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Spermatogonial Stem Cell Isolation, Storage, and Transplantation

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The past 30 years have been marked by unparalleled accomplishments in the medical treatment of malignancy. Prior to advances in chemotherapeutic and radiation treatment, many oncologic conditions had dismal survival rates. Today, medical interventions have success rates that approach complete remission for many malignancies. An inadvertent complication of these therapies, however, has been the high rate of infertility following treatment. Male germinal tissue, like many malignancies, is mitotically active and therefore is particularly susceptible to the toxic effects of both chemotherapy and radiotherapy (Meistrich et al, 1982; Meistrich, 1993). Consequently, posttreatment patients often develop severe oligozoospermia or azoospermia (Wallace et al, 1991). Potential infertility complications can be anticipated, and adult male patients interested in future procreation are counseled to cryopreserve semen before instituting treatment. With present-day capabilities of in vitro fertilization, particularly intracytoplasmic injection, male patients can maintain posttreatment fertility. Pretreatment sperm banking, however, is not a viable option for prepubescent males. These individuals have not yet begun spermatogenesis and thus lack viable spermatozoa. It is estimated that, by the end of the decade, 1 in 250 young men will be childhood cancer survivors (Blatt, 1999). For these patients, infertility has often been an accepted consequence of their life-saving treatment.

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A great deal of interest has recently been shown in testicular autologous transplantation, an intervention that may provide a future therapeutic fertility option for these individuals. Having been successfully demonstrated in rodent models, investigators have now begun to explore the possibility of using testicular autotransplantation to restore fertility in humans. This paper will review the history of spermatogonia transplantation with an emphasis on the clinical pertinence of this field of investigation. Current innovations involving the isolation of spermatogonial stem cells (SSCs) and the present capabilities of in vitro proliferation will additionally be reviewed.

Spermatogonia

Spermatogonia are male germinal progenitor cells and are composed of differentiated nonstem and stem cells. Stem cells are characterized by a capacity for self-renewal and an ability to produce differentiating cell lines (Loeffler and CS, 1997; van der Kooy and Weiss, 2000). Spermatogonia are diploid germ cells that originated from primordial germ cells (PGCs). These precursor cells originate from embryonal ectoderm. PGCs migrate to the genital ridge, where they become known as gonocytes. Gonocytes are surrounded by Sertoli precursor cells in what become the seminiferous cords. Tight junctions between adjacent Sertoli cells later become the basis of the blood testis barrier. The gonocytes undergo mitotic division, followed by arrest in the G0 phase of the cell cycle. They are mitotically inactive until after birth, when they become spermatogonia (Clermont and Perey, 1957; de Rooij and van Dissel-Emiliani, 1997).

Understanding spermatogonial nomenclature, differentiation, and regulation is important for comprehending testicular transplantation. First, distinguishing SSCs from differentiating spermatogonia has been a challenge that has been met with limited success. The term undifferentiated spermatogonia refers to As, Apr, and Aal cell types. Undifferentiated spermatogonia are considered distinct from ''differentiating spermatogonia.'' The latter group, ordered in succession, consists of A1, A2, A3, A4, Ain, and then B spermatogonia. It has been speculated that only As spermatogonia are stem cells that may divide into 2 identical daughter As spermatogonia or into 2 Apr (paired) daughter cells that are functionally committed to differentiation (Huckins, 1971). Apr cells differentiate into 4, 8, or 16 Aal cells; there is no cell division from Aal to A1; however, there is a transformation while in the

G0/G1 phase (de Rooij and van Dissel-Emiliani, 1997). Some controversy surrounds the As-based categorization of spermatogonia, and additional classification and nomenclature schemes exist. However, most concur with the As hypothesis of spermatogonia development (Russell et al, 1990). Throughout the remainder of this review, the As nomenclature will be used.

