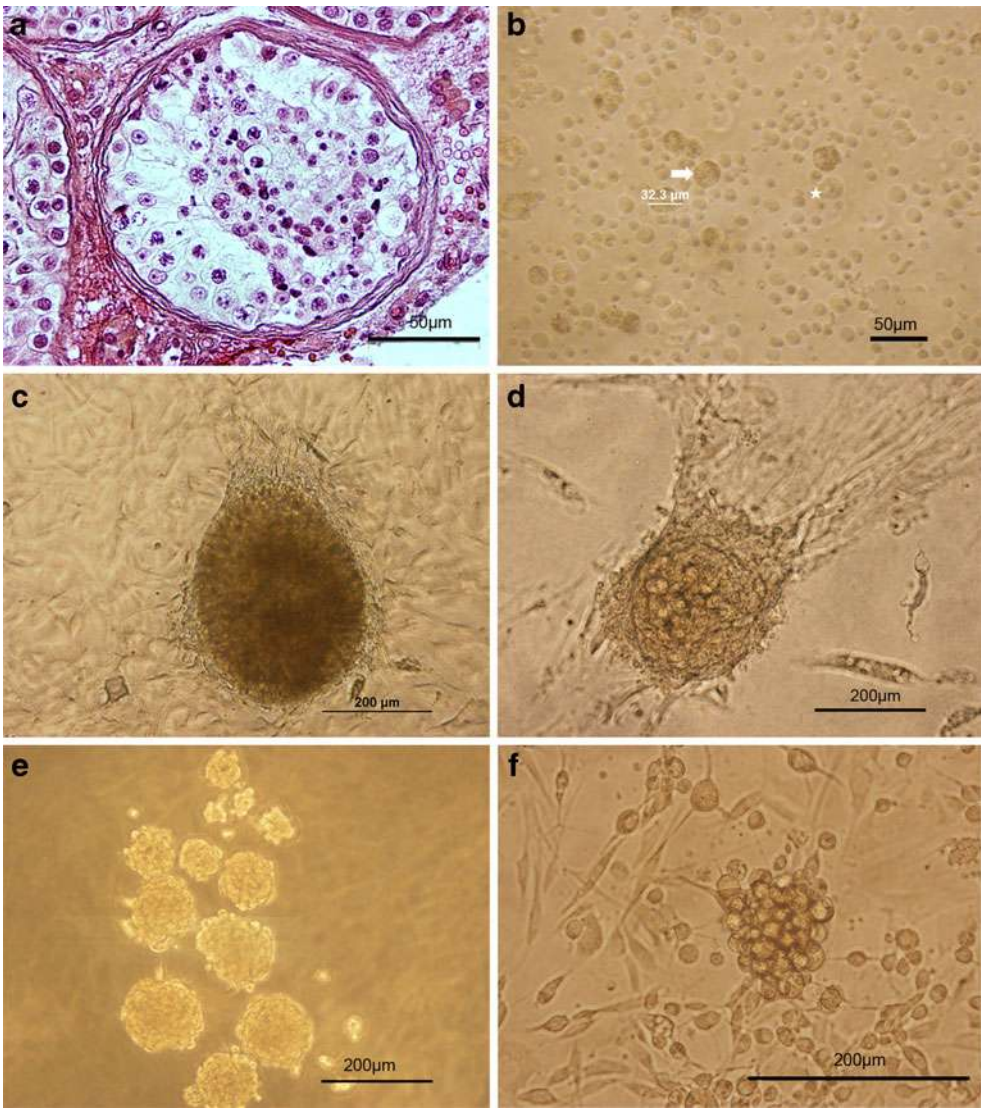

Fig. 1 Isolation and culture of SSCs from testicular tissues of non-obstructive azoospermic (NOA) patients. **a** Histological appearance of the testes biopsy obtained from azoospermic patient with complete maturation arrest that was stained with H& E and showed spermatogenic cells in tubules. **b** Cell population obtained from the seminiferous tubules after two steps enzymatic digestion contained different cell types, sizes and morphology. Spermatogonia could be identified as round cells with a large nucleus, one nucleoli and cytoplasmic inclusions (asterisk); whereas, Sertoli cells were large cells with a granular appearance (arrows). After overnight incubation, the non-adhering cells were collected and cultured. **c**–**f** The morphology of clusters growing on top of monolayer of somatic cells shows three types of colonies or clusters as observed during 2 months of cultivation. First, ES-like colonies were sharply edged and compact (**c**); while, big (**d**) and small (**e**, **f**) GSC clusters were smaller and clumpy and their cells were individually recognizable. We assayed the GSC clusters. Scale bars: A, B=50 μm and C-F= 200 μm

results showed that ≥92 % of the cells were viable. Cluster appearance varied between the different experimental groups. The clusters appeared earlier in the growth factors plus laminin group (day 22.3±3.8 of culture) and the growth factor group (day 23.7±6.4 of culture) in comparison with the control group (day ≥55). When these clusters were enzymatically dispersed and replated, their present SSC could start new clusters during 2 months of culture.

All in all, as shown in Table 2, the results indicate that the diameters of the clusters in both of the growth factor groups were varied significantly from that of the control group ($p \leq 0.01$). In terms of numbers of clusters, the growth factors plus laminin group was significantly different compared to the control group ($p \leq 0.05$). However, the diameters of the clusters in the growth factor group in the first (169.4±84.9) and second months (220.4±54.7) were not significantly different compared with the growth factor plus laminin group in the first (209.9± 53.4) and second months (173.8±80.6), respectively. Furthermore, the numbers of the clusters in the growth factor group in the first month (6±3.6) were not significantly different compared with that of the growth factor plus laminin group (8.7±4.7). However, during the second month, the number of clusters in the growth factor group (16.3±4.7) significantly varied from that of the growth factor plus laminin group (28±4) ($p \leq 0.05$). Additionally, when the number of clusters and their diameters were analyzed, a higher score was obtained after 2 months of cultivation rather than after one month ($p \leq 0.05$).

Identity confirmation of the spermatogonial cells

RT-PCR

RT- PCR was performed to analyze the expression of specific spermatogonial and germ cell markers in TESE, and the isolated testicular cells and cultured cells after the first and second months. As shown in Fig. 2, all samples expressed specific spermatogonial and germ cell genes: *DAZL, PLZF, Oct4, VASA, ITGA6* and *ITGB1*.

Colonization assay of the transplanted cells

Cell labeling with BrdU was performed before transplantation. Cell staining was examined just before xeno-transplantation. Immunoflourescent cells indicated that a lot of cells (~70 %) were labeled with BrdU before transplantation. After 2 months of cultivation, 10^5 cells from the growth factor plus laminin group were injected into the seminiferous tubules through the rete testis of the recipient testes in order to confirm the presence of SSCs in clusters and the human SSCs colonization assessment in the testis. Two months after transplantation, the cells whose nuclei stained FITC positive with BrdU were considered as transplanted cells. Two months after xenotransplantation, the transplanted cells were localized in the basal of the seminiferous tubules of the recipient testes as single cells and did not form a cluster. The non-transplanted right testis was considered as the control group.

Morphological characterization of clusters

The ultrastructures of the clusters, after 2 months of cultivation, were examined by TEM. The electron micrograph showed that cells from both types of clusters had typical morphology of human spermatogonial cells [30]. As seen in Figs. 3 and 4, both cluster cells had large spherical nuclei that contained one or two prominent nucleoli which were located along the nuclear membrane or in the center of the nucleus. The cell shapes were variable (pear-shaped or round) and contained a long irregular or round nucleus. The cytoplasm was characterized by organelles such as mitochondria, which were mostly located in the perinuclear region. Mitochondria were found singly, in pairs or in groups.

Discussion

In this study, we cultured a small number of adult human testicular cells obtained from NOA patients instead of using abundant normal testicular cells in each culture. We demonstrated that the addition of GDNF, bFGF, EGF and

Table 2 Comparison of the number (per 10^5 cells plated) and diameter of the clusters between the control and experimental groups

Groups	Day of first cluster formation	Diameters of clusters during culture		Number of clusters/(per 10^5 cells plated)	
		After 1 month	After 2 months	After 1 month	After 2 months
Control	≥55	0	112.3±29.2	0	3.3±1.5
Growth factors	23.7±6.4	169.4±84.9*	220.4±54.7*	6±3.6*	16.3±4.7*
Growth factors plus laminin	22.3±3.8	209.9±53.4*	173.8±80.6*	8.7±4.7*	28±4*, **

Results from three separate experiments were used for all groups. Values are the mean cluster numbers and diameters ± SD at different times

* Significant difference vs. control group in the same column ($p \leq 0.05$)

** Significant difference vs. growth factors group in the same column ($p \leq 0.05$)

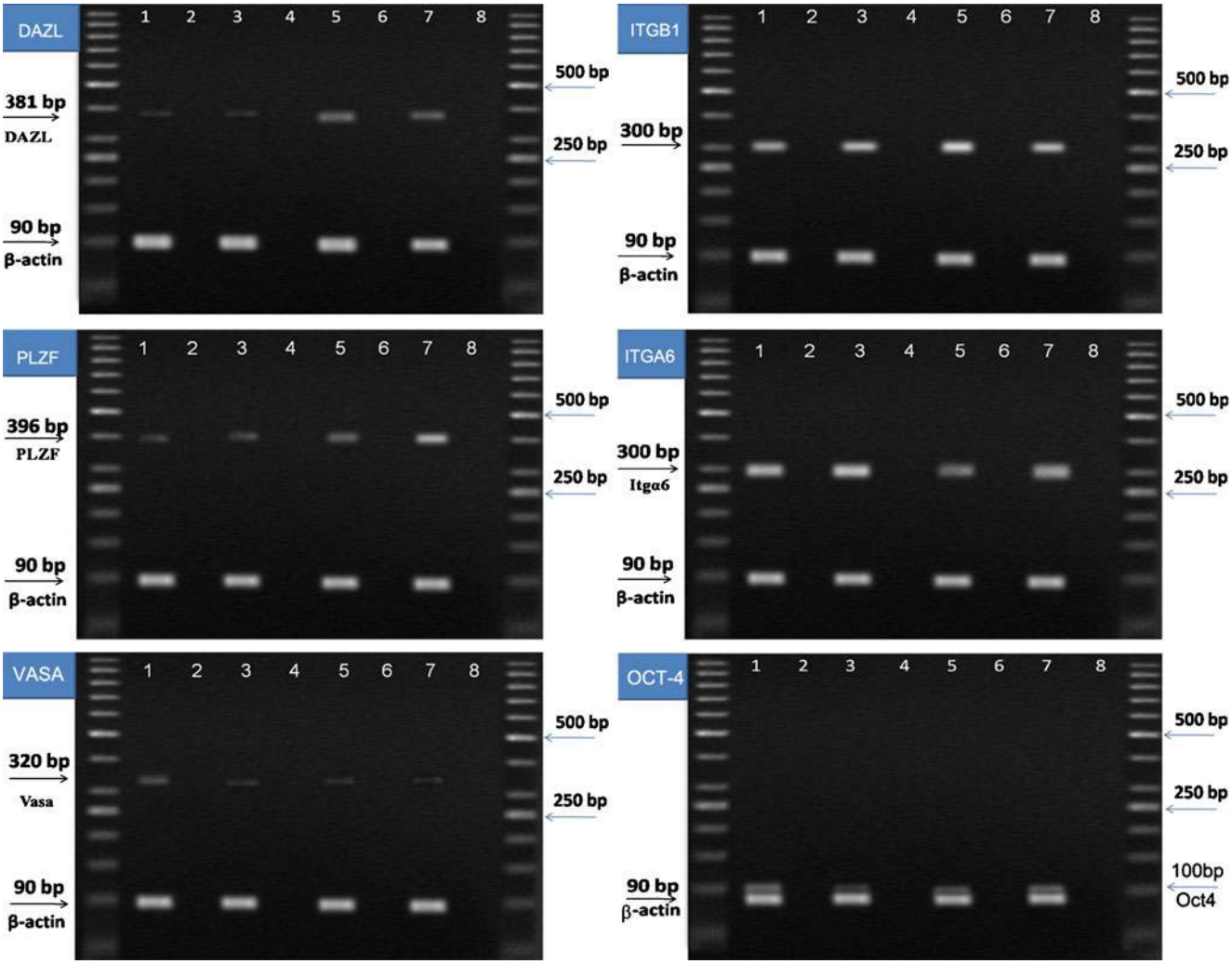

Fig. 2 Molecular characterization of spermatogonial and germ cells at the RNA level during cell culture. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine the expression of specific spermatogonia and germ cell markers. It showed that *PLZF* (396base pairs); *ITGA6* (300base pairs); *ITGB1* (300base pairs); *VASA* (320 base pairs); *DAZL* (381base pairs); and *Oct4* (100 base pairs) genes were expressed in 1) Total testis, 3) isolated testicular cells by two steps of enzymatic digestion before culture, 5) cells one month after cultivation and 7) cells two months after cultivation. 2, 4, 6, 8) Negative control, No cDNA. β-*actin* was also used as a housekeeping gene (90base pairs). As shown, all samples expressed specific spermatogonial and germ cell genes. PCR products were separated on 1.7 % agarose gel. *DAZL* (deleted in azoospermia-like), *VASA, PLZF* (promyelocytic leukemia zinc finger protein), *ITGA6* (integrin-α_6), and *ITGB1* (integrin-β_1)

LIF in the presence or absence of laminin-coated dishes significantly increased spermatogonial cell colony formation in SSCs obtained from NOA patients in comparison to the control group (neither growth factor nor laminin). It was also shown that these spermatogonial cell clusters could be successfully cultured and propagated for two months.

In vitro, SSCs form three-dimensional aggregations of germ cells on a feeder layer, termed clusters. Disassociation of clusters and serial passaging for extended periods can increase SSCs in number [16]. In our culture system, the addition of GDNF, bFGF, EGF and LIF on laminin-coated dishes increased the numbers of SSCs clusters by self-renewal in vitro. The culture medium used in this study was DMEM supplemented with essential growth factors: GDNF, bFGF, EGF and LIF. These growth factors are secreted by Sertoli cells, their receptors place on SSCs, and increase survival and proliferation in vitro [16, 18, 19, 31]. Majority of studies in humans [7, 13, 28] and animals [9, 12, 17–19, 25] have confirmed the useful effects of the aforementioned growth factors on SSCs. On the other hand, stem cells, in general, need a special microenvironment or niche to establish and maintain their stem cell properties [32, 33]. SSCs niche is provided by Sertoli cells in vivo [34] and probably this microenvironment can be reproduced in vitro [35]. Based on the previous studies, [25, 28, 35, 36]we conclude that in addition to growth factors and existence of somatic cells in culture,

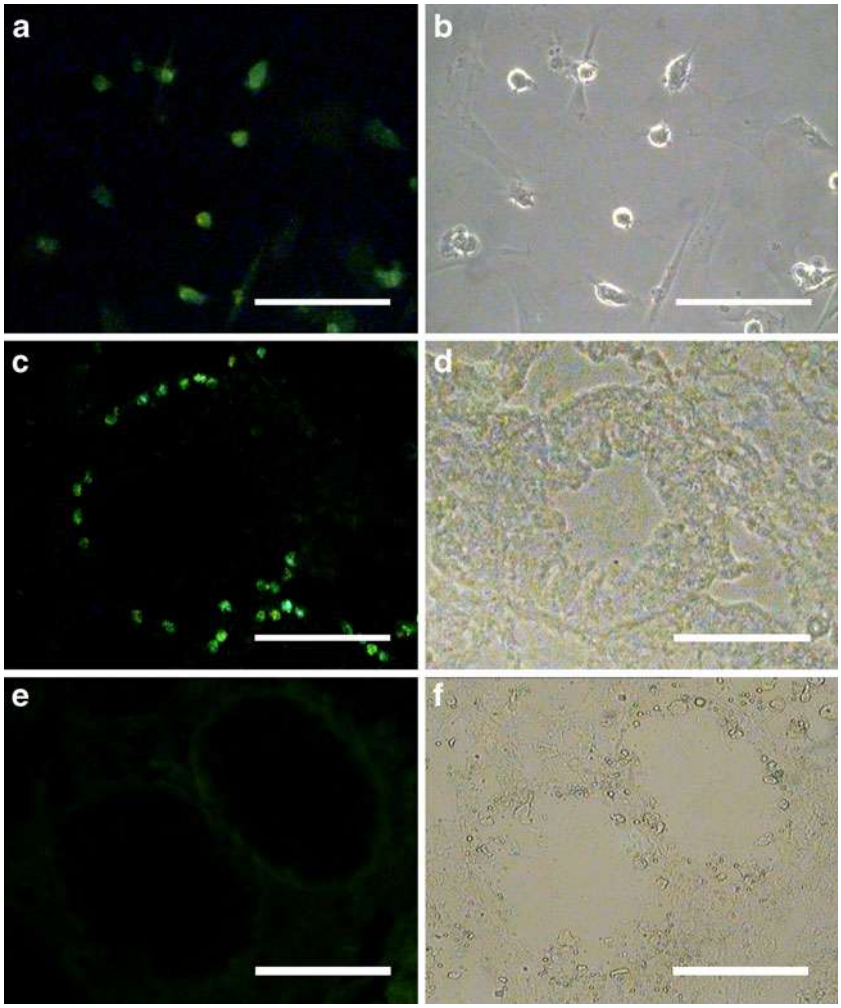

Fig. 3 Xenotransplantation of human SSCs into recipient mouse testis. In order to determine the functional spermatogonial stem cell activity of cultured cells, xenotransplantation of human SSCs into recipient mouse testis was performed. For these assay, human spermatogonial cells were harvested following culture periods of 60 days and labeled with 5-Bromo-2-deoxyuridine (BrdU). **a** BrdU was added, and staining was examined in cultured spermatogonial cells before transplantation. Labeled cells were transplanted into the seminiferous tubules of busulfan-treated adult recipient mice through rete testis. Since only spermatogonial stem cells can migrate to seminiferous tubule basement membrane recipient mouse testis. **c** Transplanted human SSCs were found as single cells or paired at the basal membranes of some of the mouse seminiferous tubules two months after transplantation. These cells were traced in the recipient testes by BrdU staining. **e** The non-transplanted right testis was considered as the control group. **b**, **d**, **f** Phase contrast. Scale bars=100 μm

likely create a testis-like microenvironment can be more effective in colony formation.

Although laminin-coated dishes could significantly increase SSCs colony formation in vitro, the small clusters GSCs were more abundant in growth factors plus laminin group. This explains why the diameter of clusters decreased after two months of cultivation (Table 2.). Kanatsu-Shinohara et al. has showed that SSCs prefer to attach to laminin [37] and can be enriched 3- to 8-fold after selection on a laminin-coated plate [38, 39]. Also, GS cells on laminin tend to form various types of colonies, ranging from chains to clumps [17]. Previous studies have shown the beneficial effects of laminin on in-vitro normal SSC proliferation [13, 40] and purification [41, 42].

In order to confirm the presence of spermatogonial cells during cultivation, RT-PCR using spermatogonial markers (*PLZF, Oct4, DAZL, VASA, ITGA6 and ITGB1*) in isolated testicular cells, cluster cells and testes tissue were performed. These markers were predominantly expressed by spermatogonial cells. To date, no SSC-specific marker has been identified for any species but the combination of expression of multiple markers can provide important information about spermatogonial cell types in rodents and other species [43].

PLZF, a marker for spermatogonial stem/progenitor cells, is well-known as a spermatogonial-specific marker in many species including humans [13, 22, 43]. *Oct4*, a general marker for stem cells, is also expressed in mouse spermatogonial stem/progenitor cells [18, 25, 31, 44, 45] and Human SSCs [7, 27, 46, 47] and may be required for the self-renewal of SSCs [48]. *Oct4* expression also reveals the presence of populations of SSCs in the human testes with pluripotent characteristics [47]. *ITGA6 and ITGB1*, surface markers for spermatogonial stem/progenitor cells, are expressed in rodents [37, 49] and humans [13, 21, 41, 47]. Our findings supported the reports by previous investigators [7, 13, 28, 47]. In our study expression of *VASA* and *DAZL* as markers of germ cell identification were also observed in isolated testicular cells, cluster cells and testes tissue. Our finding is in line with Conrad et al. [41] and Mirzapour et al. [28] who mentioned human adult GSCs and SSCs to be positive for stem cell markers such as *VASA* and *Oct4* [28, 41]. Previous studies have revealed that DAZL is presented in nucleus of spermatogonia obtained from rodents [38, 50] and adult rhesus macaque [51].

In addition to confirmation of molecular characteristics, SSCs functional assay and an ultrastructure study of the

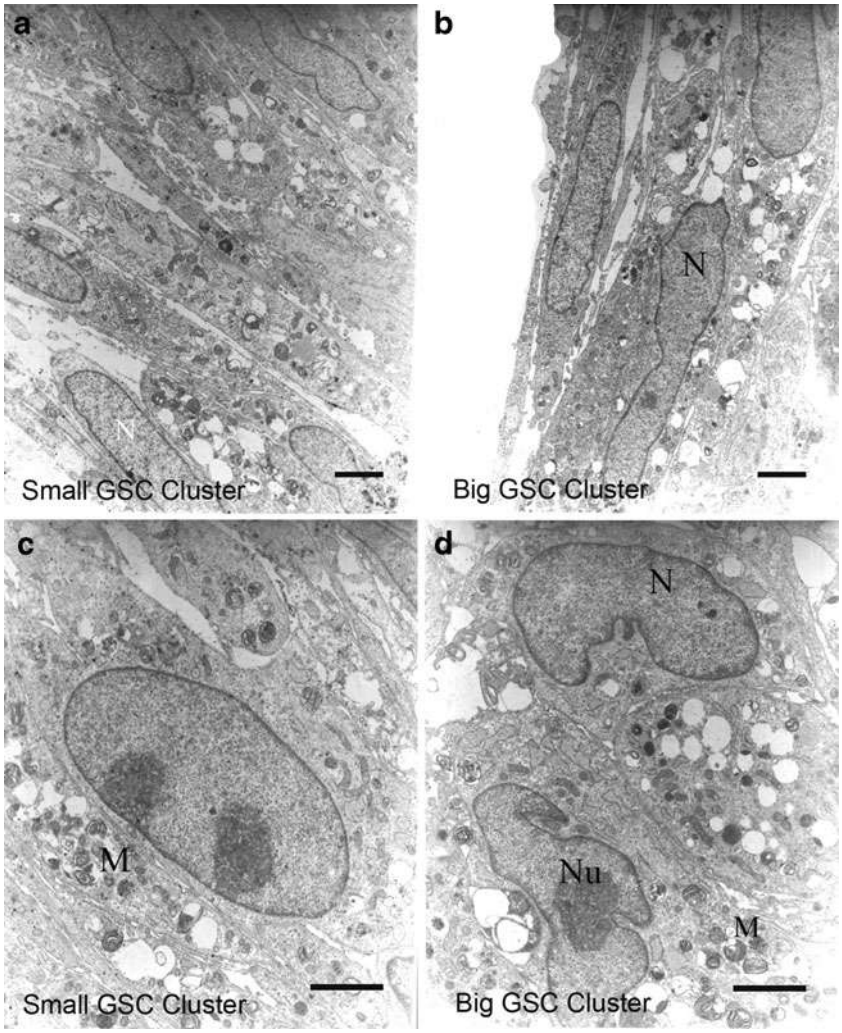

Fig. 4 Representative transmission electron micrographs from SSCs clusters. To characterize of SSCs, obtained cluster cells from culture were compared using a transmission electron microscopy (TEM). So, both types of GSC clusters were harvested during cultivation and processed for ultrastructure study. The electron micrograph showed cells from small (**a**, **c**) and big (**b**, **d**). GSC clusters had morphology typical of human spermatogonial cells. The nucleus (N) shown contains a mottled appearance with dark speckles of heterochromatin. In the nuclei, one or two small compact and highly reticulated nucleoli (Nu) were located eccentric position. Also, ratio of their nucleus to cytoplasm was very high and mitochondria (M) were positioned in the cytoplasm in abundance. Scale bar: A, B=800 nm and C, D= 500 nm

cluster cells were also performed. As there are no specific biochemical or morphological markers for SSCs in clusters [52, 53]and only the stem cells are able to colonize and repopulate in testes [29, 54], transplantation is performed as a functional assay to determine the presence of SSCs in a cell population. The cultured testicular cells were transplanted into a mouse busulfan azoospermic model. Human SSCs were found as single or paired cells at the basal membranes of some mouse seminiferous tubules. However, because of the large phylogenetic distance between mouse and human, only single or paired cells could be formed at the basal membranes of tubules. Previous reports have also shown similar results8 weeks after transplantation [13, 21, 28, 47]. Although SSCs in the clusters showed pluripotent characteristics, no tumors or teratomas were found in the three recipient mice after transplantation. This demonstrated that human SSCs remained completely committed to the germ line lineage during culture. This finding agreed with the reports by the aforementioned investigators.

Although there have been only a few ultrastructural studies on human spermatogonial cells in colonies or clusters, these studies all confirm the large nucleus to cytoplasm ratio, intensive nucleolus and high heterochromatins in humans and rodents [41, 55–57]. Similarities were found upon the comparison between the ultrastructure of the cluster cells of this study with those of previous studies.

The self-renewal and pluripotency capability of human SSCs from NOA patients in our culture system enables this system to be utilized for proliferation or differentiation of these cells from small biopsies in clinical applications, cell replacement therapy and tissue regeneration.

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REVIEW ARTICLE

Management of non-obstructive azoospermia

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Abstract Non-obstructive azoospermia (NOA) is defined as no sperm in the ejaculate due to failure of spermatogenesis and is the most severe form of male infertility. The etiology of NOA is either intrinsic testicular impairment or inadequate gonadotropin production. Chromosomal or genetic abnormalities should be evaluated because there is a relatively high incidence compared with the normal population. Although rare, NOA due to inadequate gonadotropin production is a condition in which fertility can be improved by medical treatment. In contrast, there is no treatment that can restore spermatogenesis in the majority of NOA patients. Consequently, testicular extraction of sperm under an operating microscope (micro-TESE) has been the first-line treatment for these patients. Other treatment options include varicocelectomy for NOA patients with a palpable varicocele and orchidopexy if undescended testes are diagnosed after adulthood, although management of these patients remains controversial. Advances in retrieving spermatozoa more efficiently by micro-TESE have been made during the past decade. In addition, recent advances in biotechnology have raised the possibility of using germ cells produced from stem cells in the future. This review presents current knowledge about the etiology, diagnosis, and treatment of NOA.

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Introduction

During the past few decades, a decrease of the birth rate has become a growing social problem in Japan. The live birth rate is continuously declining, and has fallen to almost half of that 40 years ago. Several factors have contributed to this trend, with infertility being one of the major problems. It has been reported that approximately 15 % of couples fail to conceive after 1 year of unprotected intercourse, and male factors are responsible for infertility in almost half of these couples [1]. Thus, development of more effective treatment for male infertility is important in this situation.

Several factors can contribute to male infertility, including decreased sperm production, abnormal sperm function, obstruction to the passage of sperm, and erectile dysfunction. Among these, non-obstructive azoospermia (NOA), which is defined as no sperm in the ejaculate due to failure of spermatogenesis, is the most severe form of male infertility. Historically, NOA patients were unable to have their own children and their only options were donor sperm or adoption. In 1978, the first live birth using in vitro fertilization (IVF) was reported [2], followed by successful live birth using the intracytoplasmic injection (ICSI) technique in 1992 [3]. Subsequently, pregnancy was reported after testicular sperm extraction (TESE) and ICSI in NOA patients [4], which allowed these patients to potentially father their own children. These advances in assisted reproductive technology (ART) have dramatically changed the management of NOA. This review

summarizes current practices and controversies with respect to the diagnosis and management of NOA.

Diagnosis of NOA

Azoospermia is diagnosed when no sperm are found in the ejaculate. It is important to note that at least two semen samples should be examined for accurate assessment [5]. In addition, absence of sperm should be confirmed by centrifugation of the semen specimen. Conducting careful microscopic examination of multiple droplets of sediment from the ejaculate has been reported to result in the detection of sperm in up to 35 % of men who were initially diagnosed as NOA [6]. When a few sperm are found after centrifugation, the condition is defined as cryptozoospermia. TESE might be unnecessary for performing ICSI in these patients, although better implantation rates have been reported using testicular sperm compared with sperm from ejaculates [7].

If azoospermia is diagnosed by semen analysis, the physician must consider whether the patient has obstructive azoospermia (OA) or NOA. The pathological basis of OA is physical obstruction of the post-testicular genital tract, while the etiology of NOA is failure of spermatogenesis due to either inadequate gonadotropin production or intrinsic testicular impairment. Taking a detailed history, physical examination, hormonal evaluation, and genetic testing are employed to establish the diagnosis. A history of factors such as anticancer chemotherapy or undescended testis leads to suspicion that the diagnosis is failure of spermatogenesis. Determining the patient's medications is also important, because some drugs can impair spermatogenesis, including steroids [8] and 5α-reductase inhibitors [9]. After taking the history, physical examination should be performed. Development of the secondary sexual characteristics is evaluated according to the Tanner stages [10]. When development of the genitalia or pubic hair is poor, this suggests the presence of hypogonadism. Measurement of testicular volume with an orchidometer or by ultrasonography is essential for making a diagnosis of NOA. The size of the testes reflects the level of spermatogenesis, so small testes indicate failure of this process. In patients with NOA, the testes are typically less than 15 cc in volume with a flat epididymis [5].

Ultrasonography is not only useful for measuring the volume of the testes, but also provides useful information about testicular pathophysiology. Testicular microlithiasis, which is defined as five or more microliths per testis [11], can be diagnosed by ultrasonography. This condition is known to be associated with failure of spermatogenesis [12], and it can be found in patients with testicular dysgenesis syndrome (TDS). Skakkebaek et al. advocated the concept of TDS, which suggests that poor semen quality, testicular cancer, undescended testis, and hypospadias are features of a single disease entity [13]. Hence, it was thought that testicular microlithiasis might be associated with testicular cancer at the end of the 1990s, but later studies did not confirm such concerns. The European Society of Urogenital Radiology only recommends follow-up ultrasonography when the following risk factors are present: previous germ cell tumor, history of undescended testis or orchidopexy, testicular atrophy (volume of $<$12 cc), and history of a germ cell tumor in a first-degree relative [14]. If testicular cancer is suspected from the ultrasonography findings, the clinician should consider further examinations such as measurement of tumor markers, MRI, and surgical orchidectomy.

Varicocele is a common condition that can be identified by physical examination. The patient should be examined in both the supine and standing positions, with the scrotum being inspected first and then palpated. Although only 20 % of men with a documented varicocele suffer from fertility problems [15, 16], this condition can cause impairment of spermatogenesis or even azoospermia. Thus, the presence of varicocele should be assessed during diagnosis of NOA patients.

Hormonal evaluation is also useful for making a diagnosis of NOA. Although NOA cannot always be excluded when gonadotropins are within the normal range (especially in patients with germ cell maturational arrest), high serum gonadotropin levels typically indicate primary testicular failure. Testicular biopsy is not usually required to make a diagnosis of NOA, since it has been reported that more than 90 % of patients with azoospermia could be accurately diagnosed as NOA or OA by combined measurement of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testicular volume [17]. Factors associated with azoospermia are summarized in Table 1.

Additional investigations for NOA patients

When NOA is diagnosed, additional investigations such as karyotyping and genetic analysis should be performed. It has been reported that an abnormal karyotype is found in 13.7 % of patients with azoospermia [18], with Klinefelter syndrome being the most frequent abnormality (10.8 %), followed by other sex chromosomal abnormalities (1.8 %) and autosomal anomalies (1.1 %) [18]. Genetic examination may also reveal another condition that is related to NOA. Several genetic defects, such as *KAL1* or *FGFR1*, are involved in Kallmann syndrome, which features hypogonadotropic hypogonadism with anosmia [19, 20]. Mutations of the androgen receptor (AR) gene, which is located on the X-chromosome, are responsible for mild-to-

Table 1 Causes of male infertility and associated factors (adapted with permission from Ref. [63])

Diagnosis	Unselected patients (%) (n = 12,945)	Patients with azoospermia (%) (n = 1446)
Undescended testes	8.4	17.2
Varicocele	14.8	10.9
Testicular tumor	1.2	2.8
Klinefelter syndrome	2.6	13.7
XX male	0.1	0.6
Primary hypogonadism of unknown cause	2.3	0.8
Kallmann syndrome	0.3	0.5
Idiopathic hypogonadotropic hypogonadism	0.4	0.4
Pituitary surgery	<0.1	0.3
Systemic disease	2.2	0.5
Obstruction	2.2	10.3
Idiopathic	30	13.3

severe androgen insensitivity [21]. While complete androgen insensitivity typically results in a female phenotype, men who have mild androgen insensitivity are more likely to present with infertility. Several genes on the X-chromosome are known to specifically act on the testis and play an important role in meiosis [22]. Recent studies have frequently detected altered copy number variants (CNVs) of X-chromosome genes in patients with failure of spermatogenesis, although further investigation is needed for clinical application of this finding [23, 24].

The most popular and significant genetic test for management of NOA is a test for azoospermia factor (AZF), which is located on the long arm of the Y-chromosome (Yq) and has three sub-regions (AZFa, AZFb, and AZFc). In Western countries, approximately 8 % of NOA patients have been reported to harbor Yq microdeletions [25]. As described below, microdeletion in the AZF region can predict surgical sperm retrieval, so it is essential to evaluate AZF microdeletion when considering TESE for NOA patients. Recently, a new molecular diagnostic kit was developed that can be used in the routine clinical setting to assess Y-chromosome deletions in Japanese patients [26].

Management of NOA

Retrieval of testicular sperm

At the present time, there is no treatment that can restore spermatogenesis in the majority of NOA patients, apart from those with secondary testicular failure. Therefore, the only way for the affected couples to achieve pregnancy without involving a donor is to retrieve spermatozoa directly from the testes for ICSI. An ideal surgical technique would achieve efficient retrieval of sperm while causing minimal trauma to the testes [27]. Several sperm-retrieval techniques have been developed, including TESE and fine-needle aspiration (FNA). TESE has been performed with multiple biopsies to increase the sperm retrieval rate (SRR) [28, 29], but removal of large amounts of tissue could lead to testicular atrophy after surgical intervention [30]. FNA is another possible technique. It was initially used for diagnostic purposes and is a less invasive method of sperm retrieval compared with TESE, but most studies have shown a significantly lower SRR with FNA than TESE [31–34]. The technique of microdissection testicular sperm extraction (micro-TESE) was first described by Schlegel in 1999 [35]. If an operating microscope (magnification of 15–25×) is employed during TESE, seminiferous tubules containing spermatozoa can be visualized. Micro-TESE has several advantages, including a higher yield of spermatozoa per biopsy, removal of less testicular tissue, and identification of blood vessels to minimize vascular injury [35]. This procedure has been widely suggested to be a better method of sperm retrieval in patients with NOA, and several studies have supported the superiority of micro-TESE for testicular sperm retrieval. In NOA patients, the sperm retrieval rate is reported to be 43–63 % when micro-TESE is employed [35–42]. It should be noted that the SRR of micro-TESE is influenced by the surgeon's experience, especially in patients with Sertoli cell-only syndrome (SCO) [43]. Experienced andrologists as well as embryologists are required to treat these patients with severe infertility.

NOA with varicocele

Treatment of NOA patients with varicocele is still controversial. Varicocele is the most common correctable cause of male infertility and surgical varicocelectomy is an important treatment for restoring fertility. Although a systematic review that included patients with subclinical varicocele or normal semen parameters concluded that there was insufficient evidence to support the efficacy of varicocelectomy for increasing the likelihood of conception [44], there have been several other reports about the efficacy of varicocelectomy in patient populations excluding men with subclinical varicocele or normal semen parameters [45, 46]. Varicocele is associated with NOA in 5–10 % of patients. Although this issue remains controversial, several articles supporting the efficacy of surgical varicocelectomy for these patients have been published [47, 48]. However, recent reports have indicated that even if there is some improvement of

spermatogenesis, the postoperative sperm concentration is still quite low and ART such as ICSI will be required [49]. Thus, it is important to decide whether to offer varicocelectomy or sperm retrieval without varicocele repair for these patients. According to a report from Cornell, even if patients have sperm in the semen after varicocelectomy, <10 % will have viable sperm at the time of ICSI and be able to avoid TESE [50]. That study also indicated the SRR was not influenced by whether the patient underwent varicocelectomy or not [50]. At the same time, improvement of the SRR [51] or improvement of the clinical pregnancy rate and live birth rate [52] have also been reported among NOA patients with varicocele. Furthermore, a meta-analysis of 233 NOA patients with varicocele showed a spontaneous pregnancy rate of 6 % following treatment of varicocele [53]. Thus, although treating varicocele shows limited efficacy in NOA patients, some of them may benefit and the physician should counsel couples with care.

NOA with undescended testis

Undescended testis is a frequent congenital disease that is usually diagnosed and treated during childhood. Its prevalence is 30 % in preterm infants and 3 % in term infants worldwide [54, 55]. When the testis is in an abnormal location (e.g., abdominal or inguinal), there is a risk of the development of testicular malignancy as well as impairment of spermatogenesis [56, 57]. This condition was thought to be associated with a 35- to 50-fold greater risk of malignant testicular tumors compared with the normal population [58], although later studies suggested a somewhat lower risk of malignancy (five- to tenfold elevation) [59, 60]. The higher temperature to which the undescended testis is exposed has a detrimental effect on spermatogenesis [61]. Given that spontaneous testicular descent cannot be expected more than 3–6 months after birth [56], early orchidopexy is recommended to promote normal testicular development in adulthood [62]. Even after the testes are relocated to the proper position, infertility is still an issue, although its frequency may be reduced. Among patients with bilateral undescended testes undergoing orchidopexy, azoospermia is still found in approximately 40 % [63]. For these patients, retrieval of testicular sperm needs to be offered as a fertility treatment. Raman et al. investigated the SRR of TESE in NOA patients with a history of orchidopexy. Sperm was retrieved in 35 of 47 attempts (74 %), which was a higher rate than in other NOA patients. The authors also reported that the age at orchidopexy was an independent predictor of SRR in these patients [64]. Unfortunately, undescended testis is sometimes not diagnosed until adulthood, and may even be found during physical examination for assessment of infertility. Previously, we reported 10 patients with bilateral undescended testes diagnosed in adulthood, all of whom had azoospermia. Micro-TESE was performed in four of these patients, but no sperm could be retrieved, indicating the severe effect on spermatogenesis when undescended testis is not treated until adulthood [65]. Because testicular function is severely impaired, orchidopexy for bilateral undescended testes in adulthood was once considered to be cosmetic and unlikely to have any effect on spermatogenesis. However, case reports have been published documenting fertility after bilateral orchidopexy [66, 67]. We also experienced a patient who achieved pregnancy by TESE with ICSI at 7 years after bilateral orchidopexy as an adult [68]. Although it is rare, it seems that improvement of spermatogenesis can be achieved by orchidopexy in some adult patients with bilateral undescended testes. After orchidopexy, self-examination of the scrotum is highly recommended for these patients to detect testicular malignancy.

NOA with chromosomal/genetic abnormalities

As described above, Klinefelter syndrome is the most frequent chromosomal abnormality among NOA patients. Men with Klinefelter syndrome tend to have small testes, less muscle, less body hair, low sex drive, and gynecomastia. Usually, the diagnosis is made during evaluation of male infertility. Approximately 95 % of men with Klinefelter syndrome have a 47, XXY chromosomal complement [69]. Micro-TESE combined with ICSI is the only approach that can be offered to NOA patients with Klinefelter syndrome. In these patients, the SRR is reported to be approximately 40–50 % [70], with a range of 21 to 72 % [71–76]. While development of micro-TESE and ICSI has allowed some of these patients to have their own progeny, it should also be noted that the spermatozoa of patients with Klinefelter syndrome may have a higher aneuploidy rate of sex chromosomes and autosomal chromosomes [77, 78]. When a patient is diagnosed as having NOA with Klinefelter syndrome, sufficient information should be given to the couple, and options such as prenatal diagnosis or preimplantation genetic screening should be presented [79, 80].

AZF microdeletion is also important when considering the fertility of NOA patients. This region on Yq has an important role in germ cell development and differentiation, and it is divided into three sub-regions which are AZFa, AZFb, and AZFc [81, 82]. The AZF region contains multiple genes required for different stages of spermatogenesis. For instance, *USP9Y* and *DBY* are located in the AZFa region, *RBMY* is in the ABFb region, and DAZ is in the AZFc region. Deletions affecting the AZF region have been reported in 8–12 % of NOA patients [63]. The most

frequently deleted region is AZFc (80 %), followed by deletion of AZFb (1–5 %), AZFa (0.5–4 %), and AZFb+c (1–3 %) [83–85]. Evaluation of microdeletion in the AZF region is clinically important because it can predict SRR during micro-TESE. Typically, complete deletion of the AZFa region is associated with the SCO phenotype, while complete AZFb deletion or AZFb+c deletion is associated with maturation arrest. Accordingly, when NOA patients have these deletions, the SRR will be virtually nil if micro-TESE is attempted [86]. On the other hand, patients with AZFc deletion, which is the most frequent abnormality, are known to have residual spermatogenesis. In these patients, the SRR is reported to range from 50 to 70 % [87, 88], although embryonic development may be impaired even if sperm are retrieved [89]. It is important to note that such Yq micro deletions will be inherited by male offspring. Therefore, genetic counseling is mandatory to provide information about the risk of conceiving a son with infertility and possibly other genetic abnormalities [86].

Hypogonadotropic hypogonadism

Hypogonadotropic hypogonadism (HH) is a condition in which secondary testicular dysfunction is caused by either hypothalamic or pituitary disease. The hyposecretion of gonadotropins results in low testosterone production by the testes and impaired spermatogenesis. HH can be classified as congenital or acquired (Table 2). Mutations of *KAL1* (X-linked recessive), *FGFR1* (autosomal dominant), and *GNRHR* (autosomal recessive) are reported to be associated with congenital HH [90], but the etiology remains unknown in approximately 70 % of patients. Although the diagnosis of congenital HH is usually made before adulthood because of the lack of puberty, a rare type of congenital adult HH has been reported, which occurs in otherwise healthy men who have completed normal pubertal development and often have proven fertility [91].

Table 2 Classification of hypogonadotropic hypogonadism (adapted with permission from Ref. [63])

Congenital
Kallmann syndrome
Idiopathic hypogonadotropic hypogonadism
Acquired
Tumors of the hypothalamus and pituitary gland
Granulomatous disease
Empty sella syndrome
Hemochromatosis
Obesity
Anabolic steroids
Aging

Although HH is a rare condition, fertility can be improved by medical treatment in these patients. When fertility is the issue, standard medical therapy is administration of gonadotropins. Human chorionic gonadotropin (hCG), with later addition of human menopausal gonadotropin (hMG) or recombinant FSH, is usually administered to rescue spermatogenesis. Detection of sperm in the ejaculate and even natural pregnancy can be expected with this treatment. If fertility is no longer an issue, administration of testosterone instead of gonadotropins could be a treatment option. Interestingly, reversal of idiopathic HH has been documented [92, 93], although lifelong hormone therapy was believed to be necessary for these patients. According to Raivio et al., 10 % (5/50) of idiopathic HH patients showed sustained reversal of their condition after discontinuation of hormone therapy [92]. Thus, brief discontinuation of hormone therapy to assess reversibility may be a reasonable approach in a subset of patients.

Future prospects

Since there is no treatment that can restore spermatogenesis in the majority of NOA patients, retrieval of testicular sperm is currently the main method of achieving pregnancy. However, spermatozoa cannot be retrieved in a certain number of patients even if surgery is performed. Various attempts to retrieve spermatozoa more efficiently have been made during the last decade. Administration of gonadotropins to NOA patients (except those with HH), particularly patients who have elevated plasma gonadotropin levels, has generally been accepted to be ineffective. Nevertheless, this treatment may have some benefit for NOA patients, although the exact mechanisms/potential effects are unclear. One possible explanation is that exogenous gonadotropins increase intra-testicular testosterone, after which spermatogonia are stimulated, leading to DNA synthesis and spermiogenesis in patients with residual spermatogenic activity [94–96]. Shiraishi et al. reported that in 20 NOA patients whose sperm could not be retrieved by micro-TESE, treatment with hCG and recombinant FSH after TESE led to sperm retrieval in 21 % (6/28 patients) during the 2nd micro-TESE attempt [97]. In that study, none of the patients who did not receive hormone therapy after the first micro-TESE attempt had successful sperm retrieval during the second micro-TESE attempt [97]. Although a definite conclusion cannot be made due to lack of well-designed clinical trials, various methods are being tried to enhance sperm retrieval.

Technical improvements using newer instruments are also being made to increase the chance of sperm retrieval during micro-TESE. Ramasamy et al. conducted a study in rodents using multiphoton microscopy (MPM) and

reported that there was a significant difference between seminiferous tubules with and without sperm [98]. The potential concern with this procedure is increased sperm DNA fragmentation by the MPM laser, but no increase was seen at the laser intensity used for imaging of the tubules [98]. The same group published another rodent study using full field optical coherence tomography (FFOCT) to identify the presence of spermatozoa in testicular tissue [99]. Because the light source for FFOCT is a halogen lamp, there is no concern about increased physical or genetic damage to sperm [99]. Recently, we reported a study performed in rodents using a narrow-band imaging system (NBI), which allowed us to distinguish spermatogenically active regions through visualization of blood vessels [100]. Although further studies need to be carried out, these new approaches could lead to better identification of spermatogenesis in humans.

Recent advances in biotechnology have shed light on possible innovations in the treatment of NOA. Successful in vitro production of spermatozoa in cultured neonatal mouse testes was reported by Sato et al. [101]. They then performed ICSI with the spermatozoa and produced healthy offspring [101]. Induction of germ cells from human-induced pluripotent (iPS) cells is also an encouraging technique in this field. It has been reported that generation of haploid round spermatids from human iPS cells can be achieved in vitro [102]. Further progress will contribute to the development of novel therapeutic techniques for NOA patients in the future.

Conclusions

Because there is no treatment that can restore spermatogenesis in the majority of NOA patients, micro-TESE is currently the mainstay for the management of NOA. Chromosomal and genetic testing should be performed in these patients because of the relatively high incidence of such abnormalities in NOA, and sufficient counseling should be provided to couples about these issues. Although various attempts have been made to establish a better sperm-retrieval system with micro-TESE, there is no other option available for patients to get their own progeny if spermatozoa cannot be retrieved. Further studies, including stem cell research, may contribute to novel therapeutic techniques for NOA.

Compliance with ethical standards

Conflict of interest Koji Chiba, Noritoshi Enatsu, and Masato Fujisawa declare that they have no conflicts of interest to declare.

Human/Animal studies This article does not contain any studies with human or animal subjects performed by any of the authors.

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REVIEW

How to improve the clinical outcome of round spermatid injection (ROSI) into the oocyte: Correction of epigenetic abnormalities

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Abstract

Background: First successful human round spermatid injection (ROSI) was conducted by Tesarik et al. in 1996 for the sole treatment of nonobstructive azoospermic men whose most advanced spermatogenic cells were elongating round spermatids. Nine offsprings from ROSI were reported between 1996 and 2000. No successful deliveries were reported for 15 years after that. Tanaka et al. reported 90 babies born after ROSI and their follow-up studies in 2015 and 2018 showed no significant differences in comparison with those born after natural conception in terms of physical and cognitive abilities. However, clinical outcomes remain low.

Method: Clinical and laboratory data of successful cases in the precursor ROSI groups and those of Tanaka et al. were reviewed.

Results: Differences were found between the two groups in terms of identification of characteristics of round spermatid and oocyte activation. Additionally, epigenetic abnormalities were identified as underlying causes for poor ROSI results, besides correct identification of round spermatid and adequate oocyte activation. Correction of epigenetic errors could lead to optimal embryonic development.

Conclusion: Correction of epigenetic abnormalities has a probability to improve the clinical outcome of ROSI.

KEYWORDS
epigenetic abnormality, oocyte activation, round spermatid injection into oocyte (ROSI)

1 | INTRODUCTION

It is said that 1 out of 100 healthy men is azoospermic[1] and that about 70–80% of those are nonobstructive azoospermia cases.[2] Micro-TESE is the sole treatment to find spermatozoa. The incidence of detecting testicular spermatozoa is about 30–60%,[3–6] but about half of those spermatozoa found are immotile or have deformities. When no intact testicular spermatozoa could be found, the patients were considered as unable to become biological fathers and sperm donation was recommended. Ogura and Yanagimachi reported the capability of fertilization with round spermatid (R-ST) using hamster R-ST in 1993 for the first time.[7] The rationale of ROSI is that R-STs develop after two times of meiosis and have the same number of chromosomes and same contents of DNA as those of matured spermatozoa. After injection into the oocyte and with proper oocyte activation, R-STs have the same ability to fertilize the oocyte as spermatozoa. (Figure 1) They reported the birth of normal offspring of mice in 1994.[8,9] There have been many successful reports in mammals except for human beings. Edwards et al. presented the idea of using spermatids isolated from men with spermatogenesis

arrest at any stage of spermatogenesis as substitutes for spermatozoa in 1994.[10] Tesarik et al. reported the birth of normal babies following round spermatid injection (ROSI) into human oocytes in 1996.[11,12] Eight pregnancies were reported after that, and seven deliveries were reported.[12–18] Doctors became gradually skeptical about its clinical usefulness and ROSI disappeared from clinical application. However, in 2015, our clinic reported in PNAS[19] 14 healthy babies born following ROSI from azoospermic patients whose first Micro-TESE conducted by urologists at other institutions, had not found any spermatozoa but a second Micro-TESE at our clinic had found round spermatids. The clinical data of 90 babies born after ROSI whose physical and cognitive abilities proved to be no significant different in comparison with normal conception was reported in Fertility and Sterility in 2018,[20] However, clinical outcome following ROSI is still low. Our studies have elucidated the main cause of poor clinical outcome as derived from epigenetic abnormalities by differences of nuclear protein in round spermatid (histone) and mature spermatozoa (protamine).[21–23]

In 2017, Kong et al. reported the beneficial effects on the correction of epigenetic abnormalities. I would like to review the history of ROSI and refer to how to correct the epigenetic abnormalities consulting recent papers[22,24–27] without being against the guidelines on genetic editing.[22,28]

2 | RATIONALE FOR ROSI

Palermo et al. developed intracytoplasmic sperm injection (ICSI) in 1992.[29] This novel technique has greatly changed the concept of fertilization. Fertilization is completed by fusion of the oocyte with the spermatozoa, and it triggered the oocyte activation. Interaction between presumptive complementary receptors on the spermatozoa and oocyte plasma membranes triggers the activation of the G-protein that activates the production of inositol triphosphate that releases Ca^{2+} from the endoplasmic reticulum.

Meanwhile, ICSI proved that the release of Ca^{2+} oscillation-releasing factor from the injected spermatozoa triggered the oocyte activation cascade.[30,31]

In either way, the release of Ca^{2+} starts to spread into all the cytoplasm and resumes the second meiotic cell division, this resulted in the extrusion of the second polar body and both pronuclei.

Round spermatid has a haploid set of chromosomes 23 and 1N DNA content just as a mature spermatozoa. So, if R-ST can be injected directly into the oocyte with the same technique as ICSI, R-ST, which has no flagellum, can fertilize the oocyte and deliver a baby.

3 | THE REASON WHY USEFULNESS OF ROSI HAS BEEN RECONSIDERED

The generally accepted theory for nonobstructed azoospermia is that whenever R-ST exists in the human testis, there are also matured spermatozoa present.[32,33] When no spermatozoa or late-stage spermatids could be found, R-ST could still be found. So, it was concluded that ROSI was not necessary clinically, or should not be conducted. However, the new facts that mouse and human male with cyclic AMP-responsive element modulator (CREM) gene[27,34–38] mutation showed the maturation arrest at R-ST

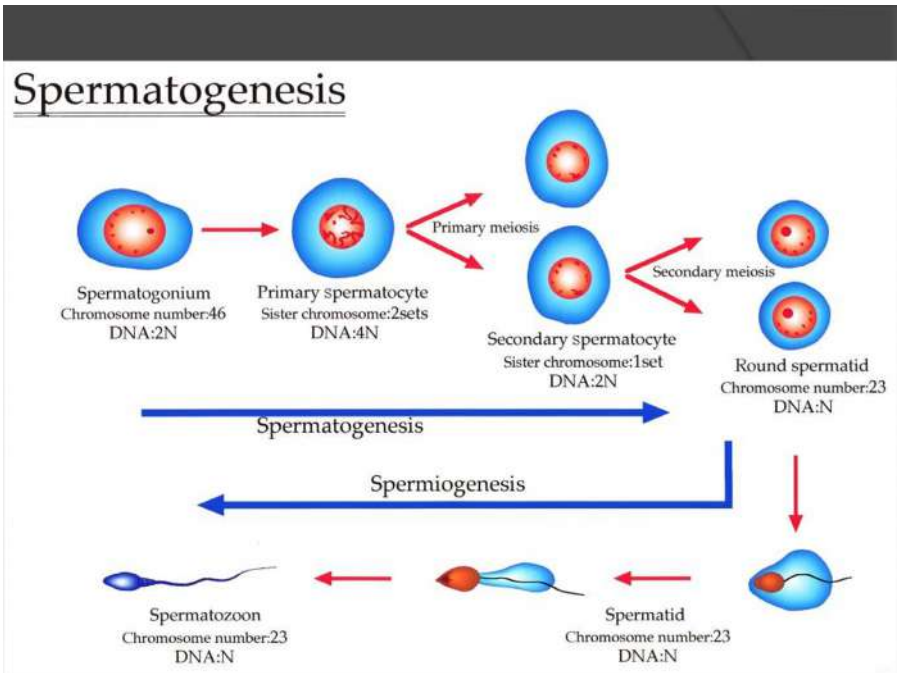

FIGURE 1 The rationale of ROSI is that R-STs develop after two times of meiosis and have the same number of chromosomes and same contents of DNA as those of matured spermatozoa. After injection into the oocyte and with proper oocyte activation, R-STs have the same ability to fertilize the oocyte as spermatozoa.

followed by the report that men with mutation genes, TAF4B and ZMYND15, showed the same maturation arrest. These facts suggest the reconsideration of the usefulness of ROSI. ROSI is now being reappraised.

4 | WHY IS ROSI NECESSARY?

One out of hundred men suffers from azoospermia and about 70% of azoospermia cases are nonobstructive. The sole way for these patients to become biological fathers is to obtain testicular spermatozoa through Micro-TESE. However, the success rate of obtaining viable testicular spermatozoa has been reported to be low. About half of collected spermatozoa are immotile or have abnormally shaped heads.[39] When intact sperm could not be found, the patients are declared to be completely infertile. However, R-ST could be found in about 30–40% of those cases. They still have a chance to become biological fathers using their own R-ST.

Sperm donation is available around the world. However, many patients continue to desire to have children with their own gamete and not with donated sperm.

5 | CLINICAL ANALYSIS OF 22 MANUSCRIPTS THAT HAD ENOUGH INFORMATION TO PROPERLY EVALUATE ROSI

Twenty-two manuscripts about ROSI with information complete enough to perform a clinical analysis were reviewed. Reports that included information about number of injected oocytes, fertilization rate, pregnancy rate, miscarriage rate, type of oocyte activation, R-ST collection method, optics used and whether the R-ST used was fresh or frozen were included.

5.1 | Identification of ROSI

The manuscripts first published on ROSI reported methods that could be questioned in terms of morphological identification or oocyte activation. However, they were correct at identifying the genetic, epigenetic, and chromosomal risks that ROSI could pose. They predicted genetic imprinting, changes in DNA association with nuclear protein, cell cycle synchronization, and DNA methylation. None of the early manuscripts addressed the mechanism of how epigenetic abnormalities may occur or offered solutions to treat them.[1]

5.2 | Actual oocyte activation

Vigorous oocyte aspiration,[12,14,15,40–47] calcium ionophore, or ionomycin,[13,48] or no,[17,49–51] electric stimulation[19,20] were reported as oocyte activation methods. Most authors used the vigorous oocyte aspiration method reported by Tesarik et al,[11] 81.8% (18/22), electric activation 9.1% (2/22), ionophore or ionomycin 9.1% (2/22), none 18.2% (4/22), and not reported 4.5% (1/22). In 2004, T. Ebner et al.[52] reported a mechanical activation with deeper insertion of injection pipette to the opposite membrane with a slight invagination. However, benefits for clinical outcome could not be recognized.

Electric stimulation is now considered to be the most effective for ROSI. The effect of each oocyte activation was examined by Ca^{2+} oscillation.

It has become clear that Tesarik's oocyte aspiration was not sufficient for full oocyte activation.

5.3 | R-ST in ejaculate or testis

Round spermatid is found in the ejaculate or testis. R-ST in ejaculate is considered to be an inadequate sample for ROSI in comparison with testicular one due to the high possibility of apoptotic change of R-ST[53] and high probability of spermatozoa existence in the same ejaculate. It has been revealed that more apoptosis occurs in the R-ST than in the testis, so the reasons that led Tesarik to use R-ST in ejaculate are still unclear. There are two assumed reasons. First was an easier collection. Second was the difficulty of the procedure of testicular biopsy at the time. So far, four cases performed ROSI with R-ST in ejaculate and the remaining 20 cases used testicular R-ST.

5.4 | Fresh or frozen–thawed R-ST

Tanaka et al conducted ROSI using cryopreserved R-ST, because there is no guarantee of 100% collection of R-ST at the Micro-TESE. However, in the initial ROSI reports only four cases used cryopreserved R-ST,[14,18,43,49] the remaining 16 cases used fresh spermatozoa. These results suggest that the cryopreservation method had not been established yet in the early days or the first researchers were not confident about the correct identification of R-ST. Two cases of delivery[14,18] were reported. Freezing method was vitrification. Now it has been proved that in terms of recovery rate, the thawing slow-freezing method is superior to vitrification (Figure 2).

