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Male germ cell transplantation: present achievements and future prospects

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ABSTRACT Germ cells are unique, since their surviving descendants can undergo meiosis and differentiate into gametes, which transmit genetic material from one generation to another. We now know that male germ cells, whether they be primordial germ cells in gonadal ridges, gonocytes, or stem spermatogonia, are transplantable. The donor cells can be transferred by direct microinjection into the seminiferous tubules, rete testis or efferent ducts, depending on the recipient species. Following transplantation, the donor cells undergo spermatogenesis in the host's seminiferous tubules in rats and mice, and have even sired offspring in mice. Interspecific germ cell transfer is possible if the recipient's immune system is defective; nude or SCID mice can even produce rat spermatozoa. However, the major obstacle restricting widespread use of this new technology is its extremely low success rate. This article discusses some ideas for improving the success rate of the transfer technique, and considers several potential applications.

KEY WORDS: *primordial germ cells, gonocytes, stem spermatogonia, transplantation, spermatogenesis*

Introduction

Germ cells are fascinating! The primary purpose of any sexually reproducing organism is to pass on its genes to the next generation *via* the germ cells, which are the only cell type to retain complete totipotency while undergoing significant phenotypic differentiation. However the cloning of Dolly has shown that under the right conditions, even somatic cells may retain their potential totipotency throughout life (Wilmut *et al.,* 1997). It is only in last 15 years that we have learnt how to purify germ cells (de Felici and McLaren, 1982; Bucci *et al.,* 1986; Pesce and de Felici, 1995), culture them *in vitro* (Matsui *et al.,* 1992; Resnick *et al.,* 1992; van Dissel-Emiliani *et al.,* 1993), transplant them into sterile recipients to produce normal donor-derived spermatogenesis (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Jiang, 1995; Jiang and Short, 1995,1998; Clouthier *et al.*, 1996) and generate donor-derived offspring (Brinster and Avarbock, 1994). In this article, we will briefly review the life cycle of male germ cells, introduce the concept of male stem germ cells, and show how they can be transplanted. We will then discuss future prospects for male germ cell transplantation.

Life cycle of male germ cells

A group of about 100 cells in the region of the epiblast at 7 days post coitum (dpc) in mice (Ginsburg *et al.,* 1990) are destined to form the germ cells, which can be identified by their alkaline phosphatase activity (Chiquoine, 1954). These cells are known as the primordial germ cells (PGCs), which are the progenitors of the male and female germline. These PGCs then migrate through the posterior primitive steak to the allantoic stalk and *via* the hindgut and dorsal mesentery to their final site, the gonadal ridges (Gardner, 1978; Tam and Snow, 1981; Lawson and Hage, 1994). In this review, PGCs are defined as mitotic germ cells during embryogenesis (Jiang *et al.,* 1997), and gonocytes as the transitional germ cells before differentiation into stem spermatogonia (Fig. 1). This definition is different from those of Hilscher *et al.* (1974) and Wartenberg (1976) whose terminology was based solely on light microscopic observations. The stem spermatogonia are Type A spermatogonia which localize in the basal compartment of the seminiferous tubules where they can either undergo mitotic self-renewal or differentiate into Type B spermatogonia, which are non-renewable and committed spermatogenesis (Meistrich and van Beek, 1993; Dym, 1994). Stem cells are cells which retain the potential for either self-renewal or differentiation. In the adult testis, PGCs and gonocytes are no longer present, and stem spermatogonia are the only self-renewing germ cells. However, both PGCs and gonocytes can be used as donor cells for transplantation. In this article, therefore, male stem germ cells have been considered as all cell types prior to differentiating spermatogonia. Because PGCs have been extensively reviewed elsewhere in this special issue, and by Jiang (1995), we will focus the discussion on some aspects of stem spermatogonia.

