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New advances on the expansion and storage of human spermatogonial stem cells

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Abstract

Purpose of review—Fertility in adult life can be severely impaired by gonadotoxic therapies, and with remarkable advancements in the treatment of childhood cancers there is a growing population of adult survivors of childhood malignancies. The aim of the review is to review the developments that have been made in spermatogonial stem cell research and potential future utility in fertility preservation.

Recent Findings—While intense interest and subsequent research surrounds the regenerative potential of spermatogonial stem cells, a recent article highlights the in vitro propagation of human spermatogonial stem cells from testicular biopsies for future transplantation and restoration of fertility. While in vitro propagation of spermatogonial stem cells has been established in animal models this is the first report in humans.

Summary—Spermatogonial stem cell transplantation began as a theoretical approach that currently is studied ardently by several research groups to make this a valid clinical option. Restoration of fertility following spermatogonial stem cell transplantation in animals suggests therapeutic potential for the technique in humans, and further research is proceeding to address the safety and efficacy of this technique.

Keywords

spermatogonial stem cells; male infertility; fertility preservation

Introduction

Spermatogenesis is an elaborate and coordinated process by which thousands of spermatozoa are produced daily within the male gonad or testis¹. This highly complex and coordinated process results in the production of spermatozoa in order to transmit male genetic information to successive generations. However, it is also a process in which terminally differentiated cells are continually produced and lost. The process of continual spermatogenesis is dependent on an adult tissue-specific stem cell population known as spermatogonial stem cells (SSC). Stem cells derived from adult tissues (somatic stem cells) also maintain the self-renewal properties necessary for the prevention of local tissue degeneration. These adult stem cells are present in many somatic tissues, including hematopoietic, hepatic, neural, epithelial, muscle, as well as the testis.

SCCs are the adult stem cell population of the testis, and they provide the capability for the high productivity of spermatogenesis. They are unique because they can be manipulated to regain the characteristic of pluripotency². When cultured under specific conditions they behave like embryonic stem cells and express transcription factors associated with pluripotency³. Although their presence was first described by Huckins⁴ in 1971, SCC transplantation was introduced in the mouse in 1994 by Brinster et al. as an assay for stem cell function^{5, 6}. These studies serve as stepping-stones for research in male germline stem cells and hope for future translation to humans.

Realistically, the potential utility of somatic stem cells is limited due to their restricted degree of potency. Therefore they cannot trans-differentiate into any cell of the body. Putative stem cells are defined by functional characteristics such as self-renewal, proliferation, differentiation, and tissue regeneration. Observing all or some combination of these attributes is difficult experimentally because this involves removing the stem cells from their niche, the highly specialized microenvironment that supports stemness. Within the niche, stem cells continuously self-renew and once they leave the niche, stem cells commit to a differentiation pathway influenced by the cellular milieu.

The SSC Microenvironment

SSCs are rare cells within the seminiferous epithelium. It is estimated that they constitute approximately 0.03% of the spermatogonia in the testis⁷. As proposed for stem cells of the intestinal crypts⁸, SSCs may include both populations of true adult stem cells, as well as potential stem cells⁹. Together these cells contribute to sustained reproductive capability in males. Evidence supports the theory that a population of potential SSCs can function as actual SSCs depending on the cell context in which they are studied⁹. This implies that investigations of the structural and molecular characteristics of the SSC niche must first define the SSC population maintained within the niche.

The niche is simply defined as the region of the seminiferous epithelium where SSCs reside and which supports their proliferation and self-renewal throughout the life of the male. Similar to other stem cells, SSC plasticity is maintained within the niche. Once they leave the niche, the SSC self-renewing capabilities cease and they begin to exhibit a differentiated phenotype and eventually become mature spermatozoa¹⁰. In the seminiferous tubule, the control of spermatogonial proliferation, self-renewal and differentiation imply the existence of regions of the basal compartment that defines the actual SSC niche. The molecular characteristics of the basement membrane provided the basis for early attempts to identify and enrich for SSCs.

The seminiferous tubules and surrounding interstitial tissue are a composite of cells, which synthesize and secrete various hormones, growth factors and cytokines to achieve spermatogenesis. These factors regulate SSC self-renewal and proliferation within their niche as well as SSC differentiation once they leave their niche. There is no doubt that Sertoli cells establish and maintain the SSC niche because fertility can be restored upon Sertoli cell transplantation into the seminiferous tubules of infertile mice¹¹.

Spermatogenesis is dependent on the functions of SSCs, whose self renewal and differentiation are supported externally through niche stimuli and internally through the regulation of specific gene expression.

Applications to Male Infertility

The tremendous regenerative potential of SSCs lead to the logical hypothetical option of restoring fertility in patients with secondary infertility due to exposure to gonadotoxins such

as chemotherapy or radiation therapy for cancer. SSC transplantation began as a theoretical approach that currently is studied ardently by several research groups to make this a valid clinical option. Certainly, adult men could cryopreserve sperm in an attempt to preserve their fertility. However, most couples would prefer a naturally conceived child without the use of costly assisted reproductive technologies. In addition this method of fertility preservation will exclude prepubertal patients. Rejuvenation of a “barren” seminiferous epithelium damaged by gonadotoxic treatment with transplantation, engraftment and colonization by SSCs offers the potential that natural fertility could be restored for these patients.