Various techniques have been suggested to distinguish As spermatogonia from spermatogonia committed to differentiation. Morphology is inadequate for this purpose. It has been asserted that undifferentiated (As-Aal) spermatogonia can be distinguished from differentiating spermatogonia (A1–4, Ain, B) because the latter cells will be in the G2 or M phase, while undifferentiated cells will not divide synchronously (de Rooij and van Dissel-Emiliani, 1997). Additionally, Apr and further differentiated spermatogonia form cellular bridges, which allow the sharing of gene products and facilitate synchronized development (Weber and Russell, 1987; Braun et al, 1989). SSCs do not have intercellular bridges. However, definitively determining that a cell lacks a bridge is a profound challenge, limited by tissue sectioning. Additionally, the capacity to distinguish As spermatogonia on the basis of the absence of intercellular bridges is limited, since it is known that some gonocytes possess intercellular bridges. There are methods to help identify the spermatogonial stage on the basis of topographical criteria (Huckins, 1971; Oakberg, 1971). However, these methods provide neither an efficient nor an effective means by which to distinguish the As or SSCs from other spermatogonia.

Lastly, spermatogonial density regulation takes place at the A2 through B spermatogonia (de Rooij and Lok, 1987). This phenomenon is influenced by programmed cell death and ensures that the number of differentiated germ cells does not exceed the organism's need (de Rooij and Grootegoed, 1998). When larger numbers of differentiated spermatogonia are present, degeneration occurs more frequently, thus reducing the cellular population. This apoptosis is similar to events occurring in somatic cells (Conlon and Raff, 1999).

History of Spermatogonia Transplantation

In 1994, Brinster and Zimmerman published their landmark findings in the field of testicular tissue transplantation. Using a mixed cellular solution obtained from dissociated testicular parenchyma, they infiltrated recipient mouse seminiferous tubules with the donor cells. Among the hallmark findings of this experiment was the discovery that donor spermatogonial cells could interact with the host environment, migrate from the adluminal compartment, and negotiate past Sertoli-Sertoli tight junctions to enter the basal compartment (Griswold, 2000). Brinster and Zimmerman (1994) demonstrated successful donor spermatogenesis from testicular tissue transplanted between different mouse subjects. They used donor testicular tissue harvested from postnatal mice between days 4 and 12 of life. The assumption was that immature mice would have the highest concentration of undifferentiated spermatozoal progenitor cells or gonocytes, thereby providing the largest quantity of viable cells for transplantation. Testicular tissue was mechanically and enzymatically dissociated into a cellular suspension. The suspension was microinjected into mice pretreated with busulfan to eliminate native spermatogenesis. Donor cells came from transgenic mice expressing the LacZ (*Escherichia coli* B-galactosidase) gene; these cells, when differentiated to the round spermatid phase, stained blue, distinguishing them from the recipient's native sperm cells. The authors identified restored spermatogenesis in the recipient mouse along with colonization and differentiation of the donor tissue (Brinster and Zimmermann, 1994). Brinster and Avarbock in 1994 reported successful spermatogenesis in a mouse allogenic spermatogonial cell transplantation experiment. They found that the donor-derived spermatogonia were responsible for generating offspring; transmission was confirmed by the presence of a donor haplotype in the resulting progeny (Brinster and Avarbock, 1994).

Cryopreservation before transplantation was first described by Avarbock and colleagues (1996). They reported successful transplantation after freezing the donor tissue for up to 156 days. Clouthier and colleagues (1996) published the subsequent landmark investigation in testicular transplantation. In this investigation, rat testicular tissue was introduced into immunodeficient mouse testis. The transgenic rat tissue was identified in the mouse seminiferous tubules, and differentiated rat germinal tissue (including spermatozoa) was recovered from the mouse epididymis (Clouthier et al, 1996). Nagano and colleagues (1998) then demonstrated the capacity to culture spermatogonial cells in vitro, followed by testicular transplantation. Their study found that spermatogonia survived in culture for up to 4 months.

Further investigations found that the intraluminal transplanted germ cells degenerated and disappeared by 1 month's time. The successfully transplanted spermatogonia localized at the basement membrane and began to show evidence of division by the first week after transplantation. Donor spermatogonia migrated to the basal compartment during the first month, and donor spermatozoa were noted by that time (Parreira et al, 1998; Nagano et al, 1999).