5.5 | Most advanced spermatogenic cell in ROSI

The three kinds of most advanced spermatogenic cells (spermatozoa, elongated, elongating spermatids) reported in Tanaka's cases were all arrested at the stage of R-ST,[19,20] but in 20 early reported groups they were mixed. Nine cases[13,14,43–47,49,50] of R-ST, 5 cases[12,15,48,51,54] of elongating spermatids, 5 cases[16–18,41,42] of spermatozoa. The strict definition of ROSI describes it as the method that uses only

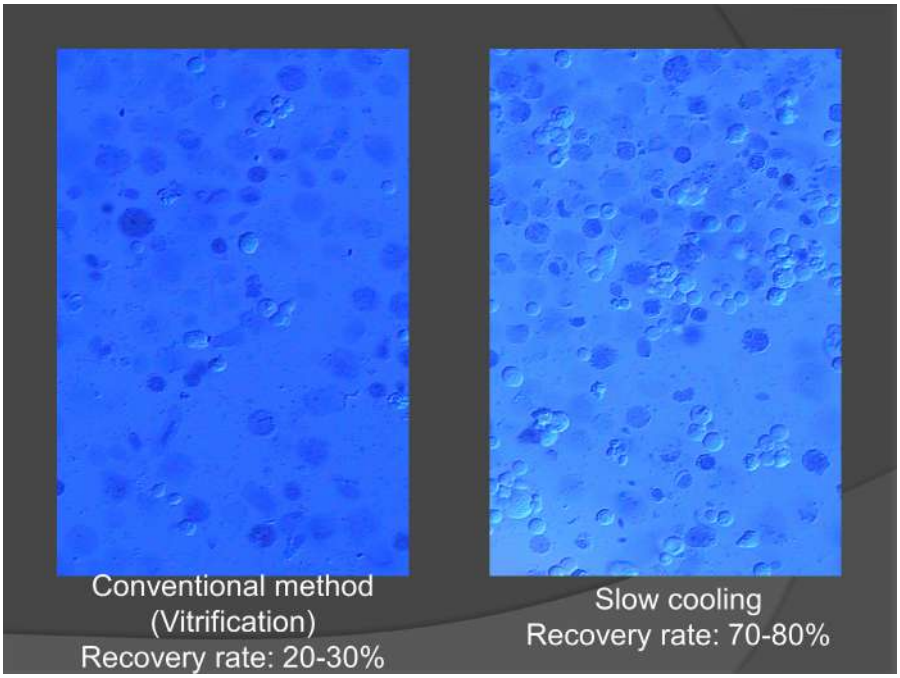

FIGURE 2 Recovery rate after thawing is significantly higher with slow cooling method than with vitrification method (Magnification: 200×).

R-ST obtained from cases of complete maturation arrest at the stage of R-ST. It has been well-known that the clinical outcome of R-ST differs greatly whether maturation arrest was complete or incomplete. ROSI results using R-ST obtained from complete maturation arrest was significantly lower than that of incomplete one. This difference could be caused by degree of transitions of nuclear protein, histone to protamine. This result made R-ST develop into elongating or elongated spermatids for the starting group of researchers doing in vitro culture.

5.6 | Clinical outcome (Table 1)

Average fertilization rate (%), clinical pregnancy rate (%), miscarriage rate (%), number of live offspring and birth rate in 20 initial ROSI reports, the precursors group,[12–18,40–51,53,55] and Tanaka group,[19,20] was (36.60 ± 15.53%) and (58.15 ± 1.95%), (18.47 ± 15.91%) and Fresh-T: 10.98 ± 7.84%, Frozen-T: 19.8 ± 5.7%, (4.76 ± 12.59%) and Fresh-T: 54.38 ± 15.02%, Frozen -T: 40.52 ± 13.41%, (9, 12 and 84) and (6.12% (9/147)), Fresh-T: 3.27% (52/1592) Frozen -T: 10.76% (44/409). There was a significant difference between Fertilization rate (%) in the precursors group and Tanaka group, and Tanaka group obtained a significantly higher fertilization rate (%) than that of the first researchers group.

The reason why there were so big differences between the two groups, the reports between 1996 to 2015 and the Tanaka's reports in 2015,[19] 2018[20] could be attributed to the following points: (1) microscopic observation of R-ST by Hoffman phase contrast microscope which has lower resolution than Nomarski differential interference contrast microscope.[56] (2) Different preparation methods for removed seminiferous tissues, simple procedures versus with or without enzymatic preparation. Preparation medium containing DNase and collagenase made it easier to isolate the spermatogenic cell in the Beginning group. (3) Almost all reports of precursors group used ooplasm aspiration which has almost no effect as oocyte activator.

6 | IN VITRO CULTURE OF HUMAN SPERMATOGENIC CELLS

A great number of studies tried in vitro culture of spermatogenic cells into mature spermatozoa but none of them was completely successful.

6.1 | In vitro culture of spermatogenic cells with Sertoli cells in culture system with follicle-stimulating hormone and testosterone

In 1998, Tesarik et al[57] reported the successful in vitro human spermatogenesis and spermiogenesis in a simple culture system (GAMATE-100) supplemented with FSH concentration of 50–100 IU/L and testosterone at concentrations of 1 μmol/L. He showed that the combination of FSH and T resumed the second meiotic cell divisions and subsequent spermiogenesis. In 2000,[53] he reported the first case of human pregnancy (twin babies, 36 weeks). The reason why he started the clinical application with some unsolved problems about development of abnormal shaped elongating/elongated spermatids remains unclear. If he had been confident on the procedure's safety he would have continued the treatment and tried to spread the method. No report indicating that the maturation arrest

TABLE 1 Clinical outcome in reviewed paper.

Year	Author	Number of injected oocytes Fresh/frozen–thawed (FT)	Fertilization rate (%)	Pregnancy rate (%) (fresh-T/frozen-T)	Miscarriage rate (%) (fresh-T/frozen-T)	Number of live offspring (fresh-T/frozen-T)	Most advanced spermatogenic cell	Oocyte activation	R-ST in ejaculate or micro-TESE	Optics		Fresh or frozen–thawed R-ST		
													Frozen	
										Hoffman	Nomarski	Fresh	Vitrification	Slow cooling
1996	Tesarik et al.	47	42.55	16.66 (2/12)	0	2	Elongated ST	Vigorous	Ejaculate	○		○		
1997	Vanderzwalmen et al.	260	21.92	14.28 (1/7)	0	1	R-ST	Ionophore	Micro-TESE	○		○		
1997	Antinori et al.	15	46.66	16.66 (1/6)	0	1	R-ST	Vigorous	Micro-TESE	○			○	
1997	Antinori et al.	135	55.55	3.57 (3/84)	33.3 (1/3)	2	Elongating ST	Vigorous	Micro-TESE	○		○		
1997	Amer et al.	251	25.09	0	0	0	Sa ejaculate Sa testis	Vigorous	Ejaculate/ Micro-TESE	○		○		
1997	Yamanaka et al.	49	69.38	0	0	0	R-ST	None	Micro-TESE	○		○		
1998	Kahraman et al.	199	25.62	3.12 (1/32)	0	1	Spermatozoa	Vigorous	Micro-TESE	○		○		
1998	Barak et al.	37	27.02	25 (1/4)	0	1	Spermatozoa	None	Micro-TESE		○	○		
1998	Bernabeu et al.	7	42.85	0	0	0	Elongating ST	Not reported	Ejaculate($n = 6$), Micro-TESE ($n = 2$)	○		○		
1999	Ghazzawi et al.	574	21.95	0	0	0	Spermatozoa	Vigorous	Micro-TESE	○		○		
1999	Al-Hasani et al.	49	18.36	0	0	0	R-ST	None	Micro-TESE	○			○	
1999	Gianaroli et al.	5	40	50 (1/2)	0	1	Spermatozoa	Vigorous	Micro-TESE	○			○	
2000	Balaban et al.	356	56.17	0	0	0	Spermatozoa	Vigorous	Micro-TESE	○		○		
2000	Levran et al	178	45.5	0	0	0	R-ST	Vigorous	Micro-TESE		○		○	
2001	Vicdan et al.	60	28.33	0	0	0	R-ST	Vigorous	Micro-TESE	○		○		
2002	Urman et al.	1021	40.54	0	0	0	R-ST	Vigorous	Micro-TESE	○		○		
2002	Sousa et al.	98/28	17.4/19.2	0/0	0	0/0	R-ST	Vigorous	Micro-TESE	○		○		
2002	Khalili et al.	42	21.42	0	0	0	Elongating ST	None	Micro-TESE	○		○		
2003	Ulug et al.	36	41.66	0	0	0	R-ST	Vigorous	Micro-TESE	○		○		
2015	Goswami et al.	13	61.53	0	0	0	Elongating ST	Ionomycin	Micro-TESE	○		○		
2015	Tanaka et al.	734	59.54	16.5 (20/121)/ 23.8 (10/42)	65.0 (13/20)/ 50.0 (5/10)	9/5 (14)	R-ST	Electrical	Micro-TESE		○			○
2018	Tanaka et al.	14 324	56.77	5.4 (80/1471)/ 15.8 (58/367)	43.8 (35/80)/ 31.0 (18/58)	48/42 (90)	R-ST	Electrical	Micro-TESE		○			○

at the stage of primary spermatocytes (Pr-Sc) resumed spermatogenesis accompanied first and second meiosis and almost complete spermiogenesis could be found.

6.2 | Developmental potential of human spermatogenic cells co-cultured with Sertoli cells

In 2002, Sousa et al.[46] reported the new co-culture system using Vero cell conditioned medium with FSH and T for 2–3 weeks. Fertilization rate (%) and blastocyst rate (%) of R-ST and elongating spermatid were (37.5, 28.6) and (30.5, 42.9). However, most of the embryos did not reach the morula stage and showed major sex chromosome abnormalities.

6.3 | In vitro culture of human Pr-Sc with Vero cells

In 2003 Tanaka et al.[58] reported that human Pr-Sc which were collected from five nonobstructive azoospermic men whose spermatogenesis were arrested completed the meiosis through in vitro coculture with Vero cells. They were cultured on the feeder layer of Vero cells in the medium of MEM + 50% human synthetic oviduct fluid +10% human serum for a week. Chromosomal analyses were performed in all cleaved cells (two cells, four cells). The generation rate of round spermatids in various types of culture medium with Vero cells was 4.4% (5/120). However, no spermatid could be developed in the group without co-culturing with Vero cells.

6.4 | Developmental potential of elongating and elongated spermatids obtained after in vitro maturation of isolated round spermatids using co-culture on Vero cells

In 2001, Cremades et al.[59] reported the results of in vitro maturation of isolated R-ST in obstructive and nonobstructive azoospermia groups. Maturation rates of elongating spermatids (%), early elongated spermatids (%), and late elongated spermatids (%) in both groups were (31.5, 13, 9.3) and (23.4, 9.8, 4.3). Normal fertilization rate (%) and blastocyst rate (%) were (40.9 and 42.9). They did not seem to transfer these developed embryos into uterus after considering the potential risks of prion transmission and low entire replicability. The newly developed gametes have not completed spermiogenesis and stopped before becoming mature spermatozoa.

In 2009, Tanaka et al.[60] reported the first differentiation of human round spermatids into motile spermatozoa through in vitro coculture with Vero cells. Coculture condition in Tanaka et al. was almost the same as that of Cremades. Maturation rate into elongating spermatids (36.0%), elongated spermatids (14.0%), spermatozoa with an immotile flagellum (0%), and spermatozoa with a motile flagellum (0%) in both groups are shown. We could confirm two spermatozoa with normal shape of head and motile flagellum with intact midpiece for the first time in the world (Video S1). Our institutional Review Board did not allow clinical application of this treatment for azoospermia due to poor clinical outcome.

7 | THE CAUSES OF POOR CLINICAL OUTCOME OF ROSI IN THE PRECURSORS GROUP

7.1 | Insufficient identification of R-ST

The most accurate identification of R-ST cannot be made by morphological findings but by chromosomal analysis. R-ST is the only round cell among the spermatogenic cell which has finished two meiotic cell divisions. So, R-ST has the haploid set of 23 chromosomes. Spermatogonia (SG) which is very difficult to be differentiated from R-ST is a somatic cell with a diploid set of 46 chromosomes. In the beginning groups, identification of R-ST was made by cytologic characters; cell and nuclear size, round shape and smooth outline, acrosome granule, crescent acrosomal vesicle. Another identification method reported by Tesarik is the aspiration of presumptive R-ST into the injection pipette and if the cell is R-ST, it is not deformed and it never separates into the nucleus and cytoplasm. Almost all of cell findings described in early reports are correct but morphological change of aspirated presumptive round cell in a pipette was not correct (Figure 3).[20] Tesarik's description is characteristic of a somatic cell. The most difficult differentiation is between R-ST and spermatogonium, but few articles have reported about it.

Acrosomal vesicles or granules are found in about 20–30% (Figure 4).[19] of R-ST and these findings are very helpful to identify them.

SG are defined as the primordial germ cells which have migrated from the gut endoderm early in the fourth week toward the gonadal ridge via the dorsal mesentery by means of amoeba like movement and ceased its movement when they reached the seminiferous tubules. However, some of them were found to be continuing amoeba like movement in the seminiferous tubules. These cells which protrude active pseudopodia were identified as SG by the immunohistochemistry with alkaline phosphatase staining and γ-H2AX conjugated with fluorescein.[61] These cells were 8–10 μm in diameter, had a high N/C ratio and had one to two prominent nucleoli that were close to a distinct nuclear membrane (Figure 5).[19,20] These morphological characteristics became the conclusive evidence of SG. However, there is a limit to make a perfect differentiation morphologically. FISH analysis is useful to differentiate round cells whether it is diploid or haploid cell. FISH does not necessarily represent the whole chromosomal picture. Whole chromosomal analyses were conducted by injection of a presumptive R-ST into metaphase-2 oocyte derived from in vitro cultured immature cell after confirming

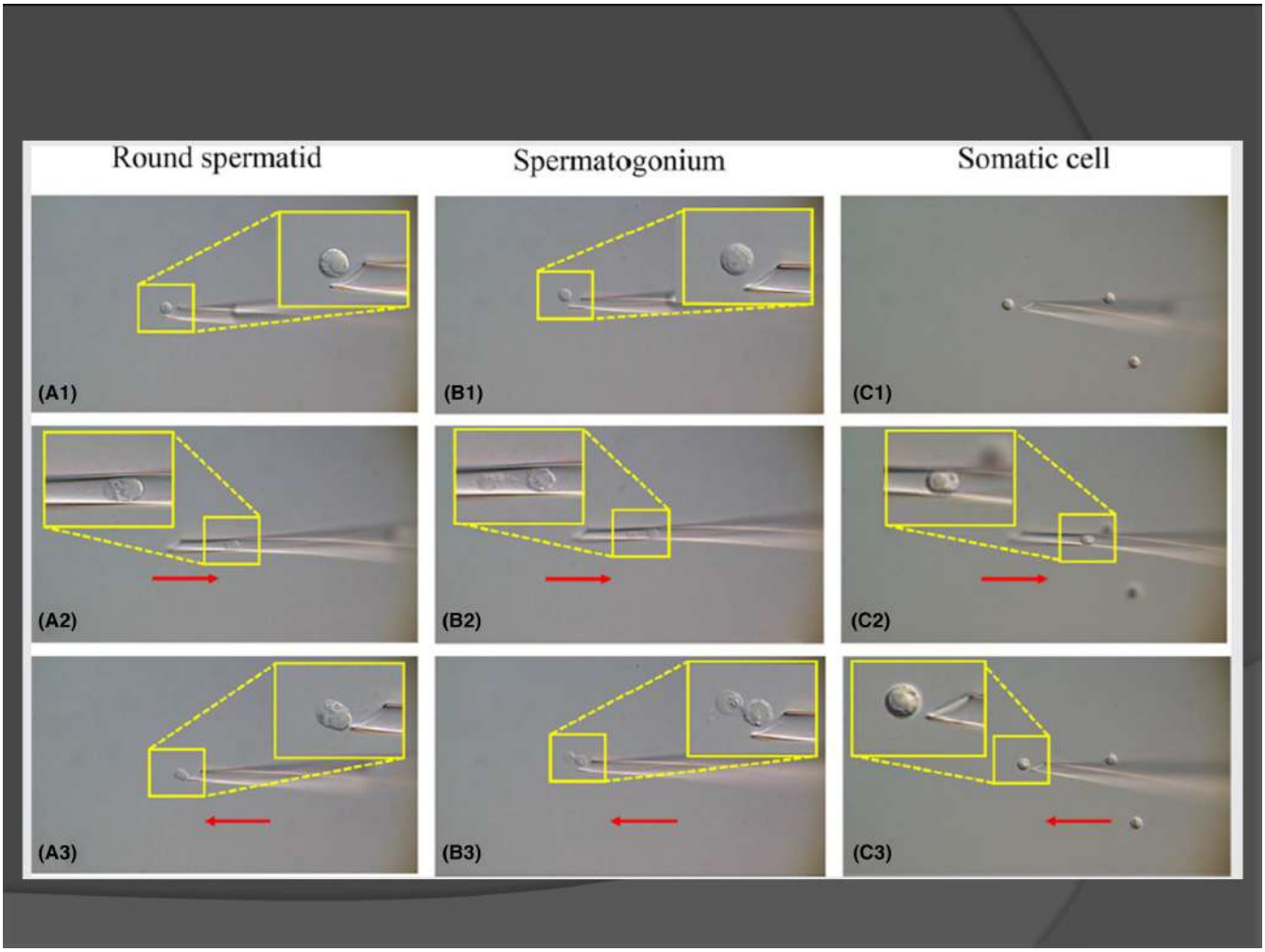

FIGURE 3 How to distinguish spermatids and spermatogonia from somatic cells. (A) When a spermatid is sucked in and out of a micropipette, its plasma membrane is readily broken and the nucleus and cytoplasm are separated. Its nucleus appears as a clear sphere. (B) A spermatogonium also can be separated into the nucleus and cytoplasm by pipetting. Its nucleus, unlike spermatid nucleus, contains clearly visible one or, more commonly, a few nucleoli. (C) Somatic cells (such as interstitial cells, fibroblasts, blood cells, and Leydig cells) released in the medium during maceration of seminiferous tubules have flexible plasma membranes which cannot be readily broken by pipetting (Magnification: 200× → zoom Magnification: 400×).

the patient's informed consent (GV,M-I staged oocyte at collection) by the effect of metaphase-promoting factor (MPF). Decisive identification of spermatogenic cells of each stage is made only by chromosomal analysis.

Spermatogonia and Pr-Sc have a diploid set of 46 chromosomes. In Pr-Sc, the crossing over that is peculiar only to Pr-Sc is found. When you could find the crossing over, you can say these cells are in the meiosis process, that is this cell is Pr-Sc. ST has a haploid set of 23 (Figure 6).[19]

There is a significant difference in the resolution of the optics used by the two groups. A Nomarski differential interference microscope has a much higher optical resolution than a Hoffman phase contrast microscope (Figure 7).[19]

One of the causes of lower clinical outcome of ROSI by the precursors group was that they used Hoffman phase contrast microscope for morphological observation, not a Nomarski differential interference contrast microscope which has much higher definition and can help obtain a more accurate identification of R-ST than when using a Hoffman phase contrast microscope.

7.2 | Insufficient oocyte activation

7.2.1 | What is oocyte activation?

The start of life begins at the encounter of the sperm and the oocyte. They fuse and develop into a new creature with the help of oocyte activation. Oocyte activation is the mother of our life.

Oocyte activation is the process by which the oocyte resumes the second meiotic division. During this process, a sperm cell triggers a series of calcium (Ca^{2+}) oscillations within the ooplasm which are involved in crucial events, such as the exocytosis of cortical granules, extrusion of the second polar body, regulation of gene expression, and the initiation of embryogenesis.[25]

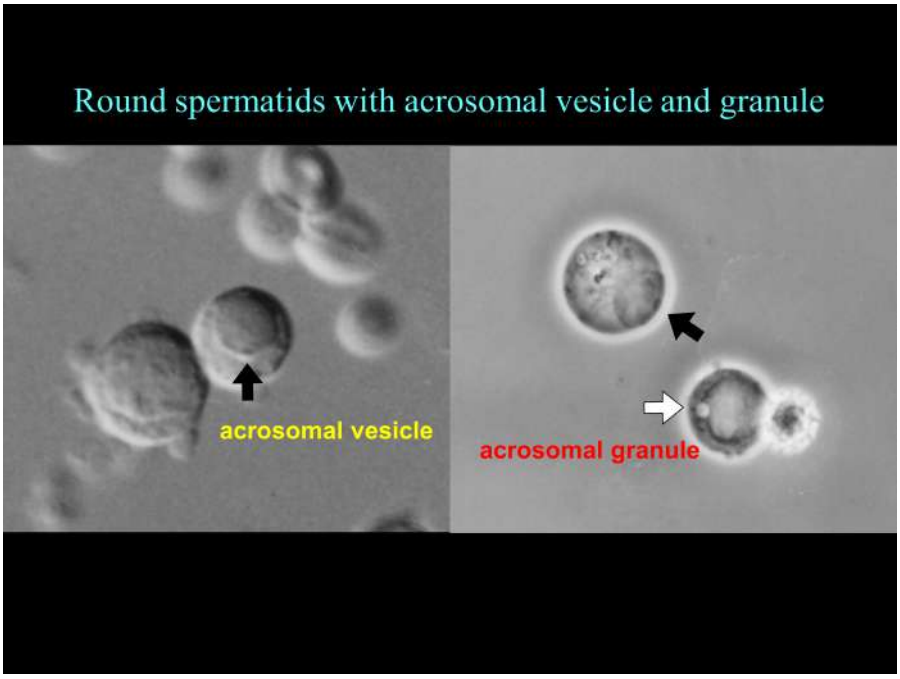

FIGURE 4 Acrosomal vesicle or granule is found in about 20–30% of round spermatids and these findings are very helpful to identify it (Magnification: 400×).

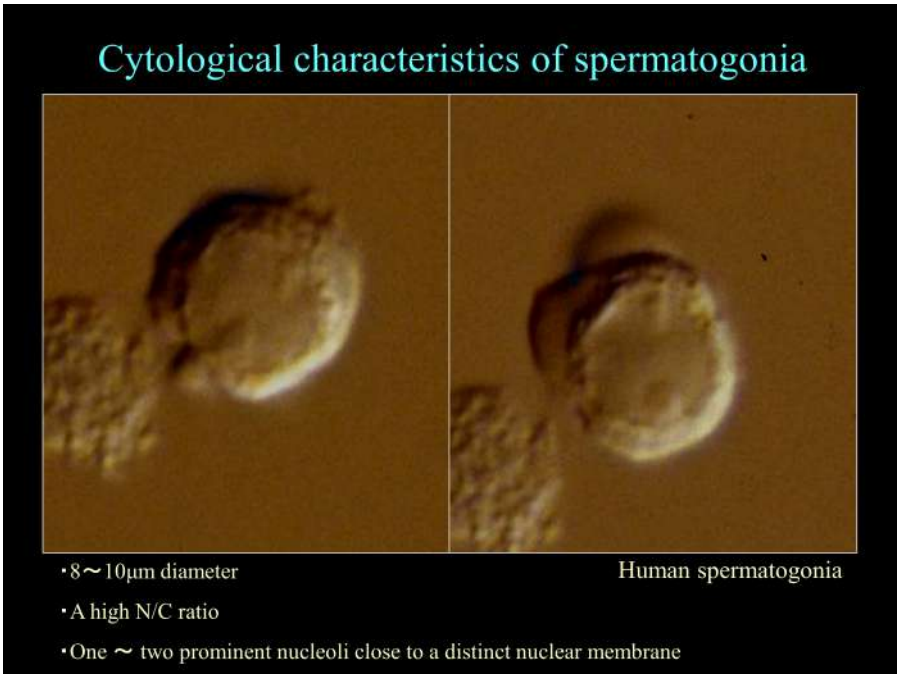

FIGURE 5 The cytological characteristics of SG is 8–10 mm diameter, a high N/C ratio one is two prominent nucleoli, close to a distinct nuclear membrane (Magnification: 400×).

7.2.2 | The mechanism of Ca^{2+} oscillation

Fusion of the oocyte with the spermatozoa is the physiological trigger of oocyte activation. Interaction between presumptive complementary receptors on the spermatozoa and oocyte plasma membranes triggers the activation of the G-protein that activates the production of inositol triphosphate (IP3) that releases Ca^{2+} from the endoplasmic reticulum. At fertilization, mammalian eggs show repetitive transient Ca rises each of which is due to Ca^{2+} release from the endoplasmic reticulum through IP3receptors. During fertilization, a factor from the sperm, the sperm factor, is released into the oocyte and induces series of Ca^{2+} spikes that are required for oocyte activation. They are known as Ca^{2+} oscillations.

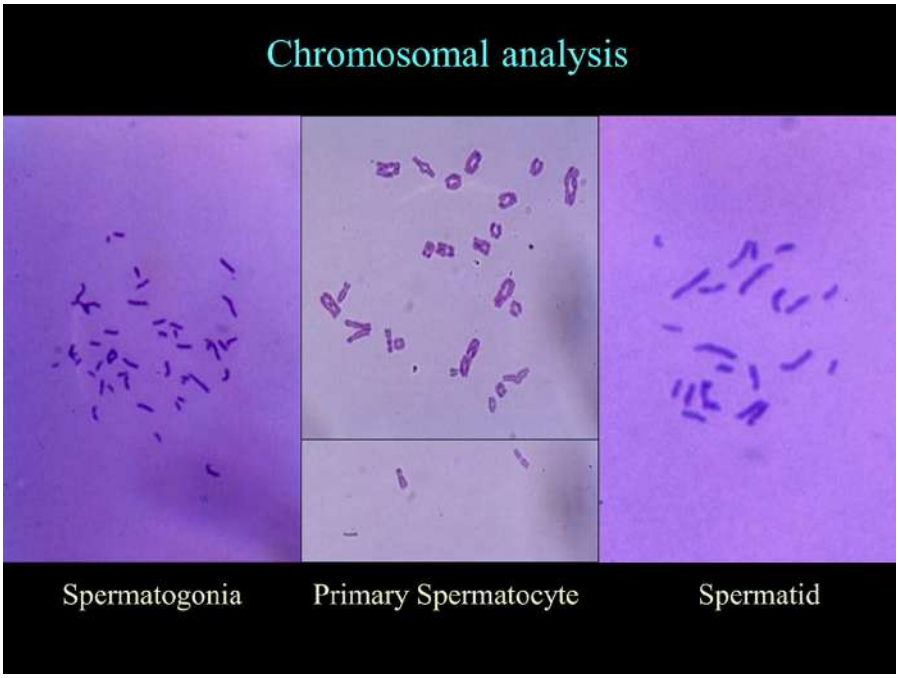

FIGURE 6 The result of chromosomal analysis. SG and Pr-Sc have a diploid set of 46 chromosomes. In Pr-Sc, the crossover that is peculiar only to Pr-Sc is found. When you could find the crossing over, you can say these cells are during the meiosis, that is, this cell is primary spermatocyte. ST has haploid set of 23 (Magnification: 400×). The center part of this figure is a composite photo put together for clarity and to keep the same level of magnification of the adjacent pictures.

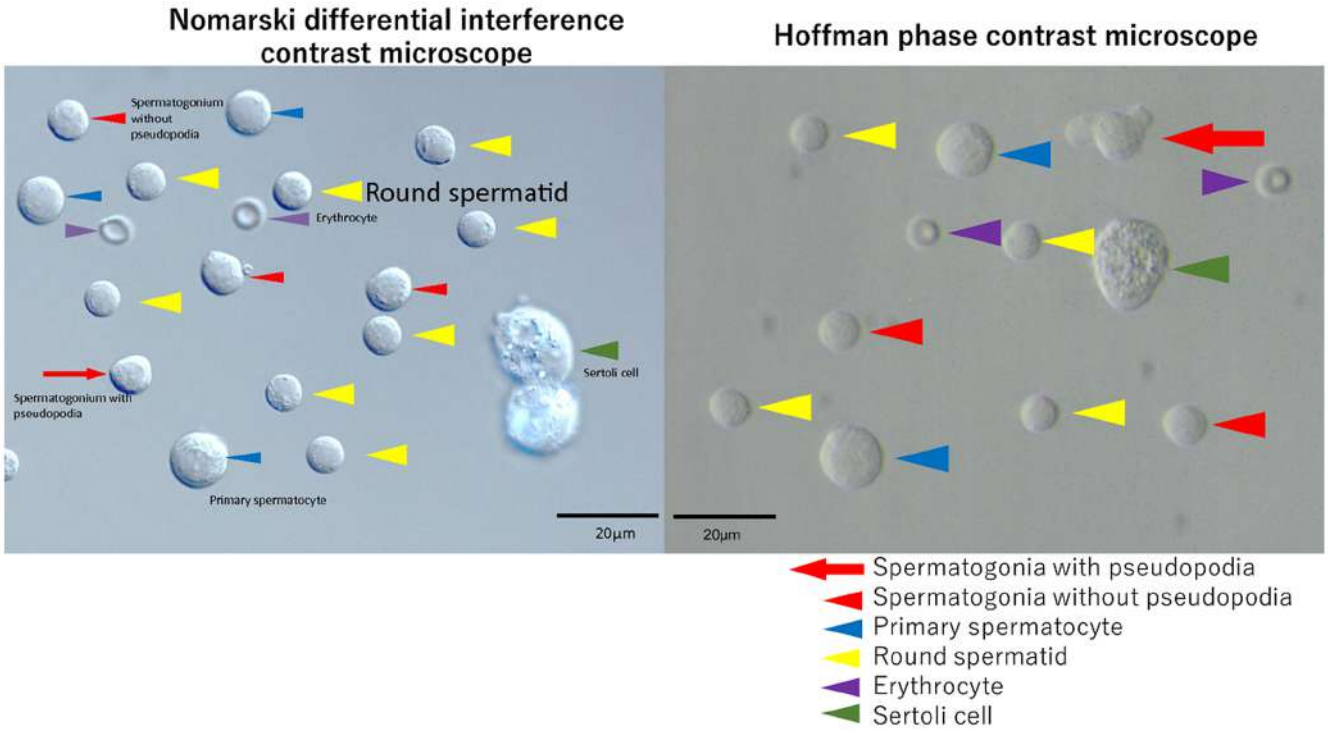

FIGURE 7 There is a big difference in the resolution of the optics used by the two groups. A Nomarski differential interference microscope has a much higher optical resolution than a Hoffman phase contrast microscope (Magnification: 400×).

7.2.3 | Which is the most effective oocyte activation?

We examined vigorous cytosolic aspiration, ionomycin, only ROSI, electric stimulation, and electric stimulation plus ROSI to find the most effective oocyte activation method. The oocytes were incubated in Ca^{2+}-sensitive fluorescent dye, Fluo8 first, and activated them in various stimulation and measured intracellular Ca^{2+} concentration using Ca^{2+} imaging. In the cytosolic aspiration and injection activation method, Ca^{2+} oscillations did not occur (Figure 8). In the ionocycin activation method, fluorescent intensity gradually decreased 5 min after treatment.(Figure 9) In ROSI alone no Ca^{2+} oscillation could be observed (Figure 10). Electrical stimulation showed the Ca^{2+} oscillation clearly about 15 min after the stimulation (Figure 11). However, height

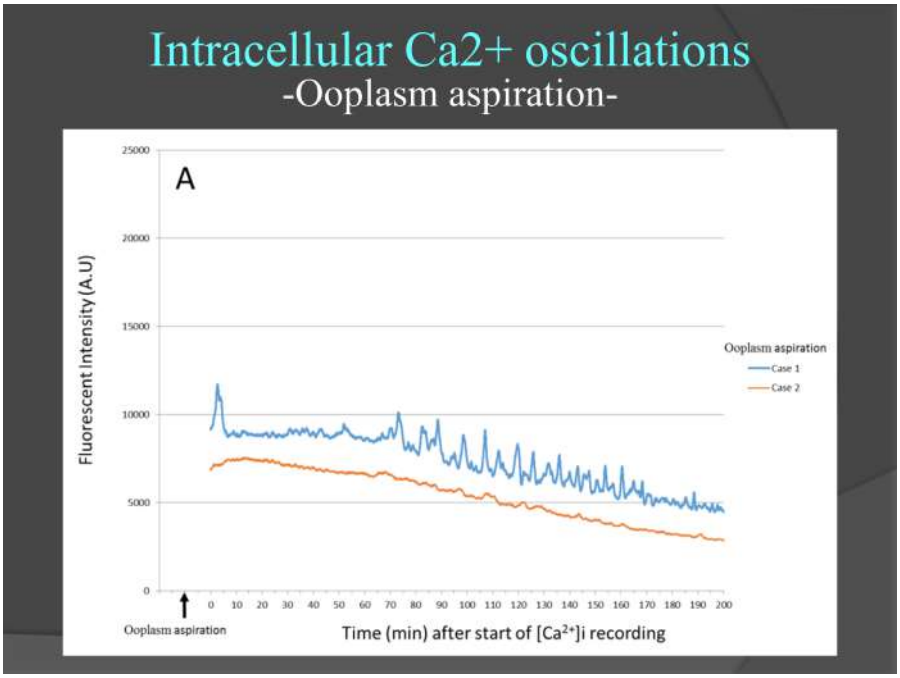

FIGURE 8 In the cytosolic aspiration and injection activation method, Ca oscillations did not occur.

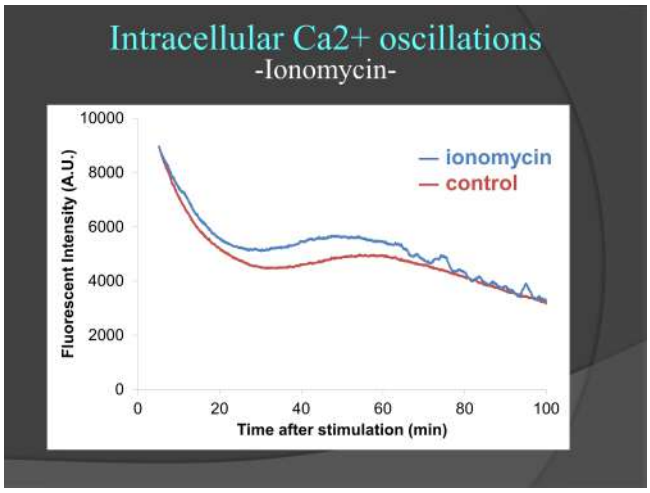

FIGURE 9 In the ionocycin activation method, fluorescent intensity gradually decreased 5 min after treatment.

interval and duration of spikes were irregular. Ca^{2+} oscillation of electric stimulation plus ROSI (Figure 12). Induced consistent large, repetitive Ca^{2+} oscillations. It was concluded that the best oocyte activation, so far, is electrical stimulation. The poor clinical outcome of precursor ROSI group could be caused by the lack of adequate aspiration oocyte activation. However, this conclusion might not always be true. A recent study reported that mouse ROSI was successful without pretreatment with SrCl2. This phenomenon might suggest the possibility that ROSI can be successful without oocyte activation. However, its likelihood might be very low. Yamaguchi et al, reported the establishment of appropriate methods for egg activation by human PLCZ1RNA injection into human oocyte in 2017,[28] PLCZ is expected to become a strong candidate for oocyte activation.

8 | EPIGENETIC MODIFICATION OF MALE GAMETE DNA

The risk of epigenetic abnormalities had been pointed out since the first successful report by Tesarik and some of following papers.

The conclusive disadvantage of ROSI seems to be incomplete transition edition of nuclear protein of histone in R-ST to protamine in matured spermatozoa.

DNA methylation and histone modification patterns, which are designed for normal embryonic development, are completely altered in male gamete chromatin before and after fertilization. Recently, abnormality of such an epigenetic modification has been a source of concern in children born after artificial reproductive technology (ART) interventions, including ROSI. Since no proven abnormality has been reported in children delivered after ROSI,[19,20] ROSI embryos with serious epigenetic abnormalities may be eliminated. However, the risk of epigenetic abnormality must be discussed when the establishment level of epigenetic modification in the round spermatids and the low implantation rate in ROSI embryos is considered. The process of epigenetic modification in mammalian male gamete is summarized as follows.

8.1 | DNA methylation

In vertebrate cell division, gene expression patterns are inherited to progeny cells with methylation of the cytosine nucleotides (5mC) in the CpG sequence, where a cytosine nucleotide is adjacent to a guanine nucleotide along 5′–3′ direction.[62] DNA methylation typically works to repress transcription by preventing the binding of transcription factors, which is required for initiation of gene

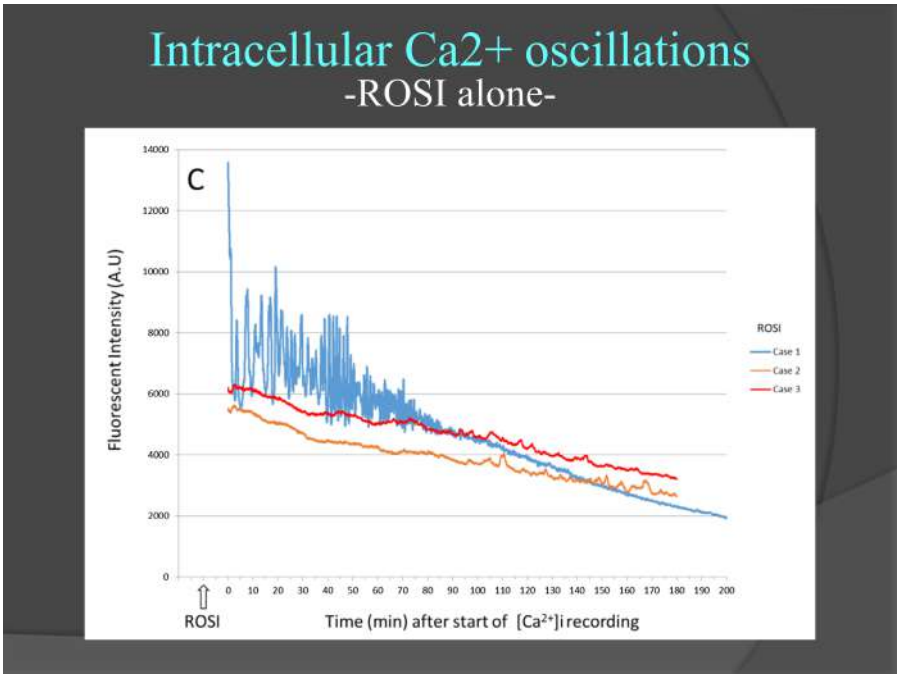

FIGURE 10 No Ca^{2+} oscillation could be observed after ROSI alone.

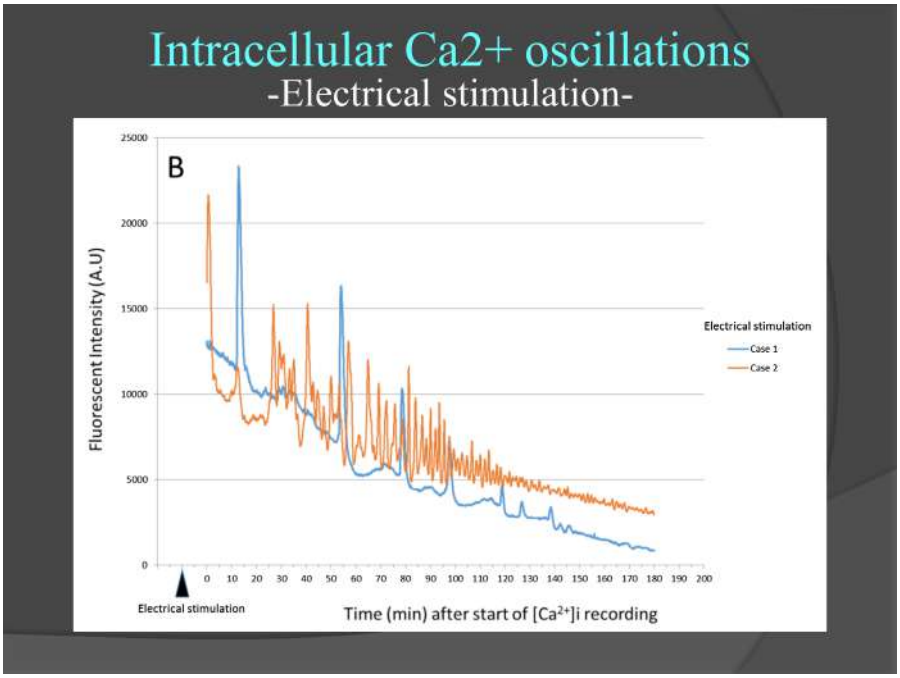

FIGURE 11 Clear Ca^{2+} oscillation after electrical stimulation. The first spike appeared about 15 min after the stimulation. However, height interval and duration of spikes were irregular.

expression.[63–65] In addition, DNA methylation is essential for X-inactivation and expression of parental imprinted genes.[66] Although the patterns of DNA methylation are maintained in proliferated and differentiated cells by maintenance methyl transferases during DNA replication and de novo methyl transferases, global demethylation and successive remodeling of methylation patterns occur in gamete genesis and postfertilization cell cleavage. In the paternal germ line, primordial germ cells undergo genome-wide demethylation during migration from yolk sack to the genital ridge[67] and then reestablishment of methylation patterns occurs through meiosis and spermiogenesis.[68] In rats, there are differential DNA methylation regions modified among the round spermatid, maturating sperm, and mature

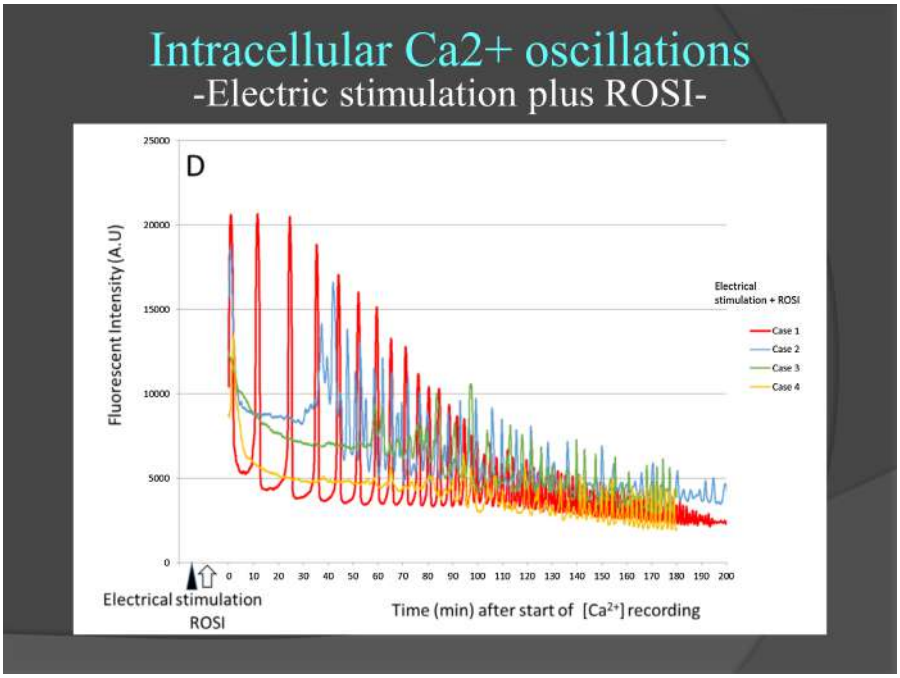

FIGURE 12 Ca^{2+} oscillation of electric stimulation plus ROSI. It induced consistent large, repetitive Ca^{2+} oscillations.

sperm stages, suggesting a possibility that epigenetic modification appears to be still incomplete in round spermatids, when meiosis has been completed.

Shortly after fertilization, another broad demethylation and successive reestablishment of methylation occurs in both sperm and oocyte nuclei. However, only paternal nuclear DNA is modified in a manner that is independent with DNA replication. 5mC in the paternal CpG sites, except for those of imprinted genes, is quickly converted to 5-hydroxymethylcytosine (5hmC) by enzymatic activity of TET proteins[69–72] before the first embryonic DNA replication. Subsequently, a gradual decrease in 5hmC is caused depending on DNA replication in the paternal DNA, although demethylation which is initiated by DNA methyltransferase 3 mainly occurs in the maternal DNA.[73–75] Zygotic DNA methylation patterns have been reestablished in the inner cell mass at the blastocyst stage.[62]

8.2 | Histone modification

In addition to DNA methylation, histone modification also regulates gene expression patterns in embryonic development. Four types of core histones (H2A, H2B, H3, and H4) form the nucleosome along with 147 base pair of double stranded DNA,[76] and 3′ terminals of each core histones extended from the histone cores are subjected to various modifications, including the acetylation or the mono-, di-, and trimethylation of lysines.[77] Histone acetylation, which is caused by histone acetyl transferases, is linked to transcriptional activation, changes the configuration of nucleosomes exposing DNA areas required for the transcription.[78] However, histone deacetylation, which is the opposite action of histone acetylation, is linked to transcriptional repression activity. The histone deacetylases are recruited to the histone tails of the nucleosomes with methylated CpG sites, resulting in chromatin condensation that diminishes accessibility for transcription factors.[77,79,80] At the deacetylated sites, histone methylation appears to follow demethylation.[79,81] Histone methylation which is catalyzed by histone methyl transferases on arginine or lysine residues of histone tails[81] controls either gene expression or repression. Methylation formed in the nucleosome tails of promotor or body regions of active genes (methylation of H3K4 or H3K36 residues) is linked to transcriptional activation. Methylation on H3K9 residues appears to play a role to suppress transcription interacting with DNA methyl transferases.[62,82]

In spermiogenesis, round spermatids replace nuclear proteins with protamines instead of the core histones to transform into mature sperm while completing DNA methylation patterning. After mature sperm with a tightly condensed nucleus penetrate oocytes, decondensation and chromatin remodeling (re-replacement of protamines into histones) of the sperm nucleus are promoted by nucleoplasmins contained in ooplasm in a few hours,[83–85] and then a male pronucleus is formed. During the specific chromatin remodeling, histone H3K9 trimethylation that is linked to gene silencing is maintained at a very low level in the male pronucleus, compared to the female pronucleus,[86,87] although high level of H3 and H4 acetylation is maintained in both male and female pronuclei.[86,88]

In ROSI embryos, the lack of methylation and histone rearrangement that must be established in spermatogenesis would have to be restored for normal embryonic development and successful delivery.

9 | EPIGENETIC ABNORMALITY IN ROSI EMBRYOS

Previous ROSI studies in mice reported that the low rate of the offspring delivery is owing to epigenetic abnormality at the early embryonic stage. In 1994, Ogura et al.[8] reported that in mice normal offspring was born with oocyte fusion of spermatids, when DNA methylation had been partially established, although the success rate was very low. A comparative study between ICSI and ROSI mouse embryos revealed that epigenetic errors are associated with the poor development of ROSI embryos.[86] In 90% of mouse ROSI embryos, significant hypermethylation was found in male pronucleus, as compared with one in ICSI embryos. DNA methylation patterns of injected spermatids appears to be directly copied by maintenance methyltransferase through DNA replication, since spermatid nucleus declines to skip demethylation process by transformation of 5mC to 5hmC which runs parallel to histone-protamine replacement. Consequently, the ROSI embryos of which male pronucleus DNA was normally demethylated by chance appears to be successfully delivered.[23] The distinct demethylation level of male pronuclear DNA was associated with body sizes of E11.5 ROSI fetuses. Zhu et al.[89] showed a possibility that decreased cell proliferation depending on hypermethylation of Rec 8 promoter regions may result in the smaller body sizes at the E11.5 stage.

However, genetic screening of ART children has revealed a possibility that similar imprinting disorders arise during in vitro culture of early cleavage embryos. In mice, serum components used for in vitro culture of early cleavage embryos decreased the expression of imprinting genes, which are generally methylated, in fetuses.[90] Many studies have also reported that demethylation of imprinted genes, such as H19, is associated with the operation of ART in mice and humans.[91–95] However, Novakovic et al.[91] showed with peripheral blood cells that ART-associated methylation disorder found in ART-neonatal children largely resolves by adulthood, suggesting no direct evidence of serious effects on their growth and health. This is a hopeful outcome that dispels a concern about abnormal methylation at the early cleavage stages. Further studies would be important to track the fate of embryonic methylation abnormalities.

10 | RECOVERY OF POOR ROSI EMBRYO DEVELOPMENT

In order to improve the success rate of ROSI, the application of several substances which inhibit abnormal DNA methylation of spermatid-derived pronucleus to the culture of mouse ROSI embryos has been attempted. Trichostatin A[96,97] and Scriptaid[21,98] inhibit histone deacetylation and successively enhance transcriptional activity and protein expression. Postfertilization treatment of mouse ROSI embryos with Trichostatin A for 20h reduced the hypermethylation level of the sperm-derived pronucleus to a level similar to that of the sperm-derived pronucleus.[86] Hosseini and Salehi[99] attempted to use mouse round spermatids exposed to Trichostatin A (100nM, 45min) prior to ROSI and confirmed similar effects that enhance blastocyst qualities (ICM number and ICM marker gene expression). Scriptaid treatment (250mM, 10h) of mouse ROSI embryos at the pronucleus stage more enhanced blastocyst formation and delivery rates restoring gene expression and abnormal DNA methylation.[21] Wang et al.[100] recently found that a compound "A366" which were selected by screening of the epigenetic modification-related small compound library was effective on normal development and delivery of mouse ROSI embryos improving epigenetic abnormalities (300nM, 20h). As Hosseini and Salehi,[99] confirmed, very brief exposure of round spermatids to these compounds may be equally effective and reduce predictable risks. In addition to such effectiveness, the studies used Scriptaid and A366 reported that the live offspring from mouse ROSI embryos were healthy and fertile. The use of these compounds must improve the success rate of ROSI in humans, although ethical concerns remain.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Self-renewal and differentiation of spermatogonial stem cells

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1. ABSTRACT

Mammalian spermatogenesis is a complex but well-coordinated process in which spermatogonial stem cells (SSC) of the testis develop to form spermatozoa. During testicular homeostasis, the spermatogonial stem cells self-renew to maintain the stem cell pool or differentiate to form a progeny of germ cells which sequentially transform to spermatozoa. Accumulating evidence from clinical data and diverse model organisms suggest that the fate of spermatogonial stem cells towards self-renewal or differentiation is governed by intrinsic signals within the cells and by extracellular signals from the SSC niche. Here, we review the past and the most recent developments in understanding the nature of spermatogonial stem cells and the regulation of their homeostasis in mice. We also review the potential clinical applications of spermatogonial stem cells in male infertility as well as in germline modification, by virtue of gene correction and conversion of somatic cells to biologically competent male germline cells.

2. INTRODUCTION

Men in most cases continue to be sexually competent until they are sixty years old, and if that limit be overpassed then until seventy years; and men have been actually known to procreate children at seventy years of age.

— Aristotle

These words by Aristotle signified that the continuity of fertility throughout life in men was noticed as early as in the 350 BC. Each day, approx. 100 million sperms are made in each human testicle, and each ejaculation releases 200 million sperms. During his lifetime, a human male can produce 10^{12} to 10^{13} sperms (1). Decades of research has led to the appreciation of the continuous nature of spermatogenesis as the reason for extended fertility in males compared to females. Mammalian spermatogenesis is a well-coordinated and a highly regulated process involving the sequential development of haploid spermatozoa from the diploid precursor germ cells in the testis. The testis is comprised of somatic cells, and a subset of undifferentiated spermatogonial cells (SSC), which can self-renew continuously or give rise to a progeny of germ cells at different stages of development until they mature to spermatocytes. Consequently, the high productivity and longevity of spermatogenesis relies primarily on the proliferation of SSCs.

The self-renewal and differentiation of SSCs during the initial steps of spermatogenesis produce heterogeneous SSC subpopulations under the regulation of multiple intrinsic and extrinsic factors, with each subpopulation differing in their stem cell properties. The extremely low number of SSCs and lack of SSC-specific markers had made the identification, isolation and study of these cells challenging. However, over the years, the development of spermatogonial transplantation techniques, efficient *in vitro* culturing, fluorescence-activated cell sorting (FACS), lineage tracing studies, single cell RNA sequencing (scRNA-seq) and mathematical modeling have made it possible to

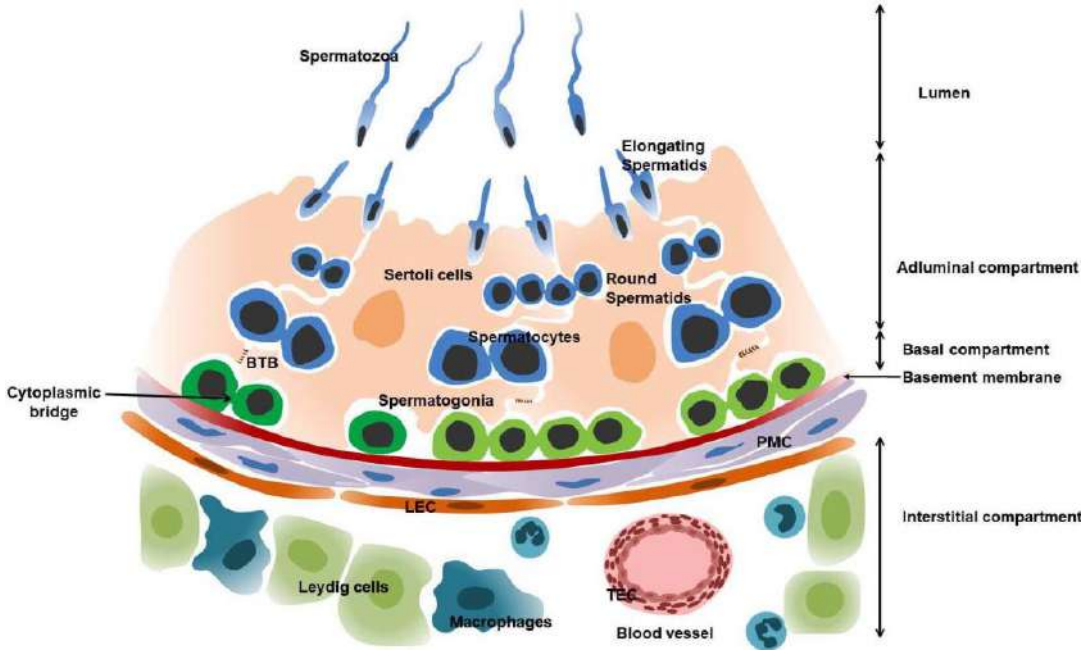

Figure 1. Schematic overview of tissue organization in mouse testis. Organization of the seminiferous epithelium shows hierarchy of germ cells supported by Sertoli cells and basement membrane. The primitive spermatogonia are localized next to the basement membrane in the basal compartment of seminiferous tubule. The basal compartment is followed by adluminal compartment, separated by the blood-testis barrier (BTB), wherein the spermatocytes derived from spermatogonia, round spermatids and elongating spermatids reside. The lumen shows presence of mature spermatozoa. The accessory somatic cells such as Leydig cells, peritubular myoid cells (PTMs), lymphatic endothelial cells (LECs), macrophages and testicular endothelial cells (TECs) of the vasculature reside in the interstitial compartment. The color key depicting different cell types is used throughout this article. Figure adapted with permission from Gauthier-Fisher *et al. (225)*.

decode the secrets of SSCs. The ease of handling and short reproductive lifespan makes the mouse a preferred animal model for reproductive biology studies. As a result, mouse testis is one of the well-studied and well-understood systems for spermatogenesis. This chapter summarizes our understanding on the self-renewal and differentiation of mouse SSCs and the possible clinical implications emerging from this knowledge base.

3. OVERVIEW OF MOUSE SPERMATOGENESIS

3.1. Site of spermatogenesis

Spermatogenesis takes place in the seminiferous tubules of the testis (2), which form long convoluted loops that pass into the mediastinum and join a network of tubules called the rete testis. The seminiferous tubules harbor the seminiferous epithelium which contains the somatic Sertoli cells supporting the male germ cells at various stages of development. Surrounding the seminiferous epithelium is a layer of basement membrane (basal lamina). Between the tubules is the interstitial space that contains blood and lymphatic vessels, immune cells including macrophages and lymphocytes and Leydig cells (Figure 1). The spermatozoa exit the testis via the rete testis and enter the efferent ductules prior to their passage through the epididymis where they undergo maturation. From the epididymis, the spermatozoa enter the vas deferens for ultimate ejaculation.

The undifferentiated spermatogonial cells lie along the basal lamina at the periphery of the tubule interspersed between Sertoli cells. Adjacent Sertoli cells form specialized tight junctions that divide the seminiferous tubule into the basal compartment, in which spermatogonia reside and the adluminal compartment that is occupied by

differentiating germ cells. The tight junctions also constitute the blood-testis barrier which is a semi-permeable barrier that prevents immune system cells from infiltrating the lumen of seminiferous epithelium; making the testis an immune-privileged site. In the adluminal compartment, preleptotene spermatocytes, derived from spermatogonia in the basal compartment, undergo meiosis and subsequently go through successive stages of primary (leptotene, zygotene, pachytene and diplotene) and secondary spermatocytes. The end-products of meiosis are round spermatids that undergo morphological changes by the process of spermiogenesis and give rise to elongating spermatozoa occupying positions closer to the lumen. The mature spermatozoa are ultimately released into the lumen of the tubule by the process of spermiation (Figure 1).

3.2. Organization and timing of spermatogenesis

Spermatogenesis in mammals is organized and timed in a manner that maximizes sperm production. The central aspect of this organization and timing is the 'wave' and the 'cycle' of seminiferous epithelium. The seminiferous epithelium is characterized by asynchronous repeating series of germ cell associations. As these cells progressively differentiate, the initial associations are observed again after a fixed interval (8.6 days in mice and 16 days in humans), as the individual cells have shifted to the next layer. This periodic change in the seminiferous epithelium is called the 'seminiferous epithelial cycle' and was first discovered in rat testes (3). The seminiferous epithelial cycle is divided into stages I through XII in mice. The repetitive patterning of the epithelial stages along the length of a seminiferous tubule is called the spermatogenic wave. The 'cycle' and the 'wave' of seminiferous epithelium represent the key for asynchronous germ cell differentiation, allowing the constant production of spermatozoa (4). Following the first wave of spermatogenesis, which proceeds at a quicker rate than the adult cycles, regular cycles of asynchronous sperm production begin, each lasting approx. 35-36 days in mice. The spatiotemporal coordination of the cycle relies on intrinsic signals from the germ cells and extrinsic signals from the somatic support cells. It is established that retinoic acid (RA) is one of the major signaling molecules responsible for regulating the distinctive cycle and wave formation that induce spermatogonial cell differentiation (5).