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In spite of the absence of specific markers for stem spermatogonia, and the difficulty in obtaining a pure population, molecular studies on the effects of growth factors and their ligands on these cells have made great progress. The product of the c-*kit* gene is uniquely expressed both in stem and differentiating spermatogonia (Wolgemuth and Watrin, 1991) and expression of the c-*kit* receptor is closely correlated with the proliferation of spermatogonia (Manova *et al.,* 1990). The intravenous injection of an anti-c-*kit* monoclonal antibody, ACK2, depleted dividing type A spermatogonia in adult mice 24-36 h later (Yoshinaga *et al.,* 1991). Dym *et al.* (1995) demonstrated that stem cell factor (SCF), also known as mast cell growth factor (MGF), kit-ligand (KL), and steel factor (SLF), significantly stimulated the phosphorylation of the c-*kit* receptor on the cell surface of spermatogonia. An excellent *in vitro* study by Rossi *et al.* (1993) showed that both FSH and its intracellular second messenger, cAMP, could increase SCF mRNA levels in mouse Sertoli cells in culture, and soluble recombinant SCF stimulated thymidine incorporation into mitotic spermatogonia in a dose-dependent manner. Autoradiography showed that SCF selectively stimulated DNA synthesis in type A spermatogonia. This is probably the first study to show that FSH-regulated SCF production in the Sertoli cells could play a major role in the regulation of spermatogenesis. Bone morphogenetic protein 8 (BMP8), a member of the transforming growth factor β superfamily of growth factors, was shown to be expressed in both type A and B mouse spermatogonia one week after birth (Zhao *et al.*, 1996). Nerve growth factor receptor (low affinity form) was also demonstrated immunocytochemically on the cell surface of proliferating spermatogonia in bovine testis (Wrobel *et al.*, 1996). The mRNA for high affinity activin receptor (ActR-IIB2) was detected on type A spermatogonia and Sertoli cells (Kaipia *et al.,* 1993), which supports the observation that activin stimulates spermatogonial proliferation in germ cell-Sertoli cell cocultures from immature rat testis (Mather *et al.*, 1990). All data suggest that multiple growth factor-receptor systems may regulate the self-renewal, proliferation and differentiation of stem spermatogonia.

Once a stem spermatogonium is formed, the process of spermatogenesis begins. It can be divided into 3 stages: the proliferative stage, the meiotic stage and the spermiogenic stage. During the proliferative stage, stem spermatogonia undergo mitotic self-renewal to maintain the stem cell pool at a constant size, and also produce differentiating spermatogonia. The latter engage in extensive mitotic proliferation, and most of the increase in cell number that characterizes spermatogenesis occurs at this stage. Ultimately type B spermatogonia are formed; they differentiate into primary spermatocytes, which initiate the meiotic process and produce round spermatids. These then undergo spermiogenesis to produce morphologically mature spermatozoa. Theoretically, when committed to spermatogenesis, a single stem spermatogonium is capable of producing 4,096 spermatids, so spermatogenesis is an extremely efficient process (Russell *et al.,* 1990).

Present achievements

Seven years ago, Brinster and colleagues and we ourselves began independently to investigate whether donor male stem germ cells could survive transplantation into the lumen of the seminiferous tubules of a recipient's testis, initiate spermatogenesis, and produce spermatozoa which were capable of siring offspring. Initially, we both chose mice as the animal model, using busulfan treatment to deplete the germ cell population in the recipient's testes prior to transplantation. Brinster's group initially chose to microinject donor germ cells into the seminiferous tubules of the recipient mouse, whereas we tried injecting donor germ cells *via* the rete testis. We soon found that it was very difficult to inject donor germ cells into the rete testis of recipient mice because there were as many as 12 small intratesticular rete channels with diameters around 100 μm, while there are only 1-2 main channels in rats with a diameter about 10 fold greater (1 mm) than in mice (Jiang, 1995).

Three years later, Brinster and Zimmermann (1994) and Brinster and Avarbock (1994) published the first results showing that mouse stem spermatogonia from prepubertal donors are capable of initiating spermatogenesis and producing viable sperm in adult recipients. Three out of over 120 recipient mice were able to father donor-derived progeny when tested mated with female mice. They also showed that mouse testicular cells could be deep-frozen, and were capable of reconstituting spermatogenesis in recipient mouse seminiferous tubules (Avarbock *et al.,* 1996). We showed that donor PGCs from rat fetuses and gonocytes from neonatal rats were capable of producing morphologically normal spermatozoa in the seminiferous tubules of adult rat recipients (Jiang, 1995; Jiang and Short, 1995). More recently, Clouthier *et al.* (1996) made the important discovery that donor rat spermatogonia can even undergo spermatogenesis and produce spermatozoa in the testes of immunocompromised mice.

