# Transplant Therapies for Male Infertility

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## Introduction

Chemotherapy and radiation treatments for cancer or other conditions, such as autoimmune diseases and myeloablative conditioning prior to bone marrow transplantation, can cause permanent infertility. Cancer survivors report that fertility status has an important impact on their quality of life [1]. Therefore, the American Society for Clinical Oncology [2], the American Society for Reproductive Medicine [3], and the International Society for Fertility Preservation [4] recommend that all patients be counseled about the reproductive risks associated with treatment of their primary disease as well as options to preserve fertility. Adult patients have the options to cryopreserve eggs, sperm, or embryos prior to treatment that can be used in the future to achieve pregnancy using established assisted reproductive technologies [5–7]. Those options are not available to all adult patients (e.g., women who cannot undergo ovarian stimulation) or to prepubertal patients who are not yet making mature eggs or sperm. This is an important human health concern because most children will survive their cancer and still have their entire reproductive life in front of them [8]. Studies show that adult survivors of childhood cancers desire to have children [9–13]. For those reasons, centers around the world are actively cryopreserving gonadal tissues for patients in anticipation that those tissues can be matured in the future to produce eggs or sperm and offspring [14–34].

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Gonadal tissue cryopreservation has been considered experimental and typically is performed at academic institutions with appropriate regulatory approval for human subject's research. The earliest documented cases of ovarian cortical tissue cryopreservation appear to be in the mid- to late 1990s in young adult women who could not undergo ovarian stimulation for oocyte or embryo cryopreservation [35–37]. The first reports of autologous transplantations of those tissues back into the patient survivors were in 2004 in Belgium [35] and 2005 in Israel [37]. Now more than 130 live births have been reported after orthotopic transplantation of frozen and thawed ovarian cortical tissues [38]. Those live birth outcomes prompted the practice committee of the American Society for Reproductive Medicine (ASRM) to recommend that ovarian tissue banking is an acceptable fertility preservation technique and should no longer be considered experimental [39]. The guidance did not distinguish between adult patient and prepubertal patients although there is only one published report of a live birth from cryopreserved peripubertal ovarian tissue (14 years old) [40] and one from cryopreserved prepubertal ovarian tissue (9 years old) [41]. Most patients who cryopreserved ovarian cortical tissues during childhood (prepuberty) are still young, and it will take many years to accumulate live birth outcomes for childhood cancer survivors. The ASRM acknowledged the limited data from childhood cancer survivors but argued that ovarian tissue cryopreservation is the only fertility preservation option available to prepubertal girls. Removing the experimental label from ovarian cortical tissue freezing could have important implications for access to care because several states in the United States have recently passed laws mandating insurance coverage for *standard* fertility preservation techniques [42].

In contrast to ovarian tissues, there are no documented live births from frozen and thawed immature testicular tissues, and testicular tissue freezing for prepubertal patients is still considered experimental [39]. Our Fertility Preservation Program in Pittsburgh (https://fertilitypreservationpittsburgh.org/) and its coordinated centers have cryopreserved testicular tissues for 371 patients since 2011 [29, 43] (STUDY19020220, STUDY19070264) with diagnoses including leukemia/lymphoma, CNS cancers (e.g., glioblastomas), sarcomas, nonmalignant diseases requiring bone marrow transplantation (e.g., sickle cell disease, β-thalassemia), and gender dysphoria (Fig. 1). Immature testicular tissues have been cryopreserved and stored for more than 1000 patients worldwide, based on published reports [30], and the actual number of cases is certainly much higher. Therefore, the research and medical communities are obligated to responsibly developing next-generation reproductive technologies that can be used in the future to mature those tissues and produce fertilization competent sperm. This chapter will briefly describe spermatogonial stem cells (SSCs) and spermatogenic lineage development, review research progress developing SSC-based therapies, and discuss the potential for application of those therapies in the human fertility clinic in the near future, as well as implications for access to advanced reproductive health care.