The limits of spermatogonia transplantation were noted in more distant, xenogeneic transplantation experiments. Although limited colonization did occur with rabbit, monkey, bull, and human transplantation, no spermatozoa or postmeiotic germ cells were found after these transplantations. Schlatt and colleagues (1999a,b) transplanted ger-

minal tissue in primates and found evidence of spermatogonial survival at 4 weeks. When bromodeoxyuridine (BrdU) was introduced into donor tissue before transplantation, immunostaining located cells in the interstitium and seminiferous tubules that were identified with the BrdU label in their nuclei at 4 weeks. Morphologic criteria indicated these were type B or differentiated spermatogonia (Schlatt et al, 1999a). In 2001, Nagano and colleagues demonstrated the transplantation of baboon testicular tissue into nude mice. In this investigation, the authors identified the survival and propagation of the transplanted cells for up to 6 months. They used a rabbit-produced anti-baboon antibody in conjunction with an anti-human antibody to identify the baboon cells. They noted that baboon cells had migrated to the basement membrane of the seminiferous tubules—indicating that mouse Sertoli cells had somehow interacted with the baboon spermatogonia and had allowed passage through the blood testis barrier. Despite this evidence of favorable interactions between the 2 tissue types, the baboon spermatogonia showed no signs of spermatogenesis (Nagano et al, 2001).

To date, successful donor-derived spermatogenesis has been primarily limited to phylogenetically similar species. In addition to mouse-to-mouse transplants, spermatogenesis has been noted in rat-to-immunodeficient mouse transfers (Clouthier et al, 1996), hamster-to-immunodeficient mouse transfers (Ogawa et al, 1999a), and mouse-to-rat transfers (Ogawa et al, 1999b). It has been theorized that evolutionary distance is primarily responsible for the failure of more distant xenogolous transplantations. Mice and rats are thought to have diverged evolutionarily 10 to 11 million years ago, and hamster and mice are thought to have diverged 16 million years ago (Catzeflis et al, 1993). Transplants with animals separated by greater evolutionary distances have been less successful. This is likely due to failed spermatogonia and Sertoli cell structural association and other functional interactions. Of note, Ogawa and colleagues (1999a) found that hamster spermatogenesis in mice resulted in morphologically defective hamster spermatozoa. This finding suggested that the recipient Sertoli cells (mouse) had influenced the final differentiation of the spermatozoa and that the species differences resulted in the morphologic errors in development. Despite morphologic dissimilarities among the various differentiated germinal tissues, transplantation between different species has uncovered much information in terms of functional similarities. The previously described cooperative interactions between the host testicular environment and the donor germinal tissue underscore these similarities.

Spermatogonial xenotransplantations using human tissue have resulted in inconsistent findings. Investigators have reported finding the survival of at least some undifferentiated spermatogonia during distant xenotransplantations. Reis et al (2000), however, reported that there was no evidence of donor tissue survival following a human-to-immunodeficient mouse testicular tissue transplantation. These investigators were using the antibody stain proacrosin to attempt to identify successful transplantation. Proacrosin is a marker of differentiated human spermatogonia (primary spermatocytes and spermatids) and would not have detected transplanted cells that had survived or propagated but not differentiated. On the other hand, Sofikitis et al (1999) reported successful spermatogenesis from xenotransplantation of human tissue into rat and mouse recipients. In 2002, Nagano and colleagues reported on the use of anti-baboon testes antibody to identify the survival of human spermatogonia in mouse recipients for up to 6 months posttransplantation. These investigators found no evidence of meiotic activity among the donor tissue. The wide range of outcomes following human-to-mouse testicular tissue transplantation warrants further investigation in this field. On the basis of the findings from other xenotransplantations, it is probable that complete spermatogonial differentiation will not be observed consistently. The large phylogenetic distance between species will likely translate into incompatibilities between the host testicular environment and donor spermatogonia that prohibit complete spermatogenesis.

What Has Been Learned From Transplantation?

Investigations of spermatogonia transplantations have provided great insight into the process of spermatogenesis and different disease states. Ogawa and colleagues (2000) found that c-kit–defective (white spotting W/W^v) mice demonstrated restored fertility when receiving transplanted spermatogonia from stem cell factor–deficient (Steel Sl/Sl^d) mice. C-kit, a receptor found on normal differentiated spermatogonia, has been associated with a variety of roles that spermatogonia can play, including situations in which substances such as mitogen (Rossi et al, 1993) and survival factor (Allard et al, 1996; Dirami et al, 1999) are involved.