It seems certain that male germ cells can be transplanted equally well by either seminiferous tubule injection, rete testis injection or efferent duct injection (Fig. 2). Brinster and Zimmermann (1994) and Brinster and Avarbock (1994) first described the seminiferous tubule injection technique for mouse testicular cell transfer. We (Jiang and Short, 1995) first reported the rete testis injection method for male rat germ cell transplantation. More recently, Ogawa *et al.* (1997) reported that in the mouse, seminiferous tubule, rete testis and efferent duct injection were all equally effective in generating donor-derived spermatogenesis. Direct injection into the seminiferous tubules is practically useful for small species such as mice, except that it is technically more demanding than the other procedures. Rete testis injection is very simple and can be used for all species with a superficial rete, such as humans, mice, rats and some marsupials (see review by Jiang, 1995). Efferent duct injection could be useful for the species with an axial rete, such as cattle, goats, guinea pigs, pigs, rabbits, sheep and carnivores (Setchell, 1978).

The stage of development of donor stem germ cells apparently affects their ability to integrate with the host's Sertoli cells. Although the underlying mechanism is completely unknown, one possibility is that the expression of unidentified adhesion molecules on the cell surface, such as E-cadherin (Byers *et al.*, 1994) determines the spatial location of donor stem cells. Donor PGCs only underwent intraluminal spermatogenesis, indicating that although these cells acquired the ability to initiate spermatogenesis in a proper physiological environment, they lacked the adhesion molecules necessary for integration with the Sertoli cells of the host. Gonocytes underwent both intraluminal and intergiditating spermatogenesis (Jiang and Short, 1998), indicating that a proportion of these cells had acquired these adhesion molecules; stem spermatogonia completely integrated with the host's Sertoli cells and underwent