With drastic improvements in the treatment of childhood cancers more attention has been given to the quality of life of cancer survivors. For all childhood cancers the 5 year overall survival rate of adolescents has considerably improved over the past 4 decades from less than 50% to nearly 80%, due to improved therapies and supportive care¹². While cryopreservation of sperm for preservation of fertility in men undergoing gonadotoxic treatment is well established, translating this effective method to adolescents and even further to prepubescent males has been more challenging. Cryopreservation of sperm before the initiation of gonadotoxic treatment is currently the best method of preserving fertility, however if the young patient is unable to provide a semen sample, electroejaculation or surgical sperm extraction can be performed¹³. Alternatively, cryopreservation of SSCs before the start of any cancer therapy followed by autologous intra-testicular transplantation of these cells after cure offers potential for preserving fertility⁵.

SSC transplantation was first introduced in the mouse in 1994 by Brinster et al. as a functional stem cell assay^{5, 6}. Spermatogonia from a fertile donor mouse were injected into the seminiferous tubules of an infertile recipient. The donor spermatogonia colonized the seminiferous tubules of the recipients and, in some cases, induced active spermatogenesis. Soon after these first studies, further experiments were performed by different groups, using both fresh and frozen-thawed samples in different species, including primates^{14–16}. These promising results suggest that this method might also be successful in the human and that, if this technique could be clinically applied, it may be able to restore fertility^{17, 18}.

Efficiency and Safety

The efficiency of SSC transplantation is associated with the number of stem cells injected¹⁹. Therefore, enrichment and propagation of stem cells *in vitro* for transplantation may be essential to allow for full repopulation of the testis in clinical translation. The initial method for transplantation in mice was intratubular microinjection at multiple sites within the seminiferous tubule⁶. Other methods of injection, such as direct injection into the efferent duct or rete testis have also been proven as efficient^{20, 21}. The most promising technique in primates and humans is ultrasound-guided injection into the rete testis²², which was later modified to include a drip feed of the solution under gravity²³. With this modified technique, after injections at multiple sites, approximately 55% of the tubules of human cadaver testes were filled.

While live offspring have been produced using this technique of SSC transplantation, safety concerns stem from the observation that litter sizes after *in vivo* conception compared to normal control mice were reduced in comparison and displayed minor developmental delay^{24, 25}. The same group also noted a decrease in motility patterns of spermatozoa analyzed after transplantation²⁶ making further investigations mandatory prior to any clinical application.

However, one major concern associated with autologous transplantation in cancer patients is the reintroduction of malignant cells. Most malignancies in children are capable of

metastasizing hematologically, making the risk of contamination of the testicular cells considerably high²⁷. The majority of studies addressing this concern involve magnetic or fluorescence-activated cell sorting and capitalize on the ability to identify specific SSC markers in rodent models²⁸. Studies in human models have reported inconsistent results, complicated by the fact that specific SSC markers in humans have not been completely established^{29, 30}.

Another aspect of this concern centers around the level of decontamination required for the testicular tissue as well identifying the threshold number of malignant cells able to cause malignant relapse when transplanted. In the rat model, transplantation with as few as 20 leukemic cells could cause malignant recurrence in the recipient model³¹. The inability to identify the level of decontamination in humans makes the detection of any level of contamination crucial. Molecular techniques, within the hematological arena have been developed with the ability to identify one malignant cell among 10⁶ normal cells³². The most sensitive of these techniques utilizes PCR³³. Patients with non-malignant hematological diseases, such as sickle cell disease would be prime candidates to potentially benefit from SSC transplantation.

Cryopreservation

In the clinical setting, treating pediatric patients makes cryopreservation of tissue or cell suspensions a necessity. The likelihood of a period more than 5 years elapsing between the removal of testicular tissue and autotransplantation is quite real. Establishing an optimal protocol for cryopreservation of tissue or cell suspensions specifically targeting SSC transplantation would be incredibly advantageous. The first successful SSC transplantation after cryopreservation in the mouse model¹⁴ was reported in 1996, followed by reports of successful transplantation with frozen/thawed testicular cell suspensions of rabbit, dog and hamster^{34–36}. Recently, other groups have looked at perfecting protocols preserving the niche cells together with the spermatogonia³⁷ as well as protocols for preserving the structural integrity of pre-pubertal human testicular tissue^{38, 39}. Defining the ideal cryopreservation protocol would be of great benefit for fertility preservation.

In vitro culture of spermatogonia

Testicular biopsies currently do not contain enough SSCs to fully repopulate the testis after transplantation making *in vitro* propagation necessary to obtain an adequate number of cells for successful transplantation. Culturing stem cells *in vitro* could potentially increase the efficiency of the transplantation technique by increasing the number of pure stem cells to be injected. Nagano et al. first reported the long-term survival of both mouse and hamster spermatogonial stem cells *in vitro*^{40, 41}. Another group later demonstrated not only long-term survival but also proliferation of SSCs⁴².

