Cryopreservation and Transplantation of Spermatogonia and Testicular Tissue for Preservation of Male Fertility

Kyle E. Orwig, Stefan Schlatt

The existence of spermatogonial stem cells in the testis offers clinically relevant options for preservation and restoration of male fertility. New approaches based on male germ cell transplantation and testicular tissue grafting can be applied to generate a limited number of sperm cells and could therefore be considered important new avenues for restoration of fertility in oncological patients. We have developed approaches to infuse germ cells into rodent and primate testes and shown that germ cell transplantation is a procedure for restoration of spermatogenesis in the testis that might be adaptable to primates. As a promising alternative, grafting of testicular tissue has been used to produce fertile sperm. The rapid progress in the development of novel experimental strategies to generate sperm by transplantation of spermatogonial stem cells or by grafting of testicular tissue should stimulate oncologists to consider the cryopreservation of testicular tissue. This review introduces the reader to the physiology of spermatogonial stem cells and summarizes the current and potential future options for fertility preservation in male oncological patients. [J Natl Cancer Inst Monogr 2005;34:51–6]

STATEMENT OF PURPOSE

The issue of fertility appears often negligible at the time of cancer diagnosis and therapy in the light of a life-threatening disease, but infertility represents one of the most devastating consequences to negatively affect the quality of life of cancer survivors (1). This review aims to inform the reader about new options for fertility preservation in the male oncological patient. Spermatogonial stem cells (SSCs) are iatrogenic targets of oncological therapies, and their depletion may be responsible for occurrence of male infertility in cancer survivors. Therefore, the reader will be briefly familiarized with the cell biology and physiology of spermatogonia. Exciting new tools using spermatogonial stem cells in basic research and clinical applications have been developed recently. These will be presented, and their future application for fertility preservation in male oncology patients will be discussed.

SPERMATOGONIAL STEM CELLS

Spermatogonial stem cells are descendants of the primordial germ cells (PGCs), which migrate from extra-embryonic sites to colonize the gonadal ridge early during embryonic life (2). In the females, the primordial germ cells proliferate extensively and enter meiotic prophase at around the time of birth. In males, cessation of germ cell proliferation and blockade of meiotic entry are important steps in the morphogenetic cascade initiated by the expression of the SRY gene (3). The male germ cells associate with somatic cells of the presumptive gonad and form testicular cords, which mark the differentiation of PGCs into gonocytes. These cells differentiate into spermatogonia during prepubertal differentiation and thereafter function as male germline stem cells. The adult seminiferous epithelium is one of the most productive self-renewing tissues in postnatal mammals, generating millions of spermatozoa each day. In adulthood, spermatogenesis is sustained by spermatogonial stem cells that balance self-renewal and differentiation in a way that maintains the stem cell pool and also precisely meets the biological demand of the testis (4).

Because the spermatogenic system is highly prolific, it is an unintended target of radiotherapies or chemotherapies designed to treat malignant cell growth (5,6). The extent of spermatogonial depletion and the speed of spermatogenic recovery depend on the type, dose, and frequency of cytotoxic insults. Since all maturing germ cells are depleted from the seminiferous epithelium within several weeks of treatment, either by maturation into sperm or by apoptosis, the starting point for recovery of spermatogenesis is the spermatogonial stem cell.

In primate testes, two types of spermatogonia can be distinguished by morphological criteria (7). The A pale spermatogonium is the active stem cell. Its regular mitotic divisions generate cohorts of differentiating germ cells but also maintain the stem cell population. The A dark spermatogonium is the reserve stem cell, which—under normal conditions—appears to be mitotically quiescent (8). As was shown in macaques, recovery of spermatogenesis after irradiation occurs from A dark spermatogonia, which transform into A pale spermatogonia before they give rise to new generations of differentiating germ cells (9). The occurrence of activation of reserve stem cells after a gonadotoxic insult explains the observation that a single cytotoxic insult is less damaging to the seminiferous epithelium than are multiple insults of lower intensity, as shown by irradiation experiments in man and monkeys (10,11). Recently, it was shown that the relatively quiescent prepubertal testis of macaques also underwent stem cell depletion when exposed to irradiation (12).