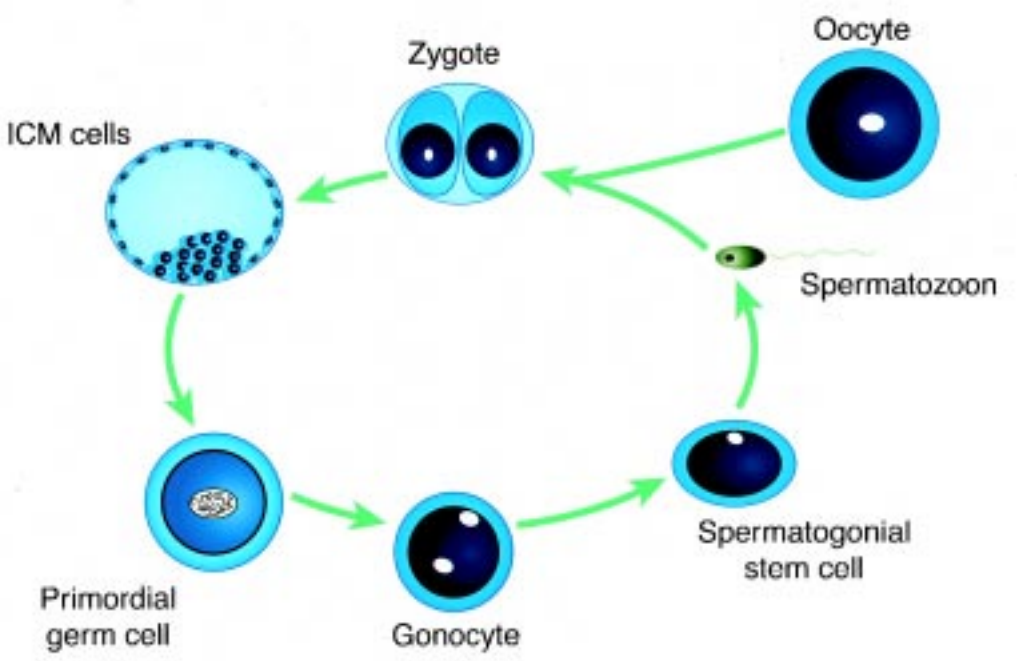

Fig. 1. The life cycle of the male germ cells in mammals. *A spermatozoon fertilizes an oocyte to give rise to a new individual starting at a zygote. After cleavage and morula formation, the early embryo becomes a blastocyst, within which an inner cell mass gives rise to all somatic cell lines and the germ cell line. The primordial germ cells (PGCs) are the founder germ cells which arise outside the gonads in the embryo, subsequently migrating and proliferating in the gonads. When they cease proliferation, PGCs are transformed into the gonocytes. Gonocytes re-commence proliferation and move to the basal part of the seminiferous cords soon after birth in rodents, where they develop into stem spermatogonia. Stem spermatogonia maintain their number by self-renewal and produce spermatozoa through proliferation, meiosis and spermiogenesis.*

spermatogenesis (Fig. 3), suggesting that they all expressed these molecules. Thus it appears that stem spermatogonia are more effective in interacting with the host's Sertoli cells than their precursors (Brinster and Avarbock, 1994).

The fact that donor spermatogenesis occurred in the lumen of the seminiferous tubules indicates that the adjacent testicular interstitial microenvironment is not necessary for the self-renewal of stem spermatogonia and the mitotic divisions of type A and B spermatogonia. Although the precise mechanism is unknown, perhaps their self-renewal, proliferation and differentiation is regulated by extracellular factors in the lumen of the seminiferous tubules, including hormones, growth factors and extracellular matrix molecules acting *via* ligand-receptor interactions, as occurs in other cell systems (Lelievre *et al.*, 1996). For example, Wang and Kim (1993) showed that retinol, the vitamin A alcohol, down-regulates male germ cell-associated kinase *(mak)* transcripts during the mitotic phase, while it up-regulates them during the meiotic phase of spermatogenesis.

We were not able to produce any evidence of offspring sired from the transplanted germ cells in 16 rats, test-mated to 213 females that produced 1392 progeny. Failure to detect donor-derived offspring may be because the fraction of donor-derived spermatozoa in the rat's ejaculate was too low to compete with the spermatozoa produced by the host's own regenerating seminiferous epithelium. On average, only about 5% of the seminiferous tubules of the recipients showed donor-derived spermatogenesis (Jiang and Short, 1995). Brinster and colleagues only produced a total of 13 donor-derived offspring from 3 recipient mice out of over a hundred subjected to transplantation of postnatal testicular cell suspensions (see Brinster and Avarbock, 1994). Therefore, male germ cell transplantation techniques are still in their infancy. This low success rate is the major obstacle to be overcome before we can regard male germ cell transplantation as a practical experimental technique.

Improving the success rate of germ cell transplantation

A priority for the future is to improve the success rate of male germ cell transplantation. The function of the host's Sertoli cells appears to affect the success of donor-derived spermatogenesis. This may be because, as mentioned previously, Sertoli cells secrete several growth factors which regulate the survival and proliferation of stem and differentiating spermatogonia. When germ cells have been severely depleted or are absent from the seminiferous epithelium, Sertoli cells eventually show signs of regression (Hatier *et al.,* 1982), and extensive junctional complexes form between neighboring Sertoli cells, extending from the basal lamina all the way up to the lumen of the seminiferous tubule (Chemes *et al.,* 1977). Normally these complexes are restricted to the basal region of the seminiferous epithelium and can be re-opened for movement of differentiating germ cells toward the lumen of the seminiferous tubules (Chemes *et al.,* 1977). Extensive tight junctions between host Sertoli cells may prevent donor stem germ cells from relocating to the basal compartment and initiating spermatogenesis. Therefore, it would be very interesting to determine which of the growth factors and adhesion molecules expressed on stem germ cells are important for donor cell colonization.