## Spermatogonial Stem Cells and Spermatogenic Lineage Development

Spermatogonial stem cells are the adult tissue stem cells in the testes that balance self-renewing and differentiating divisions to maintain the SSC pool and support continuous sperm production throughout the postpubertal life of men [44–47]. In humans, spermatogonial stem cell activity is thought to reside in the populations of $A_{dark}$ and $A_{pale}$ spermatogonia that are located on the basement membrane of seminiferous tubules (Fig. 2a, b) and are present from the time of birth through adulthood [48, 49]. Undifferentiated $A_{dark}$ and $A_{pale}$ spermatogonia may undergo 1–2 transit-amplifying mitotic divisions before giving rise to differentiating type B spermatogonia, which divide once to produce primary spermatocytes that lift off the basement membrane and enter the adluminal compartment of the seminiferous tubules [47, 50]. Two subsequent meiotic divisions give rise to secondary spermatocytes and haploid round spermatids, which undergo spermiogenesis to produce termi-

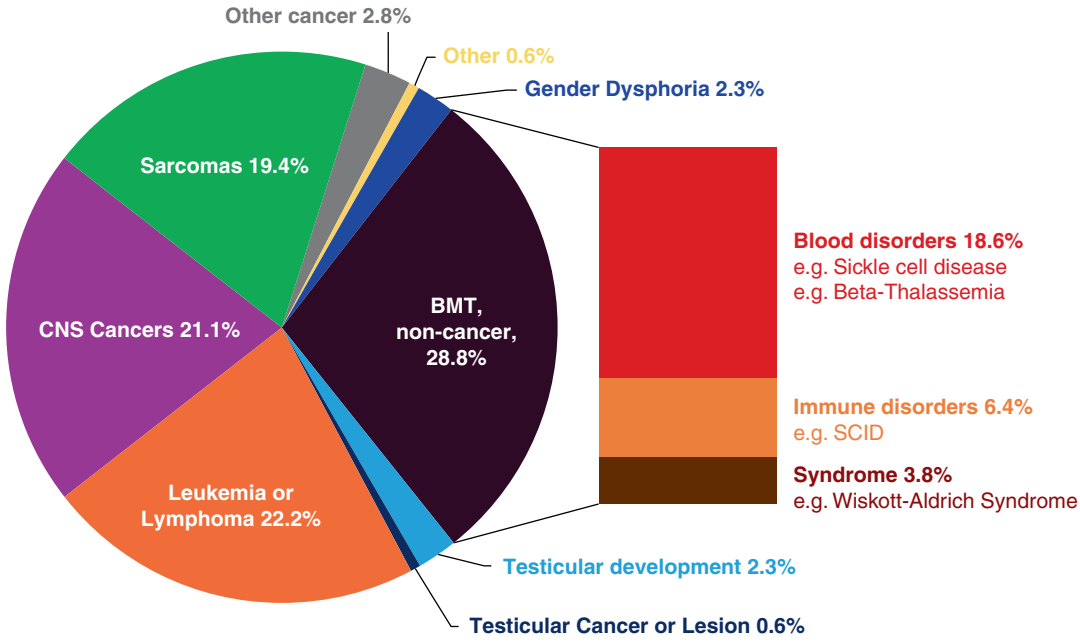


**Fig. 1** Diagnoses for testicular tissue cryopreservation. The Fertility Preservation Program in the UPMC Magee-Womens Hospital has cryopreserved testicular tissues for 371 patients from January 2011 through March 2021. Indications for testicular tissue cryopreservation and percent of total cases are indicated in the pie chart

nally differentiated sperm (Fig. 2b) [50]. Spermatogenesis occurs in the seminiferous tubules of the testes that are connected at both ends to a common collecting reservoir, the rete testis (Fig. 2a). Since spermatogenesis is a stem cell-based process and occurs in a plumbed system of tubules, reservoirs, and ducts that can be easily accessed for infusion of therapeutics, it is particularly amenable to stem cell transplant therapies.