OPTIONS FOR FERTILITY PRESERVATION IN THE MALE

Important progress in minimizing the unwanted side effects of oncological therapies has been achieved by constantly modifying and optimizing the therapeutic regimens. Without affecting the...
efficiency of oncological therapies and without compromising the cure rates of oncological patients, the modified treatment regimens allow many cancer survivors to father children following spontaneous recovery of spermatogenesis (13,14). However, some oncological diseases require rigorous treatment regimens, which will almost always lead to permanent infertility of the patient. Depending on the type of cancer and the choice of therapy, the patient has to be informed that the chances to maintain his fertility are quite limited. In contrast to the efficient treatment regimens the clinician can choose from to cure the disease, very few and limited options are available to prevent the loss of fertility.

The most reliable and time-tested options for fertility preservation in adult patients following oncological therapy are to cryopreserve gametes or embryos. Cryopreservation of semen has been used extensively in the past, and the success rates improved after the introduction of intracytoplasmic sperm injection (15,16). However, these techniques are available only for post-pubertal patients who can provide functional germ cells. Also, a retrospective study of 115 Hodgkin patients who cryopreserved sperm before receiving treatment revealed that only 33 had used the stored gametes and that only 11 of these cases culminated in live births (17). Furthermore, the cryopreserved samples are a finite resource and do not offer the possibility of restoring natural fertility.

These shortcomings are addressed, in part, by two developing assisted reproductive technologies. Testicular tissue from immature and adult patients can be collected and cryopreserved prior to cancer treatment. Since this tissue contains spermatogonial stem cells, the patients maintain their potential to use these cells at later time points to re-establish germ cell development in cases where no spontaneous initiation of spermatogenesis occurs after the patient has been cured of the disease. Two experimental procedures have been described in recent years, demonstrating how germ cells can be generated from a limited reserve of spermatogonial stem cells. In the first procedure, spermatogonial stem cell suspensions are isolated from testicular tissue and can be autologously retransplanted into the testes. The transplanted stem cells recolonize the niche on the seminiferous tubule basement membrane and ultimately generate complete spermatogenesis and mature germ cells. Each donor-derived colony of spermatogenesis arises from the clonogenic proliferation and differentiation from a single SSC (Fig. 1, A). This is the only technology that has the potential to restore natural fertility from a patient’s own germ cells. In a second procedure, testis tissue pieces are grafted to an ectopic site (e.g., under the skin) of cancer survivors or into animals. Thus, in contrast to the transplantation technique, where SSCs are removed from their cognate niches and transplanted to new niches in the recipient testes, testis grafting involves the transplantation of SSCs with their niches intact. The grafted testicular tissue is revascularized in the ectopic site and produces complete spermatogenesis. Both strategies have been developed in rodent animal models and show promise for development of clinical applications. Table 1 presents an overview of the current and the potential future applications for fertility preservation in male oncological patients reviewing the status, limitations, advantages, and disadvantages of each procedure. In the following paragraphs, the recent breakthroughs in germ cell transplantation and testicular grafting are summarized, and their potential application in a clinical setting is discussed.