The effects of donated Sertoli cells on the interaction of donor germ cells with the host's Sertoli cells also needs to be determined. We noted that the incidence of intraluminal spermatogenesis following injection of purified PGCs was lower (5/10) than with crude donor testicular homogenates (8/9) (Jiang, 1995). This could be because the purified PGCs might have interacted with the host's Sertoli cells, thus initiating donor-derived spermatogenesis which could not be distinguished from that of the host (Jiang and Short, 1995), or the injected donor PGCs may have undergone apoptosis (Pesce and de Felici, 1994) following failure to establish an interaction with the extracellular domain of SCF expressed by

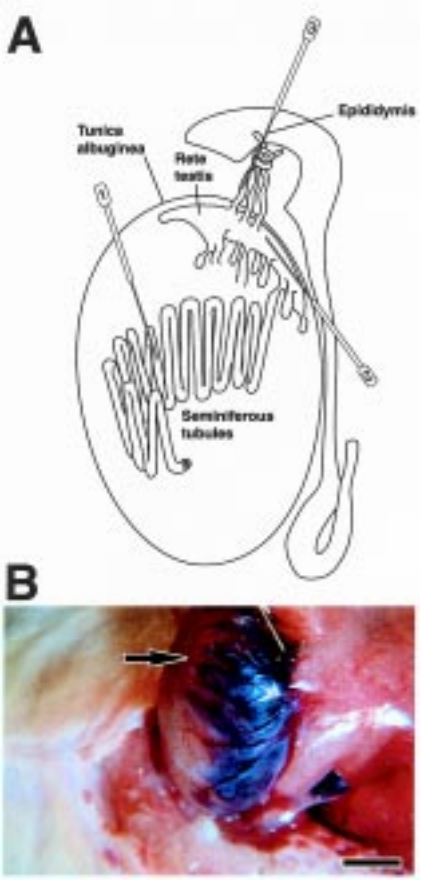

Fig. 2. Donor germ cell delivery. (A) *Three ways to deliver donor germ cells to the lumen of the host's seminiferous tubules. (1) Seminiferous tubule injection, (2) rete testis injection, and (3) efferent duct injection.* **(B)** *Presence of rete testis injection of 1% trypan blue solution. The blue dye is clearly seen in the lumen of the seminiferous tubules. Bar, 2.5 mm.*

Sertoli cells, which may be essential for their survival (de Felici and Pesce, 1994). Although Brinster and Avarbock (1994) showed that injecting additional Sertoli cells with donor testicular homogenates did not appear to increase colonisation efficiency, this could be because the number of Sertoli cells in donor testicular homogenates was already sufficient for stem spermatogonial colonization.

It was not clear whether there was any effect of the number of surviving host stem spermatogonia on donor-derived spermatogenesis. Because only a few germ cell-recipients were examined following one intraperitoneal injection of busulfan, it was difficult to compare the incidence of donor-derived spermatogenesis with that after 2 intraperitoneal injections of busulfan (Jiang, 1995). Brinster and Zimmermann (1994) and Brinster and Avarbock (1994) also did not study the effect of the number of host stem spermatogonia on the rate of colonization of the host's Sertoli cells by donor germ cells. Therefore further experiments are required to clarify this question.

Future prospects

Successful male germ cell transplantation is a major theoretical and technical breakthrough in male reproduction, since it could offer many new opportunities for the study of gametogenesis, transgenesis, male infertility and germline modification. For example, male germ cell transplantation could lead to a cure for some types of male infertility.

Male germ cell transplantation could also be a useful model for determining when the germ line acquires its potential for spermatogenesis, although all three types of male stem germ cells have been shown to be transplantable from one individual to another (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Jiang, 1995; Jiang and Short, 1995,1998). However, the precursor cells of male stem germ cells, totipotent inner cell mass (ICM)/embryonic stem (ES) cells, were unable to differentiate into stem spermatogonia and undergo spermatogenesis following transfer to the host's seminiferous tubules; instead they formed tumors (Brinster and Avarbock, 1994). The underlying mechanism is currently unclear; presumably genes which govern the differentiation of germ cells from totipotent ICM/ES cells may be switched on during their initial migration to the developing gonads, because donated gonadal PGCs are certainly capable of undergoing spermatogenesis following transplantation (Jiang, 1995; Jiang and Short, 1995,1998). This may be why gonadal PGCs showed a significantly lower potential to de-differentiate into ES-like or embryonic germ (EG) cells than migrating PGCs (Labosky *et el.,* 1994). It would therefore be very interesting to examine whether PGCs at different migration phases are equally able to undergo spermatogenesis following transplantation into adult recipients, and to determine if EG cells are more like ES cells or PGCs in their spermatogenic potential. Similarly, this transplantation model may also be useful to test which genes are critical for male germline commitment.