3.3. Types of spermatogonia

The spermatogonial cell (SPG) population is enormously heterogeneous with respect to morphology, phenotype and function. SPGs can be broadly classified as undifferentiated cells that display the stemness or the progenitor properties to varying extents, and differentiating cells that have characteristics of being committed to enter meiosis. The undifferentiated SPGs include A- type spermatogonia (A_{undiff}) which in the mouse are found as single cells (A-single, A_s) or as syncytia of typically 2, 4, 8 and 16 cells interconnected by cytoplasmic bridges (A-paired, A_{pr} and A-aligned, A_{al4-16}). While a minority among the population of A_{undiff} has stem cell activity and functions as SSCs, a subset of A_{undiff} cells (A_{al} cells) has transit-amplifying roles and functions as progenitors. The differentiating SPGs are cells that are committed to meiosis and include A_1 SPG which undergoes sequential mitotic divisions to produce A_2, A_3, A_4, Intermediate (In) and type B spermatogonia. The A_{undiff} can be distinguished morphologically from the differentiating SPG by the absence of heterochromatin in the nuclei. In-type spermatogonia contain a moderate amount of heterochromatin, whereas, B-type spermatogonia display clumps of heterochromatin around the periphery of the nuclei (6). Thus, due to numerous transit amplifying divisions, one mouse SSC has the potential to produce up to 4096 sperm cells in a single spermatogenic cycle (7), although this has been shown to be a highly overestimated calculation which has not considered the significant level of apoptosis occurring at the A_{undiff} stage (8–10).

4. SPERMATOGONIAL STEM CELLS

4.1. Origin of SSCs

In metazoans, primordial germ cells (PGC) are the progenitors for both male and female gametes, giving rise to spermatozoa and oocytes, respectively. In mice, the precursors of PGCs arise

at about embryonic day 6 (E6) from the equipotent epiblast cells in response to bone morphogenetic protein (BMP) signals emanating from the extraembryonic ectoderm (BMP4 and BMP8b) and visceral endoderm (BMP2) (11–14). B lymphocyte-induced maturation protein-1 (BLIMP1), PR-domain containing protein 14 (PRDM14) and transcription factor AP-2 gamma (TFAP2C) form a tripartite transcription factor network that facilitates mouse PGC specification by suppressing somatic gene expression of homeobox A1 (*Hoxa1)*, homeobox B1 (*Hoxb1)*, LIM homeobox 1 (*Lim1*), even-skipped homeobox 1 (*Evx1)*, fibroblast growth factor 8 (*Fgf8)* and snail family transcriptional repressor 1 (*Snai11)* genes, while initiating the germ cell transcriptional program and triggering genome-wide epigenetic reprogramming. From E7 onwards, the specified PGCs express the PGC-specific markers, viz., tissue non-specific alkaline phosphatase (TNAP), stage-specific embryonic antigen 1 (SSEA1) and developmental pluripotency associated 3 (DPPA3 or STELLA) (11, 15–17). However, PGC specification in the mouse and human exhibits some differences. The origin of human PGCs from mesodermal precursors, the requirement of the Wingless/integrase 1 (WNT) pathway along with BMP signaling for development of PGCs and lack of PRDM14 and SRY (sex determining region Y)-box 2 (SOX2) expression in human PGCs are the major contrasting differences (18, 19). PGCs proliferate while migrating through the hindgut and colonize the genital ridges (the future gonads) between E7.5 and E11. The proliferation and directional migration of PGCs are facilitated by two germ cell-soma signaling pathways: cKIT-STEEL (20) and stromal cell-derived factor 1 (SDF1)- C-X-C chemokine receptor type 4 (CXCR4) (21). Once in the genital ridges, PGCs undergo approximately five additional mitotic divisions from E10.5 to E14.5 with incomplete cytokinesis to form germline cysts. Around this time (E11.5), the testis development will be initiated by the somatic cells expressing FGF9/SRY/SOX9 proteins marking the Sertoli cell population. On the basis of the cues from these somatic cells, the germ cells also undergo sex differentiation and become developmentally restricted (22). The germ cells in the differentiating testis are now referred to as gonocytes or prospermatogonia.

In the female mice, the germ cells begin to enter meiosis at E13.5 in response to RA signaling from the mesonephros. In contrast, the gonocytes fail to enter meiosis in the developing testis due to expression of CYP26B1 in the Sertoli cells, which catalyzes the oxidation of RA into inactive metabolites. Instead, the gonocytes exit the cell cycle, get arrested at G0, and remain quiescent from approximately E14.5 until postnatal day 1-2 (P1-2). They re-enter the cell cycle on P3 and migrate to the basement membrane of the seminiferous epithelium. The subset of gonocytes that express neurogenin 3 (NGN3) transforms to form the founding SSC population in mice between P3-6. Those gonocytes which lack NGN3 expression directly differentiate into progenitor spermatogonia that undergo further differentiation, initiating the first wave of spermatogenesis at approximately P3 (23, 24). Thus, the first wave of spermatogenesis occurs without the contribution of SSC activity. Intriguingly, a recent study using scRNA-seq analysis revealed the presence of cell populations with characteristics of PGCs (referred to as PGC-like cells, PGCLCs) and SSCs (referred to as prespermatogonia, PreSPG) in human neonatal (day 2 and Day 7) testis (25). The authors hypothesized a model in which human fetal PGCs differentiate into PGCLCs and, subsequently, PreSPGs, both of which populate the human testes at birth. These neonatal germ cells are replaced by SSCs during the first year of the male child.

4.2. Kinetics of SSC cell division

It is unanimously accepted that SSCs are contained within the A_{undiff} pool. However, which cells among the A_{undiff} contribute to the SSC pool is an area of debate till date. A_s cells were traditionally regarded as the actual stem cells, whereas A_{pr} and A_{al} were thought to represent transit-amplifying progenitors (26). However, with the advent of improved experimental tools and molecular markers, it became apparent that stem cell potential may not be limited to A_s cells alone and may extend to A_{pr} and A_{al} cells also and that the developmental hierarchy of A_{undiff} cells is more complex than originally anticipated. This resulted in the proposal of three different models to explain the true identity of SSCs, viz., the A_s model, the revised A_s model and the fragmentation model, which are described in the following paragraphs.

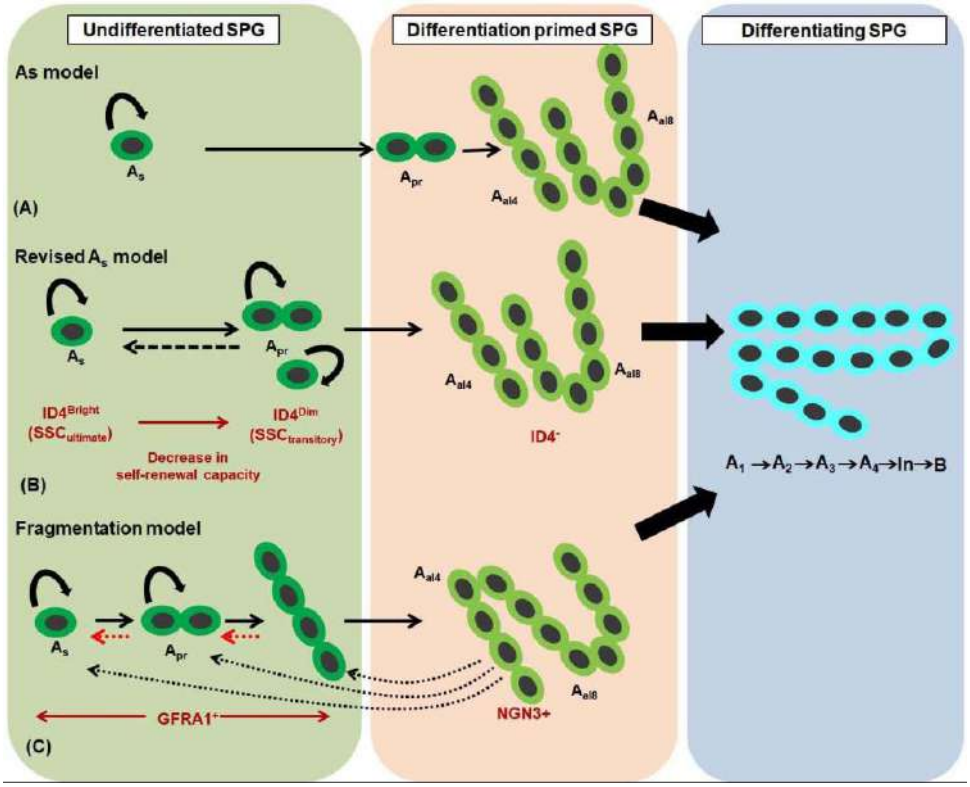

Figure 2. Kinetics of SSC division in adult mouse testis. A) According to A_{single} (A_s) model, the self-renewal (curved arrow) capacity is restricted only to A_s cells of the undifferentiated A type (A_{undiff}) spermatogonial cell (SPG) population that divide (solid arrows) into two daughter cells interconnected by cytoplasmic bridges called A_{paired} (A_{pr}) cells which subsequently divide to form longer syncytia of 4, 8 and 16 cells termed as $A_{aligned\ 4,\ 8,\ 16}$ ($A_{al4,\ 8,\ 16}$). The A_{al} cells finally differentiate (solid block arrow) into differentiating SPGs including A_1, A_2, A_3, A_4, Intermediate (In) and B type SPGs. Thus, the A_s cells constitute the SSC pool. B) In the revised A_s model the population of A_s SPGs is heterogeneous. The SSC activity resides in a subpopulation of A_s cells that express high levels of ID4 (termed as $ID4^{Bright}$ cells). In A_{undiff} cells (A_s and A_{pr}) that are produced subsequently, ID4 levels drop ($ID4^{Dim}$) and these cells exhibit a decreasing chance of self-renewal and subsequently form clones of A_{al}. The $ID4^{Bright}$ cells with the highest chance of self-renewal have been called $SSC_{ultimate}$ and $ID4^{Dim}$ cells with limited self-renewal capacity are called $SSC_{transitory}$ (with respect to the transit to progenitor state). The $SSC_{transitory}$ cells divide and give rise to $ID4^-$ differentiation-primed progenitor SPGs. Reversion of cell fate (dashed arrow) from $SSC_{transitory}$ to $SSC_{ultimate}$ state is possible but from a progenitor to stem state is strictly not possible under any conditions. C) The fragmentation or dynamic SSC model proposes that $GFRA1^+$ A_{undiff} cells continuously interconvert between equipotent single cell and short syncytial states via fragmentation (dashed red arrow). $GFRA1^+$ A_{undiff} also give rise to $NGN3^+$ progenitor cells that undergo differentiation priming. Under steady-state conditions $NGN3^+$ A_{undiff} do not typically revert back to the self-renewing state but in regenerative conditions $NGN3^+$ progenitor cells may revert to the SSC state (dashed curved arrows). The color key depicting different cell types is used throughout this article.

4.2.1. A_s model

In 1971, Huckins and Oakberg proposed the A_s model of spermatogonia multiplication, which was endorsed by most researchers in the field and was held for over 40 years (8, 9, 27, 28). This proliferation scheme was developed by studying whole-mounts of seminiferous tubules instead of sections, which enables one to observe the topographical arrangement of the spermatogonia on the tubule basal lamina. According to this model, only the A_s spermatogonia are the SSCs. SSCs divide and their daughter cells either migrate away from each other and become two new SSCs or they stay together (A_{pr}), constituting the first step along the differentiation pathway (differentiation-committed progenitors). Subsequently, the pairs can proliferate further to form A_{al} cells (Figure 2A). Thus, according to this model, there are two types of SSCs: reserve SSCs that function only in response to injury and active SSCs that divide slowly on a regular basis to maintain homeostasis.

4.2.2. Revised A_s model

The number of A_s cells in the adult mouse testis is estimated to be approximately 35,000 (29). However, following the transplantation of an unselected total donor testis cell population in recipient testes, the number of regenerated spermatogenic colonies was only 3000. (30). As this was less than 10% of the expected value, it implied that not all A_s are stem cells. Studies by Oatley *et al*. and others, using transplantation and lineage tracing experiments, concluded that SSC activity is almost exclusively contained within a fraction of A_s cells marked by expression of transcription factor known as inhibitor of DNA binding 4 (ID4), supporting a "revised" A_s model in which stem cell activity is said to be limited to a subset of A_s (termed as $SSC_{ultimate}$) while remaining A_s, A_{pr} and A_{al} cells are transiting into a differentiation-committed state (31–36). This model is also termed the 'hierarchical A_s model', as it suggests the existence of SSC hierarchy. Furthermore, this model proposes that the cells expressing high levels of ID4 (termed as $ID4^{Bright}$ population) has the greatest capacity of self-renewal and that the capacity for self-renewal decreases as ID4 expression among A_s cells regresses from bright via intermediate to dim ($SSC_{transitory}$) (35). This model also supports that some plasticity may exist for A_{undiff} at the early phase of transition from $SSC_{ultimate}$ to $SSC_{transitory}$ population which is at the progenitor state (Figure 2B). However, recent reports analyzing *Id4* expression by scRNA-seq, immunostaining and reporter assays have shown that *Id4* expression is substantially more widespread within the A_{undiff} cells than previously described, indicating that *Id4* expression may not be limited to SSCs (37–40). *Id4* expression in A_{undiff} cell fractions with the highest SSC capacity has also been disputed (40), further questioning the validity of this model.

4.2.3. Fragmentation model

Yoshida *et al* performed a series of lineage tracing and live imaging experiments to monitor SPGs in transgenic reporter mice models (41–43). They analyzed the expression of glial cell line derived neurotrophic factor (GDNF) receptor alpha 1 (GFRA1), which marks early A_s and A_{pr} SPGs that do not yet express a differentiation marker and the expression of the differentiation marker NGN3, which marks A_s, A_{pr} and A_{al} SPGs that may have taken a first step towards differentiation. Under the steady state, the SSC pool comprising of all GFRA1 expressing A_{undiff} transition into $GFRA1^-/NGN3^+$ cells, which are assumed to be the progenitor cells with increased differentiation propensity (Figure 2C). Notably, in a direct contradiction to traditional schools of thought that depicted the progenitors were irreversibly committed to a differentiating fate, it was reported that, under regenerative conditions in the testis (during restoration of spermatogenesis after transplantation into an infertile recipient testis or during regeneration after tissue injury), the $NGN3^+$ progenitor population can also experience fragmentation, with single progenitor spermatogonia breaking off from chains and reverting to a $GFRA1^+$ state to re-join the self-renewing pool (42, 44). Thus, the fragmentation model proposes that A_s cells almost always divide into A_{pr} and that the A_{undiff} syncytia (A_{pr} and A_{al}) can fragment into singles and pairs to replenish the self-renewing SSC pool (43). Due to the dynamic nature of A_{undiff} proposed here, this model is also referred to as the 'dynamic SSC model' (45). Currently, evidences for these phenomena are based primarily on observations of fragmentation using live imaging of testes in mice that are maintained in a stress condition of long-term anesthesia and computer generated biophysical models. Further, qualms also exist regarding the specificity of GFRA1 and NGN3 as markers for the SSC and progenitor populations respectively. However, the lack of functional evidence for self-renewal capacity in the fragmented cells and for the mechanisms regulating the fragmentation of A_{undiff} syncytia compels further investigation into this proposed model.

It can be argued that the differences between the revised A_s model and the fragmentation model are rather insignificant under steady state conditions as they primarily differ in the proposed mechanism for maintenance of the A_s population, i.e., by complete cytokinesis vs syncytial fragmentation respectively. Both models claim that the SSC capacity is restricted to A_s and A_{pr} cells in contrast to only A_s cells as proposed by the A_s model. In conclusion, the A_{undiff} population displays in-built heterogeneity and has their propensity for alteration or reversion of gene expression profiles in response to different requirements within the niche.

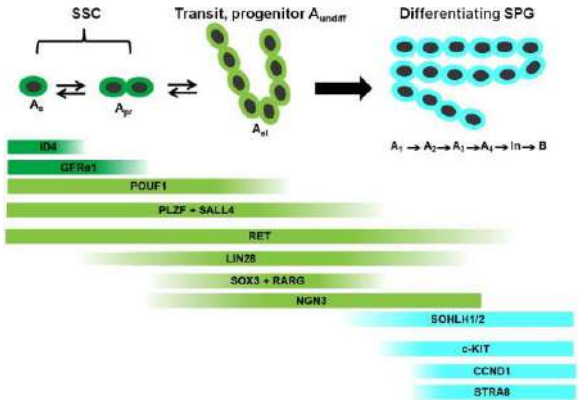

Figure 3. A schematic showing the expression pattern of markers that define different subsets of spermatogonial cells (SPGs). The transcription factor ID4 is an A_{single} (A_s) specific marker. The membrane receptors GFRA1 and RET bind the ligand GDNF, which is crucial for stem cell self-renewal. GFRA1 marks undifferentiated A type SPGs (A_{undiff}) including A_s and A_{paired} (A_{pr}) which exhibit SSC activity (marked in dark green) whereas RET expression is found across the A_{undiff} cells population (A_s, A_{pr}, A_{al}). PLZF and POU5F1 are transcription factors expressed by SSCs and A_{undiff}. The progenitor (marked in light green) A_{undiff} cells (A_{pr} and A_{al}) which arise on retinoic acid (RA) stimulation are marked by the expression of LIN28, RARG, SOX3 and NGN3. The progenitor cells under the influence of RA differentiate into differentiating SPGs (A_1-A_2-A_3-A_4-Intermediate (In)-B). The differentiating SPGs (marked in light blue) are characterized by the expression of SOHLH1/2, c-KIT, CCND1 and STRA8, which will induce them to enter meiosis and give rise to primary spermatocytes. The color key depicting different cell types is used throughout this article.

4.3. Markers of SSCs

Mice have 2-3 × 10^4 SSCs in the testis, which comprise only 0.02%-0.03% of the total germ cell population (29). Therefore, the identification of the phenotype of SSCs is critical for the functional investigation of SSCs at the single-cell level. Several methods to enrich SSCs from testes have been developed, including differential plating, density-gradient centrifugation, experimental surgical cryptorchidism, and antibody-based selection methods such as FACS and magnetic-activated cell sorting (MACS). An approach using FACS/ MACS together with a functional transplantation assay was widely used to isolate SSCs (46, 47). Currently, with the advent of fluorescence tagged proteins, cell lineage-tracing experiments are being used to study SSCs.

Flow cytometry-based transplantation experiments showed that the stem cell activity was concentrated in fractions of mouse SPGs with surface antigenic profile as follows - alpha 6-integrin $(ITGA6)^+$, beta 1 integrin $(ITGB1)^+$, thymus cell antigen 1 $(THY1)^+$, $CD9^+$, $GFRA1^+$, epithelial cell adhesion molecule $(EPCAM)^+$, $CD24^+$, E-cadherin $(CDH1)^+$, melanoma cell adhesion molecule $(MCAM)^+$, KIT^- and major histocompatibility complex class I $(MHC\text{-}I)^-$ (46, 48–51). However, considering the dynamic nature of SSC hierarchy and the SPG heterogeneity, it is ambiguous to delineate a universal array of markers for SSCs.

Hence, it is important to note that the gene expression profiles of A_s, A_{pr}, and A_{al} spermatogonia are different (39, 42), as represented in Figure 3. The expression of promyelocytic leukemia zinc finger (PLZF or ZBTB16) and CDH1 is relatively constant in the A_s, A_{pr}, and A_{al} spermatogonia, and has been used to identify all A_{undiff} (49, 52). *Gfra1*, *Id4*, *Bmi1*, *Pax7*, *Nanos2*, *Lhx1*, *Bcl6b*, *Etv5*, *T (Brachyury)*, *Sall4* are shown to be preferentially expressed in A_s cells whereas *Pou5f1 (Oct4)*, *Ngn3*, *Lin28a*, *Sohlh1*, *Sox3* and *Rarg* are expressed by A_{al} progenitor cells (34, 39, 43, 53). Later studies have shown that SSC activity is evident in progenitor A_{al} cells (43, 54). Subsequently, it was also found that the state and function of A_{undiff} is context-dependent. Thus, there are different interconvertible subsets of A_{undiff} cells that contribute to SSC activity during steady state adult spermatogenesis, postnatal testicular development and under tissue regenerative conditions (discussed in detail in section 5.5). Hence, it is important to consider the expression profiles of these functionally distinct subsets of A_{undiff} when delineating SSC activity.

5. SPERMATOGONIAL STEM CELL NICHE

The microenvironment surrounding the stem cells is called the stem cell niche, which provides juxtacrine and paracrine factors that maintain stem cell competence and decide the fate of the stem cell towards self-renewal or differentiation. The interaction of testicular “niche” cells with SSCs occurs via both cellular contact and soluble signaling. Cellular components of the niche include Sertoli cells and germ cells of the tubules, peritubular cells (peritubular myoid cells and peritubular macrophages) and interstitial cells (Leydig cells, interstitial macrophages and vasculature).

In the basal compartment, A_{undiff} cells localize preferentially to the basement membrane in areas adjacent to the vasculature network of arterioles and venules that accompany the interstitial cells, including Leydig cells (43, 55, 56). Kitadate *et al*. recently demonstrated that the self-renewal and proliferation of SSCs are favored at areas of high fibroblast growth factor (FGF) concentration, lying in close proximity to the vasculature and interstitium. However, A_{undiff} cells do not cluster to a restricted domain, but intermingle and migrate between differentiating spermatogonia and immotile Sertoli cells (43). Since the SSC localization is not restricted to any specialized area in the niche, it is designated as an 'open' or 'facultative' niche, contrary to the canonical 'closed' or 'definitive' niche observed in other stem cell systems (57–59). Although research is still ongoing to decode the complex mechanism of the coexistence of a heterogeneous SSC pool and an open niche, it is well established that germ cell-niche interaction determines the density and the fate of SSCs.

5.1. Cellular components of SSC niche

5.1.1. Sertoli cells

Sertoli cells are arguably the most important component of the SSC niche. In addition to producing a number of factors essential for SSC maintenance such as GDNF, they also physically support, nurture and protect the SSCs (60). Intriguingly, Sertoli cells that have already terminated their cell cycle before puberty expand their plasma membrane to an extreme degree and simultaneously 'hold' germ cells of all four stages (spermatogonia, spermatocytes, round spermatids and elongating spermatids) at different areas of their plasma membrane. Lack of a report describing a germ-cell-only tubular phenotype implies that SSCs and more advanced germ cells cannot exist without Sertoli cells *in vivo*.

5.1.2. Peritubular myoid cells

Seminiferous tubules are encased by contractile smooth muscle cells called peritubular myoid cells (PMCs). Besides providing structural support and propelling the flow of luminal fluid towards the rete testis, PMCs also secrete paracrine factors important for SSCs, including GDNF, leukemia inhibitory factor (LIF) and CC-chemokine monocyte chemoattractant protein-1 (MCP-1) (61–63).

5.1.3. Testicular macrophages

The role of testicular macrophages (peritubular and interstitial macrophages) within the SSC niche is not well-understood, although some reports have led to the speculation that they potentially influence SSCs proliferation and differentiation either directly via colony stimulating factor 1 (CSF1) and RA synthesis or indirectly by influencing testosterone synthesis in Leydig cells through the production of 25- hydroxycholesterol, an intermediate compound within the testosterone biosynthetic pathway (64, 65). However, these claims have not been functionally validated.

5.1.4. Leydig Cells

Leydig cells, upon luteinizing hormone (LH) stimulation via LH receptors (LHR), regulate the expression levels of steroidogenic enzymes, such as 17-beta hydroxysteroid dehydrogenase, in order to increase the production of testosterone. While testosterone is strictly indispensable for spermatogenesis, it also regulates the expression of thousands of genes in different somatic cell populations in the testis under normal conditions. One of the targets of testosterone is the Sertoli cell-controlled attachment mechanisms (66, 67). Besides testosterone, Leydig cells also produce factors that directly target SSCs such as insulin-like growth factor 1 (IGF1) and CSF1.

5.1.5. Vasculature

Vasculature cells (testicular endothelial cells or TECs) are rich sources of several cytokines and growth factors such as vascular endothelial growth factor A (VEGFA) that are required for SSC maintenance and localization. It has been proposed that $ID4^+$ SPGs (A_s cells) are mainly localized at the avascular sections of the tubule whereas the $NGN3^+$ progenitor SPG cells, derived from A_s cells, relocate to vascular areas to fulfill their new requirements for different levels of oxygen, metabolites, and various growth factors (33, 56, 64).

Vasculature associated lymphatic endothelial cells (LECs) are found at the border of

seminiferous tubules and testicular interstitium, and cover the surface of the lymphatic space. LECs located in proximity to vasculature express a number of FGFs (FGF4, 5, and 8), that are shown to regulate the density of GFRA1$^+$ A_{undiff} (40).

5.2. Signaling pathways of SSC niche

The vital soluble niche factors include GDNF, FGF, RA, follicle stimulating hormone (FSH), testosterone, CSF1, WNT and NOTCH. These somatic cell-derived factors govern multiple signaling pathways in SSCs and the resulting germ cell-soma communications are the paramount forces governing SSC self-renewal and differentiation.

5.2.1. GDNF signaling

While Sertoli cells have been considered as the primary source of GDNF during steady state spermatogenesis, TECs and PMCs are also found to be GDNF producers. GDNF is a well-defined prime factor that is required for promoting SSC renewal and maintenance, both *in vitro* and *in vivo* (68, 69). GDNF belongs to the transforming growth factor beta superfamily molecules and binds to glycosylphosphatidylinositol (GPI)-anchored GFRA1, triggering signaling via the transmembrane receptor tyrosine kinase called REarranged during Transfection (RET), which does not directly bind to GDNF.

The loss of GDNF signals from Sertoli cells or peritubular myoid cells *in vivo* results in the loss of undifferentiated germ cells, whereas overexpression leads to an expansion of the undifferentiated SSCs and the development of tumors (61, 68). Similarly, the absence of GDNF receptors (GFRA1 and RET) triggers rapid depletion of SSCs resulting in a Sertoli-cell-only phenotype (68, 70). Moreover, culturing of mouse SSCs *in vitro* requires GDNF (71, 72). These results suggested that GDNF is a *bona fide* self-renewal factor for SSCs. Furthermore, the expression of GFRA1 within the A_{undiff} cells is reduced as the syncytial length is increased. While approximately 90% of A_s spermatogonia express GFRA1, approx. 75% of A_{pr}, approx. 40% of A_{al4}, and approx. 15% of A_{al8} spermatogonia are positive for GFRA1. A_{al16} spermatogonia lack GFRA1 expression altogether (41, 45). In addition, the expression level of GFRA1 per cell is typically lower in aligned syncytia than single cells or pairs (73).

Sharma and Braun reported that GDNF is expressed cyclically in Sertoli cells and its level is at its highest during the stages when SSC self-renew (X-IV). They proposed that GDNF acts to promote self-renewal not by regulating SSC proliferation, but by inhibiting SSCs from differentiating into transit amplifying A_{undiff} SPGs by using LIN28-null A_s cells in their study (74). Additionally, the stage specific cyclic nature of GDNF availability is also associated with chemotactic migration of undifferentiated SPGs (75). Target genes of GDNF in A_{undiff} spermnatogonia include *Nanos2*, *Etv5*, *Lhx1*, *T(Brachyury)*, *Mycn*, *Bcl6b*, *Id4* and *Ccna* (76–81). Other paracrine factors involved in SSC maintenance in synergy with GDNF include FGF2, different forms of VEGFA and C-X-C motif chemokine 12 (CXCL12) (72, 81, 82).

5.2.2. FGF signaling

Fibroblast growth factors (FGF) belong to a large family of over 15 FGF members that activate receptor complexes including FGFR1, FGFR2, FGFR3, and FGFR4. FGF2 together with GDNF is crucial for proliferation of prospermatogonia and SSC *in vitro* (39, 69, 83). GDNF-independent action of FGF2 on SSC self-renewal has also been reported using transplantation assay and *in vitro* culturing (84). Intriguingly, SPGs cultured in presence of FGF2 have morphology, doubling time, and SSC activity distinct from those of SPGs cultured in presence of GDNF. FGF2 promotes survival and proliferation of SSCs through signaling pathways which are distinct from those involving GDNF. Nevertheless, studies in mice have confirmed that both GDNF levels and SSC numbers increase in FGF2-depleted testis, thereby, implying that a balance between FGF2 and GDNF influences SSC self-renewal (84).Though FGF2 has been considered as a self-renewal promoting factor, it has also been reported to induce expression of RA receptor gamma (RARG) in SPGs marking them as differentiation-primed or differentiating SPGs (85). Moreover, FGF2 also regulates the availability of RA by suppressing the expression of RA-degrading enzyme *Cyp26b1* (86). Further research is needed to better understand the role of GDNF/FGF2 ratio or FGF2 alone in SSC fate determination. It has been reported that FGF5, FGF4, and FGF8 are expressed

in LECs covering the outer surface of the tubules near the interstitium. Although the expression of FGF5 persists throughout the seminiferous epithelial cycle, its spatial availability is heterogeneous with more proximity to the interstitium. Interestingly, the distribution of A_{undiff} spermatozoa shows spatial correlation with FGF5 expression (40). It is proposed that the fate of SSCs may be determined by the competition among the SSC population for a limited supply of FGFs whose availability on the basement membrane is inversely proportional to the distance from the source and the number of A_{undiff} spermatogonia (40). However, further investigation is required to decipher the roles of different FGF ligands in determining the fate of SSCs.

5.2.3. FSH Signaling

Follicle Stimulating hormone (FSH) is a gonadotropin hormone synthesized by the anterior pituitary that acts via its cognate G-protein coupled receptor, FSH receptor (FSHR). During the perinatal period, FSH induces Sertoli cell proliferation and establishes the final Sertoli cell number. Later in development, FSH stimulates the transcriptional and metabolic activities of the Sertoli cell, which contributes to the hormonal and nutritional environment necessary for germ cell survival and development (87–89). FSH has been shown to stimulate GDNF expression in Sertoli cells and to increase the proliferation of undifferentiated SPGs *in vivo* (90). FSHR knockout male mice are fertile but display small testes and partial spermatogenic failure, with defects in sperm viability and motility (91). These data suggest that FSH plays a role in maintaining quantitatively normal spermatogenesis, but may not be absolutely required for fertility in male rodents. Interestingly, men with FSH deficiency or inactivating mutation in FSHR are infertile signifying a species specific prominence of FSH in spermatogenesis (92, 93).

5.2.4. WNT signaling

Wnt genes encode WNT ligands, which are cysteine-rich, glycosylated and lipid-modified secreted proteins that engage Frizzled (*Fzd*) receptor family members to transduce signals into target cells. In many cases, the “canonical” WNT pathway, mediated by beta-catenin, acts to maintain the stem cell pool by inhibiting their differentiation (94). On the contrary, in mouse spermatogenesis, both *in vitro* culture and *in vivo* transplantation based studies suggest that Wnt/beta-catenin signaling (activated by WNT3a) stimulates the proliferation of differentiating progenitors (95, 96). Tokue *et al*. further demonstrated that transition from stem ($GFRA1^+$) to progenitor ($NGN3^+$) state is driven by WNT6 which is prominently expressed by the Sertoli cells (97). Moreover, they identified SHISA6, a cell-autonomous WNT inhibitor, expressed by a subset of $GFRA1^+$ A_{undiff} spermatogonia. It is proposed that SHISA6 might play a role in the maintenance of the $GFRA1^+$ pool by reducing the Wnt/beta-catenin signaling strength in the $SHISA6^+$ A_{undiff} cells and preventing premature entry into the differentiation-primed state.

Interestingly, the availability of GDNF and WNT6 (a WNT family member that is abundantly expressed by Sertoli cells) during the seminiferous epithelial cycle differs, suggesting that they have distinct windows of action (96, 97). Androgen-regulated Sertoli cell gene WNT5A (an activator of beta-catenin-independent pathway) has also been implicated in control of SSC self-renewal, but the available data indicates that WNT5A is an A_{undiff} mitogen (98). Whether it supports adoption of either the stem or progenitor state is unclear.

5.2.5. Retinoic acid signaling

Genetic and molecular studies have elegantly proven that RA signaling is important for SPG differentiation, meiotic initiation, spermatid elongation, and sperm release (99, 100). Vitamin A (retinol) undergoes two oxidation steps to form RA which activates the RA receptors (RARA and/or RARG) and is then quickly (RA half-life in mouse testis is 1.3 hr) oxidized to inactive metabolites by two P450 enzymes (CYP26A1 and CYP26B1). During the first wave of spermatogenesis, RA is produced by Sertoli cells and is required for spermatogonia differentiation (101). In subsequent spermatogenic cycles, meiotic and post-meiotic germ cells become the major source of RA (101, 102). The lack of RA or vitamin A resulted in an accumulation of A_{undiff} spermatogonia, resulting from an inhibition of differentiation of A_{undiff} spermatogonia to A_1 spermatogonia. On the other hand, administration of exogenous vitamin A released this inhibition in vitamin A-deficient mice (103). Similarly, RARG is

predominantly expressed by differentiation primed NGN3$^+$ SPGs (85) and inactivation of *Rarg* in spermatogonia impairs the A_{al} to A_1 transition in the course of some of the seminiferous epithelium cycles (104). Additionally, RA has also been found to downregulate GDNF expression in Sertoli cells (resulting in the expression of differentiation-supporting factors, such as *Bmp4* and stem cell factor (*Scf*)) and antagonize the effect of GDNF in A_{undiff} (105–107). The periodic, pulsatile and stage-specific nature of RA synthesis is the prime regulator of asynchronous seminiferous epithelial cycle (108). Despite the extensive research on the role of RA in spermatogenesis, information on the molecular targets and interacting partners of retinoic acid receptors at various stages of germ cell development is still scarce.

5.2.6. NOTCH signaling

The NOTCH proteins (NOTCH 1-4) are large cell-surface receptors that are activated by membrane bound ligands on neighboring cells such as JAGGED (JAG1 and JAG2) and DELTA-like (DLL1, DLL3 and DLL4). Upon activation of the canonical pathway, the NOTCH intracellular domain (NICD) is cleaved and translocated to the nucleus, where it associates with and consequently activates a DNA-binding protein called recombining binding protein suppressor of hairless (RBPJ). The Hes/Hey family of transcriptional repressors are targets of RBPJ (109). NOTCH receptors (NOTCH 1-4) and NOTCH ligands (JAG1, JAG2 and DLL1) are reported to be expressed by spermatogonia whereas, NOTCH2, JAG1 and DLL1 are expressed by Sertoli cells as well (110). Gain-of-function mouse model that constitutively activates NOTCH1 signaling only in Sertoli cells led to a complete loss of germ cells around birth due to premature differentiation of gonocytes in fetal testis (111). Further investigations described a downregulation of *Gdnf* and *Cyp26b1* which are niche factors required for maintaining undifferentiated state of germ cells. A contrasting phenotype was observed in *Rbpj*-conditional knockout mice where NOTCH signaling was disrupted with significant increase in SSCs and overall germ cell numbers (112). The data so far is suggestive of a role of NOTCH signaling as a negative regulator of germ cell proliferation and promoter of differentiation. However, other studies reported that NOTCH blockage in germ and Sertoli cells had no effect on spermatogenesis and that NOTCH signaling is dispensable for mouse spermatogenesis (113).

5.2.7. Chemokine signaling

CXCL12, also known as SDF-1, is one of the chemokines produced by the Sertoli cells. It acts via its cognate receptor, known as C-X-C motif chemokine receptor 4 (CXCR4), a seven-transmembrane protein which signals via G-proteins, leading to MAPK activation. CXCR4 is expressed by PGCs, gonocytes and A_{undiff} SPGs. In the fetal testis, CXCL12/CXCR4 signaling facilitates the later stages of PGC migration into the genital ridge and is required for gonocyte survival (114). In the adult testis, CXCL12/CXCR4 signaling is crucial for proper homing of SSCs to their cognate niche. Evidence also suggests that CXCR4 expression is stimulated by GDNF in A_{undiff} and that CXCL12/CXCR4 signaling may promote the self-renewing state and prevent transition from A_{undiff} to progenitor state *in vitro* (81).

6. REGULATION OF SSC FATE IN MOUSE SSC NICHE

For healthy spermatogenesis to occur, it is important to maintain the number and function of SSCs during steady state and also in response to environmental and genetic insults. Frequent self-renewal of SSCs can lead to the over-accumulation, leading to defects in spermatogenesis. Conversely, SSCs get “exhausted” if there is insufficient SSC self-renewal, resulting in progressive germ cell loss. Hence, it is critical to achieve an appropriate balance of self-renewal and differentiation in the life cycle of SSCs by the niche factors. The life cycle of SSCs in the mouse testis can be described in three stages: 1) maintenance of self-renewing state 2) differentiation priming and 3) differentiation commitment. Each stage is governed by a specific network of niche factors (Table 1).

6.1. Maintenance of self-renewing state

The property of self-renewal encompasses cell proliferation, cell survival and the proportion of self-renewing cell divisions relative to differentiating cell divisions. GDNF is the key factor for maintenance

Table 1. Summary of SSC niche derived factors required for mouse SSC maintenance or differentiation.

Factor	Testicular expression	Function	Reference
GDNF	Sertoli cells, TEC, PTM	Self-renewal of SSC	(68, 69, 206)
FGF2	Sertoli cells, Leydig cells, germ cells	Expansion and induction of differentiation in A_{undiff}	(39, 69, 83, 86)
FGF4/5/8	LECs	Regulates SSCs and their niche	(40)
WNT6	Sertoli cells and Interstitial cells	WNT ligand, promotes entry of SSC into differentiation-primed progenitor state	(96, 97)
RA	Sertoli cells, spermatocytes	Differentiation of SSCs into differentiating spermatogonia and spermatocytes	(5, 99, 101)
GFRA1	Self-renewing A_{undiff} (A_s, A_{pr})	Forms GDNF receptor, SSC self-renewal.	(68, 70)
SHISA6	A subset of GFRA1$^+$ A_{undiff}	WNT inhibitor prevents premature entry of SSCs into the differentiation-primed state.	(97)
RARG	Differentiation-primed progenitor A_{undiff} (majorly A_{al})	Promotes differentiation of A_{undiff} cells into differentiating spermatogonia	(85, 104)
PLZF	A_{undiff} (A_s, A_{pr}, A_{al}), early differentiating spermatogonia	Promotes SSC self-renewal	(106, 118, 120, 121)
POU5F1	A_{undiff} (A_s, A_{pr}, A_{al})	Proliferation and maintenance of SSCs	(125, 126)
NANOS2	GFRA1$^+$ A_{undiff}	Prevents premature entry of SSCs into the differentiation state	(53, 80, 128)
ID4	GFRA1$^+$ A_{undiff}, differentiating spermatogonia	Promotes SSC self-renewal	(35)
NGN3	Differentiation-primed progenitor A_{undiff} (majorly A_{al} and few A_s and A_{pr})	Sensitizes progenitor A_{undiff} to retinoic acid signaling and mark their entry into differentiation state.	(42, 43, 85)
STRA8	Differentiating spermatogonia	Induces the entry of differentiating spermatogonia into meiosis	(5, 149, 151)
KIT	Differentiating spermatogonia	Initiates entry of differentiating spermatogonia into meiosis	(85, 150)

of self-renewal of GFRA1$^+$ SSCs. GDNF acts through two different signaling pathways to induce target genes that promote SSC self-renewal - the phosphatidylinositol 3-kinase (PI3K)/Ak strain thymoma (AKT)-dependent pathway (115) and the Src family kinase (SFK) pathway (78). The well-studied GDNF-inducible self-renewal genes include *Ets-variant gene-5* (*Etv5*), *Bcl6b, and Lhx1, Pou3f1* (Oct6), *Brachyury (T)* and *Id4*, as reviewed by Song and Wilkinson (116). *Id4* promotes the undifferentiated cell state by its ability to inhibit basic helix-loop-helix transcription factors, most of which promote differentiation. Moreover, ID4 is unique in being the only protein known to be expressed in A_s and not A_{pr} or A_{al} SPGs (31). There are many GDNF-independent and SSC-derived factors such as PLZF, FOXO1, GILZ and TAF4B that also contribute to regulate the self-renewal state of SSC (Figure 4).

Promyelocyctic leukemia zinc finger (PLZF), also known as ZBTB16 or ZFP145, is a transcriptional repressor that binds to DNA via its Kruppel-type zinc finger domains and recruits histone deacetylases (HDACs) via its POZ domain. It is expressed throughout the A_{undiff} population and therefore is widely used as a marker for A_{undiff} SPGs (117). Accordingly, loss of functional PLZF results in progressive loss of germ cells and infertility (118). In mouse SSCs, PLZF has been suggested to work in at least three different ways to ensure SSC maintenance - firstly, by modulating the activity of Sal-like protein 4 (SALL4), whose action is associated with spermatogonia differentiation; secondly, by directly and indirectly (via *Foxo1 and Etv5*) repressing differentiation genes (including *c-Kit*) and stimulating stemness genes of the spermatogonia (many of which are also GDNF targets) and thirdly, by indirectly opposing the

Self-renewal and differentiation of spermatogonial stem cells

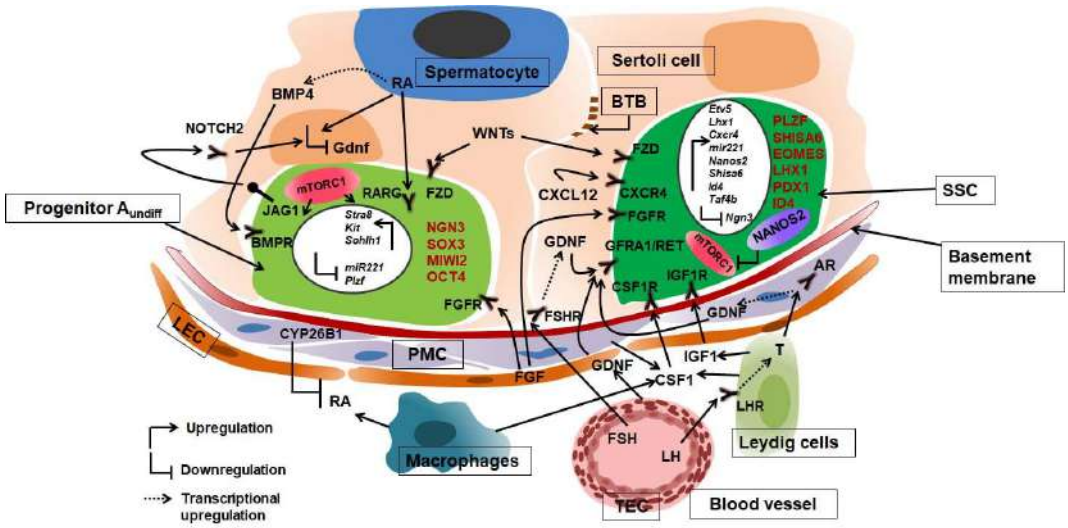

Figure 4. Molecular mechanism regulating SSC fate in adult mouse testis. The SSC niche is contributed by different juxtacrine and paracrine factors secreted by the somatic cells and germ cells respectively. Paracrine factors such as GDNF, FGF, CSF1, IGF1 and CXCL12 derived from Sertoli cells, Leydig cells, testicular endothelial cells (TECs) of the vasculature and lymphatic endothelial cells (LECs) maintain the self-renewal state of SSC via their cognate receptors by upregulating the expression of genes including *Etv5*, *Lhx1*, *Cxcr4*, *Nanos2*, *Shisa6* and *Id4*. These SSCs are marked by the expression of PLZF, SHISA6, EOMES, LHX1, PDX1, and ID4. Certain self-renewal factors function by inhibiting differentiation pathway, for example, SHISA6 is a WNT inhibitor and Nanos2 sequesters and inhibits activity of mTORC1 pathway. Follicle stimulating hormone (FSH), derived from vasculature, and testosterone (T), derived from peritubular myoid cells (PMCs), act indirectly by upregulating GDNF expression. Differentiation primed progenitor cells of A type undifferentiated (A_{undiff}) spermatogonia pool express an exclusive set of genes compared to SSCs. The progenitor cells are marked by the expression of NGN3, SOX3, MIWI2, and RARG. The major characteristic of progenitor cells is the responsiveness to retinoic acid (RA), synthesized by pre-meiotic and post-meiotic germ cells such as spermatocytes, through RARG receptor resulting in upregulation of *Stra8*, *Kit*, *Sohlh1* and downregulation of *Plzf* and *kit*-degrading miR221 making the cells vulnerable to differentiation into differentiating spermatogonia. The progenitor cells also ensure the shutting down of self-renewal pathway by RA and NOTCH signaling mediated inhibition of GDNF expression. RA degrading enzyme, CYP26B1, secreted by PMC ensures removal of RA from extratubular sources like peritubular macrophages. The color key depicting different cell types is used throughout this article. Figure adapted with permission from Mäkelä and Hobbs (45).

differentiation-promoting mTORC1 pathway through the upregulation of mTORC1 inhibitor DDIT4 (119–122). The activation of DDIT4 transcription by PLZF is likely to be important for SSC maintenance since the repression of mTORC1 signaling by DDIT4 is also necessary for maximal expression of both components of the GDNF receptor, viz., GFRA1 and c-RET in SSCs. Together, these data support a model in which PLZF operates in a molecular circuit that amplifies the responsiveness to GDNF signals as a means to maintain SSCs.

Forkhead box protein O1 (FOXO1) is a member of the forkhead transcription factor family that has a variety of functions, including regulation of glucose metabolism, insulin signaling, control of cellular growth and stem cell homeostasis. FOXO1 is highly expressed in SSCs and germ cell specific knock-out of FOXO1 results in spermatogonia arrest. FOXO1 plays a role in SSC maintenance by directly or indirectly upregulating SSC self-renewal genes including *Lhx1, c-Ret, Egr2 and Tex19*. In addition, FOXO1 regulates stem cell marker genes *Gata2* and *Dppa4* (123). In contrast to its role in GDNF induced response, PI3K/AKT pathway has a "pro-differentiation" role in gonocytes, wherein it prevents cytoplasmic FOXO1 from entering the nuclei of gonocytes and activates a cell proliferation program precociously. Hence, the precise role of FOXO1 activity in SSCs needs further investigations.

TATA-box binding protein associated factor 4b (TAF4B) is a gonad-specific subunit of transcription initiation factor TFIID, which is a component of RNA polymerase II pre-initiation complex. Mice null for *Taf4b* exhibit a unique testicular phenotype that includes normal fertility at early ages followed by a complete loss of fertility by

P84, characterized by spermiogenesis defects, loss of germ cells and testicular degeneration. This phenotype is attributed to defective perinatal germ cell development (gonocytes to spermatogonia transition) and SPG proliferation (124). Thus, TAF4B appears to be active in maintaining proliferation of gonocytes and SSCs.

POU Class 5 Homeobox 1 (POU5F1 or OCT4) is a POU-subclass homeobox transcription factor that is essential for the establishment and maintenance of stem cell activity. The major cell types that express POU5F1 in mice are the embryonic primordial germ cells, gonocytes, the precursors of SSCs that are most abundant at birth and A_{undiff} SPGs that are present after birth (125). Knock-down of POU5F1 in cultured SSC caused decrease in the proliferation rate, survival levels and SSC activity as assessed by transplantation assay (126). However, Wu *et al*. found that transient knockdown of POU5F1 did not significantly reduce SSC numbers in $Thy1^+$ SPG cultures. This discrepancy in the data can be attributed to the difference in the two studies with respect to origin of the cells, the culture conditions, and the genetic background of mice (127). Hence, further investigations are required to determine the function of POU5F1 in SSCs.

Similar to ID4 and PLZF, NANOS2 is a SSC self-renewal factor that functions by preventing premature differentiation of SSCs. NANOS2 is an RNA-binding protein that acts by sequestering and consequently inhibiting the activity of components of differentiation promoting mTORC1 pathway in a ribonucleoprotein complex (128). It was recently shown that NEDD4 (neural precursor cell expressed developmentally downregulated protein 4), an E3 ubiquitin ligase, targets NANOS2 for degradation and thus promotes differentiation (129). NANOS2 also associates with DND1 (Dead end protein homolog 1) in A_s and A_{pr}, and deletion of either results in gradual depletion of SSCs. Conditional disruption of postnatal *Nanos2* in mouse testis depleted SPG reserves, whereas overexpression of NANOS2 in mouse testis resulted in accumulation of A_{undiff} SPGs implicating the importance of NANOS2 in SSC self-renewal (53).

The stem cell property of SSCs is also governed by post-transcriptional and epigenetic mechanisms. The post-transcriptional mechanism of gene regulation include an array of regulatory noncoding RNAs (ncRNAs), such as microRNA (miRNA), long ncRNA (lncRNA), piwi-interacting RNA (piRNA) and circular RNA (circRNA), that have been observed to be involved in regulating the SSC self-renewal through forming an intricate regulatory network together with protein-coding genes (130). Dozens of miRNAs have been identified that are specifically or preferentially expressed in SSCs and have been found to modulate expression of known SSC self-renewal genes. For example, miR-21 is regulated by ETV5 (131), miR-20 and miR-106a upregulates the expression of the self-renewal factor *Plzf*; whereas, miR-221 and miR-146a suppresses the expression of the differentiation factor c-Kit (132, 133). LncRNAs are arbitrarily defined as transcripts of greater than 200 nucleotides in length that lack functional ORFs and can be localized to both the nucleus and cytoplasm. Accumulating evidence suggests that lncRNA also has substantial contributions in SSC maintenance (130). Two spermatogonia-specific lncRNA candidates, known as SPGA-lncRNA1 and 2, have exhibited a significant inhibitory effect on differentiation in an *in vitro* model (134). PiRNAs are a distinct class of small non-coding RNAs primarily expressed in the germline cells (135). These 21-31 nucleotide-long non-coding RNAs produced by a Dicer-independent mechanism are loaded into specific PIWI orthologs to form the piRNAs-PIWI complex and this ribonucleoprotein complex along with other protein components perform their function (136). PIWI proteins are composed of three proteins in mice namely, MIWI, MIWI2 and MILI. Although piRNAs-PIWI have been delineated to be involved during the meiosis of spermatocytes and spermiogenesis stages of spermatogenesis, some *in vitro* and *in vivo* (137, 138) studies are suggestive of their involvement in SSC maintenance and self-renewal. However, further research is required to explore the functions of PiRNAs in early stages of spermatogenesis. CircRNAs are an emerging class of single-stranded RNA molecules with a covalently closed loop structure generated through a special type of alternative splicing termed backsplicing, derived mostly from exons, but also from antisense,

intergenic, intragenic, or intronic regions. CircRNAs can modulate gene expression via multiple actions, including sponging miRNAs and proteins as well as regulating transcription and splicing. 5,573 circRNAs are identified so far in SSC and the average levels of circRNAs exhibited dynamic changes during male germ cell development, indicating that these circRNAs are probably involved in SSC self-renewal and differentiation (139). Nevertheless, the biological function of these circRNAs in the SSCs remains elusive.

Interestingly, the epigenome (DNA methylation at CpG sites plus histone modifications) of male germ cells undergoes profound changes during fetal development, whereas in postnatal germ cells the epigenetic marks are more stable. It has been shown that the epigenetic landscape of SSCs is plastic and is similar to that of pluripotent cell types, characterized by bivalent (both activating H3K4me3 and repressing H3K27me3) histone modifications placing promoters in a poised state capable of dynamic activation (140).

Emerging evidences have also identified many potential players of SSC maintenance. Reactive oxygen species (ROS), which were considered to be inhibitory for stem cell function, have striking self-renewal promoting effects in SSCs (141, 142). Cyclin M1 (CNNM1) protein that belongs to the Ancient Conserved Domain Protein family appears to act as a cytosolic copper chaperone. Using *in vitro* cultured mouse SSCs and spermatogonial cell lines, CNNM1 was found to be associated with SPG self-renewal (143). Recently, preferentially expressed antigen of melanoma 12 (PRAME12) protein, expressed in A_{undiff} and early differentiating SPGs, was found to be contributing to SSC maintenance. Knock-out of *Pramef12* impaired SSC self-renewal and early differentiation, resulting in a Sertoli cell-only syndrome in adult mice (144).

6.2. Differentiation priming

This stage is marked by the exit of SSCs from the self-renewing state to a differentiation primed progenitor state, wherein the A_{undiff} cells become sensitive to retinoic acid signals. The most significant molecular event triggering differentiation priming is the activation of mTORC1 pathway in SSCs (38, 121, 129, 145). In addition to mTORC1 pathway, the WNT/beta-catenin signaling also promotes transition from self-renewing to RA-responsive progenitor state of SPGs (95–97, 146). These pathways result in the downregulation of self-renewal genes including *Gfra1, Ret, Lhx1, Eomes* and *Pdx1* and upregulation of genes including *Ngn3, Sox3, Lin28* and *Rarg* (39) as shown in Figure 4. Hence, these upregulated genes are used as markers to identify progenitor SPGs.

6.3. Differentiation commitment

The $NGN3^+$ progenitor SPGs are shown to express RARG which increases their differentiation competence by making them responsive to differentiation inducing RA signaling (85, 147). Progenitor SPGs have the capacity to transition into a self-renewing state or to enter differentiation state. Accordingly, the timely onset of differentiation is regulated by managing the availability of RA and RARG expression within the seminiferous tubule. Since the meiotic and post-meiotic germ cells are the primary source of RA during spermatogenesis, the extratubular supply of RA is kept blocked by its degradation by the CYP26B1 enzyme expressed in PMCs (146, 148). An alternate mechanism of sequestration of RA precursors by round spermatids at stages II-VI has also been proposed as a mechanism to prevent pre-mature entry of progenitors into differentiation states (103). It is also believed that the somatic cell derived niche factors determine cyclic expression of RA specifically during the VII-VIII stages which dictate the spermatogenic wave (101). As a result of rise in RA levels at stages VII-VIII, the $RARG^+$ progenitor cells transit into type A_1 differentiating SPGs and expresses early markers of spermatogonial differentiation including c-KIT and stimulated by retinoic acid 8 (STRA8) as shown in Figure 4 (85, 105, 149–151).

6.4. Stage specific regulation of niche factors

The three stages of SSC life cycle mentioned above (Section 6.3) can be correlated with stages of the seminiferous epithelial cycle (Figure 5). GDNF levels are high during the stages XII-IV which

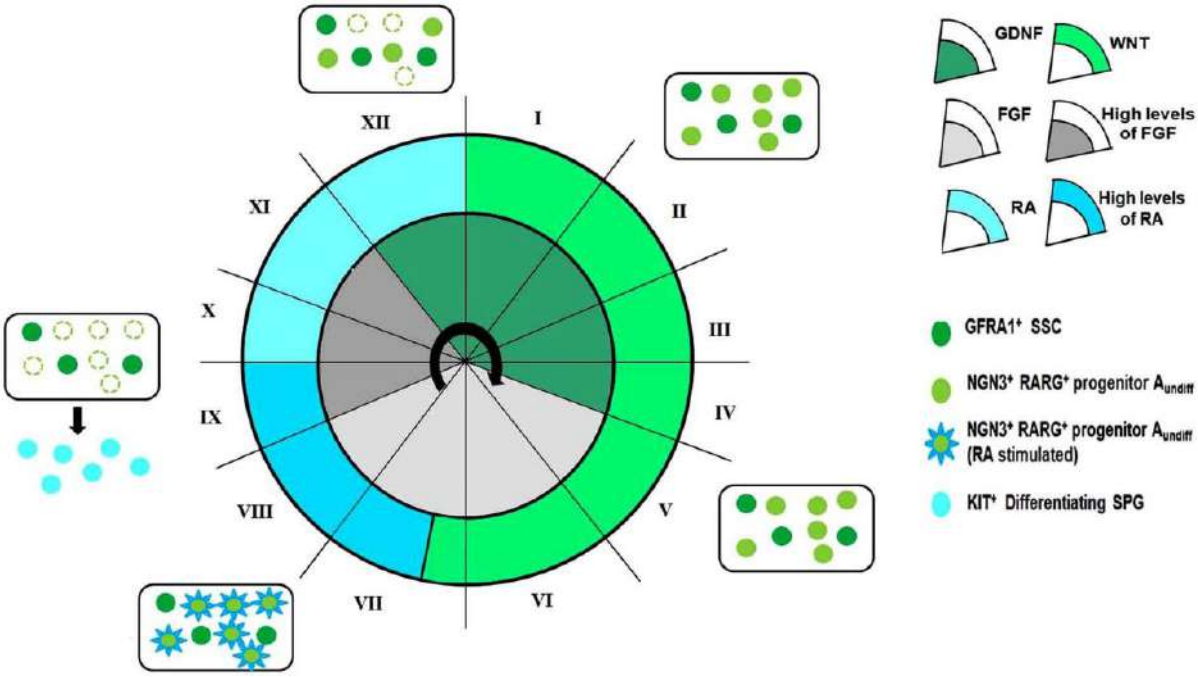

Figure 5. Regulation of SSC niche across the seminiferous epithelial cycle in adult mouse. During the steady state spermatogenesis, the high level of GDNF present at stages XII-IV is conducive for maintenance of self-renewing subset of undifferentiated A type (A_{undiff}) spermatogonial cells (SPGs) which constitute the SSC population. The presence of WNT ligands in stages II-VII ensures existence of progenitor A_{undiff}. The peak in the concentration of retinoic acid (RA) which is observed during stages VIII-IX results in the stimulation of the progenitor cells to give rise to differentiating SPGs. The reduction in the concentration of RA levels during stage X-XII and the concomitant increase in the level of FGF mark the onset of the next SSC self-renewal and differentiation cycle. The color key depicting different cell types is used throughout this article. Figure adapted with permission from Mäkelä and Hobbs (45).

are marked by proliferation of A_{undiff} and self-renewal of SSCs (29). Wnt6 signaling is strongly active in stages I-VIII (73, 97). Moreover, RA pulses start at late stages of VII. Consecutive to the action of WNT and RA signaling on SSCs, $RARG^+$ progenitor cells are observed during the stages of II-VII (85). The highest levels of RA are recorded at stages VII-IX which coincide with the appearance of KIT^+ differentiating SPGs. Mäkelä and Hobbs proposed a model, wherein the reducing levels of RA and a sharp decline in the number of FGF-consuming cells (due to A_{undiff}-to-A_1 transition) at later stages (X-II) of the cycle allow GDNF and FGF levels to rise, resulting in the next wave of proliferation of A_{undiff} (45).