Stem cell factor, a product of normal Sertoli cells, is the c-kit ligand (Schrans-Stassen et al, 1999). Findings by Ogawa and colleagues demonstrated that infertility resulting from a c-kit defect was a germ cell phenomenon. The testicular microenvironment in these animals, specifically the Sertoli cells, could still facilitate normal spermatogenesis despite never having previously supported differentiated spermatogonia. Additionally, this study showed that mutations affecting the stem cell factor, despite causing infertility, did not affect the differentiating capacity of spermatogonia when placed in a supportive environment.

The finding that mice W/W^v could support spermatogenesis has broad implications. The Sertoli cells in these

mice had no prior exposure to differentiated spermatogonia. However, they were still capable of supporting spermatogenesis from transplanted germ cells. This discovery provides credence to the use of testicular transplantation to restore fertility in cancer survivors. Select chemotherapies and radiotherapies result in a severe depopulation of germinal tissue in these patients. Like those of the W/W^v subjects, the testicular microenvironments of these posttreatment patients consist primarily of Sertoli cells. Because Sl/Sl^d spermatogonia flourished and underwent differentiation in the W/W^v testicle, it is believed that, after treatment, many cancer survivors could still reinitiate spermatogenesis if viable spermatogonia were reintroduced into their testis.

Further discoveries regarding spermatogonial transplantation have come from investigations using *jsd*-mutant mice. Testicular tissue from these infertile mice, transplanted into mice with supportive intratesticular environments, did not result in donor-derived spermatogenesis (Boettger-Tong et al, 2000). This suggested that the *jsd* mutation prohibited differentiation via a germ cell defect and not from a defect in the supporting intratubular environment.

The factors determining the timing of spermatogenesis were further clarified by transplantation experiments in the Brinster laboratory. Normal mouse spermatogenesis takes place over about 35 days, while rat spermatogenesis is 52 to 53 days. The xenologous spermatogonial transplantations showed that spermatogenesis of the rat donor germ cells, despite being in a mouse testicular environment, still followed the normal rat timeline. About 52 days transpired before rat spermatozoa were fully developed (Franca et al, 1998). This finding showed that the timing of spermatogenesis is a spermatogonia-controlled event. Furthermore, although supported by the testicular somatic environment, spermatogenesis is not directed by it.

Testicular transplantation was used by Mahato and colleagues (2001) to investigate the role of estrogen receptor (ER)-alpha gene knockout on infertility. ER-alpha gene knockout mice are known to be infertile due to a failure of spermatogenesis (Eddy et al, 1996). When spermatogonia from ER-alpha knockout mice were transplanted into normal mice, donor-derived spermatozoa resulted. The identities of these spermatozoa were confirmed by the birth of progeny carrying the donor haplotype. This discovery demonstrated that ER-alpha was required for functional spermatogonial differentiation because of its influence on testicular stroma cells, not germinal cells (Mahato et al, 2001).

Improving Transplantation

The transplantation process was modified as time proceeded to improve outcomes. The changes that facilitated improved host colonization by the donor spermatogonia included the following:

1) Enriching the quantity of undifferentiated spermatogonia within the transplanted solution,
2) Altering the host environment, and
3) Making technical modifications in the transplantation procedure.

Enriching the Transplanted Solutions—Approximately 2 in 10^4 testicular cells are SSCs (Meistrich and van Beek, 1993). Currently, most testicular transplantation cellular solutions contain an estimated 100 to 200 stem cells and yield a 7% to 20% success rate in generating colonies (Nagano et al, 1999). By increasing the fraction of spermatogonia in the transplanted solution, investigators have hypothesized that a greater quantity of undifferentiated, stem cell spermatogonia can be introduced into the host. Increasing the numerical percentage of SSCs in a transplanted solution increases the likelihood of colonization with the donor germinal line. Therefore, the isolation of spermatogonia, particularly spermatogonia As or stem cells, has been an active pursuit. Among the methods available to isolate more SSCs are 1) the use or creation of animals with spermatogonial maturation failure (without intrinsic germ cell defect), 2) the in vitro culturing of spermatogonia, and 3) the cellular isolation of stem cells on the basis of cell markers or other factors (eg, density).