The close similarity between ES cells and PGCs makes it possible that ES cells could be induced to differentiate into PGCs. The latter could then be transplanted into a recipient, resulting in the production of offspring. If this was possible, male germ cell transplantation could revolutionize transgenic technology, because it could be technically less complicated and might have a much higher success rate than conventional techniques for the production and propagation of transgenic animals. The idea would be to transfect the foreign gene into ES cells, which would then be induced to differentiate into PGCs. The transgene will be amplified by spermatogenesis and transmitted to spermatozoa following transplantation of these PGCs into a recipient male. Transgenic animals could then be produced by using this recipient male. At the moment, there are 3 methods for introducing foreign genes into animals: microinjection of DNA into the pronucleus, retroviral infection of embryos, and transfection of ES cells (Jaenisch, 1988). All these methods are very time-consuming and have a very low success rate.

Can donor XX stem germ cells interact with XY host Sertoli cells following transplantation and undergo spermatogenesis? What determines the behavior of primordial germ cells? Is it the genotype of the germ cells or the phenotype of the gonad? Recently, McLaren (1991b,1992) claimed that the sex of germ cells depends not on their own chromosomal constitution, but on the cellular environment, since XY germ cells can give rise to oocytes in an ovary and XX germ cells can give rise to spermatogonia in the testes of XX/XY chimaeric mice (Levy and Burgoyne, 1986; Palmer and Burgoyne, 1991; Lovell-Badge, 1992). However, this hypothesis does not explain why XY germ cells can undergo oogenesis in an XX somatic cellular environment, but XX germ cells fail to

survive in a testicular environment (see review by Short, 1979). Moreover, the germ cell's chromosomal constitution greatly influences its subsequent fate during oogenesis or spermatogenesis (McLaren, 1991a). XO_{Sxr} germ cells can undergo spermatogenesis in the testis, but they produce morphologically abnormal spermatozoa, showing that Y-linked genes are essential for normal spermatogenesis (Reijo *et al.,* 1995; Lahen and Page, 1997). Another good example is that genetically female mice transgenic for *Sry* develop testes but are sterile because XX germ cells cannot progress beyond the stage of gonocytes (Koopman *et al.,* 1991). This poses the question: does the abnormal differentiation of such germ cells result from their chromosomal constitution or from the somatic cell environment? The later possibility cannot be excluded because the degree of fertility in chimaeric mice is highly correlated with the proportion of XX Leydig cells (Patek *et al.,* 1991). Germ cell transplantation techniques would be an interesting way to explore the fate of female primordial germ cells after transplantation into a testicular microenvironment.

Genetic engineering of somatic cells relies on the principle of altering the genome of a few cells which subsequently increase in number by mitotic divisions. Genetic engineering of germ cells has far more profound consequences, since the altered genome will be present in all cells of the progeny. What we have called male germ cell engineering relies on the principle of altering the genome of a male stem germ cell which could subsequently proliferate *in vitro* to produce a clone of modified cells. Transplantation of these cells into the testes of a male recipient could result in the production of an infinite number of modified spermatozoa. A man may produce 10^{12} to 10^{13} spermatozoa during his life (Reijo *et al.*, 1995), demonstrating that spermatogenesis is an extremely efficient process for amplification of the modified genome. A male animal of the desired genotype could donate its stem spermatogonia to an infinite number of sterilised male recipients who would then produce viable gametes of the donor's genotype and father donor-derived offspring. If this procedure could be repeated from generation to generation, the donor's genotype could be preserved and propagated in perpetuity. Male germ cell genetic engineering therefore has far greater potential than somatic cell genetic engineering.

Interspecific male germ cell transplantation also provides a way to study some fundamental questions in male reproduction, such as what determines the duration of the spermatogenic cycle, the Sertoli cells or germ cells? It takes 35 days for a mouse stem spermatogonium to differentiate into spermatozoa, but 48-53 days for a rat stem spermatogonium to do so. It will therefore be fascinating to determine the length of the spermatogenic cycle in rat germ cells transplanted into the mouse testis. Would such mouse-derived rat spermatozoa be capable of fertilizing rat eggs? Does the acquisition of fertility depend on species-specific proteins secreted by the reproductive tract?

