Advances in mammalian spermatogonial stem cell transplantation *

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Abstract Spermatogonial stem cell (SSC) transplantation is a novel technique by which testicular cells from normal, transgenic or mutant donor are introduced into the seminiferous tubules of recipient testes through microinjection. Subsequently, donor SSCs survive, migrate, anchor and proliferate in the recipient testis, furthermore, initiate spermatogenesis and even produce sperms capable of fertilization. This technique provides a new approach for the researches of spermatogenesis mechanism, regeneration of spermatogenesis in sterile individuals and reproduction of transgenic animals. This review focuses on the methodological breakthroughs and highlights the recent findings that have substantially increased understanding of SSC biology. The article provides a comprehensive overview of this technique and its multiple applications in basic science and medicine. And the perspective direction of this field in the near future is proposed.

Keywords: spermatogonial stem cell, transplantation, application.

The maintenance of normal spermatogenesis relies on the self-renewal and differentiation of spermatogonial stem cells (SSCs) that reside in the basal compartment of the seminiferous epithelium. SSCs of normal animals, including human, can produce new stem cells by self-renewal and generate differentiated germ cells of all types. The ratio of stem cells to differentiated germ cells which both derived from one SSC relates to certain conditions of physiology and environment[1]. Individuals can be sterile owing to a lack of SSCs, the blockage of germ cells development or production of nonfunctional sperm.

The SSC transplantation technique developed recently has offered hope for these infertility patients and afforded a new way to investigate fundamental aspects of spermatogenesis. Brinster and his colleagues first transferred a germ cell mixture from a fertile mouse into the lumens of the seminiferous tubules of a sterile mouse, and the results showed that not only did stem cells colonize the recipient testis, but they also initiated spermatogenesis and produced sperms capable of fertilization. Subsequently, they introduced rat testicular cells into the seminiferous tubules of an immunedeficient mouse and discovered rat sperms in the recipient mouse testis, which was considered the breakthrough of xenogeneic sperm production[2]. The development of SSC transplantation technique has contributed to researches in SSC biology[3], the identification of cell types contributing to sterility within specific mutants[4,5], and researches of cellular interactions in the testis[6]. Moreover this technique provides some new chances for treatment of male infertility, preservation of the germ line of valuable or endangered animals and replacement of the germ line of patients whose endogenous SSCs have been deleted by chemotherapy. The present review examines what is known about SSC transplantation, details the recent technological achievements and the lessons learnt from using them, and discusses the possible future uses of this new approach.

1 Preparation of donors and recipients

Mixed germ cells containing SSCs can be microinjected into the seminiferous tubules of recipient testis in the mouse. In other animals, a micro-needle can be inserted through the efferent ductules outside the testis and passed into the rete testis. Trypan blue is added to the injected cell suspension so that filling of the seminiferous tubules can be monitored conveniently. Animals carrying genetic markers or the sperm morphology different from the recipient sperm should be selected as the sources of donor germ cells so that the development and differentiation capability

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of donor SSCs can be identified and assayed easily. At present, animals carrying fluorescent marker (GFP) or *LacZ* gene are generally selected as the donor animals in most reports. Thereby, one or more spermatogenesis cycles after transplantation, the survival, proliferation and differentiation of donor SSCs in recipient testis can be readily detected under a fluorescent microscope or with X-gal staining[7~9].

Similarly, to make the analysis of transplantation results easy, animals without endogenous spermatogenesis, such as the W/W^V mutant, or animals whose germ cells have been eliminated artificially can be used as recipients. The W/W^V mutation affects the gene—the *White spotting* (*W*) locus of the mouse, which encodes the membrane-bound tyrosine kinase, c-kit. The latter serves as a receptor for stem cell factor (SCF/kit ligand) which is the gene product of the *SL* locus. SCF is expressed in testicular Sertoli cells. The interaction between c-kit and SCF is essential for SSC proliferation within the seminiferous tubules. In the testes of mutant homozygous for either W/W^V or SL/SL^d, no germ cells and few SSC are present virtually in the seminiferous tubules. Although both W/W^V and SL/SL^d have the same phenotype, their mechanism is different. The absence of differentiated germ cells in W/W^V is due to the deletion of SSCs, whereas in Sl/SL^d is due to the environment defect, the defects of Sertoli cells[3]. Alternatively, recipients can be treated with the chemotherapeutic agent busulfan so that most germ cells can be deleted from the testis. However, a small number of endogenous SSCs persist and can reinitiate spermatogenesis. Therefore, using transgenically marked donor cells will help distinguish donor-derived spermatogenesis from endogenous spermatogenesis[7~10].