For male patients, there are several stem cell-based therapies in the research pipeline that may be used in the future to produce sperm from immature testicular tissues [51]. Those technologies include spermatogonial stem cell (SSC) transplantation [52–58], de novo testicular morphogenesis [59, 60], testicular tissue grafting/xenografting [61–67], and testicular tissue organ culture [68–71]. One day it may even be possible to produce transplantable germline stem cells or sperm from adult somatic cells (e.g., skin or blood cells) in a process called in vitro gametogenesis (IVG). For IVG, somatic cells are reprogrammed into induced pluripotent stem cells (iPSCs) that are differentiated into primordial germ cell-like cells (PGCLCs) that can be transplanted for in vivo differentiation or differentiated to sperm in vitro [72–74]. The path to the clinic for in vitro germ cells or in vitro gametogenesis techniques is long because those techniques have not been independently replicated in any species except mouse. In contrast, autologous SSC transplantation and testicular tissue grafting are mature technologies that have been replicated in numerous animal species and may be ready for translation to the human fertility clinic today. The next two sections describe the historical development of those two technologies and the state of readiness for translation to the human fertility clinic.

## Spermatogonial Stem Cell Transplantation

### History

SSC transplantation was first described over 25 years ago in mice by Brinster and colleagues [75, 76] who demonstrated that donor SSCs

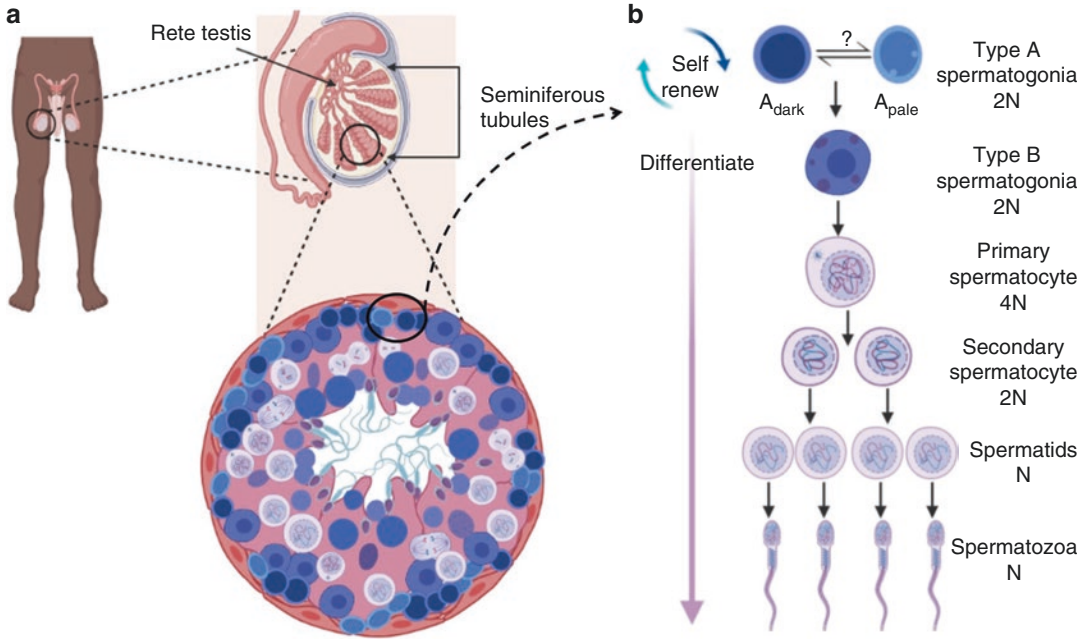


**Fig. 2** Anatomy of the testis and spermatogenic lineage development. Spermatogenesis occurs in the seminiferous tubules of the testis that are each connected to the rete testis space, a structure that can be accessed for infusion of stem cells and other therapeutics (**a**). Undifferentiated stem and progenitor spermatogonia (type $A_{dark}$ and $A_{pale}$) and differentiating type B spermatogonia are located on the basement membrane of the seminiferous tubules. Type B spermatogonia give rise to primary spermatocytes that lift off of the basement membrane and enter the adluminal compartment of the testis. Two subsequent meiotic divisions give rise to secondary spermatocytes and haploid round spermatids. Spermiogenesis produced terminally differentiated sperm (**b**)