6.5. SSC maintenance during homeostasis and regeneration

SSC niche is dynamic in nature and varies with the state of the biological system, as is observed during postnatal and pre-pubertal testis development, homeostasis and regeneration after testicular tissue injury. The dynamic SSC niche subsequently results in the dynamic interconversion of undifferentiated SPGs into different states. In developing testis, the SSC niche produces abundant growth factors and less inhibitory factors resulting in an environment that supports self-renewing proliferation. On the other hand, the SSC niche in homeostatic adult testis produces a moderate amount of mitogenic factors to maintain a stable SSC number. Accordingly, the majority of SSCs in developing testis are in mitotic state, while the SSCs in homeostatic condition are likely to be quiescent or in a slow cycling state. In regenerating testis, the SSC niche again stimulates growth factor production for SSC expansion.

It has been reported that during regenerative conditions, progenitor A_{undiff} cells (*Gfra1*$^-$ *Ngn3*$^+$) re-express the self-renewal genes and acquire SSC activity. Furthermore, during

transplantation assay which represents a regenerative condition, *Ngn3*$^{+}$ *Miwi2 (Piwil4)*$^{+}$ *Gfra1*$^{-}$ *Kit*$^{-}$ $A_{undifff}$ cells identified as differentiation primed progenitor cells also display reconstitution of stem cell activity (42). Recently, a minor subset (0.2% of testicular cells) of GFRA1^{+} cells were identified as co-expressing *Pdx1*, *Brachyury*, *Eomes* and *Lhx1* (39). Interestingly, it was observed that this subset of SPGs adopts a different expression profile signifying different cellular states in response to the niche conditions. The self-renewal state marked by *Pdx1*$^{+}$, *Eomes*$^{+}$ and *Lhx1*$^{+}$ prevails during homeostasis. However, during postnatal development and under regenerative conditions, when the niche provides excess of self-renewal signal, *Eomes* and *Lhx1* expression are upregulated and *Pdx1* is down-regulated (39). Hence, *Pdx1*, *Eomes* and *Lhx1* expression might be required for long term maintenance of SSCs under steady state spermatogenesis. Thus, replenishment of cells in the differentiation-primed state and restoration of self-renewing fractions after genotoxic damage are possible via dynamic interconversion of these A_{undiff} states

7. *IN VITRO* MANIPULATION OF MOUSE SPERMATOGONIAL STEM CELLS

SSCs are the only cells in the adult body which can transmit genetic information to subsequent generations and increase in number following birth. Thus, SSCs provide an accessible and renewable source of genetic code which can have enormous valuable applications for germline modifications in the field of medicine and molecular breeding. The development of spermatogonial transplantation techniques paved the way for *in vitro* manipulation experiments on SSCs (152). The transplantation assay for SSCs was primarily developed by Brinster and Zimmermann. They injected testis cell suspension containing SSCs into seminiferous tubules of busulfan-treated infertile mouse and congenitally infertile Kit^{W}/Kit^{W-v} mouse. The transplanted SSCs colonized the recipient seminiferous tubule and started spermatogenesis demonstrating the self-renewal and reconstitution properties of the injected cell suspension. The generated spermatozoa were able to produce offspring (153). Moreover, it was reported that one colony generated by spermatogonial transplantation is derived from a single SSC (154, 155), implying that the spermatogonial transplantation technique can be used as a biological assay for SSC identification and quantitation. The first transgenic animals using SSCs were created by transduction of mouse SSCs using a retrovirus vector containing the beta-galactosidase gene (156). Subsequent development of long-term culture systems has allowed a variety of techniques to be used for genetic modification of SSCs such as homologous recombination and gene-editing using the TALEN and CRISPR/Cas9 system (155, 157).

7.1. Establishment of germline stem cell (GS) culture

Two-dimensional (2D) culture of isolated SSCs has become a popular approach to study the influence of niche factors involved in the regulation of their proliferation and the differentiation of their progeny. The first report of culture and maintenance of mouse SSCs *in vitro* was published in 1998 (158). In this study, unfractionated testicular cells from neonatal and adult transgenic mice expressing beta-galactosidase were cultured for approximately 4 months on SIM mouse embryo-derived thioguanine and ouabain resistant (STO) feeder cells, which have been routinely used for mouse embryonic stem (ES) cell cultures, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). In transplantation assay, the cultured cells derived from neonatal testis formed spermatogenic colonies in the recipient testis demonstrating the stem cell potency of the cultured cell. However, no expansion of SSCs was observed, and the number of surviving SSCs was very low. Since the proliferation of stem cells is regulated intrinsically and extrinsically by the stem cell niche, several modifications in the culture condition were performed to identify the soluble factors which would support the maintenance and expansion of SSCs in culture. A beneficial effect of GDNF, minimal essential medium (MEM) and OP9 bone marrow stroma or fibroblast cell lines as feeder layers was observed on SSC maintenance in this short-term culture experiment (30). However, the expansion of SSC and consequent long-term

culture was not obtained using these culture conditions.

The first report of successful *in vitro* expansion of mouse SSCs was in 2003 by Kanatsu-Shnohara *et al*. The SSCs in this study were enriched by differential plating and cultured on mouse embryonic fibroblasts (MEFs) feeders in a serum-supplemented proprietary StemPro-34 (Gibco)-based medium, which contained the original StemPro-34 supplement plus 16 individual compounds and FBS with a cytokine mixture of GDNF, FGF2, LIF, and epidermal growth factor (EGF). Using the enriched culture media, quiescent SSCs resumed proliferation and formed grape-like clusters that expressed spermatogonia markers ITGA6, ITGB1 and EPCAM. The cultured cells proliferated for approx. 5 months in a logarithmic manner without losing colonization activity in transplantation assays. Moreover, the haploid male germ cells could produce offspring, proving that the cultured cells possessed the proper SSC activity (71). Although the cultured cells exhibited stem cell activity, these cells appeared (grape-like aggregates) different from the isolated SSCs in seminiferous tubules. Hence, these cultured SSCs were termed as germline stem cells. Subsequently, some studies reported comparable results regarding GS cell derivation from other mouse strains under similar conditions (69, 159). These results suggested that the combination of mouse strain and age, feeder cells used, and medium composition affected the *in vitro* expansion of SSCs.

7.2. Genome editing of GS cells

GS cells are considered to be more suitable than embryonic stem cells (ESC) for genome editing of germline lineages as GS cells have the following advantage over ES cells: 1) stable epigenetic/ genetic properties, 2) normal karyotype, 3) normal genomic imprinting status, 4) susceptible for drug selection and 5) can be maintained *in vitro* for as long as 2 years (71, 160). Transgenes can be introduced and established in GS cells through conventional gene transfer techniques such as lipofection, electroporation, and retroviral vector infection, lentivirus-, adenovirus-, and adeno-associated virus-mediated gene transductions (160–162). However, the genetic modification of GS cells has proved to be more difficult than that of ESCs, mainly due to low gene transfer and genome targeting efficiency in GS cells. The targeting efficiency of genome editing using homologous recombination has been increased by several fold using site specific double strand break producing nucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases effector nucleases (TALENs) (157, 163). Successful genome editing using clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 technology for base pair substitutions and transgene knock-in is also reported in mouse GS cells (164).

7.3. *In vitro* spermatogenesis

Although SSCs can be maintained and expanded for several months in 2D culture (section 7.1), it is difficult to induce meiosis or later stages of spermatogenesis in these conditions (71, 165). A few studies have demonstrated the ability of immortalized spermatogonia cells to differentiate into spermatocytes and round spermatids (166, 167). However, this strategy has its own limitations such as manipulation of the genome for immortalization of germ cells which is not feasible for reproduction of animals, l*et al*one for humans. The passage of SPGs into meiosis (and hence, migration through the seminiferous epithelium) depends upon the structural support of the seminiferous epithelium, interaction with the extracellular matrix (ECM) and the availability of SSC niche factors

The importance of Sertoli-germ cell interaction was also revealed in Sertoli-spermatogenic cell co-cultures established from 13- to 18-day-old mice that were able to convert pachytene spermatocytes to round spermatids capable of developing normal and fertile offspring when injected into mature oocytes (168). The requirement of spatial and temporal testicular microenvironment was understood in *ex vivo* organ culture. The first description on *in vitro* spermatogenesis was reported by Martinovitch, who used newborn mouse testis tissue cultured on a clot composed of equal parts of fowl plasma and fowl embryo extract and demonstrated the development of pachytene spermatocytes from presumably immature spermatogonia in the culture (169). Organ

culture experiments in the later years also achieved limited success till the pachytene spermatocyte stage. In 2003, Suzuki and Sato isolated seminiferous tubules from 5-day old mice and cultured it on an agarose gel block at the liquid-air interface (170). The round spermatids obtained were injected into the oocytes resulting in embryos that developed up to the 8-cell stage but no offspring was produced. This technique is also applicable to adult and cryopreserved tissues (157, 171).

Reproducing the testicular third dimension *in vitro* (three-dimensional or 3D culture) has been achieved by embedding various (dissociated) cell types of the seminiferous tubule in a collagen gel matrix. In this way, a suitable support is provided for isolated germ cells to interact with Sertoli cells and other structural and hormone-producing elements. Stukenborg *et al*. established soft agar culture system (SACS), wherein enriched SSCs from 10-day-old mice were mixed with the gel-agar medium (0.35%) and incubated on a solid-agar base (0.5%). The agar was mixed with a high glucose DMEM solution. This approach yielded enhanced viability, germ cell meiosis, and differentiation up to post-meiotic stage (172). SACS technique has reported morphologically normal spermatozoa from pre-meiotic germ cells (173, 174). Although the study of *in vitro* spermatogenesis progressed significantly over the last century, mouse tissues have been more feasible for spermatogenesis under culture conditions (175).

7.4. Germ cell induction from pluripotent stem cells

There have been attempts to generate gametes or PGCs *in vitro* from ESCs both in mice and humans by isolating cells that express a germ cell marker(s) in spontaneously differentiated embryoid bodies (176). However, these attempts were inefficient in obtaining the induced cells (less than 1.0%) and in generating induced gamete-like cell derived healthy offspring, thus unsuitable for analyzing the events that take place before the emergence of germ cell-like cells. While ESCs are pluripotent stem cells (PSCs) derived from the inner cell mass (ICM) of preimplantation blastocysts at E3.5-E4.5 *in vitro*, epiblast stem cells (EPiSCs) are PGCs derived from epiblast (which are the precursors of PGCs *in vivo*) of post-implantation embryos at E5.5-E6.5 *in vitro* (177). EpiSCs exhibit a primed pluripotency and retain attributes of the original epiblasts making them a superior source for the generation of germ cell-like cells compared to ESCs *in vitro* (178). Hence, recent studies have focused on inducing PGC like cell (PGCLCs) from epiblast-like stem cells (EpiLCs) generated from ESCs and induced pluripotent stem cells (iPSCs). *Fragilis*, *stella* (*Pgc7/Dppa3*) and *Blimp1* (also known as *Prdm1*) genes in the epiblast and BMP4 signaling from the extraembryonic ectoderm were found to be required for the specification of germ cell fate in mice (11). Subsequently, a transgenic mouse stain and ES cell line were established by Ohinata *et al*., which showed germ cell commitment by dual fluorescence reporter genes (*Blimp1-Venus*:: *Stella-Cfp* reporter mouse/ES cells) (179). Thereafter, Hayashi *et al*. in 2011 succeeded in inducing EpiLCs from ES and iPS cell lines using activin A and FGF2, and then PGCLCs were derived from aggregated EpiLCs in suspension culture by stimulation with BMP4, BMP8b, SCF, LIF, and EGF. The resulting PGCLCs were then transplanted into infertile mouse testes to produce haploid male germ cells (180).

8. CLINICAL APPLICATIONS OF SPERMATOGONIAL STEM CELLS

Infertility is a worldwide problem affecting 15-20% of couples globally with male factor involvement estimated to be present in about 50% of cases, with sole responsibility in 30% of cases and with a co-contributing female factor in 20% of cases (181, 182). Severe male infertility, including azoospermia and oligoasthenozoospermia, as well as testicular dysfunction can result from genetic or medical conditions such as Klinefelter syndrome, environmental insults such as infections, inflammation/autoimmunity or gonadotoxic medical treatments such as oncotherapies (183–185). Fertility preservation is proposed for all these health conditions, especially in pediatric cancer patients from the perspective of future interventions allowing parenthood. For adult men or adolescents, cryopreservation of ejaculated or surgically retrieved sperm is routinely proposed before gonadotoxic therapies, while for prepubertal boys,

cryopreservation of a testicular biopsy of immature testicular tissue (ITT) containing SSCs is now ethically accepted as the only way to offer a fertility preservation strategy (186).

There are at least four potential ways to theoretically use cryopreserved pre-pubertal or adult testicular tissue biopsies or germ cell preparations to obtain functional sperm. These includes the following: 1) autologous transplantation (auto-transplantation) of testicular tissue termed as testis tissue transplantation (TTT), 2) xenografting of testicular tissue under the back skin or scrotal skin of mice, 3) isolation of spermatogonial stem cells, with and without expansion, for auto-transplantation and 4) isolation, expansion, and maturation of germ cells *ex vivo* via 2D, 3D, and organoid tissue cultures.

8.1. Testicular tissue transplantation

TTT has the advantage that it retains SSCs within their niche and ensures germ cell and supporting cell interactions, providing an optimal microenvironment for cell proliferation, maturation and differentiation. However, since TTT carries a potential risk of reintroducing cancerous cells back to the patient and causing malignant relapse (187), it should only be considered for patients diagnosed with non-systemic cancer and/or non-malignant hematopoietic disorders (188). Complete spermatogenesis following auto-transplantation of ITT was first demonstrated in mice (189). Successful autologous/allogeneic TTT has been reported in rhesus monkeys (190), but not in other species including humans. In recent years, ectopic grafting of immature testicular tissues from various mammalian species under the back skin of immunodeficient mice (xenotransplantation) has been developed as a strategy for preserving testicular function and generating mature spermatozoa (189). However, complete spermatogenesis was not achieved in human xenotransplantation cases. Pachytene spermatocytes and spermatid-like cells were reported in human ITT xenotransplants placed into the scrotum of castrated immunodeficient mice (191, 192), while early spermatocytes were detected in xenotransplants under the dorsal skin (193). Intratesticular xenotransplantation also led to differentiation only up to pachytene spermatocytes stage (194). The reason for inefficient spermatogenesis in human xenotransplantation studies is believed to be the long duration of prepubertal development (8-10 years) observed in humans which may not be achieved in transplant recipient mouse systems (193).

8.2. SSC transplantation

Brinster's group was the first to demonstrate successful transplantation of testicular cell suspension containing SSCs with development of mature sperm in mice using freshly isolated and cryopreserved prepubertal or adult mouse testicular cell suspension (152, 195). Many studies thereafter have reported live offspring generation in different species including mice, rats, goats, chickens, and sheep and embryo development in non-human primates following auto-transplantation of cultured SSCs (196–200). So far, only one report has described autotransplantation of cryopreserved human testicular cell suspension in patients cured of non-Hodgkin's lymphoma, but no follow-up was published (201, 202). The small size of human testicular biopsy samples makes it difficult to isolate SSCs for preservation and transplantation, making *in vitro* expansion and maturation of human SSC critical. Other important concerns are the risk of neoplastic contamination of cryopreserved tissue with subsequent possibility of re-inducing the disease in a cured patient and the need for standardization of an efficient cell injection technique. The availability of an undamaged recipient niche which would support migration, proliferation and differentiation of the transplanted SSC is also an important factor for the success of SSC transplantation compared to TTT.

Long-term *ex vivo* propagation and expansion of pre-pubertal SSC and adult SSC from normozoospermic and infertile men (203, 204) have both been reported and these cells were shown to have stable genetic and epigenetic profiles after culture (205). Recently, Bhang *et al.* discovered that human endothelial cells secreted GDNF, basic fibroblast growth factor (bFGF), stromal cell-derived factor-1 (SDF-1), macrophage inflammatory protein 2 and insulin-like growth factor-binding protein 2 and could support SSC growth for at least 150 days (206).

To eliminate the risk of cancer-cell contamination of testicular cell suspension, attempts have been made to efficiently isolate human SSCs from cancer cells using specific markers and cell-sorting techniques (200, 207). However, these techniques did not allow complete removal of cancer cells. Alternatively, culturing the testicular cells to propagate SSCs led to elimination of all contaminating malignant cells after 26 days of culture (208).

The rete testis ultrasound-guided injection was established as the best approach for SSC transplantation into large testes with 70% of the tubules filled after an average of 30 min in the monkey testis (209). More recently, an infusion pump was used to inject SSCs in human cadaver testes, showing less variability between subjects if compared to the injection under hydrostatic pressure (210). However, leakage in the testicular interstitium was observed and further studies are warranted to improve the injection technique.

Eventually, as SSCs after being re-transplanted have to migrate to colonize the host testicular stem cell niche to gain physical and molecular support for their proliferation and differentiation, an undamaged niche is of paramount importance for a successful SSCs transplantation.

8.3. *In vitro* maturation of SSCs

Although testicular tissue or SSC transplantation are promising fertility preservation strategies, the risks related to the transplantation of residual neoplastic cells limit their application. *In vitro* maturation and differentiation of cryopreserved SSCs into haploid cells for later usage in assisted reproduction techniques (ART) would bring a promising fertility preservation option for childhood cancer survivors. Similarly, it would benefit the infertility treatment of the wide range of non-obstructive azoospermia patients who are not able to produce sperm but still have SSCs. *In vitro* spermatogenesis using human testicular cells was earlier reported in 1967 by organ culture which demonstrated differentiation of spermatocytes from preleptotene to pachytene stage (211). Many studies on organ culture in the following year showed limited success with differentiation of spermatogenic cells, that too achieved only till pre- and post-meiotic spermatocyte stage (175). Recently, development of haploid germ cells from spermatogonia cells using organotypic culture for testicular tissues from pre-pubertal cancer patients was reported (212, 213). Nevertheless, the characterization of non-cultured and cultured human SSC remains challenging/ controversial due to the heterogeneity of spermatogonia, ambiguity of human SSC-specific markers, and the inherent contamination of SSC with other testicular cells during the culture process. In addition, cell culture conditions for *ex vivo* propagation and differentiation of mouse spermatogonia have not been fully translatable to human SSC.

8.4. Pluripotent stem cells

Human ESCs and iPSCs have been considered as valuable sources of pluripotent cells to obtain germ cells *in vitro*. Human ESCs have been utilized to model and improve our understanding of human germ cell development and infertility, and have also been investigated in stem cell-based fertility preservation strategies. Germ cells or gonadal support cells have also been developed from human iPSCs derived from autologous cells such as skin biopsy-derived fibroblasts or blood cells or urine derived cells or hair keratinocytes (214–216). These strategies are encouraging for patients who lack spermatozoa or SSCs. Similar to the case in rodent species, pluripotent stem cell based strategies involve the derivation of PGCLC from a patient's somatic cell (typically dermal fibroblast, keratinocytes, or blood cells) via induction of iPSC, to be used for transplantation into the testis to induce spermatogenesis *in vivo*, or to pursue *in vitro* derivation of gametes (216, 217). These strategies have been employed for Klinefelter and non-obstructive azoospermia patients albeit with limited success (218, 219). However, direct reprogramming of mouse skin fibroblasts into embryonic Sertoli cells and Leydig-like cells has been reported (220, 221). Sertoli cell- and Leydig cell-induced differentiation of human iPSC was also recently reported (222, 223). An important limitation of human ESC and human iPSC-based approaches, apart from the legal and ethical barriers, is the high risk of accumulation of

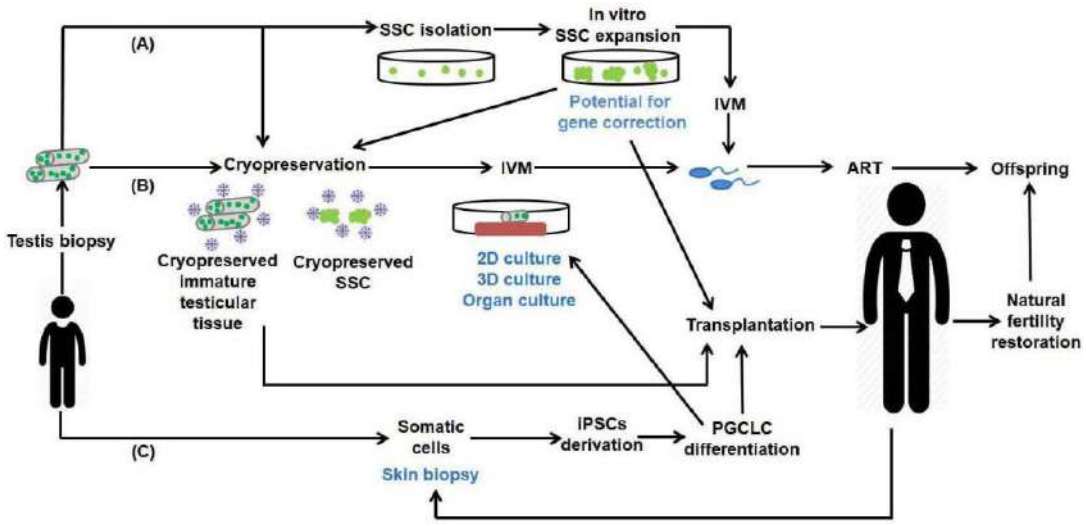

Figure 6. Schematic of potential SSC-based fertility preservation strategies in humans. Three methods for fertility preservation, especially at the prepubertal male stages, have been investigated using animal models and in few instances, using patient samples such as prepubertal cancer patients and Klinefelter patients. A) In the first method, SSCs can be isolated from testis biopsy samples and expanded *in vitro.* The expanded SSC-derived germ-line clusters can be cryopreserved for future application. B) In the second method, the tubules obtained from testis biopsy can be directly cryopreserved for future applications. In the adult stage of the male, the cryopreserved samples can be transplanted back into the testis to restore spermatogenesis and fertility. Alternatively, the cryopreserved samples can be revived by *in vitro* maturation (IVM) in 2D, 3D or organ culture, resulting in the formation of mature sperms that can be applied in assisted reproductive techniques (ART) such as intracytoplasmic sperm injection. Tissue-based approaches have the advantage of preserving the structural integrity of the seminiferous epithelium resulting in efficient restoration. C) In the third method, somatic cells such as fibroblast cells, derived from skin biopsy from prepubertal or adult male, can be reprogrammed *in vitro* to pluripotent cells (induced pluripotent stem cells, iPSCs). These iPSCs can be transdifferentiated *in vitro* into primordial germ cell like cells (PGCLCs) that can be autotransplanted into the adult male to restore natural fertility.

genetic and epigenetic mutations during reprogramming (224). Consequently human ESC or iPSC derivation of germ cells which have the potential to transmit genetic material to the offspring may not be the safest approach for fertility preservation.

9. CONCLUSION

The development of new cellular, molecular and computational technologies such as single-cell transcriptomic analysis has aided the research fraternity to elucidate the heterogeneity of mouse spermatogonial stem cells and somatic cells that contribute to the regulation of SSCs (Figure 6). This should result in a finer characterization of spermatogonia populations and development of a detailed hierarchy of successive cell states in the developmental lineages. Further, a broad-spectrum omics (including transcriptomics, epigenomics and proteomics) based study is envisaged to understand the complexity of SSC function at the molecular and spatiotemporal level. Although the rodent species have been the favored choice of animal model with the ease in handling, housing and breeding, it is important to take into consideration the interspecies differences in spermatogenesis resulting in difficulties in translating rodent data to higher species such as humans. Hence, it is critical to develop an efficient non-human primate model to study the process of spermatogenesis and to improve strategies for fertility preservation and treatment in humans. Availability of efficient manipulation techniques of SSCs would also have non-clinical applications such as improvement of molecular breeding of livestock animals. Significant advances in the area of SSC cryopreservation and *in vitro* maturation point towards the potential of SSC based clinical application to restore fertility in near future. Moreover, evolving germline genome editing research may, in the distant future, allow for the safe use of these approaches for the treatment of genetic factor-induced male infertility. We hope that future research in this line would decode the secrets of

human SSC to the level of our understanding of mouse spermatogenesis, which would enable us to do this task in a safe and efficient manner.

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Abbreviations: SSC, spermatogonial stem cell; FACS, fluorescence-activated cell sorting; RA, retinoic acid; SPG, spermatogonial cell; A_{undiff}: undifferentiated A-type spermatogonia; A_s, A-single; A_{pr}, A-paired; A_{al}, A-aligned; In, intermediate; PGC, primordial germ cell; BMP, bone morphogenetic protein; BLIMP1, B lymphocyte-induced maturation protein-1; PRDM14, PR-domain containing protein 14; FGF, Fibroblast growth factor; WNT, Wingless/integrase 1; SRY, sex determining region Y; CXCR4, SDF1; stromal cell-derived factor 1; CXCR4; C-X-C chemokine receptor type 4; NGN3, neurogenin 3; PGCLC, PGC-like cells; PreSPG, prespermatogonia; ID4, inhibitor

of DNA binding 4; GDNF, glial cell line derived neurotrophic factor; GFRA1, GDNF receptor alpha 1; ITA6, alpha 6-integrin; ITGB1, beta 1 integrin; THY1, thymus cell antigen 1; EPCAM, epithelial cell adhesion molecule; PLZF, promyelocytic leukemia zinc finger; PMC; peritubular myoid cell; CSF1, colony stimulating factor 1; LH, luteinizing hormone; LHR, luteinizing hormone receptor; IGF1, insulin-like growth factor 1; TEC, testicular endothelial cell; VEGFA, vascular endothelial growth factor A; LEC, lymphatic endothelial cells; FSH, follicle stimulating hormone; RET, REarranged during Transfection; CXCL12, C-X-C motif chemokine 12; RARG, retinoic acid receptor gamma; FSHR, follicle stimulating hormone receptor; PI3K, phosphatidylinositol 3-kinase; AKT, Ak strain thymoma; FOXO1, forkhead box protein O1; TAF4B, TATA-box binding protein associated factor 4b; POU5F1, POU Class 5 Homeobox 1; miRNA, microRNA; microRNA, long ncRNA; piRNA, piwi-interacting RNA; circRNA, circular RNA; EGF, epidermal growth factor; STRA8, stimulated by retinoic acid 8; GS, germline stem cell; 2D, two-dimensional; DMEM, Dulbecco's modified Eagle's medium; ESC, embryonic stem cell; 3D, three-dimensional; SACS, soft agar culture system; EPiSc, epiblast stem cells; EpiLCs, epiblast-like stem cells; iPSCs, induced pluripotent stem cells; ITT; immature testicular tissue, TTT; testicular tissue transplantation

Key Words: Spermatogenesis, SSC, Self-renewal, Differentiation, Review

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Spermatogonial stem cells and spermatogenesis in mice, monkeys and men

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ABSTRACT

Continuous spermatogenesis in post-pubertal mammals is dependent on spermatogonial stem cells (SSCs), which balance self-renewing divisions that maintain stem cell pool with differentiating divisions that sustain continuous sperm production. Rodent stem and progenitor spermatogonia are described by their clonal arrangement in the seminiferous epithelium (e.g., A_{single}, A_{paired} or $A_{aligned}$ spermatogonia), molecular markers (e.g., ID4, GFRA1, PLZF, SALL4 and others) and most importantly by their biological potential to produce and maintain spermatogenesis when transplanted into recipient testes. In contrast, stem cells in the testes of higher primates (nonhuman and human) are defined by description of their nuclear morphology and staining with hematoxylin as A_{dark} and A_{pale} spermatogonia. There is limited information about how dark and pale descriptions of nuclear morphology in higher primates correspond with clone size, molecular markers or transplant potential. Do the apparent differences in stem cells and spermatogenic lineage development between rodents and primates represent true biological differences or simply differences in the volume of research and the vocabulary that has developed over the past half century? This review will provide an overview of stem, progenitor and differentiating spermatogonia that support spermatogenesis; identifying parallels between rodents and primates where they exist as well as features unique to higher primates.

1. Introduction

Spermatogonial stem cells (SSCs) are the adult tissue stem cells in the testis that are at the foundation of spermatogenesis and essential for male fertility (Phillips et al., 2010). SSCs are defined by their dual potentials: 1) self-renew to maintain the stem cell pool and 2) differentiate to maintain continuous sperm production in post-pubertal males (de Rooij & Grootegoed, 1998). Similar to other adult tissue stem cells, SSCs are rare, comprising only 0.03% of total germ cells in mice (Tegelenbosch & de Rooij, 1993). However, numerous transit amplifying mitotic divisions in progenitor and differentiating spermatogonia, followed by two meiotic divisions give rise to millions of sperm each day (Phillips et al., 2010).

2. Spermatogonial stem cells and spermatogenic lineage development: lessons from the rodent

In the post-natal rodent testis, SSC activity is broadly believed to reside in the population of isolated (single) spermatogonia located on the basement membrane of the seminiferous tubules (Huckins, 1971; Oakberg, 1971a; de Rooij, 1973). These rare cells are called the A_{single} spermatogonia (A_s), which divide once every three days and make up about 0.03% of the total germ cells in the mouse testis (Tegelenbosch & de Rooij, 1993; Huckins & Oakberg, 1978a). Mitotic division of A_s produces a pair of spermatogonia (A_{paired}; A_{pr}) that will either complete cytokinesis to produce two new A_s (self-renewing division) or remain joined by an intracytoplasmic bridge and produce a chain of four $A_{aligned}$ spermatogonia (A_{al4}) at the next division (Phillips et al., 2010; de Rooij & Griswold, 2012) (Fig. 1A). The A_{al4} spermatogonia may undergo one or more mitotic divisions to form larger chains of 8, 16 and sometimes 32 A_{al} spermatogonia. Collectively, A_s, A_{pr} and A_{al} make up the population of undifferentiated spermatogonia that comprises 0.3% of germ cells in the rodent testis; A_s make up 10% of undifferentiated spermatogonia (0.03% of germ cells; Fig. 2A) (Phillips et al., 2010; Huckins, 1971; Oakberg, 1971a; Valli et al., 2015; Oakberg, 1971b). Larger clones of A_{al} spermatogonia differentiate to A1 spermatogonia. In this context, a clone is defined as the group of interconnected cells that arise from a single A_s spermatogonia. In Rodents, the clones become so large that they fill entire segments of seminiferous tubule due to sequential mitotic divisions from A1 spermatogonia that produce types A2, A3, A4, Intermediate and B spermatogonia, which divide to produce primary spermatocytes. Two meiotic divisions from primary spermatocytes give rise to secondary spermatocytes and round spermatids, which undergo spermiogenesis (morphological differentiation)

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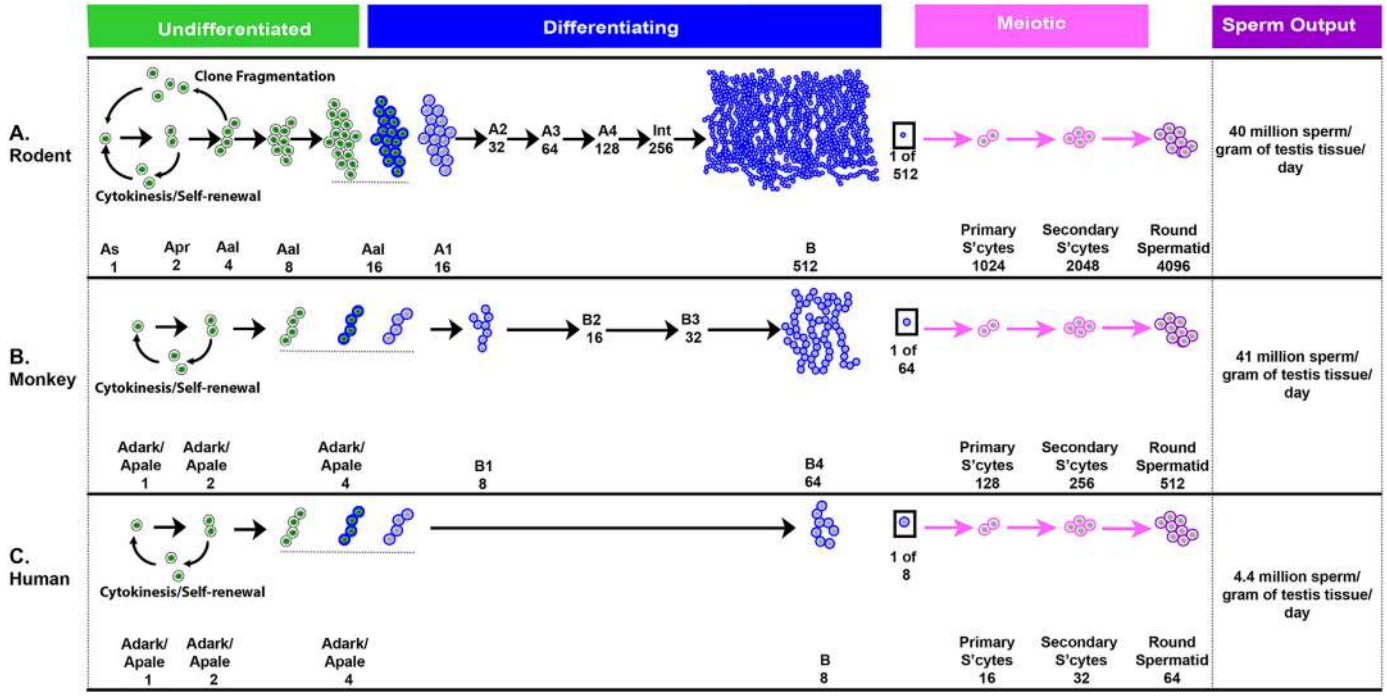

Fig. 1. Clonal development in the spermatogenic lineages of rodents, monkeys and humans. Undifferentiated spermatogonia are described as A_s, A_{pr} or A_{al} in the rodents and A_{dark} or A_{pale} in monkey and human. During spermatogenic development, A_{single} (A_s) and A_{dark} and/or A_{pale} undergo one or more mitotic divisions to give rise to cells of larger clones (chains) of interconnected cells sizes through transit-amplifying mitotic divisions. A) Clonal development in rodents features 3–4 transit amplifying divisions in the pool of undifferentiated A_s, A_{pr} and A_{al} spermatogonia followed by 6 amplifying divisions in the pool of differentiated spermatogonia (A1–A4, Intermediate, B), which give rise to primary spermatocytes. Two additional meiotic divisions produce round spermatids that undergo spermiogenesis to produce sperm. B) Clonal development of spermatogonia in monkeys features 0, 1 or 2 transit amplifying divisions in the pool of undifferentiated A_{dark}/A_{pale} spermatogonia, followed by 4 transit amplifying divisions of differented spermatogonia (B1–B4), which give rise to primare spermatocytes. C) Clonal development of spermatogonia in humans features 0, 1 or 2 transit amplifying divisions in the pool of undifferentiated A_{dark}/A_{pale} spermatogonia followed by a single a single transit amplyfying division in differentiated B spermatogonia that give rise to primary spermatocytes. Thus, there are typically 12 transit amplifying divisions in rodents; 8 in monkeys and 5 in humans between stem cell and sperm. The reduced number of transit amplyfing divisions in monkeys and humans is compensated in part by a larger stem cell pool (see Fig. 2).

to produce mature sperm. Thus, through a series of transit amplifying mitotic and meiotic divisions, a relatively small pool of stem cells in the rodent testis produces 40 million sperm per gram of testis parenchyma each day (Figs. 1A and 2A) (Tegelenbosch & de Rooij, 1993; Valli et al., 2015; Thayer et al., 2001).

Undifferentiated stem and progenitor spermatogonia in rodent testes are defined in part by clone size, as indicated above, and in part by molecular markers (e.g., ID4, PAX7, BMI1, EOMES, GFRa1, NANOS2, UTF1, ZBTB16, SALL4, LIN28, FOXO1 and others). Markers can be observed by immunohistochemistry in histological cross sections; in whole mount preparations of seminiferous tubules or by fluorescence-activated cell sorting (FACS), but clone size can only be observed in whole mount preparations of seminiferous tubules. Based on whole mount immunohistochemistry, ID4, PAX7, BMI1 and EOMES appear to have the most restricted pattern of expression, which is limited to A_s spermatogonia (Oatley et al., 2011; Aloisio et al., 2014; Komai et al., 2014; Braun et al., 2017). GFRa1, NANOS2 and UTF1 have expression limited to A_s, A_{pr} and A_{al4} (Suzuki et al., 2009; van Bragt et al., 2008; Meng et al., 2000), while ZBTB16, SALL4, LIN28, CDH1 and FOXO1 are expressed by most or all undifferentiated A_s, A_{pr} and A_{al} spermatogonia (Costoya et al., 2004; Buaas et al., 2004; Hobbs et al., 2012; Eildermann et al., 2012a; Gassei & Orwig, 2013; Tokuda et al., 2007; Goertz et al., 2011), including some overlap with cKIT+ differentiating spermatogonia (Fig. 3A). Based on the restricted pattern of expression, some have suggested that cells expressing ID4, PAX7 and/or BMI1 might be the ultimate spermatogonial stem cells ($SSC_{ultimate}$) (Helsel et al., 2017; Lord & Oatley, 2017; de Rooij, 2017). Indeed, the expression of each marker on functional stem cells has been confirmed by SSC transplantation and/or lineage tracing. However, there is little information about the extent of overlap among these markers; whether any of these proteins mark the entire population of A_s spermatogonia or whether the entire population of functional stem cells resides in the population of A_s spermatogonia. In fact, molecular heterogeneity among undifferentiated spermatogonia of all clone sizes has been repeatedly documented (Suzuki et al., 2009; Gassei & Orwig, 2013; Hermann et al., 2015; Nakagawa et al., 2010; Zheng et al., 2009).

It seems reasonable to suppose that the stem cell pool also extends to some A_{pr} spermatogonia because A_s must transit through an A_{pr} state in the process of self-renewal (see Fig. 1); this concept has been described as “false pairs” and is nicely reviewed in (de Rooij & Griswold, 2012). Furthermore, Hara and colleagues provided live video imaging of GFRa1-GFP spermatogonia data to suggest that fragmentation of larger clones (eg., A_{al4} fragmenting to A_{al3} + A_s or A_{pr} + 2 A_s or 4 A_s) was an important contributor to maintenance of the A_s pool ((Hara et al., 2014); Fig. 1A). While the fate of the fragmenting clones could not be documented in that study, clones of A_{al3} (possibly resulting from clone fragmentation) have been observed by others (Suzuki et al., 2009; Gassei & Orwig, 2013; Tokuda et al., 2007; Hara et al., 2014). However, one concern with the clone fragmentation model is that it is based entirely on observations of GFRa1 positive cells and does not account for the contribution of GFRa1 negative cells that are known to exist in the pool of A_s (Suzuki et al., 2009; Gassei & Orwig, 2013) and the pool of transplantable stem cells (Grisanti et al., 2009; Garbuzov et al., 2018). Any model that considers only a part of the stem cell pool defined by a single molecular marker is likely to be incomplete.

To date, the only way to definitively identify a spermatogonial stem cell is by observing its capacity to produce and maintain spermatogenesis long-term, by transplantation (Brinster & Zimmermann, 1994;

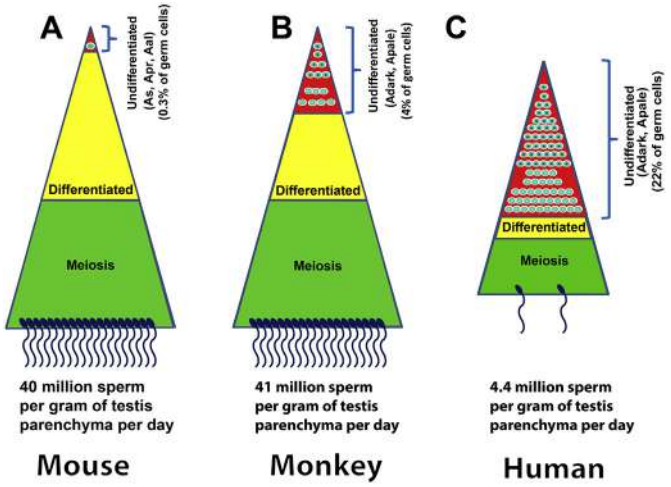

Fig. 2. Schematic comparison of the stem cell pools and sperm output in mice, monkeys and humans. A) The spermatogenic lineage in mice features a relatively small pool of A_s, A_{pr} and A_{al} undifferentiated spermatogonia (0.3% of germ cells). However, with about 12 transit amplifying divisions between stem cells and sperm (see Fig. 1), the small pool of stem cells produced 40 million sperm per gram of testicular tissue per day. B) The spermatogenic lineage in monkeys features a relatively larger pool of Adark/Apale spermatogonia (4% of germ cells) and this compensates for the reduced number of transit amplying divisions between stem cell and sperm. Sperm output in monkeys is similar to mice: 41 million sperm per gram of testicular tissue per day. C) The spermatogenic lineage in humans features the largest pool of undifferentiated Adark/Apale spermatogonia (22% of germ cells). However, with only one transit amplifying division of differentiated spermatogonia, sperm output in humans is reduced to 4.4 million sperm per gram of testicular tissue per day. Thus distinguishing features of spermatogenic lineage development in mice, monkeys and men include 1) the size of the pool of undifferentiated stem/progenitor spermatogonia; 2) the number of transit amplifying divisions in differentiated spermatogonia and 3) sperm output.

Brinster & Avarbock, 1994) or lineage tracing (Aloisio et al., 2014; Komai et al., 2014; Nakagawa et al., 2007). These are retrospective assays. There is no evidence that the mouse SSCs can be prospectively defined completely and exclusively by a specific clone size or molecular marker (see discussion above). However, it is generally agreed that smaller clones are more undifferentiated while larger clones are more differentiated and that cKIT marks the transition to differentiated type A1 spermatogonia. Differentiated type A1 spermatogonia in rodents appear to be equivalent to type B1 in nonhuman primates and type B in humans based on appearance of heterochromatin and initiation of cKIT expression (Figs. 1 and 3). Co-staining with cKIT and a marker of undifferentiated spermatogonia (e.g., PLZF, SALL4, CDH1, UCHL1, etc) in whole mount preparations of seminiferous tubules can help to define the clone size where undifferentiated stem/progenitor spermatogonia transition to differentiated spermatogonia. In mice, this transition occurs most frequently at a clone size of 16 (Fig. 1A), but can also happen at clones sizes of 8 and less frequently at smaller clone sizes (Suzuki et al., 2009; Gassei & Orwig, 2013; Tokuda et al., 2007; Hara et al., 2014). In nonhuman primates and humans, this transition occurs at smaller clone sizes (Fig. 1B & C; see discussion below).

3. Stem cells and spermatogenic lineage development in higher primates

Nonhuman primate and human testes contain two morphologically distinct types of undifferentiated spermatogonia, identified as A_{dark} and A_{pale}, based on differences in nuclear morphology and staining intensity with hematoxylin (Clermont & Leblond, 1959; Clermont & Antar, 1973; Clermont, 1966). A_{dark} spermatogonia are "relatively small, spherical or slightly ovoid" cells on the basement membrane of seminiferous tubules having dark, dense chromatin in their "uniformly stained" nuclei. A_{pale} spermatogonia are identified as "relatively larger, oval" or almost round cells on the basement membrane of the seminiferous tubules having pale, elongated nuclei with "coarser" or more "granular

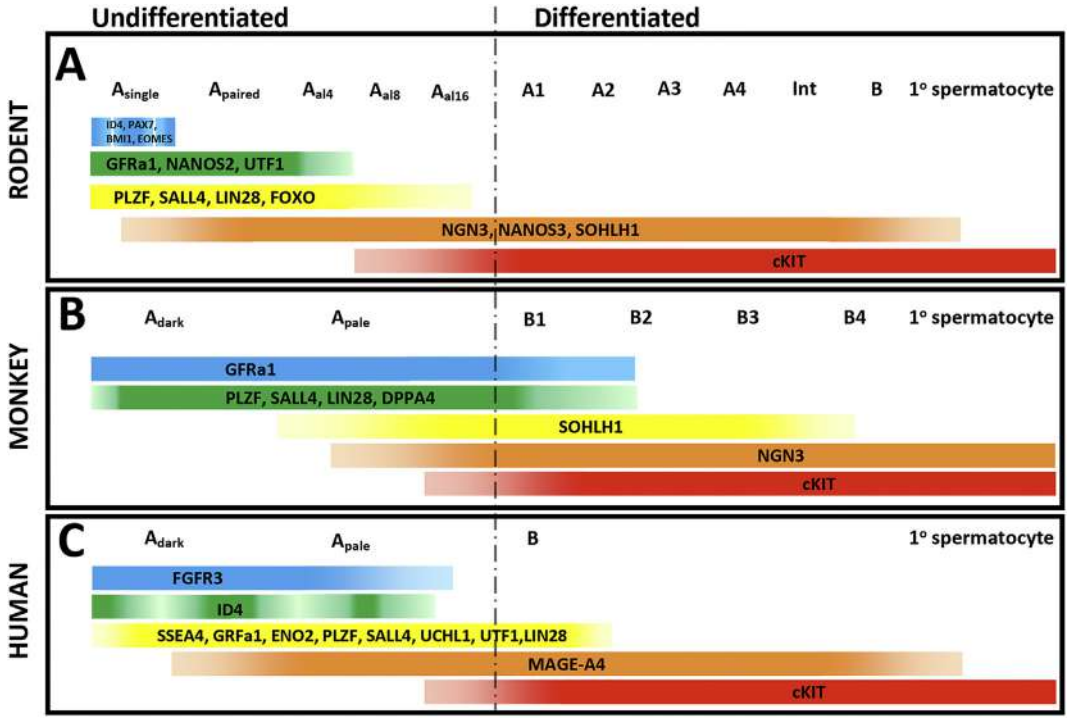

Fig. 3. Spermatogonial markers in rodents, monkeys and humans. A) Rodents; B) Monkeys; C) Humans. Several markers are conserved from rodents to monkeys to humans, suggesting their importance in spermatogenic lineage development. GFRa1, PLZF, SALL4 and LIN28 are conserved markers of undifferentiated spermatogonia. cKIT is a conserved marker of differentiating/differentiated spermatogonia. The following references describe markers in this figure that were not referenced elsewhere in the text: NGN3 (Yoshida et al., 2004); SOHLH1 (Ballow et al., 2006).

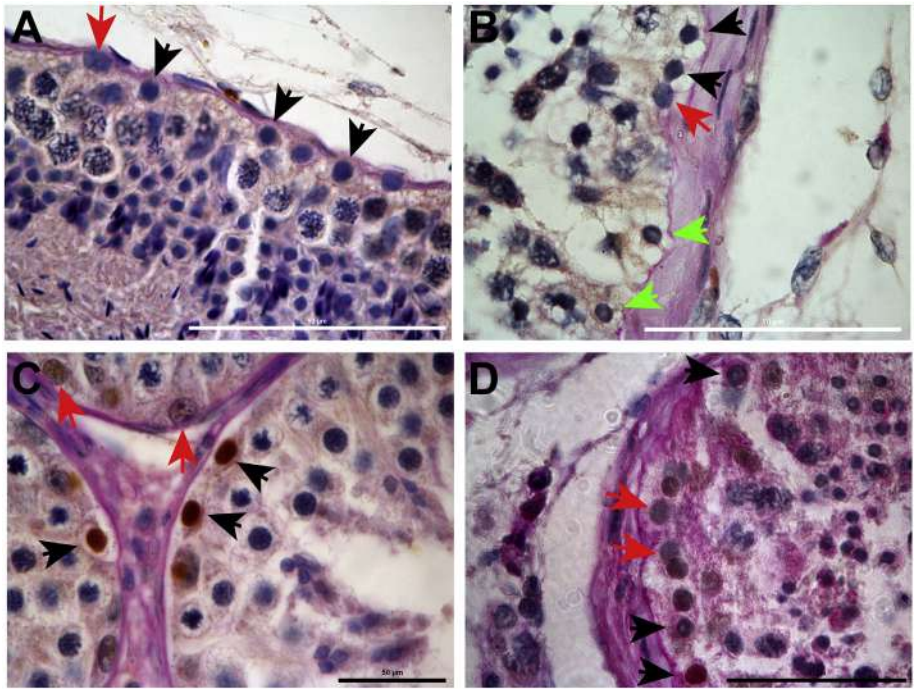

Fig. 4. Histological and immunohistochemical evaluation of A_{dark} and A_{pale} spermatogonia in monkeys and humans. Periodic acid, Schiffs' and Hematoxylin (PAS-H) staining in monkey (A and C) and human (B and D) testis section reveals A_{dark} (black arrows) and A_{pale} (red arrows) spermatogonia on the basement membrane of the seminiferous tubules. The subpopulation of A_{dark} spermatogonia with a rarefraction zone are indicated by a green arrow in (B). Colorimetric staining for UTF1 (brown color) with PAS-H staining confirms that UTF1 is a conserved marker of most, but not all A_{dark} (black arrow) and A_{pale} (red arrows) spermatogonia in monkey (C) and Human (D) testes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

chromatin". Nucleoli may be visible in both A_{dark} and A_{pale} spermatogonia (Fig. 4A). B-type spermatogonia are identified by their relatively larger size, location on or close to the basement membrane of the seminiferous tubules; clear and roundish nuclei and they are differentiated from one another by the granulation and density of heterochromatin staining. B1 spermatogonia are least heterochromatic and B4 spermatogonia are most heterochromatic (Clermont & Leblond, 1959) Some studies have identified a "rarefraction zone" (chromatin free zone) in a subpopulation of A_{dark} spermatogonia (Fig. 4B indicates examples of A_{dark} with rarefaction zone and A_{dark} without rarefaction zone). The observation of a rarefaction zone may be fixation-dependent and is used more frequently to describe A_{dark} spermatogonia in humans than in nonhuman primates (see review from von Kopylow et al. in this special issue) (von Kopylow et al., 2010; Lim et al., 2011; Paniagua & Nistal, 1984; Schulze, 1978). There are currently few researchers with the experience or patience to use the classic A_{dark} and A_{pale} descriptors of primate spermatogonia. However, researchers who do so provide a valuable link between contemporary molecular readouts and the histological descriptions in the classic literature.

In 1959, Clermont and Leblond proposed that A1 (A_{dark}) are the stem cells, which divide to either self–renew and maintain the stem cell pool or give rise to the A2 (A_{pale}) progenitor cells that may undergo one or more transit-amplifying divisions before giving rise to differentiated B1 spermatogonia. Clermont revised his model 10 years later based on in vivo labeling with ^{3}H-thymidine, which indicated that A_{pale}, but not A_{dark}, incorporated ^{3}H-thymidine in Vervet monkeys (*Cercopithecus aethiops*). Since A_{dark} did not appear to self-renew under steady state conditions, he proposed that A_{pale} are the "active" stem cells that maintain spermatogenesis in the adult testis while A_{dark} are "reserve" stem cells that regenerate spermatogenesis when it is destroyed by noxious insult (e.g., chemotherapy or radiation) (Clermont, 1969). Experimental evidence supporting this model are derived from observations in nonhuman primates and men that X-irradiation caused a striking depletion of spermatogenesis, including the entire population of A_{pale}, which were subsequently replenished from the surviving pool of A_{dark} spermatogonia (Clifton & Bremner, 1983; Oakberg, 1968; Oakberg, 1975; van Alphen et al., 1988). Ehmcke and Schlatt argued that low mitotic index and regenerative capacity of A_{dark} is consistent with the characteristics of a "true stem cell" and the regular proliferation of A_{pale} is indicative of "renewing progenitors" (Ehmcke & Schlatt, 2006). During the past 50 years, eight studies have reported on the acute labeling index of A_{dark} and A_{pale} spermatogonia. While four studies observed no labeling in the A_{dark} spermatogonia (Buageaw et al., 2005; Schlatt & Weinbauer, 1994; de Rooij et al., 1986; Simorangkir et al., 2009), consistent with the results of Clermont; four studies reported a wide range (0.06% to 18%) of A_{dark} labeling (Clermont & Antar, 1973; Fouquet & Dadoune, 1986; Ehmcke et al., 2005; Kluin et al., 1983). In all of those studies, ^{3}H-thymidine or BrdU was administered as a single bolus, and this may not effectively label a very slow cycling population of stem cells. Chronic labeling studies are needed to determine whether A_{dark} are indeed quiescent or whether A_{dark} are slow-cycling, active stem cells in steady state spermatogenesis. We have proposed that A_{dark} and at least some A_{pale} are the same population of cells that are simply at different stages of the cell cycle (i.e., A_{dark}: G0 versus A_{pale}: G1/S/G2/M) (Hermann et al., 2010). The concept that A_{dark} are in prolonged G0 is supported by observations of von Kopylow and colleagues, who found the Ki67 was expressed by A_{pale}, but not A_{dark} spermatogonia of the human testis (von Kopylow et al., 2012a). We believe this indicates that A_{dark} are slow cycling (long G0 phase), not quiescent or reserve, because when we treated adult Rhesus macaques with BrdU in the drinking water for three weeks, nearly 15% of A_{dark} incorporate label (Fayomi and Orwig, In Preparation).

4. Clonal expansion in higher primates

Three dimensional reconstruction mapping of serial cross sections and camera lucida drawings indicated that A_{dark} and A_{pale} spermatogonia in monkey testes are arranged in clones of 1, 2 or 4 cells, suggesting that there are only 1 or 2 transit amplifying divisions before differentiation to B1 spermatogonia (Clermont & Leblond, 1959; Clermont, 1969). This is fewer than the 3–4 transit amplifying divisions that occur in undifferentiated rodent spermatogonia before differentiation to type A1 spermatogonia (See Fig. 1A & B; Fig. 2A & B). Subsequent divisions from B1 spermatogonia in nonhuman primates produce types B2, B3 and B4 spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids that undergo spermiogenesis to produce mature sperm (Clermont & Leblond, 1959). Thus, the number of transit amplifying divisions from B1 spermatogonia to

spermatocyte in monkeys (four) is less than the six divisions from A1 spermatogonia to spermatocytes in rodents (Fig. 1A & B; Fig. 2A & B). Despite these differences in spermatogonial transit amplifying divisions, sperm output in rodents and monkeys is about the same (~40 million sperm per gram of testicular parenchyma per day; see Figs. 1 & 2). In contrast, men have only one generation of differentiated type B spermatogonia and sperm output is reduced to 4.4 million sperm per gram of testicular parenchyma per day (Fig. 1C; Fig. 2C; reviewed in (Valli et al., 2015)).

To summarize, there are a total of 12 transit amplifying divisions from the isolated A_s spermatogonia in rodents to the terminally differentiated sperm, which should yield 4096 sperm per stem cell that commits to differentiate (12 doublings = 2^{12} = 4096) (Russell et al., 1990). The actual yield is considerably less due to massive apoptosis (~50%) that occurs in the differentiated type A2-A4 spermatogonia (de Rooij, 1973; Huckins & Oakberg, 1978b; Huckins, 1978). By comparison, there appear to be only 8 transit amplifying divisions in nonhuman primates and 5 transit amplifying divisions in humans between the isolated A_{dark}/A_{pale} undifferentiated spermatogonia and terminally differentiated sperm (Fig. 1). Assuming similar stem cell pool sizes and spermatogenic lineage development dynamics, one might expect that sperm output in nonhuman primates (2^8 = 256) and humans (2^5 = 32) to be reduced 16-fold and 128-fold, respectively, compared with mice. However, as indicated above and in Figs. 1 and 2, this is not the case. Sperm output in monkeys is equivalent to rodents and sperm output in humans is reduced by only 10-fold compared with rodents. The contribution of apoptosis to sperm output in higher primates is not known, but the size of the stem cell pool is likely to be major contributor to differences in sperm output among species.

5. The pool of stem/progenitor spermatogonia in higher primates is larger than rodents

As described above, the precise molecular or clone size definition of functional stem cells in the rodent testis is subject to debate. However, the broader pool of stem & transit amplifying progenitors in rodents is understood to include A_s, A_{pr} and A_{al} spermatogonia with a cKIT negative phenotype. Similarly, the precise definition of functional stem cells in primate testes are subject to debate (Valli et al., 2015; Hermann et al., 2009), but the broader pool of stem/progenitor spermatogonia resides in the population of A_{dark} and A_{pale} spermatogonia with a cKIT negative phenotype. In rodents, the population of A_s, A_{pr} and A_{al} undifferentiated spermatogonia comprises 0.3% of germ cells in the testis (Fig. 2A). In nonhuman primates, A_{dark} and A_{pale} spermatogonia are present in equal numbers and comprise 4% of germ cells in the testis (Marshall & Plant, 1996). Like nonhuman primates, A_{dark} and A_{pale} spermatogonia are present in equal numbers in the human testis (Clermont, 1966; Schulze, 1978; Paniagua et al., 1987) and constitute 22% of germ cells in the testis (Paniagua et al., 1987). Thus, the larger pool of stem/progenitor cells in the testes of higher primates compensates, in part, for the reduced number of transit amplifying divisions (Figs. 1 & 2). The large pool of stem/progenitor cells in higher primates may also be a mechanism to reduce the replicative demand on each individual stem cells in longer lived species.