Increased undifferentiated spermatogonia of donor. Cryptorchid animals typically have seminiferous tubules populated with only undifferentiated spermatogonia. Furthermore, it is estimated that 1 in 2000 cells in a cryptorchid testis is an SSC (Shinohara and Brinster, 2000). An effective method for increasing the undifferentiated spermatogonial content for donor transplantation has been the use of tissue from iatrogenically derived cryptorchid mice. C57/B1 mice made cryptorchid were found, after 2 months, to have testes containing only type A spermatogonia (Aizawa and Nishimune, 1979; Haneji and Nishimune, 1982).

Steel (Sl) mutant mice are another source of testis thought to contain higher fractions of undifferentiated spermatogonia. The Sl mutation involves a defect in the production of the c-kit ligand. Phenotypical Sl mutant mice demonstrate spermatogonial maturation arrest. As a consequence, their testes are likely populated by a higher percentage of undifferentiated spermatogonia than are those of wild-type mice (Ogawa et al, 2000). However, recent investigations have called into question the exact quantity of stem cells in these subjects (Shinohara and Brinster, 2000).

Using spermatogonial transplantation as a functional assay for the presence of spermatogonial cells, Shinohara et al (2000a) reported that transplantations from crypt-

orchid donors resulted in a 25-fold increase in the number of spermatogonial colonies. Colonization was increased, but less dramatically, with donor cells from Sl mutant mice.

Additionally, the percentage of undifferentiated spermatogonia can be affected by medical intervention. Vitamin A–deficient mice and rats have only undifferentiated spermatogonia and may serve as sources for these cells (van Pelt et al, 1996). Spermatogonial arrest at Aal can be established with prolonged vitamin A deficiency (van Pelt et al, 1995). It is believed that vitamin A influences the differentiation of spermatogonia via a highly specific interaction with cells that are in stage VIII of the epithelial cycle. Interestingly, vitamin A replacement leads to synchronized differentiation (Griswold et al, 1987; Morales and Griswold, 1987; van Pelt and de Rooij, 1990).

Isolation based on markers or density. Because of the small numbers of SSCs in the normal testis, many believe that specific antibody probes or advances in cell culturing will be necessary to make spermatogonial transplantation a therapeutic option. The c-kit receptor is a marker that has been investigated in attempts to isolate undifferentiated spermatogonia. C-kit antibody studies demonstrate that undifferentiated spermatogonia are c-kit independent, while differentiated spermatogonia are dependent (Yoshinaga et al, 1991; Dym et al, 1995).

Attempts to isolate SSCs have been somewhat successful, given the light scattering qualities of these cells. Fluorescent-activated cell sorting is a cell separation technique that is based on light scattering of cells and cell surface molecules. In a crypto-orchid mouse, fluorescent sorting resulted in a 166-fold enrichment of spermatogonia stem cells, as determined by a subsequent transplantation assay. In the previously described experiment, spermatogonia were selected on the basis of low side scatter. This was because cells with low intracellular complexity, a suspected quality of undifferentiated spermatogonia, could be isolated by this technique. These cells also lacked or had low levels of alpha v integrin, and they were positive for alpha 6 integrin. Interestingly, the presence of c-kit did not increase stem cell isolation (Shinohara et al, 1999, 2000b).

The most extensive characterization of the phenotypic and functional characteristics of SSCs used a fluorescence-activated cell sorter technique to identify SSCs as a distinctive population in the adult testis (Kubota et al, 2003). Spermatogonia do not express major histocompatibility complex (MHC) class I (MHC-I) molecules, and negative selection for MHC-I (MHC-I$^-$) resulted in a sixfold enrichment of SSCs. Hematopoietic stem cells are positive for the glycosyl phosphatidylinositol–anchored glycoprotein Thy-1 (Randall and Weissman, 1998), and the selection for testicular cells in the MHC-I$^-$ population that express Thy-1 and are negative for c-kit generated a population that contained almost all of the SSC activity (Kubota et al, 2003). Furthermore, this cell population, MHC-I$^-$ Thy-1$^+$c-kit$^-$, is positive for other markers or characteristics previously used to enrich for spermatogenic stem cells, including α6-integrin and low side scatter (Shinohara et al, 2000b).