In addition, immunologic rejection between donor cells and recipient animals should also be considered when SSC transplantation performs. Animals with compatible major histocompatibility antigen can generally be used as donor and recipient when isotypic transplantation occurs, whereas immunodeficiency animals, such as nude mice, should be used as recipients or employing injection of immunosuppressive agents to inhibit immunologic rejection after transplantation when xenogeneic transplantation is performed[11].

2 Colonization of donor SSCs in recipient testis

Investigations have indicated that the colonization of transplanted SSCs in recipient testis occurs in three continuous phases. The first phase occurs during the first week after transplantation. During this period, transplanted cells are randomly distributed throughout the seminiferous tubules, and a small number of transplanted SSCs have migrated to the basement membrane of the seminiferous epithelium. When spermatogonia enter meiosis and differentiate into spermatocytes, the tight junctions are broken down and reform to allow spermatocytes to translocate apically, complete meiosis and differentiate into spermatids there. Researches have demonstrated that when germ cells are introduced into recipient seminiferous tubules, SSCs translocate through the tight junctions, towards the basement membrane of the seminiferous tubule. This result implies that Sertoli cells recognize SSCs and direct their migration in a retrograde manner[6,10,12].

The second phase occurs one week to one month after transplantation. During this phase, stem cells proliferate longitudinally in the seminiferous tubule, forming a network or chain of donor cells. The differentiation of donor cells occurs during the third phase that begins one month after transplantation. During this phase, donor SSCs have established colonies of spermatogonia and continue to expand laterally along the tubules. The production of Spermatozoon begins two months after transplantation. Spermatogonia colony expansion continues, the number of colonized sites dose not change between one and four months after transplantation, but the length and width of donor-derived colonies increase continually. After three months, about 30% tubules of the recipient testis contain donor-derived spermatogonia. Generally, 19 donor-derived colonies were generated when 10^6 cells were injected into a recipient testis. Most injected cells remained in the intraluminal compartment and were phagocytosed by Sertoli cells, only approximately 20 stem cells independently survived, colonized the recipient testis and initiated donor-derived spermatogenesis[6,10,12].

3 The factors affecting the efficiency of colonization after SSC transplantation

Many factors, such as the number of injected cells, the enrichment level of SSCs in donor cells, preparation of recipients, the usage of hormone analog and so on, can affect the colonization efficiency of SSC transplantation. Generally, the more donor cells are injected, and the higher the enrichment degree of

SSCs in injected cells reached, the higher colonization efficiency will be achieved. Applying animals of endogenous germ cells deleted by chemotherapeutic agent busulfan or the animals of artificial cryptorchidism as recipients can apparently enhance the colonization efficiency of injected SSCs[10]. It is demonstrated that using the gonadotropin-releasing hormone (GnRH) agonist, leuprolide, could greatly enhance colonization after SSC transplantation. Mouse testis injected with leuprolide had markedly enhanced donor cell colonization, not only in the efficiency of colonization but in the lateral expansion of donor-derived colonies. Leuprolide might have acted directly on germ cells or reduced the concentration of luteinizing hormone or testosterone. However, no GnRH receptors have been found on germ cells or cells associated with the seminiferous epithelium. Therefore, it is unlikely that leuprolide had a direct effect on spermatogonial colony formation and proliferation. It was noted that the suppression of testosterone by leuprolide or by the negative feedback initiated by exogenous testosterone might stimulate spermatogenesis after irradiation damage, and that high levels of testosterone might inhibit spermatogenesis. These data suggest that leuprolide might have made an environment in the testis more permissive of spermatogenesis after transplantation by decreasing the intratesticular testosterone concentration. Thus, the action mode of leuprolide is probably indirect, and a study to examine the effect of testosterone antagonists on SSC transplantation seems warranted[10, 13, 14].