**Germ Cell Transplantation**

About 10 years ago, Brinster and colleagues (18,19) harnessed the regenerative potential of SSCs and developed a spermatogonial stem cell transplantation technique in which testis cells from a fertile donor are transplanted into the testes of infertile recipients. Stem cells in the donor population give rise to complete and normal spermatogenesis in the testis seminiferous tubules of recipient animals. The extent of donor-derived spermatogenesis is dependent on the number of transplanted stem cells and the quantity and quality of stem cell niches in the transplanted testis. Therefore, in the laboratory the transplantation technique is a powerful functional assay to 1) characterize stem cell activity in donor testis cell populations, 2) evaluate the quantity and quality of stem cell niches in recipient testes, and 3) assess the effect of in vitro manipulations on stem cell function. In the clinic, the transplantation technique has potential application for restoring fertility that remains to be fully realized.
Spermatogenesis is well conserved in mammals, breeding confidence that the SSC transplantation technique that was originally described and optimized for mice might be extended to other species, including humans. While syngeneic (genetically identical) or allogeneic (same species, different individuals) SSC transplantations have been reported in mice (19), rats (20), pigs (21), goats (22), monkeys (23), and humans (24), establishment of fertility from donor stem cells has been reported only for mice (18, 25–28), rats (29, 30), and goats (31). Full implementation of the SSC transplantation technique in higher species will require careful consideration of stem cell isolation and preservation conditions as well as recipient preparation protocols to maintain the quantity and quality of stem cell niches. The best methods will be determined empirically on a species-by-species basis, but the ultimate evaluation will rest on the functional capacity to generate donor spermatogenesis and fertility in recipient testes.

Because stem cells have unlimited potential to self-renew and produce differentiating daughter cells, SSC transplantation offers the possibility of long-term restoration of natural fertility. Furthermore, evidence in mice and rats indicates that there is flexibility with respect to donor age because there are only modest differences in stem cell concentration among newborn, preadolescent, and adult testes (in fact, the highest concentration of stem cells is found in the preadolescent testis) and because the kinetics of colony expansion from each donor stem cell is equivalent, regardless of donor age (26, 30). In contrast, recipient age has a significant impact on donor germ cell engraftment. Based on the same number of transplanted donor cells, 9.5-fold more colonies of spermatogenesis are generated in preadolescent recipient mouse testes than in adult testes. In addition, each colony of spermatogenesis in the preadolescent testis was four times larger than in the adult. Thus, the overall extent of spermatogenesis in young recipient testes by two months after transplantation was nearly 40 times greater (9.4-fold more colonies × 4-fold-larger colonies) than in adult recipient testes (26). A similar situation appears to exist for the rat, although no quantitative information is available (20). However, despite the dramatic effects of recipient age on the efficiency of germ cell engraftment, previous studies demonstrate that high levels of donor spermatogenesis and fertility are generated in both preadolescent (26, 27, 29–31) and adult (19, 25) animals. Therefore, spermatogonial stem cell transplantation is a potentially viable method to preserve the fertility of men and has also great potential for prepubertal boys who currently have no fertility-preserving therapeutic options.

Capitalizing on the therapeutic potential of spermatogonial stem cells and the transplantation technique, Radford and colleagues (24) initiated a clinical trial in 1999 to test the hypothesis that “…human testicular cells might be harvested and cryopreserved before the start of chemotherapy and reintroduced into the testis on its completion.” Testis biopsies were obtained from 11 cancer patients and cryopreserved as single-cell suspensions prior to treatment. At the time of publication, the cells had been reintroduced into the testes of five patients who had successfully completed treatment. This autologous transplantation approach is feasible because spermatogonial stem cells from all species tested to date, including humans, can be cryopreserved and retain their biological activity even after long-term storage (32, 33). Moreover, in contrast to the complications associated with freezing mature gametes (eggs and sperm), spermatogonial stem cells are preserved using standard methods employed for somatic cell cryopreservation (34). The final proof in principle was provided last year when Kanatsu-Shinohara and coworkers restored fertility in infertile mice following transplantation of cryopreserved male germine stem cells (35).

Five years after the initial report by Radford et al. (24), the fertility disposition of the cancer patients of whom seven have now received cryopreserved germ cells after being cured has not been described and the follow-up of the patients is ongoing (36). Perhaps that outcome is not surprising, since the technology was in its infancy at the time and was still being optimized in rodents. However, enthusiasm for the therapeutic potential of the SSC transplant technology was enhanced by the work of Schlatt and colleagues (37), who demonstrated the feasibility of transplanting germ cell suspensions into the testes of nonhuman primates and dissected testes from men. Translation to each new species presents unique challenges but also opportunities to increase our understanding of stem cell biology and the testis environment. A preclinical study using macaques whose testes had been germ cell depleted by local irradiation (23) highlighted many of the challenges that will be encountered in the clinic. Some of the crucial steps for successful refertilization are the safe retrieval of sufficient testicular tissue before the cytotoxic insults, avoidance of ischemia, cryopreservation and thawing of cell suspensions or tissue, sorting of tumor cells or enrichment of stem cell spermatogonia, and efficient ultrasound-guided noninvasive transfer of germ cell suspensions into the rete testis. Responsible development of the transplantation technique in nonhuman primates that model the reproductive deficits of cancer survivors will provide new insights in an animal system that has relevance for human physiology. The results will be instructive for future clinical trials.
IN VITRO SPERMATOGENESIS AND TESTICULAR GRAFTING