The strategy for identifying the phenotypic and functional characteristics of SSCs along with the SSC transplantation assay provides a powerful means of identifying a unique properties of these cells, thereby accelerating the process of obtaining a pure population of spermatogenic stem cells. Currently, the surface phenotypes for mouse SSCs are side scatter^{low}, α6-integrin$^+$, β1-integrin$^+$, CD24$^+$, Thy-1$^+$, αv-integrin$^-$, c-kit$^-$, MHC-I$^-$, and CD9$^+$ and are not present in cells with the side population phenotype.

Magnetic cell sorting is another technique that may be used to better isolate spermatogonia. Von Schonfeldt and colleagues (1999) reported the isolation of c-kit–positive spermatogonia via a magnetic cell sorter. A solution of testicular cells was exposed to anti–c-kit immunoglobulin G (IgG) antibodies. Magnetic labeling was then performed using anti-IgG antibodies conjugated with ferromagnetic microbeads. A total of 25% to 55% of the isolated magnetically labeled cells were c-kit positive.

Sedimentation velocity (separating on the basis of size and shape) and differential adhesion were used by Dirami and colleagues (1999) to create an isolate of cells containing 95% to 98% porcine type A spermatogonia. Using adhesion to laminin, the isolation of SSCs has been noted to increase three- to fourfold (Shinohara et al, 1999). Morena and colleagues (1996) used sedimentation velocity in conjunction with differential adhesion to attain an 85% isolate of type A, c-kit–positive spermatogonia. Microfluidic cell sorting may also be a valuable technique for the isolation of spermatogonia on the basis of size and density (Beebe et al, 2002).

Culture of spermatogonia. Of every 1000 germ cells in the adult testicle, only 2 are stem cells (Meistrich and van Beek, 1993). It has been asserted that the low numbers of spermatogonia in prepubertal males would likely make the isolation of spermatogonia (for future therapeutic transplantation) by density and sedimentation gradients inadequate (Aslam et al, 2000). As a treatment for infertility, before proceeding with autologous testicular transplantation, it will be necessary to develop more effective ex vivo culturing techniques.

The ex vivo culture of male germinal cells has been a particular challenge, and prior attempts have met with limited success. Maekawa and Nishimune (1991) reported on a Sertoli cell co-culture from neonatal mouse germ cells that demonstrated no loss of viability at 3 days. Dirami et al (1999) found that a potassium simplex optimized

medium (KSOM) culture demonstrated up to 50% viability for boar spermatogonia at 3 days. Other groups have demonstrated further organ culture success (Boitani et al, 1993; Schlatt et al, 1999b; Meehan et al, 2000).

Nagano and colleagues (1998) demonstrated that, using testicular transplantation as a functional assay, spermatogonial survival and propagation could be demonstrated after 132 days of culture. Germ cells survived for 4 months in a serum containing media on a feeder layer of STO cells (mouse embryonic fibroblasts).

Using their ex vivo culture model that was based on STO feeder cells, Nagano and colleagues (2002) were able to transfect SSCs with a retroviral vector. They noted the greatest transfection rates (determined by the number of transplanted colonies demonstrating the transgene) were from cells cultured in STO cells with periodic exposure to the retroviral particles. They calculated that the successful transfection rate was limited to 1 in 280 SSCs. Additionally, the transfected colonies of donor spermatogonia were noted in the recipient for up to 6 months following transplantation (Nagano et al, 2002). Orwig and colleagues (2002) subsequently demonstrated transduction in rat SSCs; success was determined by donor-derived spermatogenesis demonstrating the transgene.