6. Molecular description of spermatogonia in higher primates

Based on expression of conserved molecular markers, A_{dark} and some A_{pale} spermatogonia in nonhuman primates and humans exhibit an undifferentiated phenotype, similar to A_s, A_{pr} and some A_{al} rodent spermatogonia (GFRa1+, PLZF+, SALL4+, cKIT−). Some A_{pale} have a transition phenotype similar to larger chain A_{al} spermatogonia in rodents (e.g., GFRa1+/SOHLH1+/NGN3+/cKIT+) (Hermann et al., 2009; Ramaswamy et al., 2014). Markers of undifferentiated spermatogonia that are conserved from rodents to nonhuman primates to humans include GFRa1, UTF1, PLZF, SALL4 and LIN28 (van Bragt et al., 2008; Meng et al., 2000; Costoya et al., 2004; Buaas et al., 2004; Hobbs et al., 2012; Eildermann et al., 2012a; Gassei & Orwig, 2013; Zheng et al., 2009; Hermann et al., 2009; Ramaswamy et al., 2014; Aeckerle et al., 2012; Lin et al., 2015; Di Persio et al., 2017; Valli et al., 2014; Zheng et al., 2014; Sachs et al., 2014) (See UTF1 staining of monkey and human testis cross sections in Fig. 4C and D). ID4 is conserved in the undifferentiated spermatogonia of rodents (Oatley et al., 2011) and humans (Sachs et al., 2014), but has not been described in nonhuman primates. cKIT appears to be a conserved marker of differentiated spermatogonia, marking the transition to A1 spermatogonia in rodents; B1 spermatogonia in nonhuman primates and B spermatogonia in humans (Hermann et al., 2009; Valli et al., 2014). There are no molecular markers that distinguish the entire population of A_{dark} from the entire population of A_{pale}, perhaps because both are elements of the same stem cell pool that are in different stages of the cell cycle (Hermann et al., 2010). However, a few markers have been identified that are restricted to the subpopulation of A_{dark} with a rarefaction zone (EXOSC10, FGFR3, OCT2) (Lim et al., 2011; von Kopylow et al., 2012a; von Kopylow et al., 2012b) and a few markers are restricted to A_{pale} (DMRT1, Ki67, SSX2-4) or a subpopulation of A_{pale} (NGN3, cKIT) (von Kopylow et al., 2012a; Hermann et al., 2009).

7. Cell surface markers of undifferentiated spermatogonia in higher primates

To date, no cell surface marker has been identified in any species with expression restricted to functional spermatogonial stem cells. GFRa1 is a conserved marker that appears to be most restricted to undifferentiated spermatogonia (i.e., A_s, A_{pr}, A_{al4} in rodents and A_{dark} and A_{pale} in higher primates). This marker has been used to isolate and enrich undifferentiated spermatogonia (Garbuzov et al., 2018; Buageaw et al., 2005; Gassei et al., 2010; He et al., 2012), but many investigators have reported difficulty sorting SSCs using GFRa1 antibodies (personal communications and unpublished data). It is also now clear that half of functional stem cells in the adult mouse testis are in the GFRa1 negative fraction (Garbuzov et al., 2018). This may indicate that stem cells oscillate between GFRa1+ and GFRa1− states depending on cell cycle status, signals from the SSC niche, density of germ cells on the basement membrane or other circumstances. In contrast, GFRa1 appears to be expressed by all A_{dark} and A_{pale} spermatogonia in the Rhesus macaques (Hermann et al., 2009), which presumably include the entire population of functional stem cells. ITGA6 is another robust and conserved marker that can be used to isolate and enrich SSCs from rodent, monkey and human testis cell suspensions (Valli et al., 2014; Shinohara et al., 2000; Maki et al., 2009). ITGA6 expression is not restricted to SSCs or even germ cells, but the entire population of functional SSCs can be recovered and are significantly enriched in the ITGA6+ fraction of rodent (Shinohara et al., 2000) and human testis cells (Valli et al., 2014). SSEA4 has not been used to isolate mouse SSCs, but is expressed by undifferentiated spermatogonia in monkey and human testes and has been used effectively to isolate transplantable SSCs (Zheng et al., 2014; Muller et al., 2008; Izadyar et al., 2011; Eildermann et al., 2012b; Smith et al., 2014). CD9 is expressed by a subpopulation of MAGEA4+ spermatogonia in human testes and can be used to isolate transplantable stem cells (Zohni et al., 2012). ITGA6, SSEA4 and CD9 are effective single markers for isolating primate spermatogonia because they clearly segregate the heterogeneous testis cell suspension into positive and negative fractions and have been tested functionally by xenotransplantation into infertile mouse recipients. Thus, the majority of functional SSCs are captured in the positive fractions with limited loss to the negative fractions. Other cell surface markers that have been used to isolate and enrich functional SSCs, alone or in combination with other markers, include CD90, EpCAM and GPR125 (Hermann et al., 2009; Valli et al., 2014; Kubota et al., 2003; Ryu et al., 2004; Nickkholgh et al., 2014).

8. SSC transplantation bioassay in higher primates

Similar to rodents, transplantation is the established method to quantify functional stem cells in higher primates. Of course, homologous species transplantation is not possible in humans. Homologous species SSC transplantation is possible in primates (Hermann et al., 2012; Jahnukainen et al., 2011), but not practical as a routine biological assay. Therefore, xenotransplantation to the testes of infertile, immune deficient mice has emerged as the gold standard to quantify functional stem cells from monkey or human cells populations. Human and monkey SSCs do not regenerate complete spermatogenesis when transplanted into mouse testes. However, they do migrate to the seminiferous tubule basement membrane and produce chains or networks of spermatogonia that persist for many months after transplantation (Hermann et al., 2009; Valli et al., 2014; Izadyar et al., 2011; Zohni et al., 2012; Nagano et al., 2001; Nagano et al., 2002; Hermann et al., 2007; Wu et al., 2009; Sadri-Ardekani et al., 2009; Sadri-Ardekani et al., 2011; Dovey et al., 2013; Clark et al., 2017; Durruthy Durruthy et al., 2014; Ramathal et al., 2014). It is not currently possible to recapitulate complete spermatogenesis from monkey or human cells using the xenotransplantation assays. Perhaps one day this challenge will be overcome by transplantation to more closely related species and/or using an organ culture system similar to that described for producing eggs or sperm from primordial germ cell-like cells (PGCLCs) in mice (Hayashi et al., 2012; Zhou et al., 2016).

9. Concluding remarks

Although different vocabularies have evolved to describe spermatogonial stem cells and spermatogenic lineage development in rodents, monkeys and humans, many features are conserved between species. For example, spermatogenesis emerges from isolated spermatogonia that give rise to clones of interconnected chains or networks of cells that become progressively more differentiated with each successive transit amplifying division. In all species, smaller clones are the more undifferentiated elements while larger clones are the more differentiated elements. Many markers are conserved from rodents to primates to humans as well as their association with undifferentiated versus transition versus differentiated spermatogonia. Some markers appear to be more species specific, but in some cases, this may be an artifact of antibody quality or availability for different species. There are important differences between rodents and higher primates. Rodents have more transit amplifying divisions in the pool of undifferentiated and differentiated spermatogonia than nonhuman primates or humans. Based on sperm output data, the difference in transit amplifying divisions appears to be fully compensated by a much larger pool of stem/progenitor spermatogonia in nonhuman primates. In contrast, the large pool of stem/progenitor spermatogonia in humans does not compensate for the reduced number of transit amplifying divisions and consequently, sperm output is reduced. Understanding similarities and differences between species will help to explain challenges in translating technologies such as SSC culture and SSC transplantation to higher primates and ultimately to the human clinic.

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Review

Spermatogonial Stem Cells for In Vitro Spermatogenesis and In Vivo Restoration of Fertility

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Abstract: Spermatogonial stem cells (SSCs) are the only adult stem cells capable of passing genes onto the next generation. SSCs also have the potential to provide important knowledge about stem cells in general and to offer critical in vitro and in vivo applications in assisted reproductive technologies. After century-long research, proof-of-principle culture systems have been introduced to support the in vitro differentiation of SSCs from rodent models into haploid male germ cells. Despite recent progress in organotypic testicular tissue culture and two-dimensional or three-dimensional cell culture systems, to achieve complete in vitro spermatogenesis (IVS) using non-rodent species remains challenging. Successful in vitro production of human haploid male germ cells will foster hopes of preserving the fertility potential of prepubertal cancer patients who frequently face infertility due to the gonadotoxic side-effects of cancer treatment. Moreover, the development of optimal systems for IVS would allow designing experiments that are otherwise difficult or impossible to be performed directly in vivo, such as genetic manipulation of germ cells or correction of genetic disorders. This review outlines the recent progress in the use of SSCs for IVS and potential in vivo applications for the restoration of fertility.

Keywords: spermatogonial stem cells; germline stem cells; male germ cells; in vitro culture; in vitro spermatogenesis; male infertility

1. Introduction

Spermatogonial stem cells (SSCs) are a small group of testis cells, residing at the basal membrane of seminiferous tubules. They can undergo measured mitotic divisions to balance self-renewal and formation of exponentially increasing numbers of differentiating germ cells. What makes SSCs unique among all other adult stem cells is their potential for contributing genes to the next generation. Due to their fundamental role in maintaining spermatogenesis, SSCs ensure a supply of millions to billions of sperm per day throughout the reproductive life of a male. SSCs produce cohorts of daughter germ cells to undergo synchronized, sequential, and extensive differentiation processes to be transformed from a typical spherical cell into self-propelling vehicle systems with only one mission, to deliver the male haplotype into the female counterpart, the haploid oocyte [1,2]. Considerable insight has been gained in the past three decades about the functional potential of SSCs in initiating spermatogenesis, and even their potential to be driven into pluripotency for use as an alternative to embryonic stem (ES) cells. However, we are still far from sufficient understanding of SSCs and realizing their full potential in assisted reproductive technologies and stem cell therapy. This is largely due the difficulty of unequivocal identification of SSCs and the complexity of replicating their differentiation properties and function in vitro [3,4].

Since SSCs are very rare in the testis, development of effective and efficient in vitro culture systems that support the maintenance and expansion of SSCs is crucial for their characterization and

manipulation. Moreover, optimal conditions for the in vitro culture and propagation of SSCs are also needed to help boost the potential therapeutic application of SSCs in fertility preservation or restoration. Long-term culture of rodent SSCs is well demonstrated; however, relatively few in vitro culture conditions have been examined for primate SSCs [2]. Any potential in vivo application of cultured human SSCs, such as in restoration of fertility, requires extensive studies to ensure their safety and efficacy. The establishment of efficient culture conditions for human SSCs also necessitates the availability of proper animal models, for instance, xenotransplantation techniques to assess the quality and quantity of such cultured SSCs; these assays have so far been used sporadically for testing primate SSCs [4].

Spermatogenesis is a complex process of germ cell proliferation and differentiation that requires extensive interactions among different cell types, hormones, growth factors, and various other signals, making it difficult to be replicated in vitro. There are several objectives for establishing an optimal and efficient culture system to recapitulate the process of germ cell development in vitro. This includes the study of basic requirements of male germ cell development, proliferation, differentiation, and production of haploid germ cells in a controlled in vitro environment. Additionally, such culture systems could be potentially used to produce haploid male germ cells from undifferentiated germ cells isolated from the testis of infertile adult patients and/or testicular biopsies collected from prepubertal cancer patients before undergoing gonadotoxic treatment. The establishment of culture systems for in vitro spermatogenesis (IVS) would also enable experimentations that are otherwise difficult to be performed directly in vivo such as pharmaceutical or toxicological study of new drugs or potential toxicants on human spermatogenesis. Other applications include the study of mechanisms of testicular tumors, genetic causes of male infertility, or even correction of genetic disorders causing infertility. Current approaches to IVS can be divided into three general categories including organ/tissue culture, and two-dimensional (2D) or three-dimensional (3D) cell suspension culture systems. Moreover, developing an efficient IVS model can be of benefit not only from a research perspective but also from an animal ethics point of view by using non-animal models.

This article summarizes the progress made in offering efficient methods for SSC isolation, characterization, in vitro culture, and in vitro differentiation, particularly using primate models. Moreover, in this article we review some of the salient proposed approaches for the preservation and restoration of fertility in prepubertal and pubertal patients using currently-available and potential future SSC-driven biotechnological strategies.

2. Spermatogonial Stem Cells (SSCs)

Male germline stem cells include SSCs, as a better-known component, as well as two earlier cellular stages, namely primordial germ cells (PGCs) and gonocytes. All mammalian germ cells originate from PGCs which are the primary cells of the germline lineage in both male and female embryos. PGCs first appear as a small population of alkaline phosphatase-expressing cells at ~7.5–8 days post-coitum (dpc) in rodents and at ~three weeks gestation in humans. Drawn by chemotaxis, PGCs migrate to the genital ridge and reside in the indifferent gonad at ~11–13 dpc and 4–5 weeks gestation in rodent and human embryos, respectively. Once PGCs lose alkaline phosphatase expression, they become known as gonocytes at ~14.5 dpc in rodents and ~seven weeks gestation in human embryos. After birth, gonocytes transform into spermatogonia at ~5 days post-partum (dpp) in rodents and ~three months after birth in newborn boys [5,6].

As depicted in Figure 1A, parenchyma of the postnatal testis tissue has a compartmentalized structure where germ cells along with their main supporting somatic, Sertoli, cells are enclosed in seminiferous tubules (or cords when they lack a lumen in immature testes), surrounded by peritubular myoid cells. The space between the tubules/cords is occupied by androgen producing Leydig cells, along with other components of the connective tissue. Spermatogenesis involves various interactions between somatic cells and germ cells. The process of spermatogenesis begins with spermatogonia that reside at the basal membrane which undergo a proliferation phase, comprised of a series of

mitotic divisions to exponentially increase the number of germ cells, before undergoing meiosis to be transformed into haploid germ cells, spermatids and sperm. This process is repeated regularly at pre-determined intervals to ensure a continuous supply of sperm is present at any given time during the reproductive life of an adult male. Based on morphological studies using rodent models, spermatogonia in non-primate mammals have been proposed to include stem cell (A_{single}), proliferative (A_{paired}, $A_{aligned}$), and differentiating (A_1, A_2, A_3, A_4, intermediate, and B) spermatogonia.

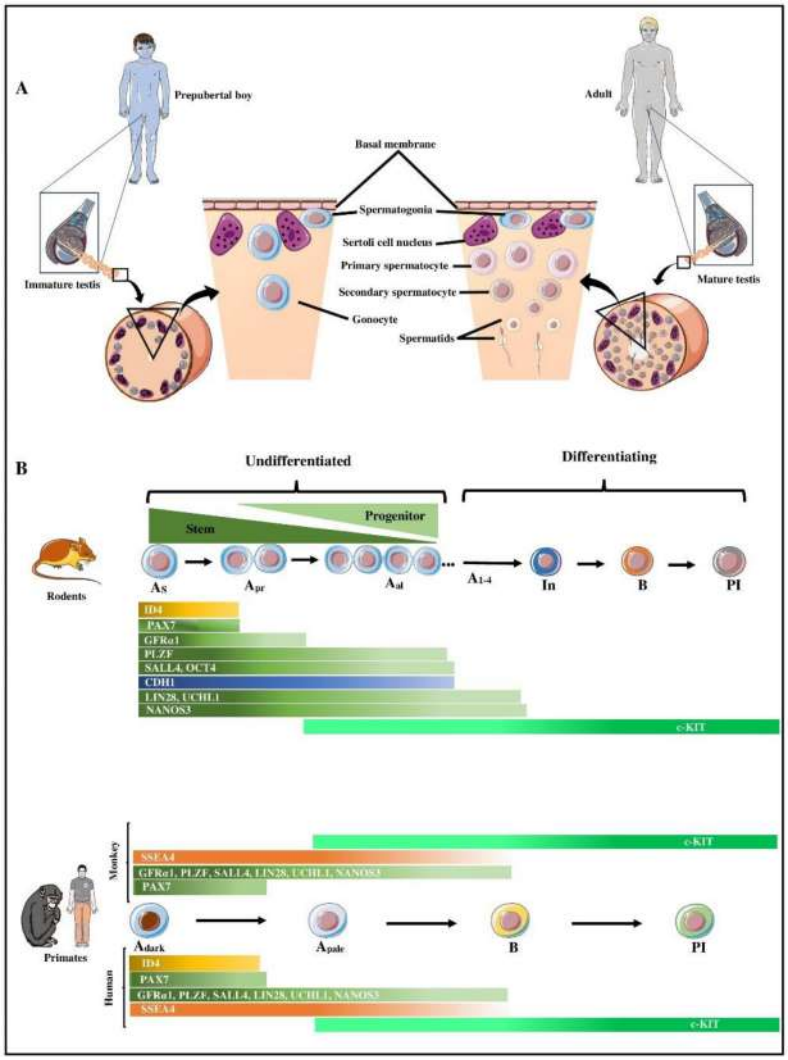

Figure 1. Schematic representation of various spermatogenic cells present in the prepubertal and adult testis, as well as comparative representation of spermatogonial markers in rodents and primates. (**A**) A schematic pie slice of a seminiferous cord/tubule of a human prepubertal and an adult showing the different types of spermatogenic cells present. Gonocytes or spermatogonia (depending on age) are the only types of germ cells present in the testis of prepubertal boys, whereas in the adult testis all stages of spermatogenic cells can be present. Spermatogonia reside at the basal membrane and are intra-tubularly surrounded by Sertoli cells. Spermatogonia undergo differentiation into spermatocytes, spermatids, and ultimately sperm. (**B**) Comparative developmental patterns and expression profile of select spermatogonial markers in rodents and primates. Rodents and primates have several common spermatogonial markers (green color-coded) with few exceptions (blue, orange, and gold-coded). For a more complete list of the currently-known molecular markers of rodent and primate spermatogonia see Table 1. A_s: Type A-single spermatogonia; A_{pr}: Type A-paired spermatogonia; A_{al}: Type A-aligned spermatogonia; A_{dark}: Type A spermatogonia with dark nuclei; A_{pale}: Type A spermatogonia with pale nuclei; In: intermediate spermatogonia; B: Type B spermatogonia; Pl: preleptotene spermatocytes.

Type A_{single} spermatogonia are present as individual half-moon-shaped cells in close proximity to the basal membrane [1,7]. Being the most primitive spermatogonia, A_{single} spermatogonia are believed to be SSCs which can undergo a symmetric division to produce two separate, but otherwise similar, self-renewing A_{single} (SSCs) to increase their numbers and form a stem cell pool, for instance to regenerate steady-state spermatogenesis after cytotoxic insult. Alternatively, and during steady-state spermatogenesis, SSCs can undergo an asymmetric division to produce a self-renewing A_{single} to maintain the stem cell pool and a progenitor A_{single}, destined for differentiation. Each progenitor A_{single} can then divide to produce two cells which remain connected, hence referred to as A_{paired} spermatogonia [8]. All subsequent generations of germ cells also remain connected through intercellular cytoplasmic bridges, thought to be important for the coordination of their progression in the differentiation process. Hence, clones of 4-16 or sometimes 32 germ cells are formed in a chain, known as $A_{aligned}$ spermatogonia [1,7].

Type A spermatogonia that belong to the A_{single}, A_{paired}, and $A_{aligned}$ stages are collectively referred to as undifferentiated spermatogonia, but transplantation studies have concluded that they are indeed a heterogenous population. An adult mouse testis contains ~35,000 A_{single} out of a total number of ~330,000 undifferentiated spermatogonia. Although A_{single} are typically designated as 'true' SSCs, functional assays have shown that only an estimated 8.5%–17% of all A_{single} have stem cell potential. In other words, >80% of A_{single} are in fact not SSCs; however, it has been suggested that stem cell activity is strongest but not restricted to A_{single}. Although an exact point of no-return has not been determined, it is believed that the SSC activity sharply declines as spermatogonia progress into A_{paired}, and especially into $A_{aligned}$. The same functional assays conclude that only ~3000–6000 'functional' SSCs exist in an adult mouse testis, comprising 0.9%–1.8% of all undifferentiated spermatogonia or 0.01%–0.02% of total cells in the seminiferous tubules [9].

Larger clones of $A_{aligned}$ spermatogonia differentiate without a cellular division to A_1 spermatogonia and initiate synchronized further mitotic divisions to produce types A_2, A_3, A_4, intermediate, and B spermatogonia. While under conventional histological preparations various undifferentiated spermatogonia show similar cell-nuclear morphology, transition of $A_{aligned}$ into A_1 signals the start of differentiation and involves major changes in morphology and mitotic behavior. Type B spermatogonia must physically pass through the Sertoli-cell barrier (also known as the blood-testis barrier), before dividing to produce primary spermatocytes which in turn undergo two meiotic divisions to give rise to secondary spermatocytes and round spermatids, before undergoing spermiogenesis to produce sperm [4,7,9,10].

In primate testes, categorization of spermatogonia also includes type A and B spermatogonia but especially for type A spermatogonia it differs and includes A_{dark} and A_{pale}. Designation as A_{dark} is primarily based on their dark nuclear staining with hematoxylin. A_{dark} are believed to comprise a population of 'reserve' stem cells since they show low proliferative activity during normal spermatogenic activity. In contrast, A_{pale} are less densely stained during histological processing and are thought to proliferate continuously. Although, both A_{dark} and A_{pale} reside at the basal membrane, they have other morphological differences; A_{dark} are characterized as being relatively small, round or slightly ovoid, while A_{pale} are relatively larger, oval or almost round cells. Subpopulations of A_{dark} have been shown to contain 1–3 chromatin-free vacuolar spaces known as nuclear rarefaction zones [11], which can be used as a background morphological indicator to help discriminate spermatogonial subtypes undergoing immunohistochemical analyses.

In 1959, Clermont proposed that A_{dark} are SSCs which undergo self-renewal to maintain their population and at the same time also give rise to A_{pale} that subsequently generate differentiating B spermatogonia. Ten years later, Clermont revised his model based on the incorporation of 3H-thymidine in A_{pale} instead of A_{dark} spermatogonia of vervet monkeys, suggesting that A_{pale} are the 'active' stem cells that maintain spermatogenesis in the adult testis [12]. In humans, a similar model has been proposed, where A_{pale} act as active stem cells to maintain their population and produce differentiating B spermatogonia. However, some believe that the original model was more accurate, stating that

A_{dark} are indeed true SSC because of their low mitotic index, while nearly all A_{pale} undergo regular divisions suggesting that these cells are the 'renewing progenitors' that amplify spermatogonial output to B1. Primate A_{pale} and A_{dark} spermatogonia are considered as counterparts of rodent undifferentiated spermatogonia (A_{single}, A_{paired}, and $A_{aligned}$), and primate type B spermatogonia as equivalents of rodent differentiating spermatogonia (A_1–A_4, intermediate and B spermatogonia) (Figure 1B). Interestingly, the rhesus monkey testis contains approximately the same total number of A_{dark} and A_{pale} as in the human [13,14].

In humans, A_{pale} divide every 16 days, the length of one seminiferous epithelium cycle, and repeat it ~4.5 times; hence the germ cells that resulted from the first division of A_{pale} become sperm after ~75 days [15]. A low proliferative activity of SSCs is deemed beneficial [16], because it decreases the chance of errors in DNA duplication during the S phase. While the number of spermatogonial divisions from SSCs to spermatocytes is comparable between the monkey and non-primates, this number is lower in humans compared with many non-primates. In humans, there is only one spermatogonial generation between the A_1 and B stages [17], while there are six divisions separating these stages in most non-primate mammals [18].

3. Characteristics of Spermatogonia in Primates

From the above discussion it can be concluded that SSCs are a rare and heterogenous subpopulation of undifferentiated spermatogonia that can only be defined by their function, not morphology. Hence, there is a need to identify specific biomarkers that allow isolation and propagation of SSCs to better gain insight into their biology and harness their potential. In response to this need, in 1994 using mouse models, a technique for transplantation of germ cells was developed which can be viewed as a unique bioassay to assess the stem cell activity of putative SSCs in any given population of testis cells.

Germ cell transplantation involves the microinjection of testis cells from a donor male into the seminiferous tubules of a recipient male. This transplantation technique has also been extended to various species, but performing primate-to-primate germ cell transplantation is more technically challenging. Although, primate-to-mouse germ cell transplantation has been shown to allow evaluation of primate spermatogonia, it provides limited information regarding the identity of primates SSCs. Nevertheless, this assay has ever since been used as a routine method for the identification, quantification, characterization, and confirmation of functional competence of SSCs. Our recent knowledge of the molecular signature and biomarkers of SSCs, especially in rodents, has allowed enrichment of testis cells for SSCs to tens- or even hundreds of folds; however, still no unequivocal and exclusive SSC marker is available. Therefore, identification of SSC markers in primates remains as one of the areas of intense research in male reproductive science and medicine [4,19–21].

Analysis of monkey spermatogonia demonstrated that they share certain germ cell and SSC markers with rodent spermatogonia (Figure 1B, Table 1). Some of these markers are expressed on the cell surface and hence can be potentially used for isolation and enrichment through cell sorting methods, such as fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS). Other spermatogonial markers are located intracellularly and can only be used for retrospective identification of SSCs.

Mouse gonocytes and spermatogonia express LIN28 throughout testicular development [22]. The analysis of newborn macaques (*Macaca mulatta)* testes also revealed that 100% of seminiferous tubules contained $LIN28^+$ germ cells; however, the number of immune-positive cells decreased during testicular development. On the other hand, although several $LIN28^+$ tubular cross-sections in adult mouse testes can be detected, only a few $LIN28^+$ tubular cross-sections can be observed in adult marmoset, rhesus, or human testes [23]. Marmoset gonocytes and spermatogonia have also shown expression for SALL4, where the proportion of $SALL4^+$ germ cells decreased during puberty and was restricted to A_{dark} and A_{pale} spermatogonia in pubertal and adult testes. The expression of SALL4 was demonstrated in the majority of gonocytes in fetal human testes and type A spermatogonia of 1-year-old boys [24]. Adult rhesus testicular tissues also showed the expression of DDX4 (VASA),

DAZL, GFRα1, and PLZF [25]. Interestingly, the number of $PLZF^+$ cells was calculated to be ~1.86 per cross-section, suggesting that the SSC population in monkey testes is a subset of either the A_{dark} or A_{pale} spermatogonia. Other known markers of non-human primate spermatogonia include DPPA4 [26], TRA-1-60, TRA-1-81, [27], and THY1 [28].

Similar to non-human primates, spermatogonia and their progenitors in humans and rodents also share some but not all markers (Figure 1B, Table 1). For instance, in mice, α6-integrin, β1-integrin [29], and THY1(CD90) [30] are well-known surface markers of SSCs/progenitor cells, while CD9 is a surface marker of both rat and mouse SSCs [31]. Surface markers including α6-integrin, CD90, GFRα1, and CD133 have also been successfully used to select human spermatogonia using MACS [32]. The expression of PLZF has also been observed in whole mounts of seminiferous tubules of human testes [33]. Additionally, ID4 [34] and GPR125 are considered markers for mouse spermatogonia and their progenitors [35], and their expression has also been observed in human spermatogonia [36]. In contrast, some other markers of rodent spermatogonia and their progenitors may not be conserved in humans. For example, it remains to be explored whether certain markers of rodent spermatogonia such as RET [37], STRA8 [38], CDH1 [39], and NEUROG3 (NGN3) [40] are also present in human spermatogonia. Conversely, certain specific markers of human spermatogonia have not been observed in rodents. For example, TSPY, a specific marker for human spermatogonia [41] is not expressed by rodent spermatogonia; however, elongated spermatids of rats are positive for TSPY [42]. Similarly, other markers of human spermatogonia such as CD133 [32] or CHK2 [43] are yet to be examined for expression in rodents. Such studies can further reveal similarities and differences between spermatogonia in rodents and primates.

Table 1. Molecular markers of undifferentiated spermatogonia in primates and mice.

Markers	Mouse	Monkey	Human
Cell Surface Markers			
THY1 (CD90)	+ [30]	+ [44]	+ [32]
β1-integrin (ITGB1, CD29)	+ [29]	+ [45]	+ [46]
α6-Integrin (ITGA6, CD49f)	+ [29]	+ [44]	+ [32]
PROM1 (CD133)	?	?	+ [32]
GFRα1	+ [47]	+ [28]	+ [32]
CDH1	+ [39]	?	?
SSEA4	?	+ [44]	+ [48]
FGFR3	+ [49]	?	+ [50]
DSG2	?	?	+ [50]
LPPR3	?	?	+ [51]
TSPAN33	?	?	+ [51]
CD9	+ [31]	?	+ [52]
GPR125	+ [35]	?	+ [36]
Intracellular Markers			
PAX7	+ [53]	+ [53]	+ [53]
DDX4 (VASA)	+ [54]	+ [25]	+ [55]
DAZL	+ [13]	+ [25]	+ [33]

Table 1. *Cont.*

Markers	Mouse	Monkey	Human
CHK2	?	?	+ [43]
LIN28	+ [22]	+ [23]	+ [23]
MAGEA4	?	+ [56]	+ [57]
NANOS2	+ [58]	+ [59]	+ [60]
NANOS3	+ [60]	+ [59]	+ [60]
SALL4	+ [61]	+ [27]	+ [24]
UCHL1 (PGP9.5)	+ [62]	+ [24]	+ [33]
PLZF	+ [63]	+ [25]	+ [33]
POU5F1 (OCT4)	+ [64]	+ [56]	+ [65]
ID4	+ [34]	?	+ [36]
NEUROG3 (NGN3)	+ [66]	+ [28]	?
DMRT1	+ [67]	?	+ [68]
UTF1	+ [69]	+ [23]	+ [70,71]
TCF3	?	?	+ [33]
SPOCD1	?	?	+ [68]
ENO2	?	+ [72]	+ [70]
SNAP91	?	?	+ [50]
CBL	?	?	+ [50]
PIWIL4	?	?	+ [51]
ZKSCAN2	?	?	+ [68]
RET	+ [37]	?	?
STRA8	+ [38]	?	?
RBM	+ [73]	?	+ [74]
TSPY	− [42]	?	+ [41]

"+" or "−" indicate that evidence is available on the presence or absence of these markers, respectively; "?" indicates that we are not aware of studies that have examined these markers.

4. Isolation and Enrichment of SSCs in Primates

Because SSCs are a very rare subpopulation of testis cells, their use in downstream applications requires optimal isolation and purification, as an important first step. In addition to the need for large numbers of SSCs for applications such as transplantation into recipient testes, access to additional SSCs is also warranted for critical analysis of cultured cells in terms of genetic and epigenetic stability as well as functionality.

Testis cells can be isolated using enzymatic digestion, usually involving the use of a combination of enzymes in two steps. In brief, after removing the tunica albuginea and excess connective tissue, the testis parenchyma is divided into smaller fragments to be first incubated with collagenase to disperse the seminiferous tubules, followed by the addition of trypsin to obtain a single-cell suspension. If necessary, DNase-I is also added to prevent adhesion of the resultant cells. Two-step enzymatic digestion protocols have been widely used for digestion of testis tissue from non-human primates [44] and humans [75]. Since this digestion method is not an optimized or cell-targeted process, it generates a mixed population of testis cells where SSCs are present at low numbers. Therefore, it would be ideal if modified protocols were to be developed for digestion of primate testis tissue to favor the isolation toward collecting more germ cells. This optimization can be similar to a three-step digestion protocol

that we developed specifically for neonatal pig testes which yields ~40% highly-viable gonocytes within an hour [76]. In the absence of optimized digestion protocols, the obtained testis cell suspensions would typically need to go through further enrichment steps to reduce the number of contaminating somatic cells or advanced stages of germ cells.

Enrichment of germ cells can be achieved with or without the use of antibodies. Specific antibodies may be used to separate SSCs (positive selection) or to exclude contaminating cells (negative selection) using FACS or MACS. For instance, MACS with an anti-GFRα1 antibody has been used for the isolation of spermatogonial subpopulations from adult monkey and human testes. Gassei et al. (2010) reported that about two thirds of GFRα1-positive cells found in the sorted fractions resembled A_{dark} spermatogonia, while one third resembled the A_{pale} [77], suggesting that most of the isolated cells were SSCs. MACS has also been successfully employed for the enrichment of human spermatogonia using antibodies against GPR125 [33] and SSEA4 [48]. Nickkholgh et al. (2014) concluded that MACS for $ITGA6^+$ and $HLA^-/GPR125^+$ can be used for efficient enrichment of human spermatogonia [78]. The use of FACS to select OCT4-positive cells has also resulted in obtaining 87% purity of human SSCs [79]. Compared to FACS, MACS is considered a more efficient, accurate, and easier method for enrichment of SSCs, especially since it does not require large numbers of cells. Thus, MACS is better suited for applications such as isolating SSCs from cells that result from small biopsies of human testis tissue.

Germ cell enrichment strategies that do not implement antibodies rely on the innate cellular characteristics that differentiate germ cells from somatic cells. Such strategies include the affinity of different cell types for adhesion to the culture plate or to extracellular matrices (ECM). Since Sertoli cells adhere to the culture plate faster than SSCs, differential planting is a widely-used technique for the separation of germ cells from somatic cells. Alternatively, differences in velocity sedimentation or density gradient centrifugation can be used to separate somatic and germ cells. Percoll density gradient for instance has been used for cell separation of human testis cells leading to ~87% pure population of SSCs [79]. However, adjusting the timing for cell sedimentation is critical to prevent the loss of large numbers of SSCs during the high activity of the sedimentation process [80].

5. In Vitro Culture of Primate Gonocytes/Spermatogonia

Culture of rodent gonocytes/spermatogonia is well demonstrated [81]; however, especially long-term culture and expansion of primate SSCs, and conclusive demonstration of their SSC potential has been challenging. Establishment of an efficient in vitro culture system to maintain both the self-renewal and proliferation capacity of human SSCs is crucial for their potential clinical applications. Culturing human SSCs, isolated from testicular biopsies of obstructive azoospermia patients (aged 22–35 years), in StemPro-34 SFM (serum free medium) containing growth factors (commonly referred to as a serum-free germline culture medium) and hydrogel, resulted in significant propagation of SSCs for two months. During the culture, expression of numerous SSC markers was maintained, suggesting that SSCs retained their undifferentiated status [82]. Moreover, similar culture conditions appear to also support the short-term in vitro culture of human SSCs from healthy individuals without altering their undifferentiated status [33].

The media initially used to culture human hematopoietic stem cells also supported the long-term culture of human SSCs [83]. The growth factors used in the latter study (i.e., GDNF, BFGF, EGF, and LIF) were previously reported to support the long-term culture of rodents SSCs [84]. In these culture conditions, human testicular cells formed two types of cell colonies; one had a flat morphology and an appearance similar to ES cells, while the other had rounded morphology. Morphologically, round cells showed an expression profile typical of spermatogonia (e.g., PLZF, ITGA6, and ITGB). The SSC clusters presented as clumps of individually visible cells, while colonies of ES-like cells were sharp-edged and compact. In these culture conditions, human SSCs could be propagated for 15 weeks, after which germline stem cell clusters no longer appeared, and ES-like cells and somatic cells started detaching from the dish. It was also found that sub-culturing human SSCs under feeder cell-free

conditions in laminin-coated culture dishes could increase the duration of propagation (up to 20 weeks). Xenotransplantation assays showed an impressive 18,450-fold increase in human SSC numbers within a time frame of 64 days [83].

The germline culture medium also supported the short-term in vitro culture of non-human primate SSCs [24], while Stem-Pro medium, supplemented with four growth factors (i.e., bFGF, GDNF, LIF, and EGF), actively maintained the SSCs isolated from prepubertal cynomolgus monkeys (aged 44–57 months) [85]. However, the lack of using functional assays to verify human SSCs in most studies, and similarities between SSC markers of human and rodents in other studies make the results controversial. Moreover, many markers used to identify human SSCs (e.g., PLZF, GFRA1, UCHL1, GPR125, and ITGA6) can also be observed in many testicular somatic cells, suggesting that these markers are not reliable. The only dependable protocol to access whether cultured SSCs are indeed functional germ cells is homologous transplantation of in vitro-cultured SSCs into a recipient testis (of another male of the same species) and subsequent generation of offspring. However, due to obvious ethical concerns, this approach is not practical in humans and difficult in monkeys. In non-human primates, different stage-specific germ cell markers have been better-defined compared with humans. Therefore, to validate the true nature of germ cells in the culture of non-human primates, monitoring of validated germ cell markers can be used to establish an efficient in vitro culture condition. Development of a definitive functional assay to evaluate the competency of human SSCs or to identify reliable SSC-specific markers would facilitate finding a culture condition for long-term in vitro maintenance of SSCs.

6. In Vitro Models of SSC Differentiation

Establishing efficient in vitro culture systems that can replicate the process of male germ cell development and spermatogenesis has several important applications. These applications include the study of basic requirements, mechanisms of action, and cell-to-cell interactions of male germ cells during development, proliferation, differentiation, and production of haploid germ cells in a controlled in vitro environment. For instance, the use of germ cells from infertile patients may help investigations into the various causes of male infertility due to stage-specific blockage of germ cell differentiation. Development of efficient culture systems for IVS would also allow experimentations that are otherwise difficult to be performed directly in vivo, such as genome editing of germ cells or correction of genetic causes of infertility. Such models can be of benefit not only from a research perspective but also from an animal ethics view by using non-animal models. Various research groups have taken different routes toward the goal of developing an in vitro culture system; these can be classified into one of three general approaches, namely organotypic culture of testicular tissue fragments, and two-dimensional or three-dimensional culture of testis cell suspensions.

6.1. Organotypic Culture of Testicular Tissue Fragments

The unique three-dimensional structure of seminiferous cords/tubules seems essential in facilitating the required interactions between germ cells and somatic cells. This partly includes providing a proper niche for SSCs to reside, and establish or maintain spermatogenesis. Thus, it makes sense to first try to culture small fragments of testis tissue or intact pieces of semiserious tubules; indeed, this is what many of the pioneering groups have done in the past and many current research labs are pursing. Organotypic culture of tissues and organs is, however, challenging because of the issues arising from the limited diffusion rates of the tissue, especially compared with monolayer cell cultures. As a result, a main hurdle in achieving IVS using testicular tissue fragments is maintenance of the tissue viability for the required length of culture.

In 1959, Trowell designed an organ culture system by employing a gas-liquid interface in which testicular fragments from adult rats could be maintained in Eagle's minimum essential media (MEM) at 37 °C with 5% CO_2 in air, albeit only for 6 days [86]. In 1964, another group using the same organ culture system was able to maintain testicular fragments of 4-day-old rats viable for four weeks, although cell differentiation was not observed [87], but in the same year they also reported differentiation of

spermatogonia to spermatocytes within 2-3 weeks of culture [88]. The effects of temperature, pH, vitamins, hormones, sera, and tissue extracts were also examined; however, developmental progress was still limited up to pachytene spermatocyte [89]. These latter culture conditions were also used to maintain human testicular tissues in vitro for several weeks [90]. It was then shown that FSH is required for germline differentiation and more specifically in the conversion of type A spermatogonia into meiotic pachytene spermatocytes [91]. In 2003, Suzuki and Sato used a gas-liquid interface culture system that was developed decades earlier [92] with minimal changes to culture 5-day-old mouse testis tissue, but interestingly, round spermatids could be observed after two weeks of culture [93].

A dramatic turn in the long-standing efforts for replicating complete IVS took place when in 2011, Sato et al. reported successful production of offspring from in vitro produced haploid germ cells [94]. Testicular fragments of neonatal mice were cultured on agarose gel (half-socked in medium) in α-MEM supplemented with knockout serum replacement (KSR) or 40 mg/mL AlbuMAX instead of fetal bovine serum (FBS). Interestingly, this culture system was also successful for in vitro production of haploid male germ cells from the testis tissue of an infertile mutant mouse model [95] as well as after cryopreservation of the testis tissue [96]. The agarose gel-based organ culture system also led to the production of haploid male germ cells from adult mouse donors; however, the efficiency was far lower than that from neonatal donor mice [97].

Subsequently, to improve the efficiency and duration of in vitro spermatogenesis (IVS), the agarose gel-based organ culture system was modified and a microfluidic technology was adopted for organ culture systems (Figure 2). The main rationale for using a microfluidic system was to encourage the exchange of substances such as gases, nutrients, and waste products by facilitating diffusion and creating conditions that are more representative of in vivo. Surprisingly, the microfluidic device allowed the maintenance of mouse testicular tissue for ~six months, prompted the induction of spermatogenesis, and led to the in vitro production of spermatids and sperm capable of giving rise to offspring after micro-insemination [98]. Ever since, pumpless microfluidic devices have also been developed (Figure 2). These systems use hydrostatic pressure and a resistance circuit to facilitate slow flow of the medium; this has led to the induction of efficient IVS for ~three months [99]. Taken together, these results suggest that the organotypic culture of testicular tissue or fragments is capable of maintaining the architecture and viability of germ cells, and induction of IVS. Moreover, the addition of a microfluidic device has shown the potential to improve organotypic culture systems, as it can lead to long-term ex vivo maintenance of testis tissues which is required for producing sperm.

6.2. Two-Dimensional Culture of Testis Cell Suspensions

Two-dimensional (2D) culture systems using enzymatically-dispersed testis cell suspensions have also been widely used for both in vitro proliferation of SSCs and production of haploid male germ cells (Table 2, Figure 2). Compared with organotypic cultures of testicular tissue, the 2D culture systems for testis cell suspensions have innate advantages and disadvantages. A 2D cell culture system can be potentially used to expand SSC numbers in vitro for downstream applications such as in auto-transplantation to the individual's testis following recovery from cancer. Using 2D culture systems of testis cells is also ideal when the objective is to investigate the role of individual cell types, putative factors, or candidate genes or gene-products on fate of germ cells. However, compared with organotypic testicular tissue culture systems, 2D culture of cells has at least two disadvantages. Firstly, it does not resemble or replicate the expected cellular interactions and structural conditions of intact seminiferous tubules, and secondly, in the absence of spatial positioning and typical cellular morphology, it is difficult to distinguish different types of cells without further analyses such as the use of flow cytometry.

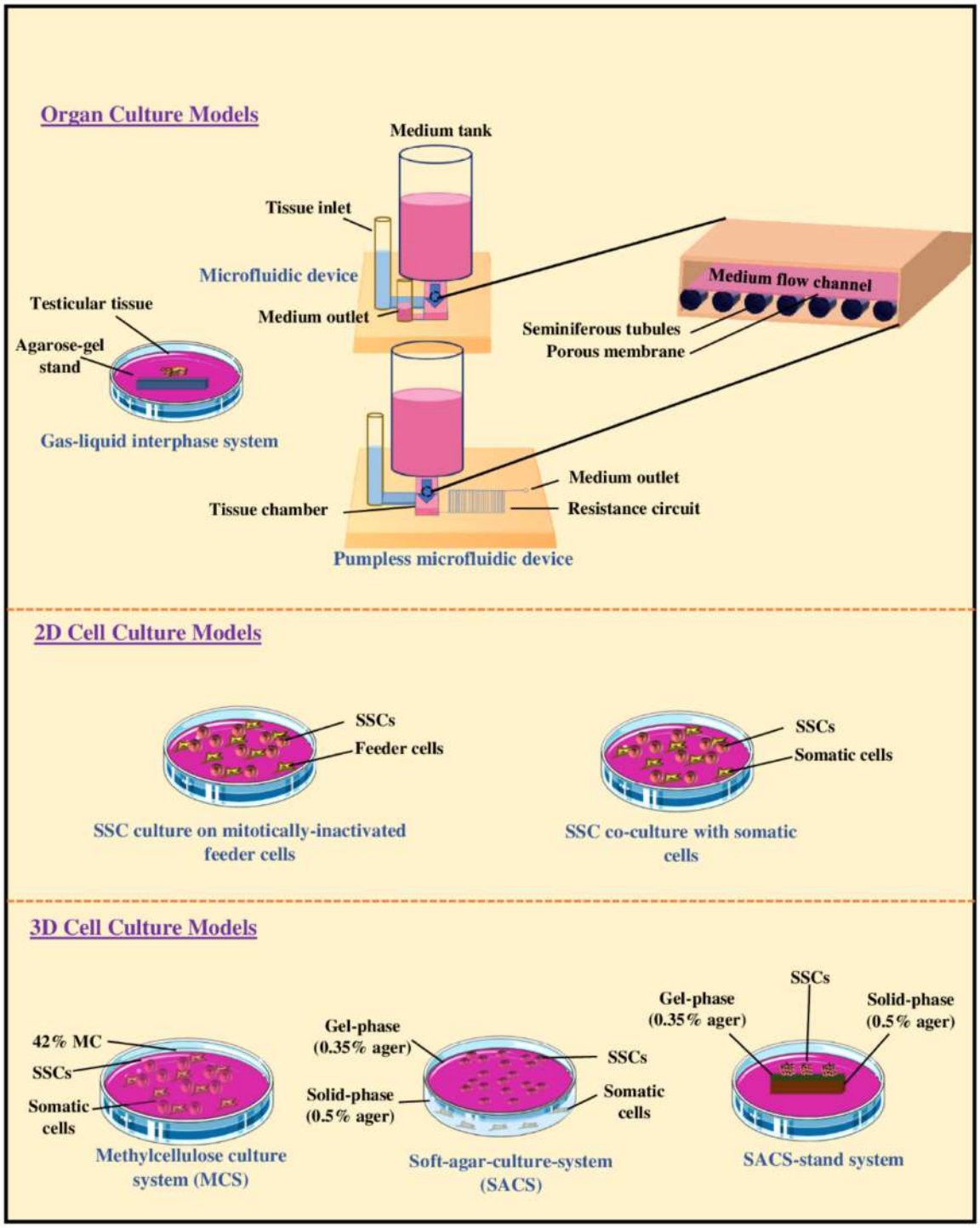

Figure 2. Schematic representation of organotypic culture of testicular tissue fragments, two-dimensional (2D), or three-dimensional (3D) cell suspension culture models for in vitro spermatogenesis. Three approaches to organotypic culture of testicular tissue fragments have been proven effective including using a gas-liquid interface system, a conventional microfluidic system, or a pumpless microfluidic device. 2D culture of cell suspensions can be achieved through two approaches, namely culturing isolated SSCs on mitotically-inactivated feeder cells (e.g., mouse embryonic fibroblast cells) or culturing on mixed populations of testicular cells (co-culturing of germ cells and somatic cells). 3D cell cultures can include using methylcellulose (MCS), a soft-agar culture system (SACS), or a SACS-stand system.

In 1983, it was reported that germ cells isolated from 6–15 wk-old mice cultured using a 2D system were able to differentiate into spermatocytes [100]. Interestingly, in 1989, haploid male germ cells were produced from testis cell suspensions of 14-day-old rats cultured on Type 1 collagen gels in a 1:1 mixture of Ham's F12 medium and Leibovitz's L15 medium with 10% FBS, epinephrine, and norepinephrine [101]. In 1993, there were two reports providing evidence that immature germ cells can be directed to form haploid male germ cells using 2D culture systems [102]; however, no follow-up studies using the same protocol have been published. In 1998, Durand's group reported that they have produced haploid male germ cells from single-cell preparations of 23–25 day-old rats cultured in DMEM-F12 supplemented with 0.2% FBS, testosterone, and FSH [103]. They attributed their success to maintaining maximal interactions between Sertoli cells and spermatogenic cells. This raised awareness among researchers as to the importance of germ cell-Sertoli cell interactions and other groups tried to maintain such cellular interactions to encourage spermatogenesis in vitro. In 2000, the same group reported further in vitro differentiation of 2D cultured isolated advanced germ cells (i.e., leptotene spermatocytes), collected from 20, 22, or 28-day-old rats to haploid male germ cells [104].

The first report of successful production of offspring from in vitro produced early-stage haploid male germ cells (i.e., round spermatids) came in 2003 [105]. Germ cells isolated from 13–18 day-old mice were cultured with Sertoli cells in a serum-free, hormone/growth factor-supplemented medium, which after 7–10 days, led to the appearance of fertilization-competent round spermatids from pre-existing spermatocytes [105]. In 2006, the importance of cell contacts between germ cells and feeder cells for IVS was highlighted, when type A spermatogonia of an immature rat (7-day-old) co-cultured with Sertoli cells differentiated to spermatids [106]. More recently, different approaches have been proposed that could direct the differentiation of SSCs all the way to haploid male germ cells. Using isolated 6-day-old mouse SSCs, production of haploid germ cells was achieved by culturing testicular cells in 10% FBS-supplemented media for 3 days, changing the media into a retinoic acid (RA)-enriched medium to induce differentiation for 2 days, and followed by changing the media again to the first medium for 6–8 days [107].

In conclusion, even a 2D cell suspension of testis cells under optimal culture conditions can induce spermatogenic differentiation; however, for this to work, cell-to-cell contact between germ cells and feeder cells appears to be important. Although, intensive research over the years has finally led to the emergence of culture conditions suitable for IVS of rodents, considerable gaps in knowledge remain in our understanding of the developmental processes involved.

Table 2. Culture conditions for long-term in vitro maintenance of rodent or primate spermatogonial stem cells (SSCs).

Culture Conditions	**Mouse**			**Monkey ***	**Human**		
Donor age	Newborn	4.5–7.5-day-old	8-day-old	3.7–4.6 years (average age)	NA	14, 34, and 45 years	22–35 years
SSC isolation method	Two-step enzymatic digestion	Two-step enzymatic digestion	Two-step enzymatic digestion	Two-step enzymatic digestion	Two-step enzymatic digestion	Two-step enzymatic digestion	Two-step enzymatic digestion
SSC enrichment method	Diff. plat.	MACS	MACS	Diff. plat./un enriched	Diff. plat.	Diff. plat. + MACS	Diff. plat. + MACS
Basic medium	StemPro-34 SFM	Alpha-MEM	StemPro-34 SFM	Alpha-MEM	StemPro-34 SFM	StemPro-34 SFM	StemPro-34 SFM
Additional supplements	StemPro supplement	Supplement	StemPro supplement	NA	StemPro supplement	StemPro supplement	StemPro supplement
Protein/serum source	FCS	BSA	Albumax II	FBS	FCS	BSA	FBS, BSA
Growth factors	GDNF, bFGF, EGF, LIF	GDNF, bFGF, GFRα1	GDNF, bFGF, EGF	NA	GDNF, LIF, EGF, bFGF	GDNF, LIF, EGF, bFGF	GDNF, LIF, EGF, bFGF
Feeder cells	MEF	STO	NA	NA/Somatic cells	NA	NA	NA
Temperature	37 °C	37 °C	37 °C	35 °C	37 °C	37 °C	37 °C
Subculture	10–14 days	5–7 days	5–6 days	NA	7–10 days	10–15 days	NA
Max. culture duration	~160 days	~180 days	~178 days	~11 days	~196 days	~120 days	~60 days
Germ cell clump formation	Yes	Yes	Yes	Yes	Yes	Yes	Yes
SSC identification	Flow cytometry	Immunostaining	Flow cytometry	Immunostaining	Immunostaining	PCR	Immunostaining
Functional analyses	Yes	Yes	Yes	Yes	Yes	No	No
Reference	[108]	[109]	[110]	[111]	[83]	[112]	[82]

* We are unaware of reports of longer SSC cultivation times than 11 days for non-human primates.

6.3. Three-Dimensional Culture of Testis Cell Suspensions

The knowledge gained through intensive research on organotypic and 2D cell culture systems directed some of the research on testis cell suspensions toward the development of artificially constructed three-dimensional (3D) structures. In 2006, testicular cells isolated from 18-day-old rats were cultured on collagen gels to mimic the composition of basal membrane of seminiferous tubules. Not only did this 3D culture system increase the viability of testicular cells but it also directed the differentiation of germ cells, suggesting that cell-to-cell interactions in a 3D culture system sufficiently support the differentiation of germ cells [113]. In 2008, a soft-agar culture system (SACS) was introduced for IVS (Figure 2), which consisted of two phases based on agar concentrations; a gel phase containing enriched SSCs, and a solid phase comprising supporting cells (e.g., Sertoli cells). The expression of meiotic stage-specific markers was observed when testicular cells obtained from 10-day-old mice were mixed with the gel-agar medium (0.35%) and incubated on a solid-agar base (0.5%) [114]. In 2012, the production of haploid germ cells from undifferentiated germ cells, isolated from 7-day-old mice, was reported using the 3D SACS; however, the fertility of in vitro produced mature germ cells was not determined [115]. This latter condition also directed the differentiation of undifferentiated germ cells isolated from 13–33 month-old rhesus monkeys; an age range corresponding to the immature through pubertal and young adulthood ages [116]. A methylcellulose culture system (MCS) has also been proposed as yet another 3D culture system which appears to support the differentiation of immature germ cells into haploid male germ cells [117].

In order to artificially reproduce the in vivo form and function of the seminiferous epithelium, a 3D engineered blood-testis barrier (eBTB) system was introduced in 2010. In essence, the eBTB is aimed at providing a condition that can maintain the interactions between somatic and germ cells, deemed crucial in achieving spermatogenic differentiation. Testicular peritubular myoid cells were first cultured on the underside of culture inserts 3 days prior to adding a mixture of Sertoli and germ cells on top of the inserts, which after an additional 22 days of culture led to the formation of haploid male germ cells [118]. Yokonishi et al. (2013) reported that under these new 3D culture conditions, dissociated immature testicular cells reconstructed into structures resembling their original tissue architecture. Their new system included using testicular cells isolated from neonatal mice to be cultured in V-shaped plate wells for 2 days to allow aggregate formation, followed by placing the aggregates on top of agarose gel blocks. Interestingly, the aggregated cells formed tubule-like structures, and haploid germ cells were observed after 30–51 days of incubation [119]. Recently, a modified 3D culture method, three-layer gradient system (3-LGS), was also introduced for in vitro generation of testicular organoids using rat testis cell suspensions. In this approach, rat testis cells were suspended in Matrigel and placed between two cell-free layers of Matrigel, which after 7 days led to the formation of testicular organoids. Additionally, self-organization of testicular cells also led to eBTB formation and Sertoli cell epithelization; however, complete differentiation of germ cells was not observed [120]. These results collective suggest that maintaining the microenvironment of testicular cells even in the form of a 3D-culture system is crucial in achieving spermatogenesis ex vivo.

7. Progress of In Vitro Spermatogenesis in Primates

The first attempt at achieving IVS using human testicular biopsies was reported in 1967, in which after three weeks of culture, differentiation of primary spermatocytes from the preleptotene to pachytene stage was observed [121]. Later in 1971, testicular tissues obtained by orchidectomy due to prostate cancer were cultured, and round spermatids were the most advanced germ cells observed in the supernatant of the media used for culturing the tissue samples at 27 day of culture [90]. However, in 1981, it was reported that the latter culture system does not support complete differentiation of spermatogonia, rather only cells already committed to meiosis at the time of culture initiation can proceed through spermatogenesis [122].

The limited success of organotypic culture systems using human testis tissue in achieving IVS diverted some of the research focus toward the use of testicular cell suspensions for in vitro

differentiation of germ cells. Culturing spermatids, isolated from testicular biopsies of obstructive azoospermia patients, in a culture medium containing FSH showed evidence of maturational process in the form of morphological changes in the spermatid nuclei and flagellar growth after only 48 h [123]. These results presented new hopes for treating infertilities related to germ cell maturation arrest. A year later, when in 1999, spermatids isolated from non-obstructive azoospermia patients were cultured on Vero cells for 5 days, round spermatids differentiated into elongated spermatids and a single mature spermatozoon was also observed [124]. Vero cell-conditioned medium supplemented with hormones also supported the in vitro meiosis and spermiogenesis of co-cultured somatic and germ cells isolated from testicular biopsies of non-obstructive azoospermic patients [125]. In 2003, the differentiation efficiency of primary spermatocytes, from azoospermic men with spermatogenic arrest, was tested after co-culture with Vero cells using various culture conditions. Primary spermatocytes co-cultured with Vero cells in MEM-based media, supplemented with 50% boar rete testicular fluid or in human synthetic oviduct fluid and 10% human serum, resulted in the generation of round spermatids at a rate of 10%. Moreover, the presence of 23 chromosomes and chromatids confirmed that the in vitro produced cells were indeed haploid [126]. In 2012, SSCs isolated from testicular biopsies of obstructive and nonobstructive azoospermic patients were co-cultured with Sertoli cells in a KSR-supplemented medium for 5 days, when fluorescence in situ hybridization (FISH) analysis of chromosomes confirmed the presence of haploid male germ cells [127]. However, a 5-day period of culture seems far too short to allow completion of the complex developmental process of differentiation from primary spermatocytes to round spermatids.

More recently, using an organotypic culture system, in vitro production of haploid male germ cells from immature human testicular tissue was also reported. Frozen-thawed 1-mm^3 testicular fragments collected from prepubertal boys (aged 2–12 years) were cultured in a KSR-based medium supplemented with 5 IU/L of FSH, which after 16 days resulted in the formation of round spermatids. Interestingly, increasing the concentration of FSH from 5 to 50 IU/L failed to induce germ cell differentiation, perhaps due to desensitization of FSH receptors or adenyl cyclase in Sertoli cells [128]. However, it should be noted that currently such in vitro produced haploid male germ cells only serve as a proof-of-principle and would need be further analyzed for their fecundability and epigenetic characteristics before suggesting these techniques for potential clinical trials.

Using non-human primates, 3D culture models of IVS have also shown promising results. Among non-human primates, the juvenile rhesus monkey is considered a suitable model for prepubertal boys, because in both species the phase of prepubertal development is characterized by a protracted hypogonadotropic and hypoandrogenic state. These characteristics provide an ideal baseline for examining putative factors involved in the initiation of spermatogenesis. To check the efficiency of SACS and MCS culture systems in supporting the differentiation of primate germ cells, testicular cells isolated form juvenile rhesus monkeys (aged 13–33 months) were cultured in either culture system for 4–8 weeks. The MCS culture system supported the differentiation of immature testicular germ cells and after 30 days of culture, haploid male germ cells were observed [116]. However, the fertilizing potential and epigenetic characteristics of the in vitro produced haploid male germ cells were not determined.

8. Limitations of In Vitro Spermatogenesis in Non-Rodents

Despite the long history of experimentations targeting IVS, an optimal and efficient system capable of inducing complete differentiation of immature human testis cells or testicular tissue to form haploid male germ cells has not yet been developed. The gradual and incremental success using rodent models of IVS; however, is encouraging and suggest that recapitulation of human spermatogenesis will be eventually possible in the future. However, when such an IVS system is provided for use with human germ cells, extensive additional safety and ethical reviews would have to be completed before such tools can be considered for potential use in reproductive medicine.

Limited availability of basic knowledge about primate SSCs and their in vitro requirements, along with the long prepubertal period in primates compared with rodents, makes achieving IVS for primates

even more challenging. Therefore, as a first step toward achieving complete IVS in primates, it is crucial to maintain the structural integrity of testicular tissue and extend the viability of isolated germ cells in long-term cultures. Moreover, obtaining sufficient amounts of donor primate testis tissue for various studies is difficult, if not impossible. Hence, the use of donor testes from other suitable non-rodent animal models such as neonatal pigs may provide an attractive alternative, given that pigs share considerably more anatomical and physiological similarities with humans than do rodents. Moreover, optimized in vitro and in vivo culture conditions for development of testicular cells and tissue from immature donor pigs have been developed to facilitate such studies [129–132].