While successful autotransplantation of SSCs was achieved in a wide range of species, these SSCs can also home to the basal membrane of seminiferous tubules of immunodeficient mice after transplantation allowing for a pre-clinical functional assay^{43–45}. While several groups cultured and propagated mouse SSCs *in vitro*, it was only until very recently that this was accomplished in humans. Extrinsic factors for self-renewal appear to be conserved among many mammalian species⁴⁶ allowing defined culture systems developed in mouse models to be applicable with modifications to humans.

Sadri-Ardekani et al.⁴⁷ isolated and cultured testicular cells from 6 adult men who underwent orchiectomy for prostate cancer treatment. The tissue was initially cryopreserved and thawed to more closely resemble anticipated future practice of SSC autotransplantation in prepubertal patients. The presence of spermatogonia was determined by RT-PCR and

immunofluorescence for specific spermatogonial markers. Xenotransplantation to testes of immunodeficient mice was performed as the functional assay and migration of SSC after transplantation was confirmed by fluorescent in situ hybridization. They were able to demonstrate culture and propagation in all men up to 15 weeks and expression of spermatogonial markers was maintained throughout the entire culture period. The ability to provide long-term culture and propagation of human SSCs *in vitro* is a remarkable leap forward in the pursuit of clinical application.

In recent years, ectopic grafting of immature testicular tissue from various mammalian species under the skin of immunodeficient mice has been developed as a newer strategy for preserving testicular function and even subsequent generation of mature spermatozoa^{48, 49}. Despite initial success with these techniques, these approaches are associated with significant risks of malignant relapse as well as potential infectious transmission to eventual patient. However, xenografting might represent a viable approach for fertility preservation in cancer patients as well as provide a model for the study of human spermatogenesis once safety, efficacy, determination of appropriate donor tissue, and optimal host conditions are addressed.

Alternatively, the generation of gametes from embryonic stem cells is currently being explored as a potential treatment for infertility. Multiple groups described how they obtained sperm-like cells from mouse embryonic stem cells *in vitro*^{50, 51} with even subsequent early embryonic development after injection of the sperm-like cells into natural mouse eggs. While the majority of the work has been established in animal models, reports of *in vitro* derivation of human sperm from embryonic stem cells have been published, though the results are still quite controversial^{52, 53}. Current investigations have also reported successful derivation of primordial germ cells and Sertoli cells from human embryonic stem cells⁵⁴. Although embryonic stem cells provide an almost limitless source of cells for regenerative possibilities and applications, ethical concerns limit their use.

Offering human SSC autotransplantation as an option for fertility preservation to patients becomes more tangible everyday. There are institutions that recognize the realistic potential of this being a valid option for patients in the near future, so much so that they have begun to offer cryopreservation of testicular tissue in hopes that within the next ten years science will have solved all of the intricacies to revolutionize the ability to preserve fertility.

Once this technique becomes a reality one of the first difficulties will be obtaining informed consent. Moreover the testicular biopsy itself is an invasive procedure with potential complications. The issue of informed consent and assent of minors and the possibility that parental decisions may not reflect the patient's wishes when they reach adulthood complicate the matter of fertility preservation in children⁵⁵. However, as always the mental and overall well-being of the child will continue to be considered a priority.

While autologous cells are recognized as the ideal transplantation resource, some patients with end-stage organ disease do not produce enough cells for transplantation. Alternative methods of obtaining stem cells and different techniques for generating pluripotent stem cells are currently under investigation and include approaches such as somatic cell nuclear transfer and altered nuclear transfer^{56, 57}. These derived pluripotent stem cells, while still largely not possible in humans, are envisioned as an alternative source of cells from which the desired germline cells can be derived.

The quest for other sources of adult stem cells has fostered interest in hematopoietic stem cells and the emergence of adipose tissue derived stem cells. Hematopoietic stem cells have long been used therapeutically as early as 5 decades ago with bone marrow

transplantation⁵⁸. More recently researchers have been able to demonstrate generation of male germ cells from bone marrow stem cells⁵⁹.

While a relative amateur in the ranks of stem cell research, it is well established that ADSCs possess all stem cell properties and are virtually identical to bone marrow stem cells⁶⁰. During the last decade intense research into their potential has progressed to where the list of ADSC differentiated cell types has expanded to include endothelial, epithelial, muscle, hepatocytes and neurons as well. With these distinctive attributes, ADSCs have been reviewed in their potential application in urology, specifically in their recovery of erectile function, male fertility and even hypogonadism⁶¹.

Conclusion

Extraordinary advances have been made over the past decade in our understanding of male germline stem-cell biology. These new techniques and methods that have been developed in both animal and human models hold great promise in treating male infertility. The next critical advances will be human SSC *in vitro* differentiation of these cultured stem cells into functional spermatozoa, as well as addressing the potential for human SSC culture in pre-pubertal patients.

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