If the testes of nude mice will produce rat sperm, could interspecific germ cell transplantation be extended to other species? Might it be possible to establish human spermatogenesis in the mouse, making use of the mouse testis merely as an *in vivo* "incubator" for human germ cells? Would this be ethically acceptable? Sensationalism aside, it would be a major scientific breakthrough since it would enable us to study human spermatogenesis in a laboratory mouse. What could we learn from these experiments? It is becoming increasingly apparent that spontaneous mutations of Y-linked

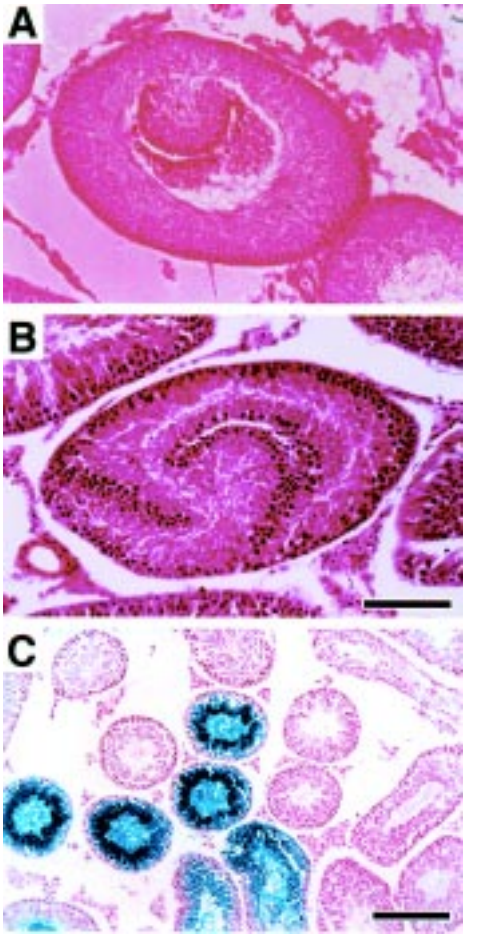

Fig. 3. Different fate following transplantation of primordial germ cells (PGCs), gonocytes and stem spermatogonia. (A) *Intraluminal spermatogenesis derived from donated PGCs.* **(B)** *Interdigitating spermatogenesis from donated gonocytes. Bar 100 μm.* **(C)** *The blue-stained tubules represent ZFlacZ donor stem spermatogonia-derived spermatogenesis following 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining. This photomicrography was kindly provided by Dr. R.L. Brinster. Bar, 200 μm.*

spermatogenesis-determining genes are a major cause of human infertility (Reijo *et al.,* 1995; Lahen and Page, 1997), and genes on the Y chromosome appear to be particularly susceptible to mutations (Short, 1997). Mutated stem spermatogonia would be obtained from testicular biopsies of infertile men, and propagated in the testes of nude mice. It might even be possible to attempt to cure the defect by gene insertion into these cells, monitoring the effect in the mouse to see if normal human spermatogenesis had been restored. If the treatment had been successful, it might then be possible to return these "repaired" spermatogonial stem cells to the seminiferous tubules of the infertile patient to restore his fertility. Currently, genetic manipulation of the germ line is a prohibited form of genetic engineering in many countries, but opinions might change if the procedure could be shown to be safe, and interspecific germ cell transplantation would enable us to monitor the procedure step by step.

In conclusion, the work of Ralph Brinster and his group has opened up the possibility of male intraspecific and interspecific

germ cell transplantation. Male germ cell transplantation has greater potential than female germ cell transplantation, since male stem cells are capable of mitotic proliferation and are present in the adult testis, whereas female stem cells have lost this potential for proliferation before birth in most species. The ability to transplant the male germ line means that we are now able to separate sex from soma –a finding of enormous fundamental significance that will challenge the intellects of the world's biologists and bioethicists for many years to come.

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