Although, there are a few reports of in vitro production of haploid male germ cells in non-rodents, it has not yet been possible to produce morphologically normal, viable, motile, and fertilization-competent sperm from undifferentiated germ cells. This implies that cells at different stages of spermatogenesis have different culture requirements, which so far have not been met. Immotile in vitro produced male haploid germ cells are infertile when used in classical in vitro fertilization (IVF) or in artificial insemination (AI). Therefore, if the ultimate goal of primate IVS is to use the resultant sperm in IVF, then the current situation is still far from ideal. Additionally, even though round spermatids, elongated spermatids, or even sperm can theoretically be produced in vitro and individually used for fertilizing oocytes using intracytoplasmic injection (as discussed below), these potential haploid cells are not always suitable for forming a zygote. Therefore, even after successful development of an efficient system for primate IVS, confirmation of normalcy and fertilization-competence of the in vitro produced haploid germ cells are necessary important steps before experimental applications or potential clinical trials can be recommended.

In order to improve the in vitro culture system for non-rodents, a growing number of factors are being explored which have traditionally been neglected in the past. This includes, for example, the potential role of extracellular matrices or paracrine factors secreted by somatic components of the testis. For instance, paracrine factors secreted by Leydig and peritubular myoid cells (e.g., peritubular factor that modulates Sertoli cell function, PModS) have been shown to modulate the effects of testosterone on Sertoli cells, in turn altering the secretion of transferrin and inhibin [133]. These factors and other novel elements may play an important role in spermatogenesis; therefore, investigating the significance of putative factors is warranted and would likely help in defining optimal culture systems for achieving IVS in primates.

9. Future Prospects of In Vitro Spermatogenesis for Fertility Preservation and Restoration

IVS has many important potential clinical applications and in certain circumstances is the only currently conceivable safe option for preserving fertility and upholding biological fatherhood. At the present time, human IVS to allow differentiation from immature stages to fully-formed sperm has not been achieved; however, given the promising preliminary results and gradual improvements in research methodologies, this technique will hopefully be applicable in future to resolve many of the problems currently linked with the male factors of infertility.

9.1. Fertility Preservation of Prepubertal Cancer Patients for Subsequent Fertility Restoration

With advances in oncotherapy, thankfully >80% of childhood cancer patients survive but, due to the gonadotoxic side effects of cancer treatments, a significant number will experience subfertility or permanent sterility as adults [134,135]. Fertility has an important impact on the post-treatment quality of life. Although cryopreservation of semen prior to cancer therapy is an established method for adult male patients wishing to preserve their future fertility, semen collection is obviously not an option for pre-adolescent boys whose testes have not started sperm production. For such immature patients, cryopreservation of testicular biopsies collected prior to the start of gonadotoxic therapies is the only conceivable option to preserve their fertility potential. This is because the testis of prepubertal individuals contains SSCs, which can be potentially used to restore spermatogenesis and ultimately produce haploid germ cells [136,137].

The pre-treatment testicular biopsies from immature boys can be cryopreserved and maintained in liquid nitrogen almost indefinitely, or until such time when the patients have recovered and been diagnosed with infertility as adults. Even if no sperm is found in the recovered individual's semen, frequently haploid germ cells can still be retrieved from the testes, using established advanced reproductive technologies. This for instance includes microscopic testicular sperm extraction (micro-TESE) to be used for intracytoplasmic sperm injection (ICSI) or round-spermatid injection (ROSI). However, if retrieval of haploid germ cells is not an option, such as when there is spermatogenesis arrest at earlier stages, then the cryopreserved testicular biopsies can be thawed and used in technologies such as IVS, which hopefully by then would be optimized and proven safe. The various scenarios and theoretical options are summarized in Figure 3 and include the potential use of testis tissue fragments or isolated cell suspensions for IVS to produce haploid germ cells, followed by their use for ICSI or ROSI. Alternatively, the cryopreserved tissue fragments can be processed to obtain testis cells containing SSCs, which could include the exclusion of potentially residual malignant cells. This is then followed by subsequent expansion of SSC numbers in culture, before offering to perform auto-transplantation of SSCs for re-colonization of the individual's testes, which can potentially lead to sufficient production of sperm to restore fertility [136,137].

Proof-of-principle studies for a number of the steps outlined in Figure 3 have been shown using various animal models, but at this time, none of the proposed approaches have been adequately tested using non-human primates to warrant even experimental use for humans. For instance, we and others have expanded the technique for male germ cell transplantation, a technique that was originally developed in mice, by showing the feasibility of autologous and homologous transplantations in various large animal models [138,139] and non-human primates, which also included successful production of fertile sperm [132]. Therefore, auto-transplantation of germ cells (containing SSCs) isolated from cryopreserved testicular biopsies is theoretically possible but not before other technical difficulties are overcome.

Firstly, it has been shown that an average testicular biopsy from prepubertal boys weighs ~31 mg and provides ~390,000 total cells, of which only ~11,700 are estimated to be spermatogonia [140]. Given the heterogenous nature of spermatogonia (as discussed above), a much smaller number of these cells would actually be expected to be functional SSCs; hence, it is unlikely that single biopsies will provide sufficient numbers of SSCs to repopulate the testis without further in vitro expansion of SSCs [140]. Therefore, an important first step toward the use of cryopreserved biopsies, as a source for auto-transplantation of testis cells, is exponential expansion of resultant SSC numbers in culture, which would take extensive additional research to optimize such an efficient culture system.

Secondly, in the process of in vitro culture, testicular cells are exposed to a number of 'unnatural' conditions including physical and chemical manipulations (e.g., enzymatic digestion, pH changes), and known and unknown factors (e.g., growth factors, cytokines), that may cause them to undergo oxidative stress or DNA methylation and/or histone post-translational modifications. Therefore, careful assessment of genetic and epigenetic modifications should be part of the further work on testis cell culture to ensure their safety for potential use in clinical applications [141–143].

Thirdly, because the biopsies are taken from immature patients before undergoing cancer treatment, they may carry malignant cells. This is especially the case for children with testicular cancer, lymphomas, leukemia, or other tumors that metastasize to the testis. In such cases, the auto-transplantation approach should not be considered due to the high risk of reintroducing malignant cells, and certainly not before the suggested methods for elimination of cancer cells using cell sorting (e.g., MACS [144]) have been optimized further and proven 100% efficient and safe [145].

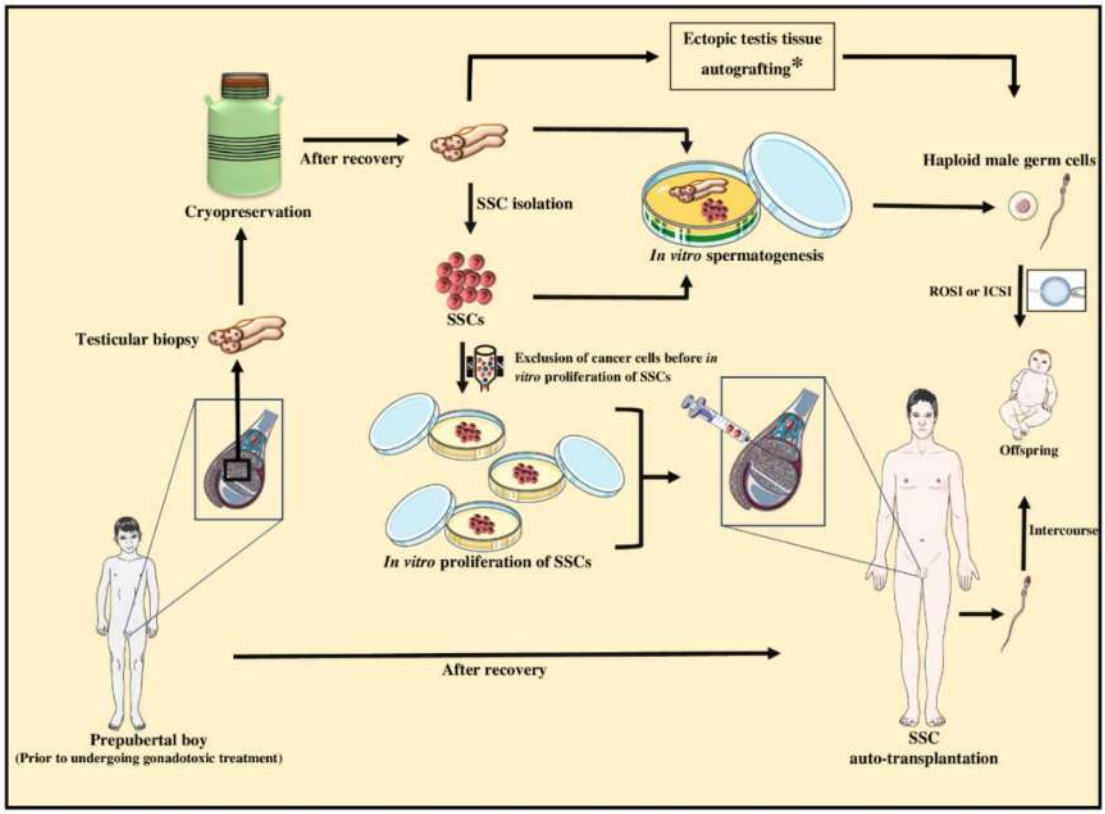

Figure 3. Schematic representation of potential fertility preservation options for prepubertal cancer patients. Testicular biopsies can be collected from prepubertal boys before commencing gonadotoxic cancer treatments. The testicular biopsies can be then cryopreserved as a source of spermatogonial stem cells (SSCs) in case the patient indeed become infertile. To preserve the fertility potential, different theoretical or experimental approaches using testicular biopsies or isolated SSCs can be used to produce haploid male germ cells. Advanced assisted reproductive technologies that can be considered after recovery from cancer include in vitro spermatogenesis, ectopic testicular tissue autografting, or auto-transplantation of SSCs into the donor testis to induce spermatogenesis. The resultant haploid male germ cells can be used for fertilization through natural conception or with the help of intracytoplasmic sperm injection (ICSI) or round spermatid injection (ROSI). Asterisk (*) in the boxed text is to emphasize that even though testis cells can be potentially processed to eliminate malignant cells using flow cytometry, this is not applicable with tissue fragments. Therefore, the option of autografting (testicular fragments) is only applicable if the patient was not suffering from testicular cancer or metastatic tumors due to high risk of reintroducing the pre-existing malignant cells back into the patient.

Autologous grafting of the testicular biopsies has also been proposed as a potential approach for restoration of fertility in childhood cancer survivors. Auto-grafting, either in the form of orthotopic (placed within the scrotum) or ectopic (placed subcutaneously outside the scrotum), relies on the ability of the frozen-thawed immature testicular fragments in undergoing extensive development, complete differentiation, and formation of sperm or other haploid germ cells to be extracted and used in ICSI or ROSI [146]. However, because of the same reasons cited above against the use of auto-transplantation of cells, autografting of intact testicular biopsies should not be considered, if there is any chance that malignant cells may still be present in the biopsies. Nevertheless, the feasibility of auto-grafting has been shown using both ectopic and orthotopic grafting of cryopreserved immature testicular tissues in non-human primates, leading to production of sperm and even recently in the birth of a healthy monkey [146,147].

It should be noted that while the use of these testicular biopsies for xenografting into host mice has also been proposed by some fellow researchers and/or clinicians, we have deliberately refrained from

including this approach as a potential option for restoration of fertility in our figures. In fact, we were first to show that fresh and cryopreserved immature testicular tissue fragments from various donor species can be grafted under the back skin of immunodeficient recipient mice where they completely develop and even produce large numbers of fertile xenogeneic sperm [148,149]. We were also among the first groups to expand the application of this testicular tissue xenografting system into primates by grafting testis tissue fragments from immature donor monkeys and mature humans [132,150,151]. Therefore, we are well-informed of the various potential applications of testicular tissue xenografting and can envision that current technical hurdles in achieving full spermatogenesis from immature human testis xenografts [152] can be sufficiently addressed. We also believe that it may even become theoretically possible to show that xenogeneic sperm from pre-treatment testicular biopsies would not carry a risk of reintroducing malignancy when used in ICSI. However, we strongly object to offering human testis tissue xenografting as a potential route to produce human sperm if it is exclusively meant to be used for human fertilization. This is because of the serious safety concerns, for instance in terms of the potential transfer of endogenous species-specific retroviral transcripts from the host mouse, as well as grave ethical and even legal limitations of using such xenogeneic sperm for human fertilization. On the other hand, if the goal is not to use the potential xenogeneic sperm for human fertilization, then further research on human testicular tissue xenografting is actually encouraged. For instance, such a system can provide a highly meritorious, novel, and unique assay for the basic research into factors that affect human testis development and spermatogenesis. It would also be a previously-unavailable tool for toxicological and pharmacological investigations into environmental toxicants or new medications that can affect human testis development or testis function [132].

Therefore, currently, the only plausibly safe approach to produce haploid male germ cells from immature human testicular biopsies for the listed potential future clinical applications is through IVS, because it does not carry a risk of reintroducing cancer cells. However, as outlined above, complete IVS has so far been only achieved using donor mice [94]. Although a similar organotypic culture system with some modifications has also been adopted to culture prepubertal human tissue, progression of spermatogonia has not been observed [153], suggesting that culture conditions developed for mouse may not directly or easily be applicable for primate IVS and would require extensive further research.

9.2. Upholding the Biological Fatherhood of Adult Infertile Patients

Meta-analysis of infertility data from various countries and continents concludes that globally an estimated 15% of couples experience infertility. Although the rate differs in different regions, overall about half (range 20%–70%) of the cases are thought to be due to the male factor [154]. Infertility in male also seems to show an uptrend, which can be due to various reasons including varicoceles, medications, obstruction, genetic disorders, or exposure to known and unknown environmental toxicants [155]. A number of infertility cases can be treated using experimental or clinical assisted reproductive techniques, as schematically shown in Figure 4. The currently applicable options include the use of ICSI for men with low numbers of sperm or the use of ICSI or ROSI following microsurgery for testicular sperm or spermatid extractions (micro-TESE) from men with obstructive azoospermia. However, in cases where the patient's testis does not contain haploid male germ cells, these latter techniques cannot be applied.

Patients lacking haploid germ cells in the testis can be divided into those with complete absence of germ cells and those with meiotic-stage, or pre-meiotic spermatogenic arrest [156]. At times, the spermatogenic arrest is treatable using conventional andrology treatments and interventions such as hormonal therapy. To uphold the biological fatherhood of the remaining patients, however, the options are currently limited and novel biotechnology approaches may need to be developed or considered in the future. These theoretical proposed options include the use of testis tissue biopsies or cells in organotypic tissue culture, or 2D/3D cell suspension culture systems, as outlined above. Using such culture systems, the cause of the spermatogenetic arrest can also be explored further and potentially

may even be overcome for instance by supplying the missing factors [95] to produce haploid germ cells in vitro for subsequent use in ICSI or ROSI.

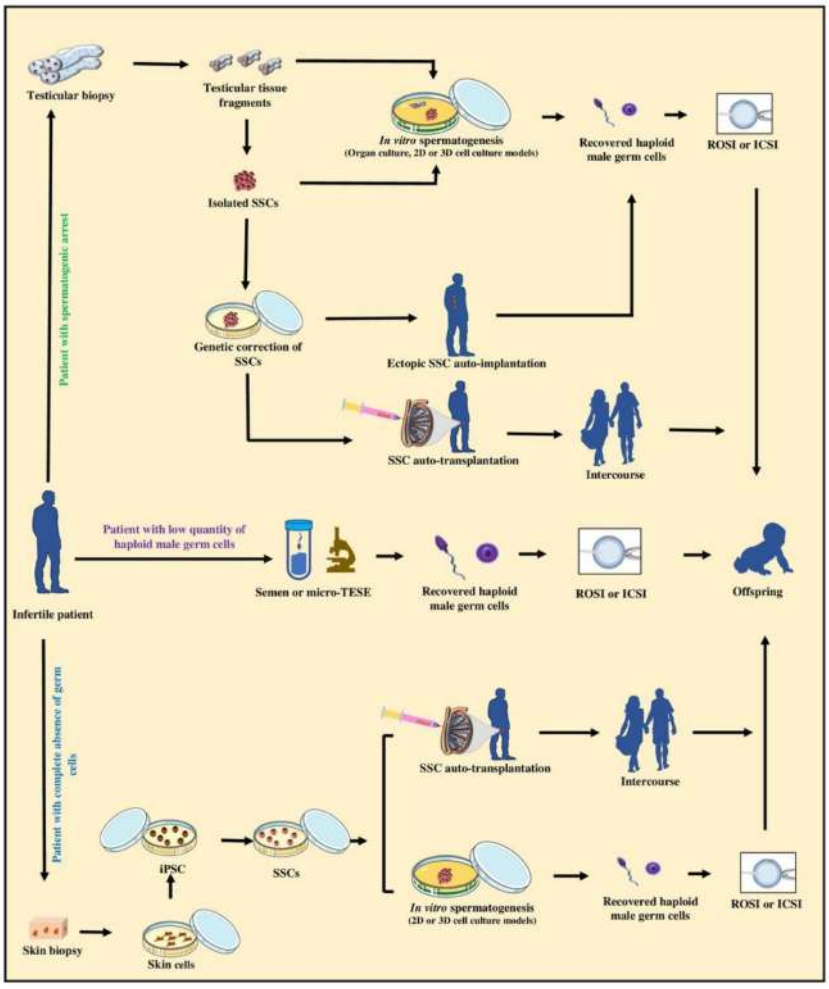

Figure 4. Schematic representation of standard, experimental, and theoretical options for upholding the biological fatherhood of adult infertile patients. The standard practice for upholding the biological fatherhood of an adult infertile patient is to use sperm from ejaculated semen or to use testicular sperm obtained by microsurgical extraction (TESE) for in vitro fertilization such as using intracytoplasmic sperm injection (ICSI) or round spermatid injection (ROSI). However, this option is applicable only when the patient's testis contains haploid male germ cells, such as in men with obstructive azoospermia. When infertility is due to spermatogenic arrest, testicular biopsies containing SSCs can be potentially obtained and used either for in vitro spermatogenesis (i.e., organotypic culture, 2D, or 3D cell culture models) in optimized culture conditions to produce haploid male germ cells. Moreover, theoretically genetic correction of isolated SSCs can also be applied in vitro before SSCs can be used for ectopic SSC auto-implantation or auto-transplantation into the patient testis to regenerate spermatogenesis and possibly lead to natural fertility. If the testis of the infertile patients does not contain normal germ cells, skin biopsies can be collected from the patient to theoretically produce induced pluripotent stem cells (iPSC), followed by differentiation of iPSC into SSCs. Such SSCs can then be potentially used for in vitro spermatogenesis (i.e., 2D or 3D cell culture models) to produce haploid male germ cells that can be used to fertilize eggs by ICSI or ROSI. Alternatively, auto-transplantation of SSCs can be potentially used to regenerate spermatogenesis and possibly natural fertility. It is important to emphasize that extensive further research is required to fully examine the various technical, safety, ethical, and legal aspects of the proposed experimental and theoretical options depicted here before they can be recommended for experimental or potential clinical trials.

Alternatively, testicular biopsies from patients who inherently have very few germ cells can be used as a source of SSCs to be expanded in vitro, followed by auto-transplantation to colonize their testes, and potentially initiate spermatogenesis to allow natural fertility. Theoretically, resultant SSCs can also be implanted ectopically under the skin of the patient for regeneration of testicular tissue to provide haploid germ cells for microsurgical extraction from the regenerated implants, followed by ICSI or ROSI. We and other have shown that testis cells collected after enzymatic digestion of immature donors of various species indeed maintain an unexpected de novo organogenic potential to regenerate functional testis tissue when cell aggregates are implanted under the skin of recipient animal models [132,157]. Interestingly, to overcome spermatogenic arrest at the level of meiotic cells using a mouse model, secondary spermatocytes have been injected into oocytes for the production of viable embryos and even offspring [158]. Intracytoplasmic injection of secondary spermatocytes has also been applied in human; however, the efficiency was reported to be low [159].

Yet, in situations where the patient's testis does not support the differentiation of germ cells or does not have any type of germ cells due to genetic or chromosomal mutations, at least theoretically, they can still be a candidate for future potential interventions. This includes the in vitro use of gene therapy or genetic correction techniques to overcome the spermatogenic arrest if in the future safe genome editing tools become available. Proof-of-principle studies using a mouse model of infertility have shown the feasibility of such approaches. For instance, due to a mutation of the c-kit ligand (KITL), spermatogenesis is completely arrested and only few abnormal SSCs are present in the testes of Steel (*Sl*) mice. However, providing the missing component, in the form of recombinant KIT, to the cultured testicular fragments resulted in a dose-dependent increase in the number of SSCs and ultimately in complete IVS [95]. Emergence of induced pluripotent stem cells (iPSC) from somatic cells, which can also be driven to form germ cells, has opened new possibilities. Although the iPSC technology in its current form cannot be used in humans, if similar but safe future alternative technologies become available, theoretically they may provide another unique opportunity to allow fatherhood for individuals who have no other options. Such newly-derived SSCs may be used in IVS or in autologous transplantation to allow parenthood (Figure 4).

10. Conclusions

SSCs are uniquely positioned among adult stem cells because of their potential for passing on genes to the progeny. However, our current knowledge of their biology is limited and so is our ability to harness their full potential for in vitro and in vivo applications in assisted reproductive technologies. Recent progress in organotypic testicular tissue culture as well 2D and 3D cell suspension culture systems may eventually lead to complete IVS using human testicular biopsies to allow preservation and restoration of fertility potential of prepubertal cancer patients undergoing gonadotoxic treatment. Moreover, such optimal IVS systems may also offer unprecedented new perspectives in the study of infertility causes and even approaches to overcome some of the currently untreatable infertility cases in men. However, it has to be emphasized that the proposed potential options discussed here are purely theoretical or are still at early experimental stages, and any recommendation for applying them in the future would require extensive safety, feasibility, and bioethical considerations.

Author Contributions: F.I. contributed to conceiving the concept, wrote the first draft of the manuscript, summarized tables, and prepared the figures. A.H. contributed to conceiving the concept, wrote parts of the manuscript, and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

Definition	Abbreviation
Artificial insemination	AI
Cadherin-1	CDH1
Casitas B-lineage lymphoma	CBL
Checkpoint kinase 2	CHK2
c-Kit ligand	KITL
Cluster of differentiation 9	CD9
Cluster of differentiation 90	CD90
Days post-coitum	dpc
Days post-partum	dpp
DEAD-box helicase 4	DDX4
Deleted in azoospermia-like	DAZL
Desmoglein 2	DSG2
Developmental pluripotency associated 4	DPPA4
Doublesex and mab-3 related transcription factor 1	DMRT1
Eagle's minimum essential media	MEM
Embryonic stem	ES
Engineered blood-testis barrier	eBTB
Enolase 2	ENO2
Extracellular matrices	ECM
Fetal bovine serum	FBS
Fibroblast growth factor receptor 3	FGFR3
Fluorescence in situ hybridization	FISH
Fluorescence-activated cell sorting	FACS
GDNF family receptor alpha-1	GFRα1
G-protein coupled receptor 125	GPR125
In vitro fertilization	IVF
In vitro spermatogenesis	IVS
Induced pluripotent stem cells	iPSC
Intracytoplasmic sperm injection	ICSI
Knockout serum replacement	KSR
Lin-28 homology A	LIN28
Magnetic-activated cell sorting	MACS
Melanoma-associated antigen 4	MAGEA4
Methylcellulose	MCS
Microscopic testicular sperm extraction	micro-TESE
Nanos C2HC-type zinc finger	NANOS
Neurogenin 3	NGN3
Octamer-binding transcription factor 4	OCT4
Paired box 7	PAX7
Peritubular factor that modulates Sertoli cell function	PModS
Phospholipid phosphatase related 3	PLPPR3
Piwi like RNA-mediated gene silencing 4	PIWIL4
POU class 5 homeobox 1	POU5F1
Primordial germ cells	PGCs
Prominin 1 (Cluster of differentiation 133)	PROM1 (CD133)
Promyelocytic leukemia zinc finger protein	PLZF

Ret proto-oncogene	RET
Retinoic acid	RA
RNA binding motif	RBM
Round-spermatid injection	ROSI
Sal-like protein 4	SALL4
Serum free medium	SFM
Soft-agar culture system	SACS
Spermatogonial stem cells	SSCs
SPOC domain containing 1	SPOCD1
Stage-specific embryonic antigen-4	SSEA4
Steel	SL
Stimulated by retinoic acid 8	STRA8
Synaptosome associated protein 91	SNAP91
Testis-specific Y-encoded protein	TSPY
Tetraspanin 33	TSPAN33
Three-dimensional	3D
Three-layer gradient system	3-LGS
Transformer-1	TRA-1
Two-dimensional	2D
Ubiquitin C-terminal hydrolase L1	UCHL1
Undifferentiated embryonic cell transcription factor 1	UTF1
Vasa gene-encoded RNA binding protein	VASA
Zinc finger with KRAB and SCAN domains 2	ZKSCAN2

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Birth Defects Research (Part C) 87:27–34 (2009)

REVIEW

Spermatogonial Stem Cells: Mouse and Human Comparisons

Martin Dym,* Maria Kokkinaki, and Zuping He

Spermatogonial stem cells (SSCs) have unique characteristics in that they produce sperm that transmit genetic information from generation to generation and they can be reprogrammed spontaneously to form embryonic stem (ES)-like cells to acquire pluripotency. In rodents, it is generally believed that the A-single (A_s) is the stem cell population, whereas the A-paired (A_{pr}) and A-aligned (A_{al}) represent the progenitor spermatogonial population. The A_1 to A_4 cells, intermediate, and type B spermatogonia are considered differentiated spermatogonia. In human, very little information is available about SSCs, except for the earlier work of Clermont and colleagues who demonstrated that there are two different types of A spermatogonia, the A_{dark} and A_{pale} spermatogonia. The A_{dark} spermatogonia were referred to as the reserve stem cells, whereas the A_{pale} were considered the renewing stem cells. In this review, we outline several spermatogonial renewal schemes for both rodents and primates, including man. We also compare phenotypic markers for spermatogonia/spermatogonial stem cells in rodents and humans and address SSC potential and therapeutic application. **Birth Defects Research (Part C) 87:27–34, 2009.** © 2009 Wiley-Liss, Inc.

Key words: pluripotency; spermatogonial stem cells; renewal patterns; phenotypic markers

STEM CELL RENEWAL PATTERNS IN RODENTS AND HUMAN

Spermatogenesis is the process of sperm production and it begins at puberty (5–7 days after birth in rodents and 10–13 years after birth in man) and maintains throughout life. It is a complex cell differentiation process that involves several germ cell types residing in the testis. The generation of sperm is initiated and also maintained by a few number of spermatogonial stem cells (SSCs) that are attached to the basement membrane of the seminiferous tubules (Clermont, 1972). Spermatogonia can be recognized and characterized morphologically. In whole mounts of seminiferous tubules from rodent testes, spermatogonia have clear cytoplasm, a high nucleus to cytoplasm ratio, and basal localization (Clermont and Bustos-Obregon, 1968). There are more spermatogonial subtypes in rodents compared with human. In rodents, early studies (Clermont and Bustos-Obregon, 1968; Dym and Clermont, 1970) have demonstrated that there are two populations of stem cells, namely the type A_0 (reserve stem cells) and the type A_{1-4} spermatogonia (renewing stem cells). A proposed scheme for stem cell renewal in rodent testis is presented in Figure 1. In this scheme, the type A_4 spermatogonia divide and form more differentiated intermediate spermatogonia, and notably they can also revert back to new stem cells, new type A_1 spermatogonia. Another proposed scheme of rodent spermatogonial renewal is presented in Figure 2, and in this model, only the A-single (A_s) spermatogonia are considered the stem cells of spermatogenesis (Huckins, 1971; Oakberg, 1971). As illustrated in Figure 2, the A_s spermatogonia can divide symmetrically either into new A_s stem cells or into differentiated A-paired (A_{pr}) spermatogonia that are characterized by an intercellular bridge (Dym and Fawcett, 1971; Greenbaum et al., 2006). The A_{pr} spermatogonia divide and form chains of 4, 8, 16, and even 32 A-aligned (A_{al}) spermatogonia. The A_{al} spermatogonia then form type A_1 to A_4 spermatogonia and are followed by intermediate (In) and type B spermatogonia, the primary spermatocytes, secondary spermatocytes, and eventually spermatids. This latter stem cell renewal scheme is now generally accepted by most investigators.

In contrast, very little information is known about human spermatogonial renewal mechanisms. Clermont (1963) first character-

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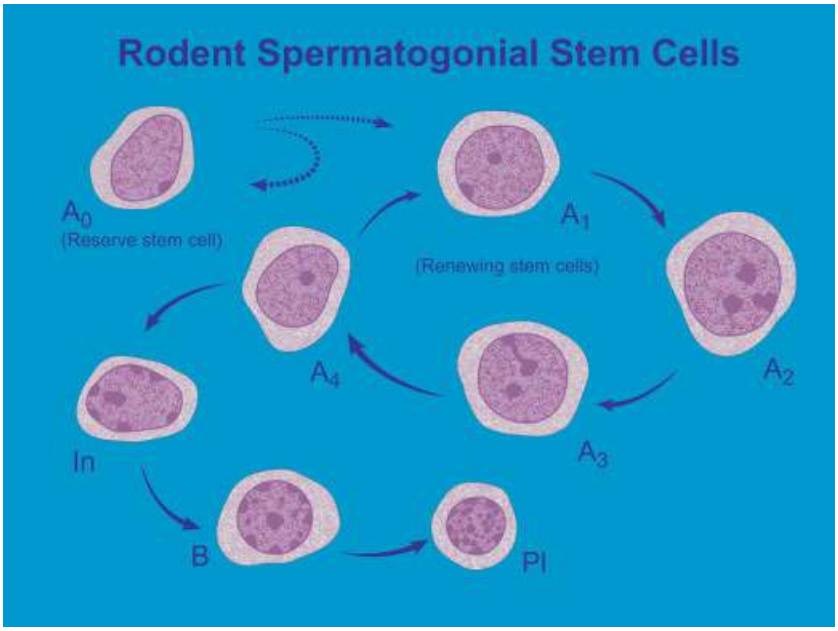

Figure 1. A proposed scheme of stem cell renewal in the rodent testis. The type A_0 spermatogonia are considered the reserve stem cells of rodent testis, whereas the type A_{1-4} spermatogonia are the renewing stem cells. The type A_{1-4} can divide and cause the intermediate (In) spermatogonia, type B spermatogonia, preleptotene spermatocytes (Pl), and sperm, successively. It was also suggested that some of the type A_4 spermatogonia can also cause new stem cells (Modified from Clermont and Bustos-Obregon, 1968; Dym and Clermont, 1970; Dym, 1994).

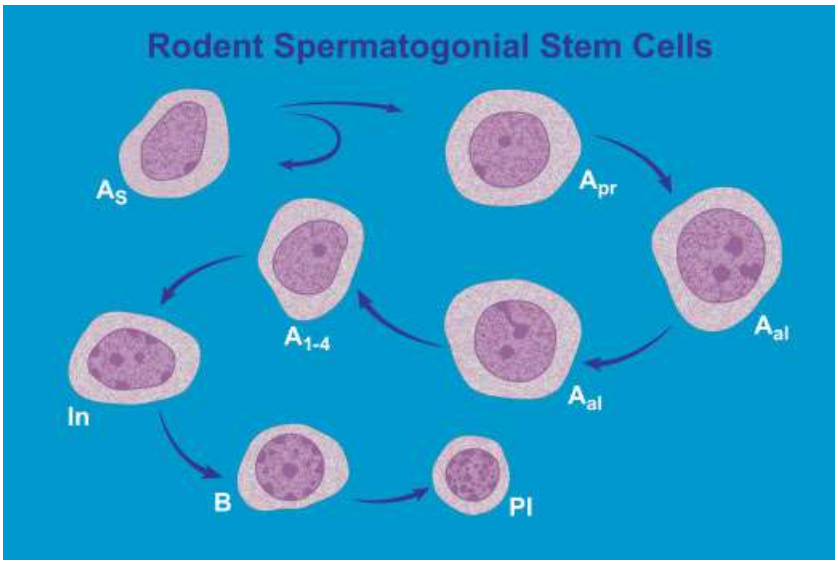

Figure 2. A second proposed scheme of stem cell renewal in rodent testis. The A_s spermatogonia are thought to be the stem cells in the testis, whereas the A_{pr} and A_{al} spermatogonia are the progenitor cells. In this scheme, the A_{pr} and A_{al} are also considered morphologically undifferentiated spermatogonia, whereas type A_{1-4} spermatogonia, intermediate (In) spermatogonia, and type B spermatogonia are differentiating spermatogonia (Modified from Huckins, 1971; Oakberg, 1971).

ized and identified the A_{dark} and the A_{pale} spermatogonia, and both of them are considered undifferentiated stem cells. Clermont et al suggested that the A_{dark} spermatogonia were the reserve stem cells, whereas the A_{pale} spermatogonia were the renewing stem cells (see Fig. 3) (Clermont, 1963; Clermont, 1966a,b; 1972). In this scheme of human spermatogonial renewal, the A_{pale} spermatogonia can divide and form type B spermatogonia that further divide and cause the primary spermatocytes. This classification for human spermatogonia has been adopted by most researchers. Other classifications have been proposed by certain investigators including Ehmcke and colleagues (Ehmcke and Schlatt, 2006); nevertheless, in 2009, more than forty years after Clermont presented his initial scheme for human spermatogonial renewal, the identity of the true human spermatogonial stem cell and the mechanisms of renewal of human SSCs still remains unknown. It is speculated that the human spermatogonial stem cell is a subpopulation of the A_{dark} and/or the A_{pale} spermatogonia.

STEM CELL PHENOTYPIC MARKERS IN RODENT AND HUMAN SPERMATOGONIA

Exploring markers for human spermatogonia help to identify the stem cell pool in the testis; however, this aspect of work in man is extremely limited. Work on monkey spermatogonia demonstrated that certain rodent markers for germ cells and SSCs are also present on the monkey spermatogonia; these phenotypic markers are VASA, DAZL, PLZF, and GFRA1 (Hermann et al., 2007). Notably, the frequency of PLZF positive cells in monkey testis was around 1.86 per tubule cross section. This observation indicates that the spermatogonial stem cell population in the monkey testis is a subset of either the A_{dark} or the A_{pale} spermatogonia. More and more information is available on the phenotypic identity of SSCs in rodents. Our laboratory and others have demonstrated that GFRA1 is a marker for mouse SSCs and probably their progeny (Meng et al., 2000; Buageaw et al., 2005; Hofmann et al., 2005; Naughton et al., 2006; He et al., 2007), and that KIT is a hallmark

for the more differentiated spermatogonia, including type A_{1-4} spermatogonia (Yoshinaga et al., 1991). Because there is not a unique marker available to distinguish the SSCs and other undifferentiated spermatogonia, called A_{pr} and A_{al}, it is helpful to use two or three antibodies to characterize their phenotypes. We conducted a double staining procedure, using antibodies to GFRA1 and POU5F1, demonstrating that the same subset of mouse spermatogonia stains for POU5F1 and GFRA1 (He et al., 2007). GPR125 is also believed to be a marker for mouse spermatogonial stem/progenitor cells (Seandel et al., 2007). It is speculated that some of these markers as mentioned above will also be applicable to human spermatogonia. Data from our laboratory have recently shown that GPR125 may be a marker for human SSCs, as it is for mouse SSCs (see Fig. 4).

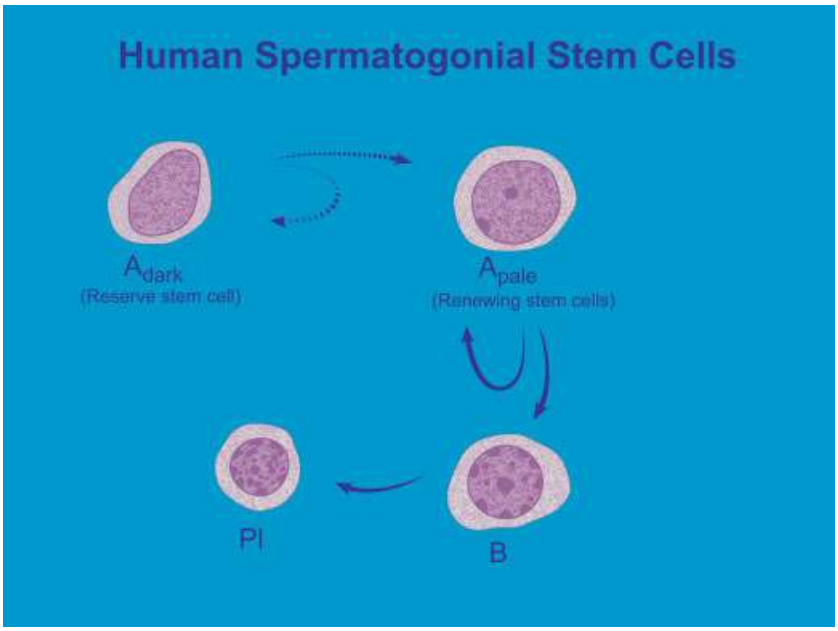

Figure 3. A proposed scheme of stem cell renewal in the human testis. The A_{dark} spermatogonia are believed to be the reserve stem cells in the testis, whereas the A_{pale} are the renewing stem cells. The A_{pale} can divide and either cause new A_{pale} or to the more differentiated type B spermatogonia.

A comparison of the markers for spermatogonia and their progenitors in human and rodents indicates that human and rodent spermatogonia share many but not all phenotypes (Table 1). In rodents, α6-integrin (CD49f), β1-integrin (CD29), and Thy-1 (CD90) are surface markers for mouse spermatogonial stem/progenitor cells (Shinohara et al., 1999; Kubota et al., 2003), and CD9 is a surface marker for mouse and rat spermatogonial stem/progenitor cells (Kanatsu-Shinohara et al., 2004b). GFRA1 and RET are coreceptors for GDNF and markers for spermatogonial stem/progenitor cells (Buaas et al., 2004; Costoya et al., 2004; Buageaw et al., 2005; Hofmann et al., 2005; Naughton et al., 2006). In human, α6-integrin is expressed in spermatogonia and their progenitors and was used to isolate and purify human spermatogonial cells by magnetic-activated cell separation (MACS) (Conrad et al., 2008). Other rodent surface markers,

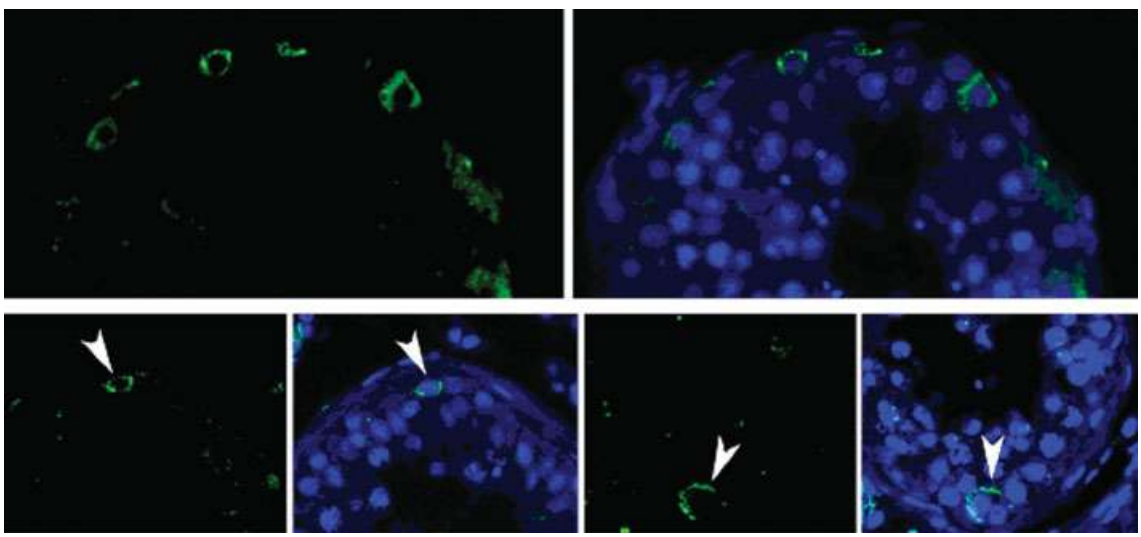

Figure 4. Phenotypic characteristics of human SSCs. Immunohistochemistry reveals that GPR125 is expressed in a subpopulation of spermatogonia (arrowheads) along the basement membrane of adult human seminiferous tubules. GPR125 seems to be a potential surface marker for human SSCs. GPR125 staining–green; DAPI nuclear staining–blue.

TABLE 1. A Comparison of Markers for Human and Rodent Spermatogonia

Marker	Human	Rodent	References
α6-integrin (CD49f)	+	+	(Shinohara et al., 1999; Conrad et al., 2008)
β1-integrin (CD29)	−	+	(Schaller et al., 1993; Shinohara et al., 1999)
CD9	?	+	(Kanatsu-Shinohara et al., 2004b)
CD133	+	?	(Conrad et al., 2008)
CDH1	?	+	(Tokuda et al., 2007)
CHEK2	+	?	(Bartkova et al., 2001)
GFRA1	+	+	(Meng et al., 2000; Conrad et al., 2008)
GPR125	+	+	(Seandel et al., 2007; Dym et al., 2009)
KIT	−	+	(Yoshinaga et al., 1991; Rajpert-De Meyts et al., 2003)
MAGE-A4	+	?	(Rajpert-De Meyts et al., 2003)
Neurogenin3	?	+	(Yoshida et al., 2004)
NSE	+	?	(Rajpert-De Meyts et al., 2003)
PLZF	+	+	(Buaas et al., 2004; Costoya et al., 2004)
POU5F1	−	+	(Looijenga et al., 2003; Ohbo et al., 2003)
RET	?	+	(Naughton et al., 2006)
STRA8	?	+	(Giuili et al., 2002)
Thy-1 (CD90)	+	+	(Kubota et al., 2003; Conrad et al., 2008)
TSPY	+	−	(Schnieders et al., 1996; Kido and Lau, 2006)

Markers known to be expressed (+) or absent (−) and unknown (?) in human and rodent spermatogonia are listed.
Abbreviations: CDH1, cadherin 1, type 1, E-cadherin; CHEK2, CHK2 checkpoint homolog; GFRA1, GDNF family receptor alpha 1; GPR125, G protein-coupled receptor 125; MAGE-A4, melanoma antigen family A, 4; NSE, neurone-specific enolase; PLZF, promyelocytic leukemia zinc finger protein; POU5F1, also known as Oct-4; STRA8, stimulated by retinoic acid gene 8; TSPY, testis specific protein, Y-linked 1.

such as CD90, GFRA1, and CD133, were also used to select human spermatogonia by MACS and comparable results to α6-integrin were obtained (Conrad et al., 2008). PLZF is characterized as a hallmark for mouse spermatogonial stem/progenitor cells (Buaas et al., 2004; Costoya et al., 2004). In adult monkey, the expression of PLZF is confined to the A_{dark} and/or A_{pale} spermatogonia (Hermann et al., 2007). We have recently demonstrated, using whole mounts of seminiferous tubules, that PLZF is localized to human spermatogonia (unpublished data). GPR125 has been demonstrated to be expressed in mouse spermatogonia and their progenitors (Seandel et al., 2007), and we have recently reported that GPR125 is also present in human spermatogonia (Dym et al., 2009). Collectively, the above studies suggest that some spermatogonial markers are conserved between rodents and humans.

In contrast, some other rodent markers for spermatogonia and their progenitors are not applicable to humans. This can be illustrated by the fact that β1-integrin (CD29), a marker for rodent spermatogonial stem/progenitor cells, is not expressed in human spermatogonia but present in spermatocytes, spermatids, and spermatozoa in normal human testis (Schaller et al., 1993). Another example is that POU5F1 (Oct-4), a marker for mouse spermatogonial stem/progenitor cells (Ohbo et al., 2003; Ohmura et al., 2004; Hofmann et al., 2005), is not detected in adult human spermatogonia (Looijenga et al., 2003). Similarly, KIT is regarded as a marker for mouse differentiating spermatogonia (Yoshinaga et al., 1991; Schrans-Stassen et al., 1999; Dolci et al., 2001), but it is undetected in human spermatogonia (Rajpert-De Meyts et al., 2003). Notably, some human markers for spermatogonia are also not applicable to rodents. As an example, the TSPY protein is preferentially expressed in elongated spermatids but not in spermatogonia of adult rat testis (Kido and Lau, 2006), unlike the expression pattern of the TSPY in adult human spermatogonia (Schnieders et al., 1996). Other rodent markers, including CD9 (Kanatsu-Shinohara et al., 2004b), CDH1 (Tokuda et al., 2007), neurogenin3 (Yoshida et al., 2004, 2007), RET (Naughton et al., 2006), and STRA8 (Giuili et al., 2002), were demonstrated to be expressed in spermatogonia and their progenitors; however, whether these rodent markers are present in human spermatogonia remains to be clarified. Similarly, some human markers, such as CD133 (Conrad et al., 2008), CHEK2 (also known as chk2 tumor suppressor protein) (Bartkova et al., 2001; Rajpert-De Meyts et al., 2003), and NSE (Neurone-specific enolase) (Rajpert-De Meyts et al., 2003), are also awaiting further studies to explore whether they are present in rodent spermatogonia and their progenitors. Such investigations would uncover further similarities and/or differences in spermatogonial phenotypes between human and rodents.

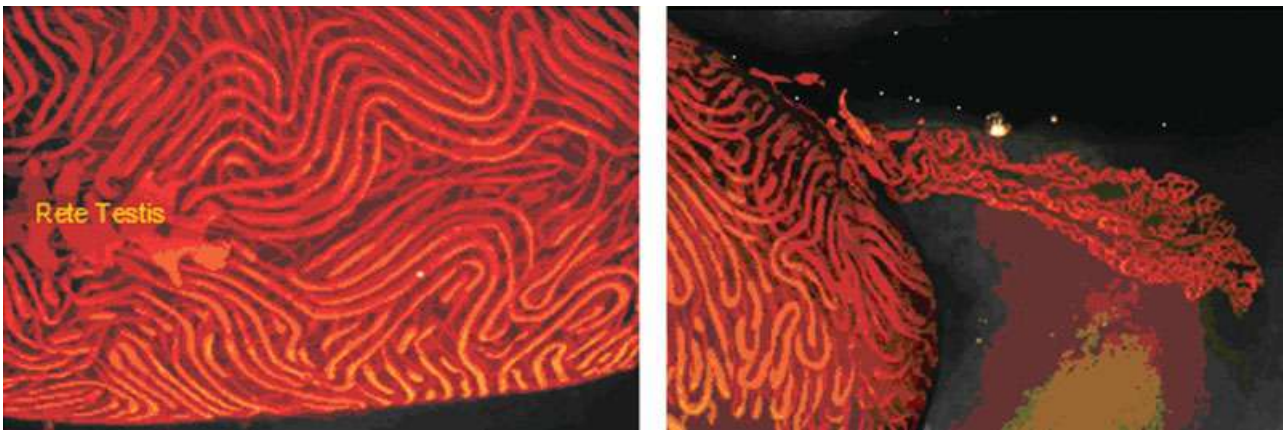

Figure 5. Transplant assay method shows the rete testis injected with Microfil, an orange dye. Many of the seminiferous tubules are filled with the Microfil dye. After dehydration in ethanol and clearing in methyl salicylate, the parallel course and undulating pattern of the seminiferous tubules are clearly seen (left panel). The right panel confirms that the injection was into the rete and seminiferous tubules, because in this photograph, fluid can be seen leaving the testis and entering the epididymis via the filled ductuli efferentes (arrows).

A FUNCTIONAL ASSAY FOR SPERMATOGONIAL STEM CELLS

The use of phenotypic markers alone is not sufficient to demonstrate a functional stem cell. We and others have adopted the procedure of using multiple markers on the same cell population to provide further evidence of stemness. Transplant of SSCs into seminiferous tubules and repopulation of sterile seminiferous tubules, a functional assay, was developed by Brinster in the mid-nineties (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994) and was recently reviewed (Brinster, 2007). The procedure involves introducing presumed SSCs in a small amount of media into the rete testes and then forcing the cells and media back into the seminiferous tubules. Figure 5 (left panel) shows rat seminiferous tubules filled with a dye solution (Microfil) after injection into the rete testes and then the passage of the fluid into the ductuli efferentes (Fig. 5, right panel). If indeed the cells are SSCs, then the tubules eventually produce sperm. Nagano et al. (2002) demonstrated the colonizing capacity of human SSCs in a human to nude mouse xenotransplantation assay. Recently this transplant assay was expanded to the primate testis in a monkey to nude mouse xenotransplantation assay (Hermann et al., 2007). Putative monkey SSCs were transplanted into seminiferous tubules of nude mice pretreated with busulfan and they could colonize in the seminiferous tubules of the recipient mice. In the monkey and human testis, the transplanted germ cells resulted in colonization of the mouse testis, but spermatogenesis was incomplete and became arrested at the stage of spermatogonial division (Kim et al., 1994; Dobrinski et al., 1999, 2000; Nagano et al., 2001, 2002).

PLURIPOTENCY OF ADULT SOMATIC CELLS AND STEM CELLS

In 2006 and 2007, a series of novel articles were published showing that somatic skin cells could be reprogrammed to ES-like cells, the so-called induced pluripotent stem (iPS) cells. Each report on the induction of pluripotency in mouse and human skin fibroblasts used retroviral delivery of key pluripotent stem cell genes such as, Oct-4 (Pou5f1), Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka, 2006; Hanna et al., 2007; Meissner et al., 2007; Okita et al., 2007; Takahashi et al., 2007; Wernig et al., 2007). In a second step, transformed iPS cells were identified and selected by expression of pluripotent markers including Nanog (Okita et al., 2007; Wernig et al., 2007) or Oct-4 (Wernig et al., 2007), or by ES-specific morphology (Meissner et al., 2007). These iPS cells had unique characteristics as they were germ-line competent and indistinguishable from ES cells derived from the embryo at the epigenetic level. Additionally, recent work has demonstrated that patient-autologous skin iPS cells can be genetically modified and used after differentiation by induction to cure a mouse model of sickle cell anemia (Hanna et al., 2007). Although this research paves the way toward stem cell therapy, it seems impractical for the iPS cells to be used in clinical application because of their instability and potential retroviral infection. As a result, it is essential and necessary to figure out more physiological methods to induce pluripotency from adult somatic cells or adult stem cells. More recent efforts have been taken to generate iPS cells from neural stem cells using only two transcription factors, Oct-4 and either c-Myc or Klf4 (Kim et al., 2008). It has also been demonstrated that iPS cells can be produced from adult cells by nonintegrating adenoviruses transiently expressing Oct-4, Sox2, Klf4, and

c-Myc (Stadtfeld et al., 2008). However, it is still possible that these factors could somehow find their way into the DNA of the iPS cells. Therefore, reprogramming of adult cells without the use of oncogenes would be very useful and safer as a means to produce ES-like cells.

The proof of principle that spermatogonial stem cells/progenitor cells could be reprogrammed to pluripotency by biochemical means alone was first shown by Shinohara and colleagues (Kanatsu-Shinohara et al., 2004a). However, Shinohara and colleagues could not derive ES-like cells from SSCs of adult mice. Then in 2006, a group headed by Hasenfuss (Guan et al., 2006) demonstrated that mouse adult spermatogonia, possibly the spermatogonial stem cells and/or their progeny, were able to reprogram biochemically to pluripotent ES-like cells. This was confirmed in mouse by Rafii's group (Seandel et al., 2007) showing that adult spermatogonia and/or their progenitors could indeed form pluripotent ES-like cells. We and others have recently demonstrated a similar phenomenon in male germ cells and spermatogonia of the human testis (Golestaneh et al., 2007; Conrad et al., 2008; Kossack et al., 2008). It is important to note that in the testis SSC/progenitor cell reprogramming, there is neither the addition of oncogenes nor the use of retroviruses. The SSCs/progenitor cells appear to reprogram spontaneously to pluripotency when removed from their niche and when ES cell media is added. Thus, SSCs/progenitor cells have great potential to be used as a safe means to generate ES-like cells that eventually can be used for clinical therapies of human diseases.

Human ES cells are pluripotent stem cells that have the potential to differentiate into all the types of cell lineages and tissues in the body, and thus they are ideal cell sources for cell transplantation and gene therapy. However, the major concern is the ethical issues associated with obtaining human ES cells from IVF clinics. The iPS cells have major advantages over human ES cells because there are no ethical issues involved and, more importantly, the iPS cells appear to be similar to ES cells in morphology, proliferation, and pluripotency, as evaluated by teratoma formation and chimera contribution. In contrast, the iPS cells have some disadvantages, e.g., safety is a major concern due to the potential of cell transformation or tumor formation because of the oncogenes from the transfected iPS. It may be possible to overcome these issues by generating pluripotent stem cells directly from spermatogonial stem cells by testicular biopsy as there is no oncogene transfer. Thus, the generation of human ES-like cells from SSCs may offer a safer means of obtaining pluripotent stem cells than from the iPS cells.

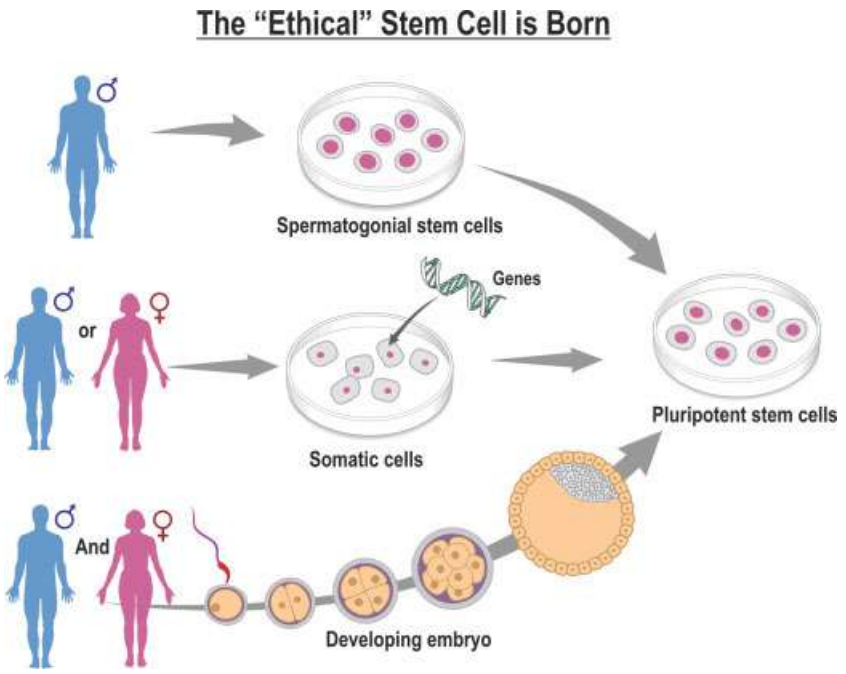

Figure 6. A schematic diagram illustrating three sources of human pluripotent stem cells.

The identification of the true human SSC is now especially important in view of the discrepancy between the Skutella report (Conrad et al., 2008) and the report by the Reijo-Pera group (Kossack et al., 2008). The Skutella group concluded that the ES-like cells derived from human spermatogonia (Spga) were in fact as pluripotent as true embryonic stem (ES) cells. The Reijo-Pera group noted that their cells differ from true ES cells in gene expression, methylation, and in their ability to form teratomas. Comparisons are difficult because the Skutella group used isolated Spga to get their ES-like cells, whereas the Reijo-Pera group used the entire testis biopsy without separating the Spga. It is possible that ES-like cells derived from isolated spermatogonial stem cells yield superior ES-like cells compared to using whole testis, but this remains to be determined.

As summarized in Figure 6, there are now three means to generate human pluripotent stem cells: (1) from a fertilized embryo, the traditional method; (2) from adult somatic cells, the iPS cells; and (3) from adult SSCs and/or their progeny. One major advantage of the third approach is that the production of the ES-like cells is spontaneous, unlike method 2, where several genes, some cancer causing, is employed. Thus, human SSCs and/or their progeny have great potential for cell- and

tissue engineering-based medical regeneration for various human diseases. It is possible that in the near future men could be cured of their diseases using a biopsy from their own testes.

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Novel methods to enhance surgical sperm retrieval: a systematic review

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ABSTRACT

Objectives: To explore the use of novel technologies in sperm retrieval in men with azoospermia due to a production defect.

Methods: We performed a Preferred Reporting Items for Systemic Reviews and Meta-Analysis (PRISMA)-compliant systemic literature review for manuscripts focussed on novel sperm-retrieval methods. We identified 30 studies suitable for qualitative analysis.

Results and Conclusions: We identified multiple new promising technologies, each with its own distinct set of benefits and limitations, to enhance chances of sperm retrieval; these include the use of multiphoton microscopy, Raman spectroscopy, and full-field optical coherence tomography during a microdissection-testicular sperm extraction procedure. ORBEYE and ultrasonography technologies can also serve to better visualise areas of sperm production. Finally, artificial intelligence technology can play a role in the identification of sperm and, perhaps, better-quality sperm for use with assisted reproduction.

Abbreviations: AI: artificial intelligence; ANN: artificial neural network; ART: assisted reproductive technology; 3D: three-dimensional; DNN: deep neural networks; FFOCT: full-field optical coherence tomography; H&E: haematoxylin and eosin; ICSI: intracytoplasmic sperm injection; IVF: *in vitro* fertilisation; MESA: micro-epididymal sperm aspiration; MeSH: Medical Subject Heading; MPM: multiphoton microscopy; (N)OA: (non-)obstructive azoospermia; SCO: Sertoli cell-only syndrome; SRR: sperm retrieval rates; TESA: testicular sperm aspiration; (micro-)TESE: (microdissection-) testicular sperm extraction; (CE)US: (contrast-enhanced) ultrasonography

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Introduction

For couples struggling with infertility, male infertility can be the sole contributing factor in 20–30% of cases and the partial contributing factor in 10–20% [1]. Infertility in males can be attributed to a variety of factors related to the production, transport, or function of sperm. Semen can exhibit decreased concentration of sperm (oligospermia), decreased motility of sperm (asthenospermia), decreased morphology of sperm (teratospermia), and finally, absent sperm in the ejaculate (azoospermia) [2]. In cases of azoospermia or absent ejaculate altogether, treatments can be focussed on obtaining adequate sperm samples for use in assisted reproductive technology (ART). Traditionally, these treatments have included vasal, epididymal, or testicular aspiration. In the present review, we will discuss recent advances in surgical sperm retrieval for non-obstructive azoospermia (NOA), focussing primarily on microdissection-testicular sperm extraction (micro-TESE)

The joint American Society of Reproductive Medicine (ASRM) and AUA 2020 male infertility guidelines highlight that for men with NOA undergoing sperm retrieval, micro-TESE should be performed [3]. Furthermore, in a meta-analysis of sperm retrieval rates (SRR) for men with NOA, micro-TESE was found to result in successful extraction 1.5-times more often than non-microsurgical TESE. Additionally, TESE was twice as likely to succeed when compared to testicular sperm aspiration (TESA) [4].