could regenerate spermatogenesis and produce donor-derived offspring after transplantation into the testes of mouse recipients that were rendered infertile by chemotherapy treatment. SSC transplantation is a robust technology that has now been replicated in rats, pigs, goats, bulls, sheep, dogs, and monkeys with donor-derived embryos or offspring produced in mice, rats, goats, sheep, and monkeys [52, 54, 56–58, 77–83]. SSCs from donors of all ages, newborn to adult, are competent to regenerate spermatogenesis [54, 84], and SSCs can be cryopreserved and retain spermatogenic function upon thawing and transplantation [58, 85, 86]. Wu and colleagues reported that mouse SSCs were competent to regenerate spermatogenesis and produce offspring after 14 years of cryostorage [87]. Thus, it appears feasible that a testicular tissue biopsy (containing SSCs) could be obtained from a prepubertal boy prior to gonadotoxic therapy, frozen, thawed at a later date, and transplanted back into his testes to regenerate spermatogenesis.

Radford and colleagues reported the first SSC transplantation in human patients in 1999 [88] and in 2003 [89]. Briefly, testicular cell suspensions (including SSCs) were cryopreserved for a total of 12 patients with Hodgkin's disease. Seven of those patients returned to have their frozen and thawed testis cells transplanted back into their testes. The outcomes of those transplants were not reported, but the study provides insights into the motivation of men who were willing to undergo an early-stage experimental procedure for the possibility of having a biologically related child. Homologous species SSC transplantation had only been performed in mice and rats when Radford and colleagues reported the first autologous human SSC transplantations in 1999. The technique has now been replicated in numerous mammalian species, demonstrating safety and feasibility that supports application in the human clinic.

## Methodology

In mice, SSC transplantation is a surgical procedure. Testes are accessed through a mid-abdominal incision and oriented under a dissecting microscope to visualize the efferent ducts that connect the rete testis space to the head of the epididymis. A pulled glass capillary pipet is passed along the efferent ducts until the tip of the pipet emerges into the rete testis space, which can be visualized on the surface of the mouse testis [90]. By infusing a cell suspension or other therapeutic into the rete testis space, it is possible to fill all seminiferous tubules of the testis at the same time.

The rete testis space in monkeys and humans is located in the center of the testis, and therefore, it cannot be targeted by visual inspection of the surface of the testis. However, the rete testis space is echo-dense and can be visualized by ultrasound. Schlatt and colleagues pioneered the method of ultrasound-guided rete testis injection into dissected bovine, monkey, and human testes as well as in vivo injection into cynomolgus monkey testes in 1999 [91]. This method has now been used to infuse testis cell suspensions to the seminiferous tubules of several large animal species with regeneration of spermatogenesis and in some cases embryos or offspring [56–58, 79–82]. Unlike the approach used for SSC transplantation in rodents, ultrasound-guided rete testis injection in larger mammals does not require surgery. A hypodermic needle is simply inserted through the base of the scrotum and through the testicular parenchyma until the needle emerges into the rete testis space. The injection needle and the rete testis space are both echo-dense and visible on ultrasound (Fig. 3a–c). Infusion into the rete testis space fills all seminiferous tubules at the same time because all seminiferous tubules are connected to the rete testis (Fig. 3c). In our experience and others, about 250–500 μL of fluid or cell suspension can be injected into the seminiferous tubules of prepubertal rhesus macaques, and 500–1000 μL can be injected into the seminiferous tubules of adult rhesus macaques [58, 83, 92]. It is important not to overfill the tubules because this can impede blood flow and cause ischemia to the testis. We believe the ultrasound-guided rete testis injection can also be applied in humans because the rete testis can be easily visualized by ultrasound inspection of human testes (Fig. 3d, e).