Under the best conditions, it was estimated that only 10% to 20% of the SSCs survived for 7 days or longer using STO feeder layers and an undefined medium. The identification of the factors that regulate SSCs and the ability to use these factors to induce SSC proliferation in culture have obvious advantages for understanding molecular mechanisms regulating these cells and using them for genetic modification. Jeong et al (2003) demonstrated that SSCs can be cultured for 4 to 12 weeks, including passages when culture medium was supplemented with Kit ligand, leukemia inhibiting factor (LIF), basic fibroblast growth factor (bFGF), insulin-like growth factor 1, interleukin-11, 2 mercaptoethanol, sodium pyruvate, oncostatin, and platelet-derived growth factor in addition to 10% fetal bovine serum (FCS). The number of donor-derived colonies from SSCs cultured with these factors increased as time progressed from 4 to 12 weeks. In another study, the long-term culture of SSCs in a medium containing factors known to affect the proliferation and maintenance of PGCs, including glial cell line–derived neurotrophic factor (GDNF), LIF, bFGF, epidermal growth factor, and 1% FCS, resulted in an increase in the number of SSCs as time progressed (Kanatsu-Shinohara et al, 2003). The primary cultures consisted of clusters of round cells that formed on the top of the feeder layer that continued to form on new plates of feeder cells after passage. The number of SSCs increased for 5 months, and the transplantation of the cultured SSCs restored fertility to congenitally infertile mice. An interesting distinction of this study was that the researchers reported successful culture and donor-derived spermatogenesis of SSCs from C57Bl/6 × DBA/2 F1 mice only, but not from the SSCs of mice with C57Bl/6 or 129/Sv backgrounds using this protocol (Kanatsu-Shinohara et al, 2003). This indicates there may be important differences in the culture conditions for germ cells derived from different strains, and this may affect the conditions needed to culture nonrodent SSCs.

Culture of SSCs with STO feeder cells but no serum in the medium demonstrated that the stem cells require GDNF, GDNF-family receptor alpha-1 (GFRα-1), and bFGF to support replication (Kubota et al, 2004). The SSCs formed clumps in culture, expressed Oct −4, and doubled every 5.6 days. SSCs could be maintained under these conditions for 6 months and generated normal donor-derived spermatogenesis when transplanted into infertile recipients. These data support the hypothesis that GDNF is important in maintaining the undifferentiated population of spermatogonia in the mouse testis. Transgenic mice that overexpress GDNF in the testis are known to accumulate undifferentiated spermatogonia (Creemers et al, 2002). This foundation to investigate the basic biological activity of SSCs will accelerate our understanding of the mechanisms regulating the stem cell population in the testis.

Ex vivo culturing and transfection provide a powerful tool for potential gene therapy. This methodology could allow genetic deficiencies to be corrected from the patient's germ line. An effective culturing mechanism for SSCs would allow a small quantity of tissue extracted from the testicle to grow into a quantity sufficient for transplantation. Such an option would allow a patient before therapy with sterilizing side effects to preserve his germinal tissue. Following therapy, this cultured quantity of his native spermatogonia would be retransplanted. The success of the intervention would be measured by the repopulation of the testicle with germinal tissue, effective spermatogenesis, and fertility as measured by semen analysis and fecundity rates.

Altering the Host—The initial investigation by Brinster and Zimmermann (1994) determined that the host testicle pretreated with busulfan was more amenable to accepting transplanted spermatogonia than the testis of animals with genetically defective spermatogenesis. Specifically, they found a 37% success rate among busulfan-treated mice compared with an 18% success rate among mice with genetic spermatogonia deficiencies, although these differences may have been related to varying methods for the detection of successful transplantation.

Radiation therapy potentially causes severe depopulations of spermatogonia from the testis. Exposure of a primate to 1 Gy or more of x-ray therapy has been associated with a loss of all spermatids by 31 days (de Rooij et al, 1986). Radiation applied to the testicle results in cellular

death, particularly of the more rapidly dividing cells. Radiation kills in greatest quantity the differentiated spermatogonia, while the As (stem cells) are the most radioresistant (van der Meer et al, 1992a,b). Radiation-induced sterilization has not been commonly employed in the preparation of recipient testes. Among the concerns is the potentially greater local fibrosis with radiotherapy compared with chemotherapy; this would likely limit the utility of this pretransplant preparation technique.

Using a gonadotropin-releasing hormone (GnRH) analog, Ogawa and colleagues (1998) demonstrated improved transplant colonization of recipient testes. GnRH analogs cause a loss of sensitization of the luteinizing hormone–releasing cells of the anterior pituitary, ultimately leading to diminished testosterone production from testicular Leydig cells. The testosterone-deficient testicular environment was found to be more supportive of transplanted germinal tissue (Ogawa et al, 1998).