The efficacy of micro-TESE is limited by the ability of the surgeon to identify seminiferous tubules containing spermatozoa, especially with patients who have Sertoli cell-only syndrome (SCO). When micro-TESE is employed in patients with NOA, the SRR are reported to range from 43–63% [5–7]. The seminiferous tubules are currently evaluated by subjective assessment of their size and opacity, utilising the operating light microscope. Although micro-TESE has become first-line in sperm retrieval in men with NOA, there are some challenges with the procedure, including difficulty differentiating between seminiferous tubules with normal and abnormal spermatogenesis, as well as extensive tissue dissection that can sometimes lead to lifelong testosterone deficiency [7]. Some of the latest advances on the horizon, such as multiphoton microscopy (MPM), Raman spectroscopy (RS), and full-field optical coherence tomography (FFOCT) have demonstrated the potential to better identify areas of spermatogenesis and improve sperm extraction success [8]. We will also elaborate on the use of ORBEYE (a novel 4 K three-dimensional [3D] surgical exoscope), ultrasonography (US), and artificial intelligence (AI)

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technology to maximise success with both identification of sperm, as well as strategies to enhance sperm selection for ART.

Methods

The search strategy was conducted according to a modified Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [9] (Table 1). A literature search was performed using PubMed, the Medical Literature Analysis and Retrieval System Online (MEDLINE), the Excerpta Medica dataBASE (EMBASE), and Cochrane electronic databases to identify studies investigating novel sperm retrieval and identification methods utilising micro-TESE, US, and AI for the years 1999–2020. The search was executed using the following keywords: 'novel', 'surgical sperm retrieval', 'sperm retrieval', 'mTESE', 'microdissection testicular sperm extraction', 'on obstructive azoospermia,' 'NOA' 'Azoospermia', 'ORBEye', 'Artificial intelligence' and 'sperm identification'. Medical Subject Heading (MeSH) phrases included: ('Artificial Intelligence'[MeSH]) AND 'Azoospermia/therapy*'[MeSH], AND ('sperm retrieval'[MeSH]) AND ('1999'[Date-Publication]: '2020' [Date – Publication]).

Results

The search identified 769 records; an additional five reports were identified via searching the references of relevant manuscripts, and recent published abstracts were considered for inclusion. Inclusion criteria for technologies used to enhance the micro-TESE technique included the following: manuscripts describing the use of novel technologies in the setting of sperm extraction with mice or human subjects. Studies that evaluated solely the theory of these technologies, and not test efficacy on human or mice subjects were excluded. Manuscripts describing novel technologies specific to procedures other than sperm extraction, and manuscripts in languages other than English were excluded.

Following the literature search and application of exclusion criteria, 30 studies were included in the final qualitative synthesis (Figure 1). The final studies were divided up as follows:

The section on MPM included three studies, two on *ex vivo* human tissue and one in rats.

The section on RS included studies on mitochondrial tissue of human and rat spermatozoa.

The FFOCT section included three new studies, which focussed solely on analysing murine tissue.

Due to the novelty in the following technologies some studies were included that did not directly pertain to the field of urology or sperm retrieval as the current literature is limited. However, we felt that in order to gain a full understanding of the technologies these studies should be taken into account.

The ORBEYE section included two studies in the final manuscript. One study compared the ORBEYE with traditional operating microscopes in surgeries with human patients. The other study focussed on results from vasectomy reversal procedures done on rats.

The US section included 12 studies in the final manuscript, 11 of which applied directly to the field of Urology and one from other subspecialties. Of the 12 studies, 11 explored US as a tool for aiding sperm retrieval in human patients. The remaining study focussed on results focussed on global use of US on human patients.

The AI section included eight studies in the final manuscript; six of which applied directly to the field of Urology and two from other fields. Five of the cited studies compared the use of AI with data from traditional methods of extraction with human patients. One study involved identification of spermatozoa parameters in domestic cats. The remaining study focussed on general knowledge regarding AI.

Discussion

Multiphoton microscopy

MPM has several advantages over other forms of microscopy. MPM uses a near infrared femtosecond pulsed laser with two or three low-energy photons to produce the excitation of intrinsic fluorophores causing autofluorescence (Figure 2) [10]. The MPM's near infrared light passes relatively unhindered through tissue, without the need to use additional dyes (that can damage sperm), and enables deeper imaging than other imaging techniques. The penetration depth for MPM is up to 400 µm below the surface, allowing the surgeon to image the lumina of seminiferous tubules. The underlying tissue is optically sectioned, allowing for real-time high-resolution images without the need for physical extraction [10]. Additionally, MPM-guided testis biopsies could potentially prevent the risk of iatrogenic male hypogonadism by optimising the ability to identify only sperm containing tubules and prevent loss of Leydig cells in interstitial testicular tissue [11].

The photons can be combined and scattered in non-centrosymmetric tissue, such as collagen and oriented microtubules, allowing for the visualisation of peritubular fibrosis, typically present in testes with severely defective spermatogenesis [12]. In a pilot study by Najari et al. [13], MPM demonstrated a 92% concordance rate of diagnosis compared to haematoxylin and eosin (H&E) staining in men with NOA, and accurately differentiated normal from abnormal

Table 1. PRISMA 2009 checklist.

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	2
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	2
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g. Web address), and, if available, provide registration information including registration number.	3
Eligibility criteria	6	Specify study characteristics (e.g. PICOS, length of follow-up) and report characteristics (e.g. years considered, language, publication status) used as criteria for eligibility, giving rationale.	3
Information sources	7	Describe all information sources (e.g. databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	3
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	3
Study selection	9	State the process for selecting studies (i.e. screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	3
Data collection process	10	Describe method of data extraction from reports (e.g. piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	3
Data items	11	List and define all variables for which data were sought (e.g. PICOS, funding sources) and any assumptions and simplifications made.	3
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	3
Summary measures	13	State the principal summary measures (e.g. risk ratio, difference in means).	3
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g. I^2) for each meta-analysis.	-
Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g. publication bias, selective reporting within studies).	3
Additional analyses	16	Describe methods of additional analyses (e.g. sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	-
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g. study size, PICOS, follow-up period) and provide the citations.	3
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	-
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	-
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	-
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	-
Additional analysis	23	Give results of additional analyses, if done (e.g. sensitivity or subgroup analyses, meta-regression [see Item 16]).	-
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g. healthcare providers, users, and policy makers).	10
Limitations	25	Discuss limitations at study and outcome level (e.g. risk of bias), and at review-level (e.g. incomplete retrieval of identified research, reporting bias).	10
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	10
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g. supply of data); role of funders for the systematic review.	12

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spermatogenesis in human testicular tissue. That study validates the potential impact MPM could have on sperm retrieval in men with NOA; however, there are still some impediments.

Although MPM has shown promising results in enhancing the identification of seminiferous tubules with sperm, further studies must be done to ensure safety of the laser intensity and ethical issues regarding assisted reproduction. MPM safety concerns include thermal and non-linear damage to DNA that can potentially induce genetic abnormalities in gametes used for *in vitro* fertilisation (IVF). Although rodent models showed minimal phototoxicity, these findings have yet to be validated in a human model.

Raman spectroscopy

RS is a laser-based, optically label-free probe derived from the principle of inelastic scattering from molecular vibrations. RS utilises the molecular fingerprints of different tissues and transforms the biochemical information into a characteristic Raman spectrum [14]. In reproductive medicine, RS was first utilised to evaluate sperm DNA integrity and to distinguish between spermatozoa that could bind to the zona pellucida [15]. This technique was 96% sensitive and 100% specific in distinguishing the presence of spermatogenesis in rat models with SCO histology (Figure 3) [16]. Given that the sensitivity and specificity of RS are greater than any other techniques discussed thus far, RS-guided micro-TESE could have the potential to improve SRR [16]. While this technique is non-invasive and non-destructive, the overall safety of this laser-based technique needs to be assessed in human models. While this is a real-time analytical tool, each analysis takes ~2 min and results can be skewed by light pollution [17].

Full-field optical coherence tomography

FFOCT is a technique that uses a simple tungsten halogen lamp and is based on the principle of white-light interference microscopy to produce high-resolution images of unprocessed and unstained tissue [18]. Some of the advantages of FFOCT are the speed (1 frame/s) and ease with which images can be obtained from relatively large areas of tissue. One specific advantage that the FFOCT harbours over MPM and RS is the use of very safe incident light coming from a 150-W halogen lamp, making it ideal for IVF with intracytoplasmic sperm injection (ICSI), as it decreases the potential of thermal DNA damage in extracted sperm. In a pilot animal study, Ramasamy et al. [19] demonstrated that FFOCT successfully distinguished between tubules with and without spermatogenesis, by imaging testicular specimens from a busulfan-treated rodent model (Figure 4) [19]. Normal adult rats exhibited tubules with uniform size and shape (mean [SD] diameter 328 [11] µm), while busulfan-treated rats showed marked heterogeneity in tubular size and shape (mean [SD] diameter 178 [35] µm), with only 10% containing sperm within the lumen. FFOCT defined spermatogenesis as the presence of a bright signal, which emanates from the unique microtubular structure in sperm tails. Unlike MPM, FFOCT has considerable limitations, including the absence of cellular details, limited depth of imaging below the specimen surface, and the fact that this system can only image *ex vivo* specimens [19,20]. Unfortunately, further studies have not been carried out in human testicular tissue, and it is unknown if whether FFOCT displays the same efficacy for identifying spermatogenesis given the complex cellular microtubular structure.

ORBEYE

The ORBEYE 4 K 3D microscope is a surgical exoscope or 'camera' that can be used to enhance urological microsurgical procedures such as micro-epididymal sperm aspiration (MESA) or micro-TESE [21]. It consists of two Sony 4 K (4096 × 2160 pixels) Exmor R CMOS image sensors, which help provide high sensitivity, low noise, and a wide colour range image. The exoscope is placed over the surgical field and the image is projected on to two 140-cm (55-inch) monitors that allow for active 3D viewing with lightweight passive light 3D glasses (Figure 5).

Historically, surgeries done with state-of-the-art microscopes allowed for high magnification and detailed views of the surgical field. However, they did necessitate frequent repositioning due to the shallow depth of field and required surgeons to constantly fixate their eyes into the eyepieces of the microscope [22]. The ORBEYE overcomes both difficulties with the use of wider fields of view and longer depths of field. The use of video monitors eliminates the need for eyepieces, which forces surgeons to hold uncomfortable postures at awkward angles. It also allows other members of the operative team, including staff and students who may not be participating in surgery, to be able to learn from and follow the surgical steps in as well, by wearing the 3D glasses [23].

The ORBEYE is already widely used in surgical fields with microsurgical subspecialties. It was first reviewed to address its advantages and disadvantages in micro-neurosurgery in 2018 [24]. In urology, ORBEYE was compared to the traditional operating microscope for vasectomy reversal in a prospective randomised controlled animal trial on rats in 2019 [25]. The study concluded that there was no difference with respect to patency, operating time, or granuloma formation. Another study analysed the differences in operating time and surgeon fatigue for urological microsurgery, including MESA and micro-TESE, between the two scopes [21]. Although the difference was not statistically significant (P = 0.092), operating times for varicocelectomies appeared to be shorter with ORBEYE than traditional microscopes. The logistics of transport, draping, and operating seem to be advantageous with the ORBEYE, which can be attributed to its manoeuvrability, compactness, and simple plug-and-play interface. The ORBEYE also appears to have an ergonomic advantage over traditional microscopes, allowing surgeons a more natural heads-up posture. This is especially important

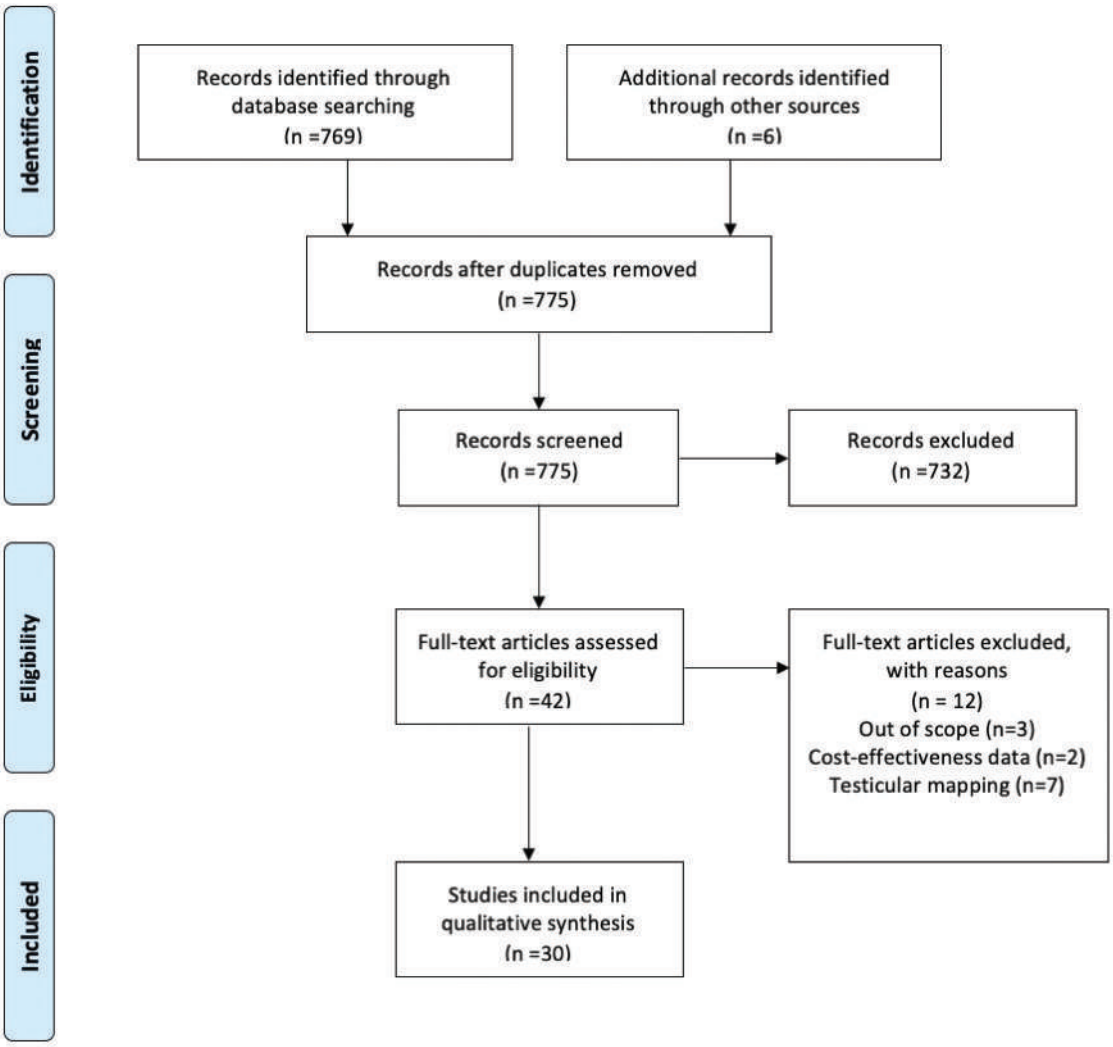

Figure 1. PRISMA flowchart of screening and selection procedure.

in urology, with a relatively large amount of time spent by surgeons in high-risk neck positions [26]. Similar outcomes were reported in neurosurgery and vascular surgery [24,27]. A potential disadvantage comes with the cost of the ORBEYE, which runs in the range of 400,000 USD (American dollars), about twice as much as traditional operating microscopes. Another potential disadvantage lies in operating from the point of view of surgical assistants, as a rotated view of the surgical field will be projected on to the monitor from the assistant's position.

Ultrasonography

Due to the invasive nature of biopsies and poor predictive ability of clinical characteristics, testicular imaging of men with NOA is an area of great interest. US has been investigated as a non-invasive and widely accessible method for evaluating a patient during testicular sperm retrieval [28].

Sperm retrieval success rates in patients with NOA range from 42–62% and appear to be related to the method of retrieval. Earlier research has shown that isolated regions of spermatogenic tissue may exist in testes of men with NOA [29,30]. Currently, the location of TESE biopsies are chosen arbitrarily, and as a consequence, a large portion of the biopsies yield negative results [31].

In men with NOA, previous studies have shown that testicular structure, including testicular blood flow is severely altered and strongly modified, showing decreased or absent intratesticular arterial flow compared to normal testes. In contrast, men with obstructive azoospermia (OA) exhibit uniform flow, compared to the controls. Previously it has been reported that in young boys, testicular blood flow is correlated to testicular volume, and that flow increases when the maturation process leading to spermatogenesis appears, thus suggesting a relationship between blood flow and testicular tubal function [32]. Furthermore, spermatogenesis is not uniform

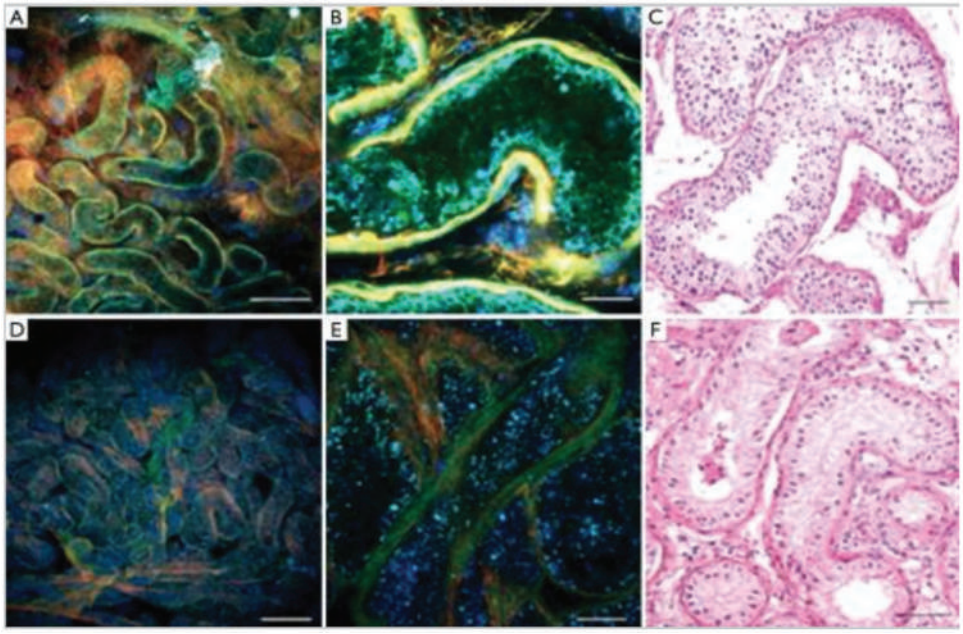

Figure 2. Seminiferous tubular histology patterns imaged by MPM at low (a and d) and high (b and e) magnification compared to high magnification stained tissue (c and f). Normal spermatogenesis is shown by green areas in A to C and seminiferous tubules with SCO pathology is shown by blue areas in D to F. H&E (c and f). Scale bar represents 500 µm (a and d) and 80 µm (b,c,e,f). Permission for reproduction obtained from Elsevier Publishing, Ramasamy et al. [10].

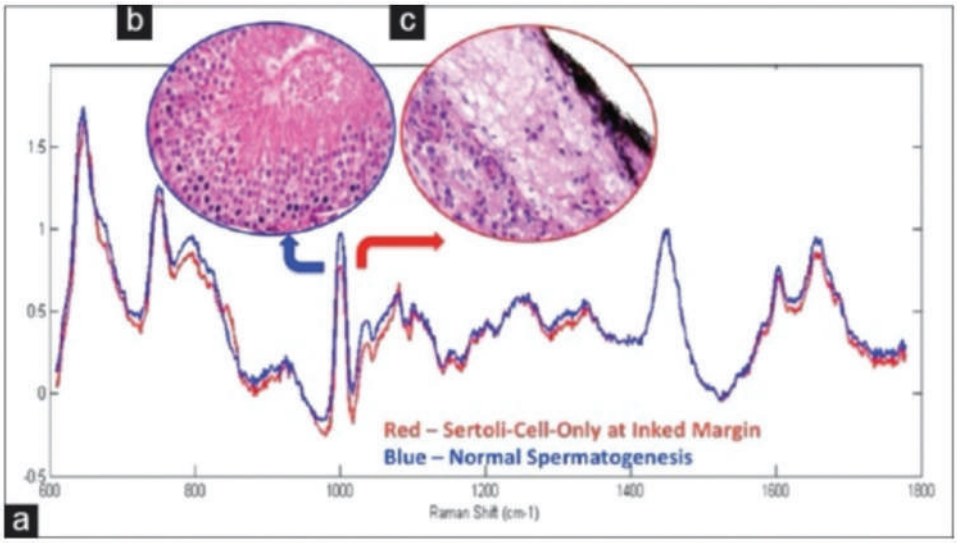

Figure 3. (a) Mean processed spectra for SCO (red curve) and active spermatogenesis (blue curve) with 1000 and 1690 cm^{-1} discriminatory Raman peak intensity, respectively. (b) Representative testicular biopsy shows active spermatogenesis. (c) Representative testicular biopsy shows SCO. (b and c) H&E, reduced from ×200. Permission for reproduction obtained from Elsevier Publishing, Osterberg et al. [16].

throughout the testis. Studies of patients with NOA showed that sperm quality was highest in areas with high sperm perfusion. Herwig et al. [33] reported in their study that indeed high levels of perfusion matched with a qualitatively and quantitatively high level of sperm retrieval from TESE. Additionally, researchers developed a method of non-invasive testicular screening using Doppler US that possesses low sensitivity (47.35%) but a high specificity (89.8%), suggesting that their technique would better predict the absence of spermatozoa than their presence, which would still favour the use of Doppler US to avoid areas of absent spermatogenesis [31]. Therefore, Doppler US could be an advantageous technique compared to standard of care in locating foci of high perfusion and assumed spermatogenesis, excluding areas of absent spermatogenesis, and subsequently improving rates of sperm retrieval in patients with NOA.

Earlier research (2001) by Belenky et al. [34] established that US-guided TESA compared to 'blind' TESA was a safe and accurate method for sperm retrieval in

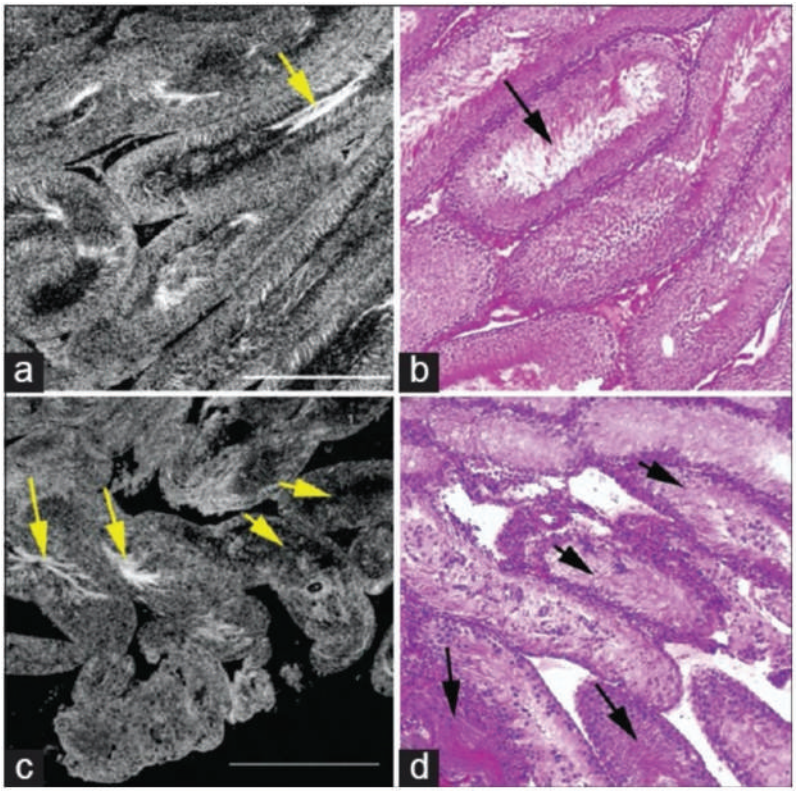

Figure 4. Comparative FFOCT and H&E-stained histology. (a) Testis of a normal rat shows seminiferous tubules with relatively uniform size and shape, (b) H&E histology stain of the same specimen. Arrows point to the sperm within the tubule lumen. (c) Seminiferous tubules in the testis of a rat treated with busulfan, showing thinner tubules and a greater degree of heterogeneity in size and shape with ~10% normal spermatogenesis. (d) H&E staining of the same specimen. Field of view in each panel: 1 mm^2 Permission granted under the creative commons attribution license, Ramasamy et al. [19].

Figure 5. Operating room setup with the ORBEYE™ surgical microscope. The microscope coming from the surgeon's left-frontal side is held over the surgical field resulting in no obstacle between the surgeon and the monitor. The operator, the assistant and the entire operating staff using 3D glasses have the same view as the operator.

patients with NOA, but no differences were found between the two groups in pregnancy rate in the patient's female partner. Another study in 2019 utilised contrast-enhanced US (CEUS) 10 days prior to TESA. The study assessed 70 men, 46 with NOA and the remaining with OA. The group reported that CEUS-guided TESA with cognitive fusion did not yield improved sperm retrieval outcomes of TESA in patients with NOA, potentially due to imprecise correlation between biopsy sites and main perfusion areas

analysed by CEUS [35]. Conversely, a 2018 study that utilised CEUS in 120 men with NOA and subsequent micro-TESE, demonstrated improved success rates for micro-TESE potentially due to CEUS's ability to locate the best perfusion areas. This work suggests that CEUS can highlight microvascular distribution in testicles, aid in locating areas of best perfusion over the maximal longitudinal section, and improve success rates [36]. Lastly, work by Herwig et al. [37] with patients with azoospermia undergoing TESE biopsy for retrieval of sperm showed high sperm quality in areas of high tissue perfusion. Furthermore, their results correlated the number of motile sperm isolated from tissue samples with the intensity of tissue perfusion using colour Doppler US.

Whilst showing much promise, the strength of any prognostic tool is truly known only with external validation and some limitations exist to the use of US for sperm retrieval. For US, outcomes are highly dependent on the skills of the operator. For example, Nariyoshi et al. [38] included patients from two separate clinics in Japan in their research with US-guided sperm retrievals. This could have allowed a variety of factors to potentially impact the results. In addition, successful examination and interpretation of the testicles requires training and experience. Furthermore, in examining testicular perfusion with Doppler US, previous studies were only able to locate main arteries in the testes and were unable to resolve the microvasculature. Despite this, high-density microvasculature greatly contributes to high blood testicular perfusion and this was correlated with focal spermatogenesis [31]. Nonetheless, whilst some limitations exist, Doppler US can be a potentially effective tool in aiding sperm retrieval procedures.

Artificial intelligence

Identification of sperm parameters, selection, and assortment are an essential task when processing human testicular samples for cryopreservation or IVF. The quality of spermatozoa is one of the most important parameters for oocyte fertilisation and embryo quality. Studies have shown that abnormalities in the quality of spermatozoa correlate with cleaving embryo morphology at later stages [39].

Problems in sperm maturation cause abnormalities in sperm morphology, which need to be identified to ensure proper egg fertilisation. Assessment of sperm parameters such as semen pH, sperm morphology, viscosity, concentration, and motility can help determine male factor infertility. While some of these factors can be consistently and objectively assessed, manual assessment of other factors such as sperm morphology and motility are subjective, operator dependent, and error prone. Development of standardised and automated methods is vital for accurate and consistent results.

AI is a large 'umbrella' term that encompasses methods that mimic the intelligence or behavioural patterns of humans or any other living entity. Machine learning is a technique by which a computer can 'learn' from data, without using a complex set of different rules. This approach is mainly based on training a model from datasets. Further on, 'deep learning', a revolutionary method pioneered in 2012 by George E. Dahl, is a technique to perform machine learning inspired by our brain's own network of neurones.

Artificial neural networks (or ANNs), are biologically inspired computational models developed to simulate the way in which the human brain processes data [40]. The network operates based on interconnected virtual neurones that can accept input features and produce an output decision on the basis of its 'existence'. These networks are capable of learning under certain training instructions, generally without task-specific instructions and can make decisions on the basis of this experience. For example, in image recognition, such techniques might learn to identify images that contain spermatozoa by analysing example images that have been manually labelled as 'spermatozoa' or 'no spermatozoa' and using analytical results to identify spermatozoa in other images. Such ANNs have been shown to classify and recognise patterns accurately [41].

Built on previous concepts of neural networks, deep neural networks (DNN) are ANNs with multiple layers between the input and output layers [42]. DNNs can model complex non-linear relationships. For example, a DNN that is trained to recognise histological cell types will study the given image and calculates the probability that the cell in the image is of certain type. The user can review the results and select which probabilities the network should display and return the proposed label. Each mathematical manipulation as such is considered a layer, a complex DNN, may have many layers, lending itself the name 'deep' networks.

When addressing male infertility, the main challenges are prediction of sperm presence, identification of sperm on biopsy extraction, and qualification of sperm integrity after extraction. Currently, these processes remain unaided by AI systems, are not automated, and are operator dependent.

One fundamental advantage of ANN exists in its ability to predict outcomes based on previous data. In a retrospective analysis of data collected from physical examinations Samli and Dogan [43] developed an ANN for predicting spermatozoa prior to testicular biopsy in men with NOA and compared it to a standard logistic regression model. Using factors such as age, duration of infertility, serum hormone levels, and testicular volumes, the group was successful in creating a model with a significantly higher

sensitivity than the logistic regression model and was able to correctly predict outcomes and achieve clinically-acceptable sensitivity in 59 of 73 patients in the test set.

An alternative method to assess the probability of finding sperm was used by Ramasamy et al. [44]. Their method consisted of a retrospective analysis of men who underwent micro-TESE. Rather than training ANN with images their method consisted of using readily available clinical features to model and predict the chance of identifying sperm with micro-TESE in men with NOA. The proposed model demonstrated 59.4% success in correctly predicting the outcome of sperm retrieval based on pre-existing clinical evidence. Although showing promising data, these results were not generalisable and other studies are required for external validation.

In the field of reproductive medicine, there are no existing computer-aided sperm analysis systems for testicular biopsies. This process is very operator dependent and relies on manual image analysis for sperm identification. To tackle this problem, Wu et al. [45] proposed a deep convolution neural network method in which a dataset of 702 de-identified images from testicular biopsies were collected from testicular biopsies of 30 patients. The group was able to achieve a mean average precision of 0.741 with an average recall of 0.376 on their dataset, suggesting that deep learning is an efficient method of finding sperm in testicular biopsy samples.

In a clinical setting, ANNs, and deep learning methods hold true potential for innovation in automatic assessment of human sperm due to the ability to work with low resolution images and unstained sperms, in real time and with high accuracy. Javadi and Mirroshandel [39] suggested a deep learning method for selecting the best sperms in an ICSI procedure. The proposed model extracts features of the acrosome, head shape, and vacuole from sperm images gathered in real time. The method was able to select the best fresh sperm for injection, and ultimately achieved a better accuracy than existing state-of-the-art methods in acrosome and vacuole abnormality detection on the proposed benchmark. Experimental results showed high accuracy of the proposed deep learning model.

Similarly, work by McCallum et al. [46] focussed on assessing the quality of sperm DNA using deep learning-based methods. Traditionally, sperm quality has been assessed by skilled clinicians to select the best sperm based on various morphological and motility criteria, but without direct knowledge of their DNA cargo. The group developed a DNN approach that is directly compatible with current, manual microscopy-based sperm selection and complementary to current clinical selection. Overall, the team was able to rapidly predict DNA quality (<10 ms/cell) and sperm selection within the 86th percentile from a given sample.

Motility estimation is another essential step in evaluation of male fertility. Because it can be considered as a functional test, it is a direct measurement of the energy status of the mammalian sperm. An AI-assisted method of evaluating sperm motility could thus be another beneficial tool for clinicians to select sperm after retrieval. To address this task, Contri et al. [40] focussed on ANNs for the definition of kinetic subpopulations and epididymal spermatozoa in domestic cats. This study prospectively collected electro-ejaculated samples from seven adult cats. The motility pattern of the feline semen was evaluated using a computer-assisted sperm analyser (CASA) system IVOS 12.3 (Hamilton-Thorne Bioscience, Beverly, MA, USA). The results of their study demonstrated the ability of ANNs to differentiate significant kinetic differences in electro-ejaculated vs epididymal samples.

The role of AI, neural networks, and deep learning, in the realm of fertility and reproduction still remains to be determined. While some technologies have shown promise, to our knowledge, studies have not yet determined the best ways to use AI for sperm extraction and reproduction. However, several have reported the use of ANN in medicine, mainly for the diagnosis and prognostic evaluation of several pathologies [41,43]. For example, work by Berlin et al. [47] has demonstrated the ability of machine learning to improve the efficiency and consistency of the automated planning method for prostate volumetric arc radiation therapy. Recently, the field of ophthalmology established itself as a paradigm shifter in the use of clinical AI. The IDx-DR (Digital Diagnostics, formerly IDx) is an autonomous AI designed to detect diabetic retinopathy and diabetic macular oedema. In 2018, it became the first United States Food and Drug Administration (FDA)-approved autonomous AI in any field of medicine.

Nonetheless, the use of AI, to tackle problems of sperm identification is a challenging task. For example, the number of sperm images available in real time can be a limiting factor for the AI training phase. The normal and abnormal sperm classes are highly imbalanced, thus making the problem harder. Furthermore, the images that are available in real time are taken using a low-magnification microscope and the details of these images are not clear, the pictures are very 'noisy' [39]. Overall, while some barriers exist to the use of AI and deep learning in reproductive medicine, overcoming these barriers will allow rapid predicting capabilities, identification mechanisms, and analysis of sperm integrity done in real time, without the need of samples being stained for identification purposes (Table 2) [4,7,10,11,13,14,17–24,28,31,33,36–39,41,43].

Table 2. Advantages and disadvantages of different retrieval/identification methods.

Retrieval/identification method	Advantages	Disadvantages
Micro-TESE	Good chance of sperm recovery [4]	Risk of damage to testis architecture [7] Success may depend on surgical skill [7]
Multiphoton microscopy	3D *in vivo* histological images Depth of penetration up to 400 µm Real-time analysis [10] Lower energy laser = minimal damage [11] Decreased operating time	Human studies not conducted to determine risk of genetic damage [13]
Raman spectroscopy	Flexible probe for surgical ease Non-destructive near infrared light source [14]	Safety not assessed in human models Specimen preparation takes 2 minutes and can be influenced by light pollution [17]
Full-field optical coherence tomography	Fast and easy to obtain images [18] Safe light source from 150-W halogen lamp [19]	Absence of cellular details Limited depth of imaging below cellular surface [20] Only images *ex vivo* specimens [20] Efficacy not proven compared to other techniques [19]
ORBEye	Elimination of eyepieces allows for better surgical posture [21] Simple to use and easy to transport May allow for shorter surgery times [21] Wider FOV and longer DOF eliminates need for frequent repositioning [22] 3D viewing monitors provides optimal teaching/demonstration environment [23]	Cost Surgical assistants have rotated surgical view, which can lead to confusion during operation [24]
Ultrasonography	Fast, easy, portable [28] Widely available [28] Ability to locate areas of high perfusion [33] Help rule out areas of absent spermatogenesis [31] Improve success rates of sperm retrievals [36,37]	Operator dependent [38] Requires training and experience [38] Difficulty with resolving microvasculature of the testicle [31]
Artificial Intelligence	Automated assessment of extracted sperm [37,38,44] Real-time analysis of sperm [37] Ability to work with unstained images [37] High accuracy [37] Ability to predict outcomes prior to extraction [41] Avoid operator dependence [43]	Training phase of AI can be limited by the number of sperm images available [39] Limited by quality of microscopy images [39] Technology is not widely available Requires collaboration between computer scientists and clinicians

FOV: field of view; DOF, depth of field.

Conclusion

Multiple new promising technologies have emerged recently to assist urologists during sperm retrieval for a male with infertility. The use of MPM, RS and FFOCT during a micro-TESE procedure can help distinguish tubules with and without spermatogenesis, a role that can also be potentially played by Doppler US. ORBEYE technology can be used as a valid alternative to the traditional microscopy technique. Finally, some studies have also shown promising results for the use of AI and neural networks to enhance sperm identification in surgically extracted sperm samples.

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Testosterone signaling and the regulation of spermatogenesis

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Spermatogenesis and male fertility are dependent upon the presence of testosterone in the testis. In the absence of testosterone or the androgen receptor, spermatogenesis does not proceed beyond the meiosis stage. The major cellular target and translator of testosterone signals to developing germ cells is the Sertoli cell. In the Sertoli cell, testosterone signals can be translated directly to changes in gene expression (the classical pathway) or testosterone can activate kinases that may regulate processes required to maintain spermatogenesis (the non-classical pathway). Contributions of the classical and non-classical testosterone signaling pathways to the maintenance of spermatogenesis are discussed. Studies that may further elaborate the mechanisms by with the pathways support spermatogenesis are proposed.

Androgens are essential for male fertility and the maintenance of spermatogenesis.[1,2] Testosterone is the androgen in the testis that is responsible for supporting spermatogenesis. In the absence of testosterone or functional androgen receptors (AR), males are infertile because spermatogenesis rarely progresses beyond meiosis.[3-5]

Testosterone is produced by Leydig cells in the interstitial space of the testis. As a result of the local production, testosterone levels in the testis in men are 25 to 125-fold greater in the testis (340 to 2,000 nM) as compared to serum (8.7–35 nM). Testosterone levels are similarly elevated in rodent testes.[6-10] Thus far, the specific physiologic requirements for high levels of testosterone in the testis are not known. However, it has been established that spermatogenesis does not proceed in the absence of relatively high levels of testosterone (>70 nM in the rat).[11]

Cellular Targets of Testosterone in the Testis

AR is present in the somatic Leydig, peritubular and Sertoli cells. The localization of AR to germ cells is controversial with some studies finding AR positive germ cells and other studies showing that there is no AR in germ cells (reviewed by Wang and colleagues).[12] Functional evidence suggests that if AR is expressed in germ cells it is not required. Specifically, chimeric male mice having both AR defective and wild type germ cells produced pups from the AR defective germ cells.[13] Also, AR defective germ cells transplanted into the testes of azoospermic male mice were able to form colonies of cells undergoing spermatogenesis.[14] Finally, cell-specific knock out of AR in germ cells such that AR is not expressed during or after meiosis did not alter spermatogenesis or fertility indicating that AR is not required in later stage germ cells.[15]

Sertoli cells are thought to be the major cellular target for the testosterone signaling that is required to support male germ cell development and survival.[16,17] AR expression levels rise and fall in adult Sertoli cells in a manner corresponding with the cyclical stages of the seminiferous epithelium. In the rat, the expression of AR protein is low and difficult to detect except during stages VI–VIII when AR levels increase dramatically.[18] AR expression is similarly cyclical in men.[19] It is during stages VI–IX that the lack of testosterone or AR most affects processes required for spermatogenesis.[5,20,21]

Regulation of Spermatogenesis Control Points by Testosterone and AR

Testosterone deprivation studies performed in rodents have established that testosterone is required for germ cells to progress beyond meiosis and that testosterone is required for the release of mature spermatids during stage VIII in rats (reviewed by Sharpe).[1] Thus far, evidence of direct testosterone support of meiosis is lacking as there are few meiosis-specific processes that are known to be directly regulated by AR-dependent actions. Instead, testosterone may act indirectly to permit germ cells to complete meiosis.

Withdrawal of testosterone or knock out of AR in Sertoli cells results in three major impairments to fertility. First, the integrity of the blood testis barrier (BTB) is compromised, which exposes post meiotic germ cells, formerly in a secluded specialized environment, to autoimmune attack and cytotoxic factors.[22,23] Second, there is a block in conversion of round spermatids to elongated spermatids due to a defect in cell adhesion that causes the premature detachment of round spermatids from Sertoli cells.[21,24,25] Third, fully mature spermatozoa cannot be released from Sertoli cells and the germ cells are phagocytized by the Sertoli cells.[21]

The use of Cre-Lox conditional knockout techniques to create mice in which the loss of AR is restricted to Sertoli cells (SCARKO mice) has allowed for a more precise determination of

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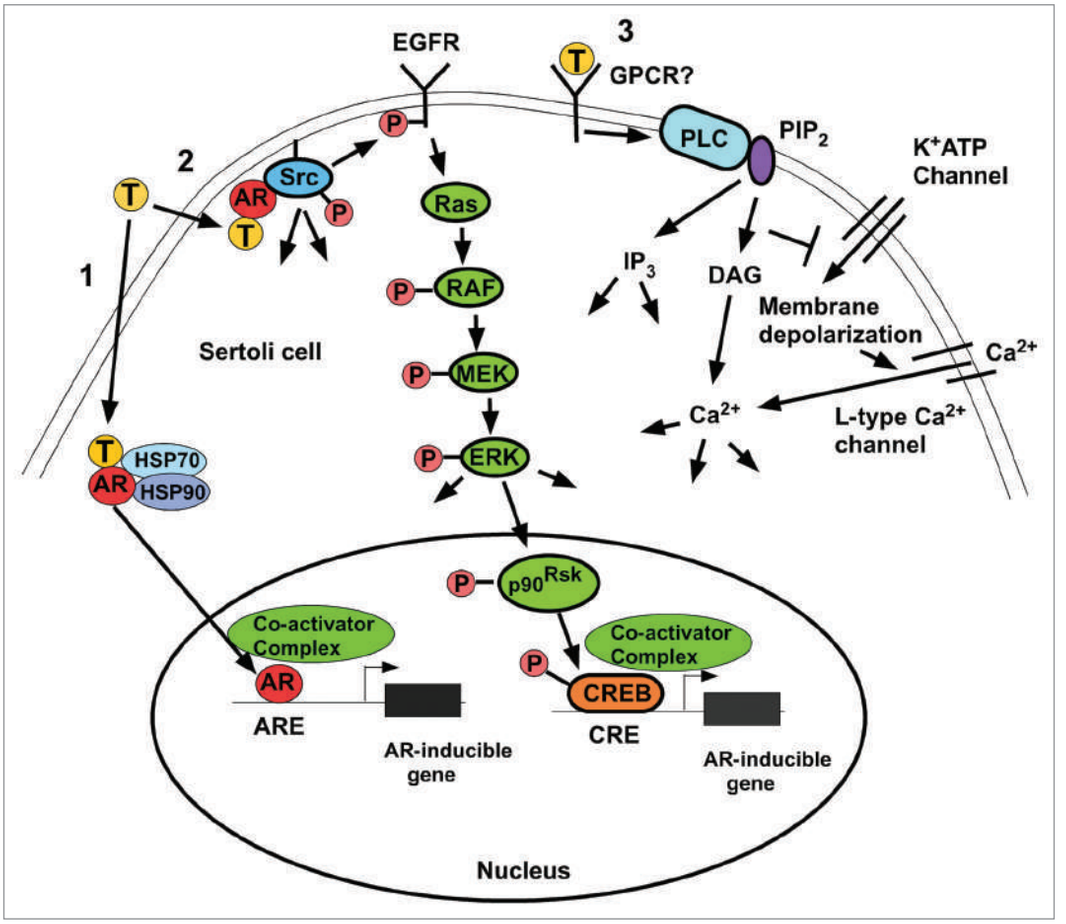

Figure 1. Testosterone signaling pathways. (1) The classical testosterone signaling pathway. Testosterone diffuses through the plasma membrane and binds with the AR. The AR undergoes an alteration in conformation allowing it to be released from heat shock proteins in the cytoplasm. AR then is able to translocate to the nucleus where it binds to specific DNA sequences called androgen response elements (AREs). AR binding to an ARE allows the recruitment of co-activator and co-repressor proteins that alter the expression of genes to alter cellular function. (2) The non-classical kinase activation pathway: testosterone interacts with the classical AR that then is able to recruit and activate Src, which causes the activation of the EGF receptor via an intracellular pathway. The EGF receptor then activates the MAP kinase cascade most likely through Ras resulting in the sequential activation of RAF and MEK and then ERK that activates $p90^{Rsk}$-kinase, which is known to phosphorylate CREB on serine 133. As a result, CREB-regulated genes such as lactate dehydrogenase A (LDH-A) and early growth response 1 (Egr1) and CREB can be induced by testosterone.[42] (3) The non-classical Ca^{2+} influx pathway: Testosterone interacts with a receptor in the plasma membrane that has characteristics of a G_q coupled G-protein coupled receptor (GPCR). Phospholipase C (PLC) is activated to cleave PIP_2 into IP_3 and DAG. Lower concentrations of PIP_2 inhibit K^+_{ATP} channels causing membrane depolarization and Ca^{2+} entry via L-type Ca^{2+} channels.

the effects of testosterone action on Sertoli cells in an otherwise normal testis. These strategies determined that, in the absence of Sertoli cell AR, spermatogenesis in mice does not progress beyond the pachytene or diplotene stages of meiosis[5,15] and the integrity of junctional complexes making up the BTB are not maintained.[20,26] Specifically, studies of SCARKO mice indicate that androgens regulate the expression levels of BTB tight junction-associated proteins and their localization.[22] Studies of cultured Sertoli cells have determined that testosterone stimulation increases the rate at which integral membrane adhesion proteins are endocytosed and then recycled to the membrane suggesting that testosterone may assist in the cyclical reformation of the BTB after the passage of leptotene spermatocytes through the barrier.[27] More recent studies have identified AR expression in Sertoli cells as a factor that limits the expression of differentiation markers in spermatogonial germ cells[28] and that Sertoli cell nuclei show signs of immaturity and are abnormally localized away from the basal lamina.[22,26,28,29] The implications of these last two characteristics of AR deficient Sertoli cells for maintaining spermatogenesis have not yet been investigated.

Classical and Non-Classical Testosterone Actions in Sertoli Cells

The classical testosterone signaling pathway. Testosterone has been shown to act via two pathways: the classical and the non-classical.[30,31] In the classical pathway (**Fig. 1**, left), testosterone diffuses through the plasma membrane and binds AR that is sequestered by heat shock proteins in the cytoplasm. A conformational change in AR causes the receptor to be released from

heat shock proteins. AR then translocates to the nucleus where it binds to androgen response elements (AREs) in gene promoter regions, recruits co-regulator proteins and regulates gene transcription. Activation of the classical pathway requires at least 30 to 45 min to initiate changes in gene expression.[32]

Several microarray studies using various models have been performed to survey testicular gene expression in the presence and absence of testosterone signaling (reviewed by Verhoeven and colleagues).[33] A broad spectrum of genes in the testis were found to be regulated by testosterone, but the number of Sertoli cell-specific genes that are regulated by testosterone make up a small subset. Furthermore, the genes identified in the microarray studies performed thus far show relatively little overlap and the number of genes displaying a two-fold or greater change in expression are limited.[33] Interestingly, a relatively high percentage of the regulated genes are inhibited by testosterone. Although one study determined that 65% of AR-regulated genes were linked to a conserved ARE within 6 kb of their transcription start sites, only the *Rhox5* (Pem) homeobox transcription factor encoding gene, has been shown to be induced in Sertoli cells by androgens through AR binding to ARE promoter elements.[34] Presently, there is no evidence that any one AR-regulated gene is critical for the completion of spermatogenesis; however, it is likely that spermatogenesis would be disrupted as a result of the mutation or elimination of multiple AR-regulated genes.[28] Further work is required to characterize the AR-regulated genes regulated by testosterone via the classical pathway as being essential or nonessential for spermatogenesis.

Because testosterone and AR are essential for spermatogenesis and male fertility, it is surprising that the gene survey studies have not identified more testosterone-regulated genes expressed in Sertoli cells that are required for spermatogenesis. One explanation for the lack of identified genes responsible for spermatogenesis may lie in the animal models used to obtain the microarray data. Thus far, gene expression data has been obtained from either prepubertal rats and mice or from AR knock out mice in which AR expression is eliminated before birth. In both models, the testes lack full complements of germ cells, which decreases the complexity of the signals received by Sertoli cells. One solution to the problem may be to selectively knock out AR in Sertoli cells in adult mice and obtain gene expression profiles prior to the loss of germ cells. Fortunately, at least one group is developing an adult mouse model in which the AR gene can be inducibly extinguished.[35] Further confirmation of the importance of AR-regulated genes for maintaining fertility in mice may be obtained in the future through comparisons to genetic surveys of mutated genes found in infertile men.

The non-classical testosterone signaling pathway. There are at least two non-classical mechanisms of testosterone action in Sertoli cells. In the testosterone-mediated [Ca^{2+}] influx pathway (**Fig. 1,** right), testosterone rapidly induces the influx of [Ca^{2+}] into Sertoli cells within 20–40 sec through L-Type [Ca^{2+}] channels.[36,37] Testosterone also is thought to cause the activation of an unidentified G_q type G protein coupled receptor and the activation of phospholipase C (PLC) that then hydrolyzes PIP_2 in the plasma membrane to produce IP_3 and diacylglycerol (DAG). The decrease in the levels of PIP_2, an inhibitor of ATP-mediated activation of K^+_{ATP} channels, promotes the closing of these channels causing an increase in membrane resistance and depolarization of the cell. As a result, voltage dependent L-type Ca^{2+} channels open and allow the influx of Ca^{2+}, which may alter many cellular processes.[38] Thus far, potential cellular targets and spermatogenesis processes regulated by the testosterone-mediated [Ca^{2+}] influx pathway have not been investigated.[39]

Testosterone also has been shown to rapidly activate a series of kinases in Sertoli cells that are known to regulate spermatogenesis. Stimulation of Sertoli cells with levels of testosterone (10–250 nM) that are similar to or lower than those found in the testis causes AR to transiently localize to the plasma membrane and results in AR interacting with and activating Src tyrosine kinase (**Fig. 1** and middle).[40] Androgen stimulation triggers the direct association of the proline rich region of AR (amino acids 352–359) and the SH3 domain of Src.[41] Testosterone-mediated activation of Src causes the phosphorylation and stimulation of the EGF receptor (EGFR) via an intracellular pathway. Stimulation of EGFR is required to activate the MAP kinase cascade (Raf, MEK, ERK) that causes $p90^{Rsk}$ kinase to phosphorylate the CREB transcription factor.[40] Activation of the non-classical pathway has been shown to induce the expression of CREB-mediated gene expression.[42] In contrast to the classical pathway, induction of ERK and CREB phosphorylation by testosterone is rapid (within 1 min) and can be sustained for at least 12 hr.[42] The regulation of additional gene expression by other transcription factors downstream of ERK and Src remains to be investigated.

The activation of Src and Erk kinases by non-classical testosterone signaling was found to alter processes that are critical to maintain spermatogenesis. Testosterone stimulation of Sertoli cells co-cultured with germ cells from adult rats increased the numbers of germ cells attached to the Sertoli cells by 50%. However, the addition of inhibitors of Src or ERK kinase reduced germ cell attachment below basal levels.[40] Additional studies were performed in which AR-defective Sertoli cells were infected with adenovirus constructs expressing wild type AR or AR mutants that selectively activated only the classical pathway or the non-classical pathway. In these studies, testosterone stimulation could only increase the attachment of germ cells to Sertoli cells expressing wild type AR or mutated AR that can stimulate the non-classical pathway.[40] These findings suggest that testosterone can act via the activation of Src and ERK kinases to facilitate Sertoli-germ cell attachment. It is possible that testosterone signaling that increases dramatically in stages VII–VIII of the cycle may be responsible for the remodeling of Sertoli-germ cell adhesion complexes that occurs during these stages when round spermatids begin to elongate. Furthermore, in testosterone deprived or AR deficient Sertoli cells, the lack of non-classical pathway-induced kinase activation may be responsible for the sloughing off and loss of spermatids that occurs during stages VII–VIII in the absence of testosterone signaling.

The release of mature sperm from Sertoli cells was also shown to be regulated by Src kinase that is activated by non-classical signaling. Seminiferous tubule fragments were micro dissected to isolate fragments containing only stages VII–VIII having mature

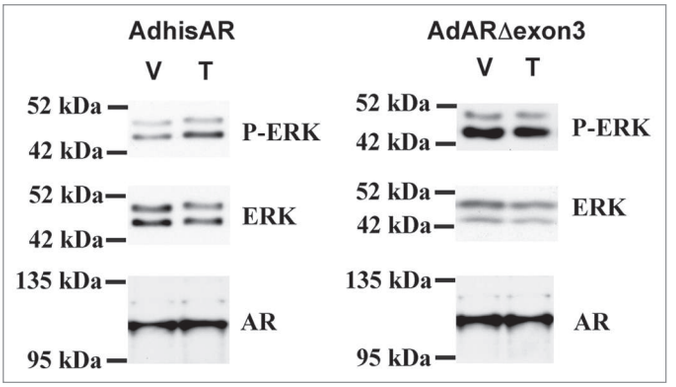

Figure 2. Deletion of exon 3 eliminates non-classical AR activity. 15P-1 Sertoli cells were infected with adenovirus constructs expressing wild type AR (AdhisAR) or exon 3-deleted AR (AdARΔexon 3). P-ERK, ERK and AR levels were determined by western blot after a 10 min stimulation in serum free media with vehicle (V) or 100 nM testosterone (T).

elongated spermatids that were ready to be released. Culturing the seminiferous tubule fragments in the presence of a Src kinase inhibitor resulted in the release of 45% fewer sperm. These results are consistent with earlier studies showing that during stages VII–VIII when sperm are released, activated Src levels increase in the vicinity of the specialized Sertoli-elongated spematid adhesion complex called the ectoplasmic specialization (ES).[43-45] Furthermore, Src is structurally associated with cell adhesion regulatory proteins at the ES.[46] Src also is known to phosphorylate focal adhesion kinase (FAK), β-catenin and N-cadherin proteins that contribute to the formation of the adhesion complexes between Sertoli cells and the mature elongated spermatids.[47-49]

It has been proposed that only the classical pathway is required for spermatogenesis because spermatogenesis is halted during meiosis in transgenic mice in which exon 3 of the AR containing a portion of the DNA binding domain was removed.[50] However, the non-classical activity of the exon 3-deleted AR mutation was not tested in the study. Recreation of the AR mutant lacking exon 3 and analysis of non-classical testosterone signaling in a Sertoli cell line lacking AR activity revealed that the mutant did not permit the phosphorylation of ERK in response to testosterone stimulation (**Fig. 2**). This result indicates that the removal of more than 50 amino acids of AR in this model may alter the structure of AR to eliminate both non-classical and classical activity.

Applying Lessons Learned to Future Studies

Work is underway to better characterize the spermatogenesis processes in vivo that are regulated by the classical and non-classical pathways. Transgenic mouse models are being created in which the endogenous AR gene is removed while simultaneously initiating the expression of previously characterized mutant AR genes that are capable of only activating one of the testosterone signaling pathways (Walker WH, unpublished data). Analysis of these mouse models will determine the extent to which spermatogenesis progression is allowed by each of the pathways independently. Furthermore, the genes that are regulated by each pathway will be identified and the effects of each pathway on the maintenance of the BTB, germ cell adhesion and the release of mature sperm will be determined.

It is likely that both pathways will be found to contribute important independent regulatory actions required to maintain spermatogenesis. Signals from the two pathways also may act in concert or synergy. Data obtained from studies of progesterone, glucocorticoid and estrogen receptor function suggest that the classical and non-classical pathways cross-talk and interact in their target cells. Specifically, stimulation of cells with the steroid hormones resulted in rapid phosphorylation of their cognate receptors, which permitted the receptor to recruit co-factors resulting in the increased stimulation of specific endogenous target genes.[51,52] Also, rapid signaling through AR has been shown to phosphorylate paxillin, which was found to contribute to testosterone-mediated transcription in prostate cells.[53] It is possible that the phosphorylation and activation of kinases by the non-classical pathway in Sertoli cells may contribute to gene expression regulation via the classical pathway. In addition, important gene targets may be regulated independently downstream of the kinases that are activated via the non-classical pathway as exemplified by the androgen-mediated activation of the CREB transcription factor that is required for spermatogenesis.[42,54]

If the non-classical pathway is found to be required to maintain spermatogenesis, then it is expected that new targets for the regulation of spermatogenesis will be identified. One potential target for contraceptive development could be the testosterone-induced interaction of AR and Src kinase that initiates the non-classical pathway. Peptides that have already been identified to block AR-Src interactions[55,56] and corresponding peptidomimetic molecules are being assessed for blocking the non-classical pathway and spermatogenesis (Walker WH, unpublished data). It is possible that the partnering of factors that block the non-classical pathway with a Sertoli cell-specific delivery system could provide a hormone independent, reversible male contraceptive.

Conclusion

Although testosterone has been known to be essential for male fertility for at least 70 years,[57,58] the molecular mechanisms by which testosterone acts to support spermatogenesis are only now being identified. The identification of testosterone-regulated genes and kinases in Sertoli cells has allowed for the discovery of the precise targets of testosterone action and a better understanding of the how testosterone regulates the process of spermatogenesis. As the molecular mechanisms of testosterone signaling continue to be revealed, we will accumulate the intellectual resources required to produce therapies for specific male infertility conditions and targets for contraceptive development.