4 Application of SSC transplantation

Great progress in research of SSC transplantation was the regeneration of spermatogenesis from cryopreserved cells. Studies have indicated that testicular cells from both repubertal and adult mice, frozen for 4 ~ 156 days with the techniques similar to those generally used for somatic cell cryopreservation, could establish spermatogenesis after thawing and transplantation[10, 15]. The potential value of the application of this technique lies in that: (1) cryopreservation of SSCs from valuable or endangered animals would allow regeneration of the germ line at any time in the appropriate host; and (2) clinical chemotherapy or radiation treatment often produces reversible or irreversible depression to the spermatogenesis of human individuals. Thus, testicular biopsies of these patients can be cryopreserved before treatment and transplanted at a later date to regenerate spermatogenesis.

SSC transplantation can also serve as a functional assay system to evaluate the success of the long-term culture of germ cells so that the self-renewal and differentiation capabilities of SSCs after culture can be determined exactly. Researches indicated that mice germ cells maintained on STO (SIM mouse embryo-derived thioguanine and ouabain-resistant fibroblast cell line) cell feeder layers of 19 ~ 132 days successfully generated donor-derived spermatogenesis in recipient testis after transplantation. These feeder layers provide an environment that supports SSCs survival *in vitro*[10, 16, 17]. Studies on SSC transplantation suggested that germ cells in many animals maintained on STO cell feeder layers for some time can successfully initiate donor-derived spermatogenesis in recipient testis after transplantation.

Combining the SSC culture techniques with SSC transplantation, genome of germ cells can be manipulated to produce transgenic animals or to treat some genetic diseases by gene therapy. At normal body temperature, differentiated or differentiating germ cells die, whereas somatic cells and SSCs in testis are unaffected. Thus, cryptorchid testes contain a higher proportion of SSCs compared with wild-type controls. In the testis of neonatal animals, there are only SSCs and Sertoli cells in the seminiferous epithelium because SSCs are still undifferentiated. Thus, the testes of neonatal animals also possess a higher proportion of SSCs. Several approaches have been used in the transgenic researches of germ cells from the testes of neonatal animals, and from cryptorchid testes of adult animals mediated by the retroviral vectors. All methods resulted in stably transfected stem cells capable of colonizing recipient testes, and with the periodic infection system producing the highest level of infection. Donor-derived spermatogenesis continually produced positive sperms carrying the transfected gene[18]. In addition, co-injection of retroviral particles and fresh germ cells into recipient testes also resulted in integration of the reporter gene. These results demonstrate that foreign genes can be introduced into the male germ line directly.

SSC transplantation can also be used for the isolation and characterization of SSCs. Up to now, the mechanisms of SSC proliferation and differentiation are still poorly understood due to a lack of a proper method to detect, identify and separate these unique cells. The establishment and development of SSC transplantation now provides a functional assay for SSCs. It allows conclusive verification of the presence

and quantification of SSCs within a sample based on the number and area of colonies derived from injected germ cells. Therefore, we can firstly hypothesize that SSCs express a certain kind of molecular marker, then screen SSCs from the cell suspension of seminiferous epithelium based on this marker, subsequently verify the presence and quantification of SSCs according to the number of colonies and the area of the seminiferous tubule colonized after the transplantation of screened SSCs, and finally determine whether this molecule can serve as a SSC marker. Using this strategy, it is demonstrated that the antigenic phenotype of SSC is Thy-1$^+$, α_6 and β_1 integrin$^+$, CD24$^+$, CD9$^+$, (MHC-I)$^-$, c-kit$^-$, α_v integrin$^-$, Sca-1$^-$, CD34$^-$. Using SSC transplantation as a functional assay for the presence of SSCs, cells recovered from cryptorchid testis were enriched for stem cells 25- and 50-fold based on the number of colonies and on the area the seminiferous tubule colonized, respectively[1, 5, 19, 20].

5 Xenogeneic SSC transplantation

The most remarkable aspect of this research is the ability of the testis of one species to support spermatogenesis of germ cells from another species in varying degrees. Thus, xenogeneic or cross-species SSC transplantation provides a new approach to further investigation of spermatogenesis mechanism. This technique can also be employed to determine whether the cycle of spermatogenesis is controlled by germ cells or Sertoli cells in xenogeneic transplants. Generally, the spermatogenesis cycle takes about 35 days in mice, whereas 52 ~ 53 days in rats. It is demonstrated that rat germ cells developed at their characteristic rate of 52 ~ 53 days in the mouse testis, while mouse germ cells developed at their characteristic rate of 35 days in the same testis. These results suggest that the rate of spermatogenesis is controlled by germ cells and a testis can maintain spermatogenesis at two different rates simultaneously[2, 8, 10].