Technical Improvements—Improved technical modifications were achieved by injecting the donor cellular solution retrograde through the efferent ductules; previously, investigators used direct injections into the seminiferous tubules. In larger host species, ultrasound-guided needles effectively introduced the donor cellular solution directly into the rete testis. Schlatt and colleagues (1999a,b) reported on these techniques used on bulls and primates. They described the process as more efficient than microtubule injection (used commonly in mouse recipient transplants). These investigators noted that transplantations into normal, large mammal testes were limited in terms of infused volume. They attributed this characteristic to high intertubular pressures. In contrast, large animal testes that were regressed or immature had a much greater filling of the seminiferous tubules during infusion. This was attributed to a decreased quantity of Sertoli cell fluid, marked by a diminished seminiferous tubule diameter (Schlatt et al, 1999a).

Germ cell transplantation of testicular cells from donor goats transgenic for human alpha-1 antitrypsin expression into the testes of sexually immature recipient goats resulted in 2 of the 5 recipient goats producing sperm positive for the transgene. The germ cells in a volume of 5 mL were injected into the sexually immature recipients by an ultrasound-guided technique. Mating of one of the recipient goats produced 1 offspring (of 15) that was positive for the human transgene (Honaramooz et al, 2003). To our knowledge, this research represents the first time that donor-derived spermatogenesis resulted in the transmission of a donor haplotype to the offspring. It also demonstrates that sexually immature recipients, similar to rodent species, may represent the best hosts for donor-derived spermatogenesis in livestock species. This may be due to the structure of the seminiferous epithelium and the lack of Sertoli cell–tight junctions before puberty.

Future Clinical Applications and Caveats

The most likely clinical application of testicular transplantation will involve prepubertal males facing systemic chemotherapy with sterilizing side effects. These individuals could undergo a pretreatment testicular biopsy. The extracted tissue could be used for SSC isolation and cryopreservation or cultured ex vivo. On completion of treatment with no evidence of recurrence, these patients could undergo an autotransplantation with their own cryopreserved or cultured spermatogonia. The subsequent repopulation of their testes with germinal tissue would result in spermatogenesis and fertility. While the above-described scenario may be technically feasible today or in the near future, there are numerous caveats that must be considered before clinical application.

Malignant diseases that are blood related, such as leukemia, sarcomas, and lymphomas, should be considered a great risk for reintroduction of malignancy into the patient. On the other hand, nonblood-related malignancies such as Hodgkin lymphoma may not pose a serious risk for patients undergoing autologous transplantations (Aslam et al, 2000). Jahnukainen and colleagues (2001) reported on the transmission of rat T-cell leukemia via testicular transplantation from diseased donors. These findings demonstrate the profound importance of developing accurate ex vivo spermatogonial isolation and quantification assays. The investigators noted that as few as 20 lymphoblastic cells introduced into the recipient testis were capable of transmitting acute leukemia into the healthy hosts (Jahnukainen et al, 2001).

Additional caution must be exhibited with the application of human xenogeneic transplantation; as a clinical adjunct, it is an unlikely prospect. In the past, porcine retroviruses have infected human kidney cells (Patience et al, 1997). Thus, the possibility of introducing a xenologous viral genotype into human germ line makes the risk of such clinical investigations imposing.

Among the topics requiring further research is the pretransplant preservation of human spermatogonial cells. Currently, there is no standardized cryopreservation protocol for human spermatogonia. The method employed for previous murine transplantations used dimethylsulphoxide (DMSO) as the cryoprotectant (Avarbock et al, 1996). There have also been reports of using ultrarapid freezing and/or vitrification to cryopreserve testicular tissue and SSCs. However, the lack of investigation in the area of SSC cryobiology is likely indicative of reduced success. It can be anticipated that future research on the cryobiology of SSCs will define optimal combinations of cryoprotectants and rate of temperature change during the freezing and thawing process. This will aid in making the entire process more efficient.

These caveats, as mentioned, must be systematically

and experimentally addressed before SSC isolation, cryopreservation, and transplantation become a clinically feasible technology. For these reasons, continued basic studies of SSC biology are essential and require significant attention, considering the future translation and importance of these technologies in the preservation of reproductive potential in young male cancer survivors.

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