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The Role of Testosterone in Spermatogenesis: Lessons From Proteome Profiling of Human Spermatozoa in Testosterone Deficiency

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Testosterone is essential to maintain qualitative spermatogenesis. Nonetheless, no studies have been yet performed in humans to analyze the testosterone-mediated expression of sperm proteins and their importance in reproduction. Thus, this study aimed to identify sperm protein alterations in male hypogonadism using proteomic profiling. We have performed a comparative proteomic analysis comparing sperm from fertile controls (a pool of 5 normogonadic normozoospermic fertile men) versus sperm from patients with secondary hypogonadism (a pool of 5 oligozoospermic hypogonadic patients due to isolated LH deficiency). Sperm protein composition was analyzed, after peptide labelling with Isobaric Tags, *via* liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) on an LTQ Velos-Orbitrap mass spectrometer. LC-MS/MS data were analyzed using Proteome Discoverer. Criteria used to accept protein identification included a false discovery rate (FDR) of 1% and at least 1 peptide match per protein. Up to 986 proteins were identified and, of those, 43 proteins were differentially expressed: 32 proteins were under-expressed and 11 were over-expressed in the pool of hypogonadic patients compared to the controls. Bioinformatic analyses were performed using UniProt Knowledgebase, and the Gene Ontology Consortium database based on PANTHER. Notably, 13 of these 43 differentially expressed proteins have been previously reported to be related to sperm function and spermatogenesis. Western blot analyses for A-Kinase Anchoring Protein 3 (AKAP3) and the Prolactin Inducible Protein (PIP) were used to confirm the proteomics data. In summary, a high-resolution mass spectrometry-based proteomic approach was used for the first time to describe alterations of the sperm proteome in secondary male hypogonadism. Some of the differential sperm proteins described in this study, which include Prosaposin, SMOC-1, SERPINA5, SPANXB1,

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GSG1, ELSPBP1, fibronectin, 5-oxoprolinase, AKAP3, AKAP4, HYDIN, ROPN1B, ß-Microseminoprotein and Protein S100-A8, could represent new targets for the design of infertility treatments due to androgen deficiency.

Keywords: sperm, hypogonadism, proteomics, LH deficiency, testosterone

INTRODUCTION

Testosterone (T) is the androgen in the testis that is required for initiating and maintaining spermatogenesis, and the production of mature sperm is intimately dependent on androgen action within the testis. Therefore, in the scenario of an absence of T, or its receptor, spermatogenesis does not proceed beyond the meiosis stage and results in male infertility (1). In men, Leydig cells are the responsible for producing T after being stimulated by luteinizing hormone (LH), a glycoprotein hormone secreted from the pituitary gland in response to the pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus (2).

Male hypogonadism is a clinical syndrome that results from the failure of the testis to produce physiological concentrations of T and/or a normal number of spermatozoa due to pathology at one or more levels of the hypothalamic–pituitary–testicular axis (3). For example, an impairment of the pituitary or hypothalamic function can lead to LH deficiency, which is a form of secondary hypogonadism. Secondary hypogonadism, also named hypogonadotropic hypogonadism, is characterized by low T concentrations in serum, reduced spermatogenesis, and inappropriately low concentrations of gonadotrophins (LH and follicular stimulating hormone (FSH)) (4). LH deficiency usually occurs in conjunction with FSH deficiency. However, isolated LH deficiency is a rare clinical condition, originating from a dysregulation in the hypothalamus-pituitary GnRH-LH-testosterone axis, which results in an isolated dysregulation of LH secretion, with a normal or low-normal FSH secretion (5). Both pituitary gonadotrophins LH and FSH, together with a high LH-stimulated intratesticular T concentration, are considered crucial for spermatogenesis and are required for quantitatively normal sperm production (6). FSH is particularly important for establishing a normal and functional Sertoli cell population, LH for promoting the production of T, whereas androgen action is needed for the fulfillment of germ cell development. The stimulation of spermatogenesis by androgen needs a direct action on androgen receptors (AR) in Sertoli cells. T is, in fact, essential for maintaining qualitative spermatogenesis (2) and it is required for at least four critical processes: maintenance of the blood-testis barrier (BTB) (7), meiosis (8), Sertoli-spermatid adhesion (9), and sperm release (10). Although T is essential to maintain good spermatogenesis, a study performed in genetically modified mice displaying a strong FSH stimulation, together with a minimal T production, showed near-normal spermatogenesis (11). Thus, this result reflects that a strong FSH signaling can maintain spermatogenesis also in presence of low levels of intratesticular T.

The molecular mechanisms of T action in spermatogenesis have not been completely revealed until recently. Mice lacking Sertoli cell androgen receptors (AR) show late meiotic germ cell arrest, suggesting Sertoli cells transduce the androgenic stimulus coordinating this essential step in spermatogenesis. Specifically, the loss of T was found to alter the expression and post-translational modifications of meiotic cells proteins involved in oxidative damage, DNA repair, RNA processing, apoptosis, and meiotic division (12).

With the advances obtained using high-throughput techniques, such as proteomics, we may now target the role of T in spermatogenesis. Proteomics represents a state-of-the-art, technology-driven science, which allows studying, in a high-throughput mode, proteins, protein modifications, and protein interactions. This powerful tool is currently widely used to elucidate complex biological processes, including fertility and infertility (13, 14). Therefore, proteomics might represent a novel platform for clinical research to investigate the *in vivo* effect of hormones on the protein expression of cells, tissues, and biological fluids. However, in humans, no studies have been yet performed to confirm the T-mediated expression of sperm proteins and their functional importance.

Therefore, to better understand the impact of T in the sperm proteome profile, the aim of this study is to evaluate human sperm protein composition by high-resolution mass spectrometry in patients with male hypogonadism displaying low intratesticular T as a condition reported in isolated LH deficiency.

MATERIALS AND METHODS

Experimental Rationale

The rationale for studying only men with secondary hypogonadism due to isolated LH deficiency was to select a condition of a severe reduction in both blood and intratesticular T levels, without other confounding risk factors. Primary hypogonadal patients were excluded because of their high LH levels, which can represent a confounding factor, since it may maintain minimal intratesticular T levels, although blood T levels are reduced. Furthermore, patients with primary hypogonadism often show normal or increased serum estrogen levels, which might represent a confounding factor. On the other side, patients with secondary hypogonadism and reduced values of both FSH and LH values have generally azoospermia since low FSH is associated with an impairment in spermatogenesis. We, therefore, selected a rare clinical model (secondary hypogonadism due to isolated LH deficiency), in which we may observe LH and testosterone deficiency associated with a normal or low-normal FSH secretion, and presence of spermatogenesis.

Subjects

Six male hypogonadic patients aged between 25- and 55-year-old with secondary hypogonadism due to isolated LH deficiency were consecutively enrolled for this study. The diagnosis of secondary

hypogonadism due to LH deficiency was done in presence of symptoms of male hypogonadism (e.g.: loss of body hair, reduced sexual desire and activity, decreased spontaneous erections, erectile dysfunction, and gynecomastia) and low levels of blood T and LH (3), which were further confirmed by the GnRH test (15). A pituitary magnetic resonance imaging (MRI) was performed in all patients to confirm the diagnosis of pituitary disease. Inclusion criteria were as follows (1): total T < 2.5 ng/ml (2), LH < 1.0 mUI/ml, and (3) clinical symptoms of hypogonadism. Exclusion criteria included (1): age <18 yrs and >55 yrs (2), primary hypogonadism or associated testicular diseases (3), residual adenoma (4), smoking (5), diabetes mellitus (6), previous androgen replacement therapy (7), varicocele, and/or (8) genital tract infections.

In addition, five normogonadic normozoospermic fertile men, whose partners were pregnant when the study started, were enrolled as a control group. None had a history of fertility problems. All female partners conceived within 3 months before the start of the study.

Hormonal Study

A blood sample was collected at 08.00 hours in the andrological clinic of Fondazione Policlinico "A. Gemelli" IRCCS in Rome (Italy), for the determination of T, estradiol (E2), sex hormone–binding globulin (SHBG), LH, and FSH. T and E2 were assayed in duplicate by radioimmunoassay (RIA) with the use of commercial kits by Radim (Pomezia, Italy). LH, FSH, and SHBG were assayed by immunoradiometric methods on a solid-phase (coated tube), which is based on a monoclonal double-antibody technique. The reference values of the studied hormones are reported in **Table 1**. Results have been reported as average ± standard deviation. Statistical analysis has been carried out with SPSS v18.0 (IBM Corp., Armonk, NY, USA). All data have been first analyzed for normality of distribution using the Kolmogorov–Smirnov test of Normality. The appropriate parametric test (t-test) was used to assess the significance of the differences between groups. P-value < 0.05 was considered significant.

The diagnosis of male secondary hypogonadism was moreover confirmed by the GnRH test (100 μg i.v., LHRH, Ferring Pharmaceuticals, Saint-Prex, Switzerland) for FSH and LH hormones. Additionally, the hormonal diagnosis of secondary hypogonadism was further corroborated by pituitary MRI performed in all patients.

Semen Sample Collection and Analysis

Human semen samples were obtained from 6 male patients diagnosed with secondary hypogonadism due to LH deficiency and 5 fertile controls at the andrological clinic of Fondazione Policlinico "A. Gemelli" in Rome (Italy). All subjects gave informed consent according to the guidelines of the Declaration of Helsinki. Complete semen analysis for all individuals was performed according to World Health Organization (2010) classification (16). Only seminal samples from secondary hypogonadism individuals with sperm presence were used for the current study.

Sperm Preparation and Purification

The ejaculates were washed with PBS and the sperm cells were selected after 50% Percoll™ gradient (GE Healthcare, Uppsala, Sweden), as previously described (17, 18). Briefly, the sperm samples were centrifuged through a 50% Percoll gradient at 400 g for 30 min at room temperature (RT) without brake. The recovered sperm cells were then resuspended in phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA) and subjected to a residual leukocyte depletion using Dynabeads™ CD45 (Invitrogen, Carlsbad, CA, USA). Basically, 1 ml aliquots of samples were incubated with 50 μl washed dynabeads for 1 hour at RT, with constant shaking. Samples were washed twice by applying magnetic force for 2 min, and the efficiency of the procedure was checked using phase-contrast microscopy (Olympus BX50, Olympus, Tokyo, Japan).

Quality Control to Assess Sperm Contamination

The absence of leukocytes was further confirmed both by microscopic observation after Diff-Quick staining and by performing a real-time PCR for the leukocyte-specific marker Protein Tyrosine Phosphatase Receptor Type C (PTPRC). For Diff-Quick staining, sperm smears were prepared by placing 5 μl of the sample onto a slide and pulling it out into a smear using a second slide followed by air drying for 20–30 s. The staining kit used was Diff-Quick (Medion Diagnostics AG, Düdingen, Switzerland), and smears were stained according to the manufacturer's instructions.

For sperm RNA analysis, sperm RNA was individually extracted and purified following the RNeasy Mini Kit protocol (Qiagen, Hilden, Germany) with some modifications previously established (19). The possible somatic contamination from leukocytes was further assessed by reverse transcription reaction using SuperScript III RT (Invitrogen, Carlsbad, CA, USA) and oligodT primers (Invitrogen, Carlsbad, CA, USA), and a subsequent real-

TABLE 1 | Clinical, hormonal and seminal parameters in secondary hypogonadism patients and normogonadic controls.

	Hypogonadic patients (n=5)	Fertile controls (n=5)	Range values/Lower reference limit
Testosterone (T)	1.93 ± 0.31*	5.2 ± 0.8	2.5-8.4 ng/ml
Estradiol (E2)	26.92 ± 5.16	26.8 ± 9.3	15-44 pg/ml
FSH	2.14 ± 0.70	2.5 ± 1.2	1.0-8.0 mU/ml
LH	0.81 ± 0.12*	2.5 ± 0.9	2.5-10.0 mU/ml
Seminal volume	2.20 ± 1.15 ml*	4.10 ± 1.85 ml	1.5 ml
Total sperm count	21.00 ± 10.19 x 10^6*	98.60 ± 49.94 x 10^6	39 x 10^6
Total sperm motility	59.00 ± 12.45%	63.0 ± 19.87%	40%
Normal morphology	3.10 ± 2.4%*	21.2± 7.91%	4%

P-value < 0.05 is indicated in the table (*).

time PCR using PowerUp™ SYBR™ Green PCR (Applied Biosystems, Foster City, CA, USA) targeting the leukocyte-specific marker Protein Tyrosine Phosphatase Receptor Type C (PTPRC). The absence of amplification of the leukocyte-specific marker *PTPRC* PCR product at cycle 40 in sperm RNA isolated from the individual samples included in the proteomics study confirmed the absence of leukocyte RNA contamination (**Supplementary Table 1**). Likewise, to assess sample RNA integrity, protamine 1 (*PRM1*) was targeted as a positive control of a sperm-specific intact RNA (**Supplementary Table 1**).

In conclusion, only sperm samples with no visible contamination with other cells and negative for *P*TPRC at the mRNA level were used for further proteomic analysis. Consequently, one sample from an individual with secondary hypogonadism was discarded due to leukocyte contamination at both microscopic observation and specific RNA-leukocyte analysis.

Protein Solubilization

Protein solubilization was independently performed on each sperm sample. Briefly, after sperm purification, the sperm pellet was solubilized in a 2% SDS lysis buffer. Lysates were centrifuged at 16,000 g for 10 min, and the proteins present in the supernatant were quantified using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA).

Differential Proteomics

A comparative sperm proteomic analysis was performed comparing two pools: a fertile control pool (n=5) versus a secondary hypogonadic patients pool (n=5). Due to the low sperm count and limited material in the patients diagnosed with secondary hypogonadism, a sample pooling strategy was considered for the current study, despite the potential limitations of this approach.

To prepare the two pools, 25 µg of each sample were used (125 µg of total protein for each pool). The TMTduplex™ Isobaric Mass Tagging Kit (TMT 2-plex; Thermo Fisher Scientific, Rockford, IL, USA) was used for the peptide labeling, following the manufacturer's instructions with minor modifications (13).

Peptide Labeling With Isobaric Tags (TMT 2-Plex)

For the preparation of the TMT labeling, 80 µg of proteins from each pool were suspended in 100 mM TEAB (triethyl ammonium bicarbonate, pH 8.5) and the same concentration (0.85 µg/µl) and volume were acquired for both pools. Proteins were reduced in 9.5 mM TCEP (tris (2-carboxyethyl) phosphine) for one hour at 55 °C. Then, proteins were alkylated with 17 mM iodoacetamide for 30 min at RT avoiding light exposure. Proteins were precipitated overnight at -20°C with 80% (v/v) cold acetone. Afterward, samples were centrifuged at 17,500 g for 10 min at 4°C, and the acetone-precipitated protein pellets were resuspended in 80 µl of 100 mM TEAB. For protein digestion, trypsin was added to the protein pellets at a 1:20 protease-to-protein ratio and incubated overnight at 37°C with constant shaking. Regarding peptide labeling, 30 µg of peptides from each pool were labeled with TMT isobaric tags (the control group was labeled with TMT-126, and the secondary hypogonadic patients with TMT-127). Specifically, TMT label reagents (0.8 mg each) were equilibrated at RT, dissolved in 41 µl of anhydrous acetonitrile (ACN; Sigma-Aldrich, St. Louis, MO, USA), and added to the reduced and alkylated peptides of each pool. After 1 hour of incubation at RT, the reaction was quenched with 8 µl of 0.5% hydroxylamine for 15 min at RT under shaking. Then, the TMT-labeled pools were combined at equal amounts in a single tube. After, labeled peptides were dried in a vacuum centrifuge and re-suspended in 20 µl of 0.5% trifluoroacetic acid (TFA; Sigma-Aldrich, St. Louis, MO, USA) in 5% ACN before processing with Pierce C18 Spin Columns (Thermo Scientific, Rockford, IL, USA) following manufacturer's instructions.

LC-MS/MS

The dried peptides pellet was reconstituted in 0.1% formic acid and injected for analysis by reverse-phase chromatography-MS/MS (Tandem mass spectrometry). Specifically, tryptic peptides were separated by using a reversed-phase nano LC-MS/MS setup comprised of nano-LC Ultra 2D Eksigent (AB Sciex, Brugg, Switzerland) attached to an LTQ-Orbitrap Velos mass spectrometer (Thermo scientific, San Jose, CA, USA). Peptides were loaded onto a C18 trap column (5 µm, 120 Å, 100 µm i.d. x 2 cm in length, Nanoseparations). For analytical separation, a gradient was applied on line with an analytical column (3 µm, 100 Å, 75 µm i.d. x 15 cm in length, Thermo Scientific, San Jose, CA, USA), and the following buffer system was used: buffer A (97% H2O-3% ACN, 0.1% Formic acid) and buffer B (3% H2O-97% ACN, 0.1% Formic acid). For the peptide mixtures the following gradient was applied: 0–5 min 0% to 5% B, 5–285 min 5% to 40% B, 285-290 min 40% to 100% B, 290-300 min 100% to 100% B at a flow rate of 400 nl/min. MS/MS analysis was performed using an LTQ-Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA, USA) with a nanoelectrospray ion source with precursor ion selection in the Orbitrap at 30,000 of resolution, in a range of 400-1700 m/z, selecting the 15 most intense precursor ions in positive ion mode. MS/MS data acquisition was completed using Xcalibur 2.1 (Thermo Fisher Scientific, Waltham, MA, USA). Higher energy collisional dissociation (HCD) for MS2 was set to 40%.

Database Searching and Data Interpretation

Data were processed using Proteome Discoverer 1.4.1.14 (Thermo Fisher Scientific, Waltham, MA, USA). For database searching, processed data were submitted to the Homo sapiens UniProtKB/Swiss-Prot database with Sus scrufa Trypsin (HUMAN_PIG_UniProt_R_2017_01.fasta; released January 2017; 21,484 protein entries) added to it using SEQUEST, version 28.0 (Thermo Fisher Scientific, Waltham, MA, USA). The percolator search node was used for re-scoring. The following search parameters were used: five maximum missed cleavages for trypsin, TMT-labeled lysine in N-terminal (+225.156 Da) and methionine oxidation (+15.995 Da) as dynamic modifications, cysteine carbamidomethylation (+57.021 Da) as a static modification, 20 ppm precursor mass tolerance, 0.1 Da fragment mass tolerance, and 100 mmu peak integration tolerance was applied, and most confident centroid peak integration method was used.

Criteria used to accept protein identification included a false discovery rate (FDR) of 1% and at least 1 peptide match per protein. In addition, proteins have been treated as "ungrouped" to avoid any possible ambiguity among the different isoforms of the same protein. The relative quantification of proteins was achieved by dividing the intensity of reporter ions of HCD MS2 spectra for secondary hypogonadism patients pool (TMT-127) with the controls pool (TMT-126), which were obtained using Proteome Discoverer software. Purity correction factors provided by the manufacturer were applied to the isotopic purities of the reporter ions.

For protein quantification purposes, only unique peptides were used, and protein ratios (TMT-127/TMT-126) were normalized to the protein median. The normalized protein ratios were log2 transformed and the 95% confidence interval was calculated (mean ± 1.96 SD), and the protein ratios outside the range were defined as significantly different (p-value ≤ 0.05). Log2 values were reverted to normal values, and the cut-off for up-regulated proteins was ≥1.264, and for down-regulated proteins was ≤ 0.768.

Proteins differentially expressed in hypogonadic patients and controls were classified according to their main cellular function(s) using the information available at the UniProtKB/Swiss-Prot database (http://www.uniprot.org). The sperm proteomic datasets were uploaded to the Gene Ontology Consortium database (http://www.geneontology.org/) (20), based on PANTHER v17 database (Release date 2021-10-01), to predict the functional involvement of the deregulated sperm proteins in male hypogonadism. The significance of enrichment analyses was calculated by a Fisher's exact test. P-values < 0.05 after FDR adjustment were considered statistically significant.

Western Blot

In order to validate the proteomics results, western blot analyses were performed in protein extracts of 4 hypogonadic sperm samples used for the proteomics study, and 4 controls. For western blot analysis, sperm cells were diluted in 2% SDS lysis buffer. The protein concentrations were measured using a BCA protein assay. Samples were resuspended in SDS Laemmli sample buffer and boiled for 5 min at 95°C. SDS–PAGE was transferred onto PVDF membranes (Millipore, Burlington, MA, USA). Membranes were developed using enhanced chemiluminescence (ECL Amersham - GE Healthcare, Little Chalfont, UK). For immunostaining, the anti-AKAP3 rabbit monoclonal antibody (Dilution 1:1000; Abcam, Cambridge, UK) and the anti-PIP rabbit monoclonal antibody (Dilution 1:1500; Novus Biologicals, Minneapolis, MN, USA) were used. The constitutively expressed tubulin protein [mouse anti-Tubulin monoclonal antibody (Dilution 1:5000; Sigma-Aldrich, Saint Louis, Missouri, US)] was used as loading controls for quantitative western blotting.

RESULTS

Clinical and Seminal Parameters

The results for clinical, hormonal, and seminal data are reported in **Table 1**. Total T, LH, sperm count and sperm morphology resulted significantly reduced in the hypogonadic patients, compared to the control group (p-value < 0.05). Thus, all patients with hypogonadism enrolled in this study were oligozoospermic. The analysis of seminal parameters in those patients confirmed that spermatogenesis was conserved because of FSH levels, since sperm could be observed in the ejaculate.

GnRH test confirmed the diagnosis of LH deficiency in the individuals with hypogonadism. Pituitary MRI demonstrated the presence of partial empty sella in all patients. Empty sella syndrome is a condition in which the pituitary gland shrinks or gets flattened. Partial empty sella is suggestive that, to some extent, the pituitary gland is still visible on the MRI scan (21), as observed in our hypogonadic patients.

Therefore, the comparative proteomic study included 5 oligozoospermic hypogonadic patients and 5 normogonadic normozoospermic fertile controls. All subjects had a sperm count >10 x 10^6 sperm/ml and leukocyte contamination was excluded at both microscopic and RNA levels.

Differentially Abundance of Sperm Proteins in Hypogonadic Patients

LC-MS/MS of the 2 pools of sperm samples, comprising both the hypogonadic patients and the fertile control individuals, resulted in the identification of a total of 986 proteins. The comparison of the sperm proteomic profiles from hypogonadic patients and controls resulted in the detection of 43 differentially abundant proteins. Of those, 32 proteins were observed to be under-regulated, and 11 up-regulated, in the pool of hypogonadic patients, compared to controls (**Table 2**).

The categorization of proteins with altered abundance in hypogonadic patients according to the information available at the Uniprot Knowledgebase revealed that 13 proteins were related to reproduction (18% of the total) (**Figure 1**). Worth mentioning, some of these proteins had more than one main cellular function, and the 43 proteins were involved in 71 cellular functions. Specifically, within the reproduction-related group, 8 proteins were associated with fertilization or sperm-oocyte interaction (42%; SERPINA5, AKAP4, PRSS37, AKAP3, ROPN1B, SMCP, ELSPBP1, and GSG1), 7 with sperm motility and capacitation (37%; SEMG2, SEMG1, HYDIN, AKAP4, ROPN1B, SMCP, and ELSPBP1) and 4 with spermatogenesis (21%; SERPINA5, ROPN1B, SPATA19, SPANXB1) (**Figure 1**). Proteolysis and protein metabolism (15%), signaling (13%), immune system (11%), and metabolic process (8%) were the other prevailing cellular functions enriched for the sperm proteins with differential abundance in hypogonadism.

To unravel whether the deregulated sperm proteins in male hypogonadism were involved in explicit biological processes, we have performed GO term enrichment analysis using the Gene Ontology Consortium database based on PANTHER. The outcomes obtained confirmed that the sperm deregulated proteins in male hypogonadism were involved in fertilization, sperm capacitation, reproductive process and regulation of proteolysis (P-value and FDR adjusted P-value < 0.05). Concerning the GO Cellular Component, the deregulated sperm proteins were localized in the sperm fibrous sheath, acrosomal vesicle, extracellular exosome, extracellular matrix, and extracellular space (P-value and FDR adjusted P-value < 0.05).

TABLE 2 | List of up-regulated and down-regulated sperm proteins in male hypogonadism (n=32 proteins down-regulated; n=11 proteins up-regulated).

Accession	Gene name	Description	Peptide count	Unique Peptides	Ratio Hypo/Ctl
Down-regulated proteins in male hypogonadism sperm samples (n=32)					
Q9Y272	RASD1	Dexamethasone-induced Ras-related protein 1	1	1	0.425
P12273	PIP	Prolactin-inducible protein	3	3	0.514
P05164	MPO	Myeloperoxidase	1	1	0.532
P02768	ALB	Serum albumin	15	11	0.579
P05109	S100A8	Protein S100-A8	1	1	0.597
Q08380	LGALS3BP	Galectin-3-binding protein	1	1	0.604
Q9UBC9	SPRR3	Small proline-rich protein 3	1	1	0.632
P05154	SERPINA5	Plasma serine protease inhibitor	1	1	0.633
O14607	UTY	Histone demethylase UTY	1	1	0.640
Q02383	SEMG2	Semenogelin-2	22	18	0.644
P08118	MSMB	Beta-microseminoprotein	1	1	0.647
P07288	KLK3	Prostate-specific antigen	1	1	0.647
P25311	AZGP1	Zinc-alpha-2-glycoprotein	2	2	0.656
Q15843	NEDD8	NEDD8	1	1	0.663
P04279	SEMG1	Semenogelin-1	18	14	0.664
Q8TCT9	HM13	Minor histocompatibility antigen H13	1	1	0.666
Q99963	SH3GL3	Endophilin-A3	1	1	0.688
O15127	SCAMP2	Secretory carrier-associated membrane protein 2	1	1	0.702
Q6W4X9	MUC6	Mucin-6	1	1	0.705
Q9Y5C1	ANGPTL3	Angiopoietin-related protein 3	1	1	0.705
Q4G0P3	HYDIN	Hydrocephalus-inducing protein homolog	1	1	0.709
Q5JQC9	AKAP4	A-kinase anchor protein 4	34	33	0.710
P07602	PSAP	Prosaposin	2	2	0.711
A4D1T9	PRSS37	Probable inactive serine protease 37	2	2	0.724
Q4W5G0	TIGD2	Tigger transposable element-derived protein 2	1	1	0.733
Q9UIA9	XPO7	Exportin-7	2	2	0.733
O75969	AKAP3	A-kinase anchor protein 3	25	25	0.742
Q9H4F8	SMOC1	SPARC-related modular calcium-binding protein 1	1	1	0.745
Q0VFZ6	CCDC173	Coiled-coil domain-containing protein 173	1	1	0.750
Q9BTW9	TBCD	Tubulin-specific chaperone D	1	1	0.750
Q9NZL4	HSPBP1	Hsp70-binding protein 1	1	1	0.759
Q9BZX4	ROPN1B	Ropporin-1B	6	1	0.768
Up-regulated proteins in male hypogonadism sperm samples (n=11)					
P02751	FN1	Fibronectin	13	13	1.272
Q7Z5L4	SPATA19	Spermatogenesis-associated protein 19, mitochondrial	1	1	1.280
Q12765	SCRN1	Secernin-1	1	1	1.283
O14841	OPLAH	5-oxoprolinase	1	1	1.289
P62750	RPL23A	60S ribosomal protein L23a	1	1	1.296
Q9NS25	SPANXB1	Sperm protein associated with the nucleus on the X chromosome B1	4	3	1.386
P49901	SMCP	Sperm mitochondrial-associated cysteine-rich protein	2	2	1.387
Q96BH3	ELSPBP1	Epididymal sperm-binding protein 1	2	2	1.468
Q2KHT4	GSG1	Germ cell-specific gene 1 protein	1	1	1.656
Q17RY6	LY6K	Lymphocyte antigen 6K	1	1	1.672
Q9H3G5	CPVL	Probable serine carboxypeptidase CPVL	1	1	1.987

The criteria used to accept protein identification included at least 1 unique peptide and an FDR of 1%. Results are expressed as the protein ratio of sperm proteins from secondary hypogonadism patients (Hypo) to controls (Ctl).

Western Blot Validation

To validate the proteomic results, a Western blot analysis was performed for two of the proteins with differential abundance: A-Kinase Anchoring Protein 3 (AKAP3) and Prolactin Inducible Protein (PIP). Sperm protein extracts from 4 out of the 5 hypogonadic patients used for the proteomic analysis, and 4 out of the 5 fertile controls, were used for the Western blot analysis (two samples were completely used for proteomic analysis). As expected, significantly decreased levels ($p<0.05$) of PIP and AKAP3 were found in the secondary hypogonadic patients (**Figure 2A**), confirming the proteomics data. Indeed, the ratios for the 2 proteins among hypogonadics and controls, obtained from Western blot data (ratio 0.41 and 0.77, respectively), were very similar to the ones observed by proteomics data (ratio 0.51 and 0.74, respectively) (**Figures 2B, C**; **Table 2**).

DISCUSSION

The maintenance of spermatogenesis in humans requires an adequate secretion of LH, resulting in the maintenance of high intra-testicular testosterone (ITT) (2). In men and rats, the intratesticular T concentration is far higher than its concentration in the peripheral circulation (22). It is known that intratesticular T can be reduced substantially without an effect on spermatogenesis (5). However, there is a minimally required T concentration below

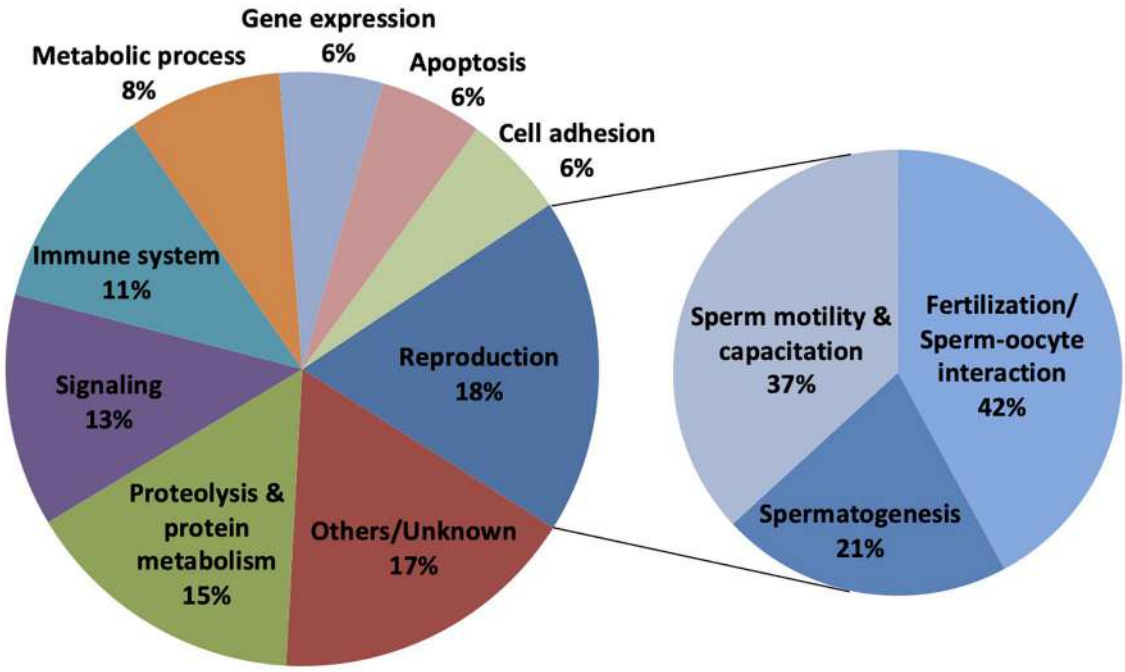

FIGURE 1 | Classification of the differentially expressed sperm proteins in male hypogonadism (n=43) according to their Cellular function. Protein classification was made according to UniProtKB/Swiss-Prot database (http://www.uniprot.org).

which spermatogenesis is affected, leading to male infertility (23). T levels are approximately 40-fold higher in the testes than in the serum in healthy men with normal reproductive physiology. Noteworthy, it has been reported that T concentration in the testes in the absence of LH was still 4–5 times higher than serum T concentrations, since intratesticular T concentrations may still support spermatogenesis in men even if serum T is reduced (24).

In order to study if the reduction of serum T, although mild, might modify the qualitative protein composition of spermatozoa, we selected five patients affected by hypotestosteronemia due to isolated LH deficiency.

In previous studies, we reported the effect of male secondary post-surgical hypogonadism on modulating accessory gland function and protein secretion (25). In fact, in 2014 we published the first experimental proteomic study, using high-resolution mass spectrometry, aimed at studying the seminal proteome of patients affected by secondary hypogonadism, before and after 6 months of testosterone replacement treatment (26). More recently we reported a quantitative high-resolution proteomic research in seminal plasma samples of hypogonadotropic hypogonadism patients, before and after only 3 months of testosterone therapy (27), demonstrating the effect of male hypogonadism on modulating a panel of eleven seminal proteins.

In order to design adequate therapies for male infertility, it is essential to broaden our knowledge of the mechanisms underlying the role of testosterone in the initiation and maintenance of the poorly understood process of spermatogenesis, and in the pathogenesis of its disturbances.

Here, for the first time, we have quantified the changes occurring in the sperm proteome in patients with secondary hypogonadism, identifying 43 sperm proteins differentially expressed due to reduced serum T concentration. Thirty-two of these proteins were less abundant while 11 were more abundant in the hypogonadic patients.

Interestingly, many of these proteins have been previously reported to be involved in spermatogenesis, spermiation, sperm motility and capacitation, sperm-oocyte interaction, and/or fertilization. The reduction in hypogonadic patients of Prosaposin (PSAP), Ropporin-1B (ROPN1B), Plasma serine protease inhibitor (SERPINA5) and SPARC-related modular calcium-binding protein 1 (SMOC-1) might, in fact, reflect a dysregulation of the molecular machinery involved in spermiogenesis (28, 29), Sertoli cell junctions and blood-testis barrier (30, 31), spermiation (32), and epididymal shedding of cytoplasmic droplets (32–34). The increased abundance in hypogonadic patients of Sperm protein associated with the

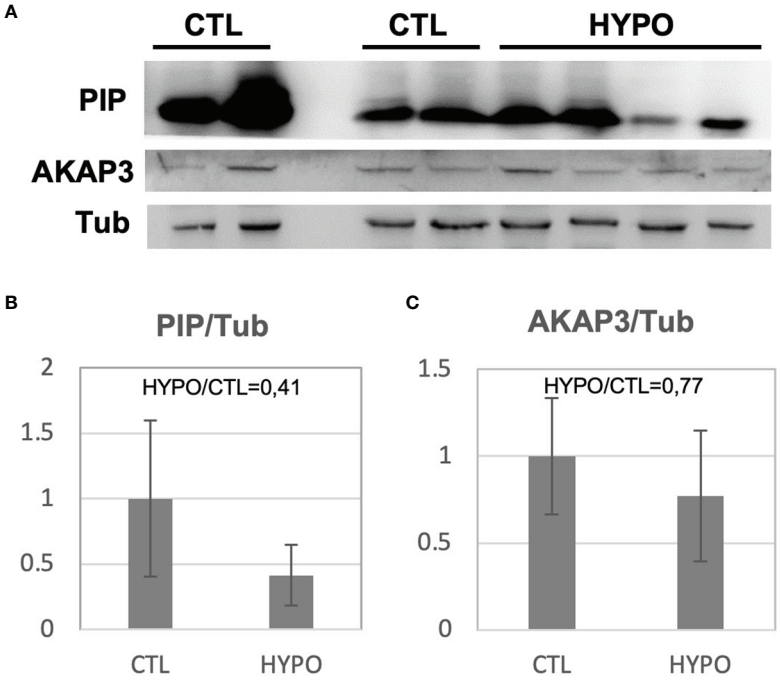

FIGURE 2 | Western Blot analysis for the PIP and AKAP3 proteins obtained from sperm samples of patients with secondary hypogonadism (HYPO) and controls (CTL) **(A)**. The histogram shows the ratio of densitometric values of PIP **(B)** and AKAP3 **(C)** to Tubulin (Tub), a loading control. The ratio from control (CTL) was arbitrarily set to 1. Mean ± SD of patients is shown.

nucleus on the X chromosome B1 (SPANXB1) and Germ cell-specific gene 1 protein (GSG1) proteins, which have been previously reported as markers of defective spermatogenesis (35–37), and of Epididymal sperm-binding protein 1 (ELSPBP1) and fibronectin (FN1), which are considered markers of sperm damage and low sperm quality (38, 39), underlines the physiological role of testosterone in the qualitative control of sperm production.

The increase of 5-oxoprolinase (OPLAH) in hypogonadism might reflect a deficiency in the biosynthesis of glutathione, which is an important scavenger for reactive oxygen species in man (40). In addition, it is known that male hypogonadism is associated with oxidative stress, both at systemic (41) and at sperm level (42). The reported increase in OPLAH levels might be an indirect marker of oxidative stress in sperm of hypogonadic patients. We, therefore, further support the previously reported evidence in mice about the role of T in modulating the expression of testicular proteins involved in oxidative damage (11).

Moreover, we observed that male hypogonadism may impact sperm motility through the down-regulation of some specific proteins such as A-kinase anchor protein 3 (AKAP3), A-kinase anchor protein 4 (AKAP4), Hydrocephalus-inducing protein homolog (HYDIN) and ROPN1B, which are constitutive of the microtubule (43, 44) or necessary for the movement of the sperm tail (45–48).

Furthermore, ß-Microseminoprotein (MSMB) and Protein S100-A8 (S100A8) were reduced in sperm samples of hypogonadic patients. These proteins, although not annotated in UniProt Knowledgebase as involved in reproduction, have been previously reported in the literature as involved in preventing spontaneous acrosome reaction (49, 50).

Importantly, we reported in the panel of down-regulated proteins 9 seminal plasma proteins (Semenogelin-1 (SEMG1), Semenogelin-2 (SEMG2), myeloperoxidase (MPO), MSMB, SERPINA5, Prolactin-inducible protein (PIP), Prostate-specific antigen (KLK3), PSAP and Zinc-alpha-2-glycoprotein (AZGP1)). These proteins might derive from a residual quote of seminal plasma, might be attached to the cell membrane, or might be imported into the sperm cells through exosomes (51). However, it is interesting that we have previously reported in previous studies 8 out of 9 of these proteins (SEMG1, SEMG2, MPO, MSMB, PIP, KLK3, PSAP and AZGP1) to be downregulated in seminal plasma of patients with secondary male hypogonadism (26, 27). We, therefore, confirmed our previous data about the modulation of these seminal proteins in male secondary hypogonadism.

Overall, our data revealed the physiological role of intratesticular testosterone in modulating a molecular machinery directly involved in spermatogenesis and spermiogenesis, spermiation, sperm quality control, sperm motility and oxidative damage.

This *in vivo* model of intratesticular T deficit in humans allowed us to revisit the role of testosterone in spermatogenesis and in sperm function. Our findings suggest that Leydig cell dysfunction represents a mechanism responsible for the

infertility of these patients. As a consequence, fertility can be effectively restored in these patients by hCG treatment. Thus, the identified proteins in this study might represent a target of responsiveness for hCG stimulation on sperm quality and fertility outcomes.

Furthermore, to date there is no approved serum biomarker for low intratesticular T. Intratesticular T can only be measured *via* invasive testicular biopsy or aspiration. We also speculate that the identification of these deregulated sperm proteins related to low intratesticular T may represent, if validated by further studies, putative non-invasive indirect biomarkers of intratesticular T.

CONCLUSIONS

Despite the limitations of this study, performed on a small sample scale and pooled samples, this is the first application of high-resolution MS-based proteomics aimed to reveal an array of sperm proteins reflecting an impairment of spermatogenesis in testosterone deficiency. We performed our study in a clinical model – male hypogonadism due to isolated LH deficiency, in the presence of spermatogenesis – which is very rare. Thus, it is very difficult to enroll these patients. However, this rare clinical model permitted us to study spermatogenesis in presence of reduced T levels.

Further studies will be required to compare hypogonadic oligozoospermic samples with idiopathic oligozoospermic samples, in order to confirm the role of testosterone in the differentially expressed proteins.

The identification of a panel of proteins involved in androgen deficiency provides us a lesson on how androgens act under normal circumstances in the process of spermatogenesis and in the control of sperm function. Therefore, the identified proteins might represent new clinical biomarkers in androgen deficiency conditions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: ProteomeXchange Consortium *via* the PRIDE partner repository (52); PXD032270.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the International Scientific Institute "Paul VI", Rome - Italy. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GG, DM, FB, and RO contributed to conception and design of the study. GG, SC, and DM collected clinical data. GG, FB, AS-V, MJ, and FM performed formal analysis. GG, FB, and MJ performed data analysis. GG, FB, and DM wrote the first draft of the manuscript. RO and AP performed the supervision of the study. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.852661/full#supplementary-material

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Biological therapy for non-obstructive azoospermia

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REVIEW

Biological therapy for non-obstructive azoospermia

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ABSTRACT

Introduction: Most male patients with non-obstructive azoospermia (NOA) have no therapeutic options outside of assisted reproductive techniques to conceive a biological child. If mature sperm cannot be obtained from the testes, these patients must rely on options of donor sperm or adoption. Several techniques are in the experimental stage to provide this patient population alternatives for conceiving.
Areas covered: This review discusses three of the experimental techniques for restoring fertility in men with NOA: spermatogonial stem cell transplantation, the use of adult and embryonic stem cells to develop mature gametes and gene therapy. After this discussion, the authors give their expert opinion and provide the reader with their perspectives for the future.
Expert opinion: Several limitations, both technical and ethical, exist for spermatogonial stem cell transplantation, the use of stem cells and gene therapy. Well-defined reproducible protocols are necessary. Furthermore, several technical barriers exist for all protocols. And while success has been achieved in animal models, future research is still required in human models.

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Gene therapy; non-obstructive azoospermia; spermatogonial stem cell transplantation; pluripotent stem cell

1. Introduction

Infertility affects approximately 15% of couples of reproductive age. Male factor infertility plays a role in 50% of these cases. Among patients with male factor infertility, approximately 10–15% have azoospermia [1]. Azoospermia is defined as the absence of spermatozoa in the ejaculate. This condition is typically classified as obstructive azoospermia (OA) or non-obstructive azoospermia (NOA). After confirmation of NOA with a second semen analysis, the workup of azoospermia involves a detailed history and physical examination along with hormonal and genetic testing. Testicular biopsy allows for a histologic diagnosis although the underlying cause of the azoospermia may never be determined. OA is characterized by an obstructed flow of spermatozoa at any point along the male genital tract. Spermatogenesis in the testis is usually normal in these patients. The etiology of NOA is more varied. Normal spermatogenesis requires intact and functioning seminiferous tubules as well as functioning Leydig cells for hormonal support of spermatogenesis by producing testosterone. NOA can be broadly classified into primary testicular failure and secondary testicular failure. Primary testicular failure refers to pathology localized to the testis resulting in elevated Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). Known genetic causes of infertility such as Klinefelter's syndrome and Y chromosome microdeletion will cause primary testis failure. Local effects from chemotherapy or radiation can also cause primary testis failure and result in NOA. A significant proportion of primary testicular failure cases are diagnosed as idiopathic, which limits therapeutic options.

Secondary testis failure is caused by impaired pituitary secretion of gonadotropins, which leads to insufficient stimulation of the Sertoli and Leydig cells in the testis. Kallmann syndrome results from a lack of hypothalamic Gonadotropic Releasing Hormone (GnRH) secretion, which leads to insufficient production of LH and FSH. This subsequently leads to secondary testicular failure as the Sertoli cells are unable to support spermatogenesis and the Leydig cells are insufficiently stimulated to provide local testosterone secretion for spermatogenesis. Men who take supplemental testosterone will also develop secondary testicular failure because the supplemental circulating testosterone suppresses FSH and LH production.

Men with NOA have limited options for reproduction. Testicular sperm extraction with Intracytoplasmic Sperm Injection (ICSI) is possible in these patients, but it is expensive and associated with high morbidity in the female partner. If sperm cannot be retrieved by testicular sperm extraction, there are no current options to maintain the reproductive potential of these individuals. Therefore, there is significant clinical demand for alternate therapies for NOA that would allow the production of mature spermatozoa either using stem cells or by gene therapy for patients with idiopathic causes of infertility. Such a technique might allow for natural conception in a population that is otherwise relegated to assisted reproductive techniques. This review will cover three experimental approaches for restoring fertility in men with NOA: spermatogonial stem cell (SSC) transplantation, *in vitro* spermatogenesis using adult or embryonic pluripotent stem cells, and gene therapy.

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Article highlights

- Existing therapeutics for non-obstructive azoospermia (NOA) are limited and depend on the ability to harvest mature sperm from the testis for ICSI
- Spermatogonial stem cell transplantation is a promising option to repopulate the germinal epithelium in men with NOA
- The use of embryonic and adult stem cells to develop mature male gametes may provide patients with an alternative therapeutic option.
- At present, a complete understanding of the stem cell niche is still lacking
- For cases of NOA caused by a to a mutation at a single gene locus, gene therapy may be a definitive treatment for infertility.

This box summarizes key points contained in the article.

2. SSC transplantation

SSCs are precursors to mature spermatids that reside in the basal compartment of the seminiferous tubules of the testis. SSCs have the capability to self-renew and enter into meiosis to become a spermatocyte [2]. The spermatocytes then differentiate into haploid spermatids, which are transformed into mature spermatozoa. The SSC population is therefore critical in the production of sperm.

Sertoli cells surround the SSCs and provide signaling molecules and factors to support both the self-renewal and differentiation process. This microenvironment is referred to as the stem cell niche [3]. Recreating the SSC niche in the laboratory setting is a critical step in the development of SSC transplantation. The concept of transplanting SSCs to enable normal spermatogenesis in an otherwise sterile individual has promising clinical applications in men with NOA.

Transplantation of SSCs was first described by Brinster and Zimmerman in 1994 when suspensions of testicular cells from fertile mice were transplanted into infertile mice resulting in fertility restoration and progeny [4]. Subsequently, it has been shown that cryopreservation of SSCs does not impair their ability to produce healthy spermatozoa capable of fertilization [5]. Currently, two strategies exist for transplantation of SSCs – testicular tissue harvest and grafting and SSC isolate injection.

Testicular tissue grafting retains the natural stem cell niche, which may be optimal. However, this approach has not yet been optimized for cancer patients due to concerns for cancer cell contamination. Cryopreservation protocols for testicular tissue are well established with most centers using a slow freezing protocol with dimethyl sulfoxide [6]. Keros et al. found that a slow freezing protocol with dimethyl sulfoxide led to improved tubule integrity, fewer damaged spermatogonia, and better maintenance of the lamina propria when compared to cryopreservation protocols with glycerol and propanediol [6]. Testosterone production was also better maintained in the Dimethyl sulfoxide (DMSO) protocol [6]. However, ideal concentrations of dimethyl sulfoxide remain to be established.

Xenograft models have been used to store and support SSCs and testicular tissue in several animal models [7]. Wyns et al. describe using a mouse xenograft model for cryopreserved immature testicular tissue harvested from prepubertal males [8]. This work still requires a major advancement to be clinically relevant: the transfer of the tissue back into the patient postpubertally with successful regeneration of spermatogenesis.

Testicular tissue grafting can be performed in several locations. The ideal location for graft transplantation is the scrotum because its temperature is lower than that in other sites that have been applied in animal models such as the peritoneum or under the skin on the back [9]. Grafting in ectopic sites also requires that the patient use Assisted Reproductive Techniques (ART) for conception. Some authors recommend grafting to several sites to optimize yield [10]. Timing of grafting, prepubertal versus postpubertal, has also been debated. High FSH and LH concentrations are required for successful proliferation of the graft; therefore, the peri-pubertal period may be an ideal target time, but this has not been explored. Because prepubertal boys do not yet make enough FSH and LH to support spermatogenesis, the ideal timing for grafting may be after patients have reached sexual maturity [10].

Schlatt et al. performed a grafting procedure in neonatal mouse model after castration to maintain high levels of FSH and LH [11]. This method, of course, cannot be replicated in humans, but it provides evidence that high levels of gonadotropins are necessary for successful grafting.

SSC injection is the other method of transfer to the recipient. The rete testis, efferent ducts, or the seminiferous tubules have been identified as injection targets for SSCs in several species [12]. Upon injection, the SSCs migrate to the basement membrane of seminiferous tubules and subsequently self-renew and differentiate to establish spermatogenesis in the recipient. This process should theoretically allow for conception without the requirement of assisted reproductive techniques, which is a major advantage. Hermann et al. injected adult rhesus monkey SSCs into the rete testes of recipient prepubertal rhesus monkeys and showed that mature spermatozoa were present in the ejaculate when they reached maturity. These spermatozoa demonstrated fertilizing capabilities when used with ICSI [13].

At present, SSC transplantation is experimental and has not been done in human models, but research thus far is promising. Several challenges have plagued the transformation of this idea into a real clinical therapy for patients: identifying the SSC population in the testis, culturing the SSCs, storing of the SSCs, and reintroducing the cells safely into a recipient. Several groups have been able to isolate human SSCs and culture and store the cells. However, well-defined protocols that are easily replicated are needed. Additionally, confirming the successful introduction of the SSCs into a human recipient is difficult as one must distinguish between native SSCs and transplanted SSCs as well as native mature spermatozoa and transplanted spermatozoa [14]. Finally, after successful SSC transplantation, it is essential to prove the fertilization potential of the mature spermatozoa in the recipient. Because there is some evidence of SSC loss after transplantation, a sufficient population of SSCs must be harvested. However, the results have poor reproducibility and the process is inefficient as demonstrated by a study by Wyns et al. showing 14.5% of the spermatogonial population remained at three weeks after grafting in a mouse population [15].

SSC transplantation resulting in mature gametes with fertilization potential has not yet been replicated in the human

population, but research thus far has been promising. If it could be developed successfully for humans, several patient populations could potentially benefit, including men facing gonadotoxic treatment for malignancy who desire biological children in the future. Additionally, prepubertal boys with a cancer diagnosis might benefit from this option, particularly given the fact that obtaining mature spermatozoa prior to treatment is not an option. Similarly, adult azoospermic men with Sertoli cell-only syndrome could be treated with transplantation of SSCs to repopulate the germline epithelium. Patients with Klinefelter's syndrome demonstrate progressive fibrosis of the seminiferous tubules over their lifetime and may also be candidates for SSC transplantation if harvested early in life. For patients with genetic defects causing NOA such as Y-microdeletions, genomic editing would be required to remove the mutation. If SSC transplantation combined with genomic editing was successful, this procedure could also be applied to fertile patients with genetic diseases at a single locus to prevent transmission to offspring [16].

3. *In vitro* spermatogenesis using pluripotent stem cells

An alternative method for developing mature gametes in men with NOA is the use of pluripotent stem cells. This technique could be applied to adult males with idiopathic NOA. In order to develop germ cells from pluripotent stem cells, several discrete steps are required, each with specific stimuli required for progression to the next stage of development. In addition, the differentiation process that occurs *in vitro* does not always emulate the process that occurs *in vivo*. Despite these challenges, pluripotent stem cell lines may enable development of disease models for infertility to allow for development of new therapeutics.

There are two stem cell sources (in addition to SSCs) that may be used for generating germ cells: human embryonic stem cells and adult pluripotent stem cells [17]. Human embryonic stem cell lines have been studied for several years. Toyooka et al. generated male germ cells from mouse embryonic stem cells. However, the fertilization potential of the mature sperm was not studied [18]. Subsequent studies have shown that haploid spermatids developed from mouse embryonic cells have fertilization potential as demonstrated by live births [19]. Studies with human embryonic stem cells have yet to yield functional haploid spermatozoa [17]. In addition, the use of embryonic stem cells is limited by the ethical concerns, further limiting the propagation of this technique toward clinical application.

Pluripotent adult stem cells are able to self-renew and differentiate into all three germ-layer cell types. These stem cells are developed from somatic cells, making them easily accessible. Zhu et al. demonstrated that adult mouse pluripotent stem cells can differentiate into SSCs and late-stage male germ cells with the combination of *in vitro* and *in vivo* harvest [20]. These authors could not demonstrate that these cells progressed to form mature spermatozoa; therefore, fertilization potential was not shown. Hayashi et al. successfully developed primordial germ cells from both embryonic stem cells and adult pluripotent stem cells in mice and were able to show fertilization potential with ICSI [21]. In order to prompt differentiation into germ cells from adult pluripotent stem cells, oncogenic factors must be used. Until another protocol is developed, adult pluripotent stem cells are not an option as a therapy for male infertility because of their tumorigenic risks [17].

4. Gene therapy

Gene therapy has already been applied in the research setting to many human disease processes. The technique involves adding a 'normal' gene to a patient's genome, removing an 'abnormal' gene, or mutating an 'abnormal' gene to allow it to function appropriately. This requires a thorough understanding of the specific genetic defect underlying the pathologic process. Unfortunately, such knowledge is lacking in the majority of cases of NOA.

Germline genetic defects pose a unique challenge for gene therapy in that spermatogenesis originates from stem cells. Therefore, the SSCs would need to be genetically altered in order to pass the altered genetic material to all mature spermatozoa and progeny. SSC transplantation with genetic modification, as discussed previously, would be required.

Although gene therapy for male infertility has been successful in mouse models resulting in live progeny, it is not yet applicable for clinical practice in infertile patients with NOA for a number of reasons. As stated earlier, we currently do not have enough knowledge to fully understand the single gene defects that cause NOA. For example, altering germline genetics is illegal in humans at the present time in most countries, and insertion of genetic material into the germ cell line could lead to oncogenesis. Finally, it is evident that gene therapy – which is a very expensive therapeutic – would provide therapeutic benefit only for a small subset of patients with NOA. This includes some men with NOA caused by known genetic defects and those with primary ciliary dyskinesia, a genetic disorder that results in nonmotile spermatozoa. However, men with Klinefelter's syndrome and Y-microdeletions would not be candidates for gene therapy due to the amount of DNA that would require addition or deletion. Without the knowledge of single gene defects that cause infertility in humans, gene therapy is not yet applicable for clinical practice. Substantial ethical and safety hurdles must be surpassed as well [22].

Promising research is being conducted to determine other genetic bases for NOA such as DNA methylation patterns and sperm mitochondrial genome deletions [23]. As further developments occur, more targets may develop for gene therapy. With time, the future may bring answers to these patients as to the cause of their fertility [24].

5. Conclusion

Existing therapies for patients with NOA who wish to conceive a genetically related child are limited and require harvesting mature spermatozoa from the male while the female endures the morbidity of assisted reproductive technology. The use of SSCs is promising, although their entrance into the clinical landscape awaits further optimization to ensure safety and reproducibility. The use of SSCs has the broadest applicability to subsets

of the infertile male population and has been demonstrated successfully in animal models. The hurdles that remain include identifying ideal protocol for harvest, storage, and transfer in humans. Additionally, this option is not available to cancer patients, given the risk of oncologic recurrence. Further limiting the use of stem cell therapies for some patients with NOA is the potential requirement to manage the underlying genetic defect that lead to the disorder in addition to the stem cell transplantation. Stem cell therapy using embryonic stem cells or adult pluripotent stem cells is further from the clinical horizon as mature spermatozoa with capability to fertilize have not yet been developed, likely due to incomplete understanding of the stem cell niche required for spermatogenesis.

Gene therapy has been very promising to treat certain genetic diseases and does have some applicability to the male fertility stage for patients with single gene defects resulting in azoospermia. Until further single gene defects are identified and the ethical concerns over gene therapy for the germline are ameliorated, this option will not be widely applicable to the NOA population.

All novel therapeutics are expensive. Stem cell therapy at present is used off-label. Insurance does not usually provide any coverage, and the services can be cost prohibitive for many patients. If the biologic therapies discussed in this review reach the clinical setting, the procedures will undoubtedly be extremely costly, limiting the patients who can take advantage of these options. As supply increases and protocols are optimized, novel treatments tend to become more affordable. However, many existing therapeutics for both male and female fertility remain uncovered by insurance plans and require a significant payment from the patient. Not only will the treatments need to be more affordable, but the viewpoint that fertility treatments need not be covered by insurance in the United States will need to be modified. With regard to assisted reproductive techniques, data suggest that improved insurance coverage results in better outcomes in terms of fewer fresh embryos transferred and fewer multiple gestation pregnancies [25,26].

Despite the hurdles that remain, an impressive amount of progress has been made toward novel therapeutics for men with NOA who desire biological children.

6. Expert opinion

NOA is amenable to ICSI if mature spermatozoa can be obtained from the testicle. Unfortunately, not all men with NOA are candidates for ICSI, and the burden for the female is significant in terms of cost and morbidity. Men with maturation arrest and Sertoli cell-only pathology would benefit from the novel therapeutic options discussed here. The ability to repopulate the germinal epithelium of the seminiferous tubules with SSCs would drastically change the field of male infertility. Similarly, propagating adult or embryonic stem cells into mature spermatozoa would provide a therapeutic option for these patients. Gene therapy is applicable in a smaller population of patients but nonetheless would be a major advancement in the field. Despite the major advances made in the field of biologic therapy for male infertility, several obstacles exist to bring these to the clinical realm.

Ethical limitations create a significant barrier to performing the appropriate studies in humans. In the prepubertal patient population, storage of testicular tissue, SSCs, or stem cells is controversial, given the patient's inability to provide his own consent or truly understand the implications of the procedure. Moreover, the use of embryonic stem cells is surrounded by controversy. Obtaining embryonic stem cells requires destruction of the embryo. The struggle to balance the need to cure disease with the moral obligation to protect future human life is inherent to the use of embryonic stem cells. Gene therapy for male infertility is similarly wrought with ethical concerns as manipulating the germline could have significant implications as a therapeutic option but also as a gateway to selecting characteristics to alter the genetics of populations.

Standardized protocols for tissue and cell culture and storage must be established prior to the use of these therapies in the clinical landscape. Although SSCs have been used with success in several animal models as well as across species, the results are not easily reproducible and the optimal site, method, and timing of injection or grafting are not yet well understood. The genetic stability of the cells after transplantation is critical as any acquired mutations will be passed to progeny. Pretransplantation culture and storage must be perfected to obtain genetic stability.

Future research is essential for further development of this promising field. Eliminating oncogenic risks must occur before studies in human subjects can take place. Of the existing novel therapeutics discussed, it is our opinion that SSC transplantation has the most promise and fewest limitations. It is reasonable to offer testicular tissue harvesting to prepubertal patients undergoing gonadotoxic treatments, ideally under a clinical research protocol, with the hope that these technologies will evolve to the clinical realm when these patients reach reproductive age.

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Declaration of interest

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