Culture and in vitro maturation of ovarian follicles have successfully been developed in animal models and present clinically relevant approaches for fertility preservation in female oncological patients (38,39). In the male, spermatogonial stem cell expansion and meiotic entry appear to be blocked in cultures of testicular cell suspensions, and it is therefore impossible to apply the existing in vitro approaches to generate sperm for tumor survivors whose only remaining germ cells are diploid spermatogonia.

In females, cryopreservation and grafting of ovarian tissue have become an experimental tool to provide fertility protection in oncological patients (40–42). Grafting can be considered a specialized form of tissue culture in which the ectopic grafting site acts as a bioincubator for the grafted tissue. In contrast to in vitro approaches, blood supply to the grafted tissue is fully restored. We and others have shown that grafting presents a tool for generation of sperm from dissected pieces of immature and adult testicular tissue (43–49). In all studies using mouse-to-mouse grafts, the newborn mouse testicular tissue matured up to the level of complete spermatogenesis independent of the grafting site being ectopic (43–48) or homotopic (49). Most studies used castrated male recipients as hosts. However, spermatogenesis in syngeneic mouse grafts was restored to a similar degree when intact recipients of either sex were used (48). Histological analysis of the grafted tissue shows that first elongating spermatids are present after 4 weeks of grafting and that up to 20% of the seminiferous tubules contain elongated spermatids 3–4 months after grafting (43). A dilation of the lumen and sloughing of differentiating germ cells indicate an accumulation of fluid in the seminiferous tubules (43), an effect that was less obvious in female recipients (48). These features are similar to the damage seen after efferent duct ligation and indicate that an optimal time window exists when mature sperm can be retrieved from mouse grafts (43). Sperm retrieved from grafted mouse tissues was used to generate healthy offspring with assisted fertilization techniques (44,49). Grafting was also successful when we and others used tissue from hamsters, pigs, goats, calves, rabbits, and monkeys (43,45–47,49). In contrast to mouse grafts, the xenografts showed a lesser tendency to undergo tubular atrophy associated with the accumulation of fluid in the seminiferous tubules.

We and others described an excellent efficiency of spermatogenic restoration in testicular tissue grafts from various nonprimate species, which had been cryopreserved prior to grafting (43,45,49). These results indicate that the cryopreservation of testicular tissue is a powerful tool when combined with the grafting procedure. In a recent attempt, we cryopreserved and grafted testicular tissue from a juvenile rhesus monkey. Figure 1, B, shows the histology of the grafted testicular tissue, which was retrieved 9 months after the grafting procedure and had been cryopreserved for several months prior to grafting. At this stage of development, the tissue shows initiation of spermatogenesis up to the level of primary spermatocytes. In light of a previous study analyzing the timing of initiation of spermatogenesis in xenografts of noncryopreserved juvenile monkey tissue (46), we conclude that the combination of cryopreservation and grafting will allow the generation and collection of sperm from primate species.

In contrast to immature tissue, grafting of adult testicular tissue appears to be less promising (45). Although adult tissue grafts contained sperm, it remains questionable whether these sperm were generated after partial recovery of spermatogenesis or whether they remained in the grafted tissue while most of the other germ cells degenerated. Since the data on the success of

![Flowsheet showing the decision tree to determine the appropriate application for fertility preservation in male oncological patients.](image-url)
grafting of adult tissue are limited, more studies are needed to judge the efficiency of restoring spermatogenesis in adult testicular tissue. The testicular tissue of all species with the exception of the marmoset (45) replaced the castrated host with androgens, indicating that not only the spermatogenic but also the steroidogenic function of the testicular tissue is restored in the grafts. In conclusion, grafting as an autologous or xenologous procedure has the potential to become a clinically relevant approach for fertility preservation in prepubertal boys.