However, xenogeneic SSC transplantation is also limited by some factors, especially the kinship distance between donor and recipient. Hamster germ cells injected into immunodeficient mouse testes colonized the recipient testis and hamster spermatogenesis developed. However, the donor-derived spermatogenesis abnormally developed in the seminiferous tubules, and hamster sperms were present in the epididymis without acrosomes, heads and tails. After transplantation of rabbit and dog testicular cells into immunocompromised mouse testes, the colonization and spermatogenesis derived from donor SSCs also occurred, but cells from either species did not progress into later stages of spermatogenesis. In goat, bull, monkey and human testis, ultrasound guided intratesticular rete SSCs injection is performed generally. Testis cells were transplanted from donor goats carrying a human alpha-1 antitrypsin gene to the testes of sexually immature wild-type recipient goats. After puberty, sperms carrying the donor-derived transgene were detected in the ejaculates of 2 out of 5 recipients. Mating of one recipient resulted in 15 offspring, one of which was transgenic for the donor-derived transgene[21]. Cynomolgus monkeys were treated with a GnRH antagonist before germ cell injection, and then the recipients were injected with testicular cells labeled with BrdU. Labeled cells were identified four weeks after transplantation in the testicular interstitium and in some seminiferous tubules of the recipient[22].

6 Infertility and gene knock-out studies

SSC transplantation can also be used for the investigation of causes of infertility occurring as a result of a natural mutation or targeted gene deletion. Sometimes gene knockout experiments will lead to a lack of SSCs, blockage of germ cell development or production of nonfunctional sperms, thereby resulting in male sterility. It is difficult to identify testicular cell type(s) in that the disrupted gene is phenotypically important before the establishment of SSC transplantation technique. Employing this novel approach, transplantation of germ cells carrying the disrupted gene into wild-type recipients can provide this information. Ogawa et al. transplanted SSCs from infertile mice carrying the *SL* mutation into infertile, white spotting (W/W^V or W^V/W^{54}) mutant mice; the recipient mice were shown to be fertile[10, 23]. It implies that fertility of recipient was restored after transplantation of SSCs from a sterile donor into an infertile recipient that had normal Sertoli cells.

Another naturally occurring mutation in mice that affects spermatogenesis is the juvenile spermatogonial depletion (*jsd*) mutation. A mutant mouse undergoes a single wave of spermatogenesis, followed by a failure of SSCs to replenish the testis. When germ cells from *jsd* animals were injected into W/W^V or busulfan-treated recipients, no donor-derived spermatogenesis was observed. In contrast, the injection of non-*jsd* germ cells into *jsd* recipients indicated

that *jsd* animals could support donor-derived spermatogenesis. These data suggest that the *jsd* phenotype results from a defect in the germ cells, but not in the testis somatic cells[10, 24].

The first report of using SSC transplantation to correct sterility owing to gene knockout was achieved in animals in which estrogen receptor α (ERα) had been deleted. Male ER deficient (αERKO) mice are sterile; however, when germ cells from αERKO mice were injected into wild-type testis, normal donor-derived spermatogenesis developed and functional sperms were produced that carried a normal copy of the gene encoding αERKO. Thus, these results demonstrated that a gene knockout that disrupts spermatogenesis has no deleterious effects on germ cells[10, 25].

7 Perspective

SSC transplantation will contribute to a better understanding of spermatogenesis mechanisms. Its clinical applications might provide a new method for the treatment of male infertility. This technique will also allow scientists to combine various germ cell and Sertoli cell mutants so that somatic and germ cell's function can be examined exactly. It is also useful to apply this technique to preservation of the germ line of economically valuable animals, older animals unable to breed naturally and endangered species. And what is more, SSCs are the only cells in the postnatal animals that undergo self-renewal throughout life and transmit gene to subsequent generations. Thus, the ability to cryopreserve, culture, and transplant these unique cells provides a powerful biological tool for studying testicular function, SSC biology, preserving individual genomes, and modifying germ lines.

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