Before this procedure can be applied routinely in patients, preclinical studies and clinical trials using testicular tissue from monkeys and humans have to be performed to reveal whether autografting or xenografting might be efficient and safe approaches to generate male gametes from immature testicular tissue.

**CONCLUSIONS**

From our current knowledge, we can conclude that recovery of spermatogenesis in cancer survivors will depend on the ability of mitotically quiescent stem spermatagonia to transform into actively dividing stem and progenitor spermatogonia. Since the absence of proliferation renders the reserve stem cell less vulnerable to cytotoxic drugs, the capacity to recolonize the testis depends on the number of reserve stem cells that pass the quality control checkpoints for genetic integrity and successfully undergo self-renewal to replenish the population of actively dividing spermatogonia. In those areas of seminiferous tubules in which the stem cell pool is replenished, spermatogonia will start to produce differentiating progeny. In this regard, further improvement of oncological therapeutic regimens towards less-cytotoxic drugs will allow more patients to preserve their testicular stem cell pool and enable spontaneous recovery of spermatogenesis. However, in patients exposed to intense chemotherapeutic regimens patients will initially generate small foci of spermatogenesis, generating only limited amounts of sperm that do not reach the rete testis, the epididymis, or the ejaculate. These patients may be able to father children by retrieval of sperm through application of testicular sperm extraction and in vitro fertilization of oocytes from the partner. In patients showing partial recovery, the areas of ongoing spermatogenesis may grow by ongoing self-renewal and migration of stem cells, leading to a more extended recolonization of seminiferous tubules and finally restoration of fertility. The enormous capacity of the spermatogonial stem cell to recolonize the testis is the reason that on some rare occasions fertility is re-established even many years after oncological therapy.

If all germine stem cells have been depleted after intense cancer therapy, no chances for spermatogenic recovery are available as yet. In principle, generation of spermatogonial stem cells could be achieved by germine transformation of other types of stem cells. Recently, generation of germ cells has been achieved from embryonic stem cells, opening new and fascinating scenarios for future research (50–52). However, the strategies to generate new germine stem cells are as premature as they are exciting, and it will take several years to explore whether safe, efficient, and ethical methods can be established to apply this tool in a clinical setting. If germ cell transplantation and testicular grafting become available, the oncologist will have several choices for the protection of male fertility. The decision on the most appropriate gonadal protection treatment could then be made according to a decision tree as depicted in Fig. 2.

In those patients whose oncological therapy regimens let the clinician predict a complete depletion of testicular stem cells, the outlined approaches of germ cell transplantation and testicular grafting might soon be available to provide options for fertility preservation. Although these techniques must still be considered experimental, the promising results in rodents and nonhuman primates indicate the potential for clinical application. While further research is needed especially in nonhuman primate models, the cryopreservation of testicular cells and/or tissue should be considered an important aspect of current oncological therapy. Responsible development of these new reproductive technologies will increase the fertility options for men following successful cancer treatment. Furthermore, preservation of testis tissue for today’s prepubertal cancer patients will allow them to consider the various fertility options that will be available in 5–15 years and to use their cryopreserved samples to generate sperm and father children with their own genetic heritage.

**REFERENCES**


NOTES

The authors appreciate the support of the Pittsburgh Development Center of Magee-Womens Research Institute, the Deutsche Forschungsgemeinschaft (Scho 394/3, 394/6), and the University of Pittsburgh School of Medicine. Kyle Orwig is supported by the National Center for Research Resources grant 1 R01 RR018500–01, NIH, and the Pennsylvania Department of Health. We appreciate the collaboration with Ina Dobrinski (1 R01 RR17359–01) and Tony M. Plant (R01 HD 13254 and U54-HD-08610).