## Other Considerations

The tissue biopsies from young patients are usually small and may not contain enough SSCs to produce robust spermatogenesis after transplantation. Thus, it may be necessary to expand SSC numbers in culture before transplantation. SSC culture has been firmly established in rodents [93–98], including development of conditions that do not require supporting feeder cells [99, 100], which may be an important consideration for clinical application. SSC culture has been extended to rats, hamsters, and rabbits [95, 101, 102], but extension to larger animal species has been a challenge, perhaps due to species-specific differences in factors that regulate SSCs [103–105]. Many laboratories have described protocols for human SSCs culture [14, 15, 106–121], but definitive evidence of long-term SSC expansion in higher primates is lacking, and no methods have been independently replicated among laboratories [114, 122, 123]. In the absence of a robust method to expand human SSCs in culture, the best recourse may be to transplant the cells to their native environment in the seminiferous tubules of the testis with the proper structural support and niche factors that support human SSC proliferation, self-renewal, and differentiation.

In mice, the efficiency of SSC engraftment and regeneration of spermatogenesis are better in 5–8-day-old mouse pups than in adult recipients [54]. Mice do not have a prolonged prepubertal period like humans. The spermatogonial stem cells or prospermatogonia migrate to the basement membrane of seminiferous tubules within a few days after birth and initiate spermatogenesis [124–126]. Therefore, testis development in a teenage boy may be similar to a 5–8-day-old mouse pup where the testis is growing under the influence of gonadotropic hor-

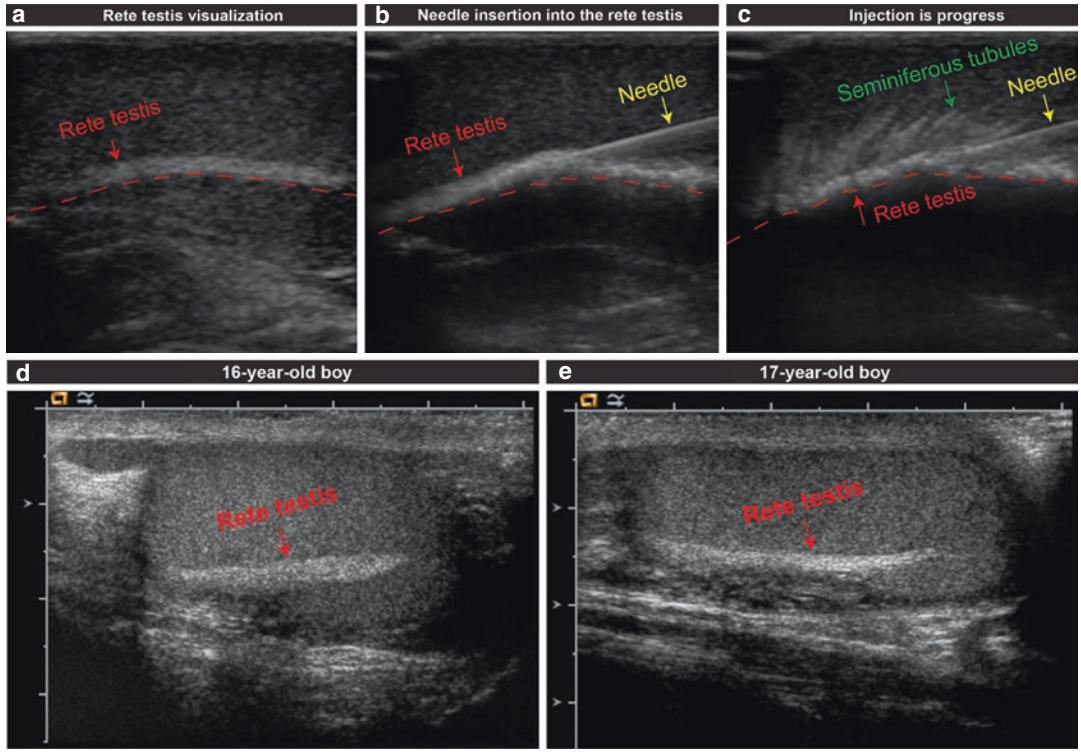


**Fig. 3** Spermatogonial stem cell transplantation by ultrasound-guided rete testis injection. All seminiferous tubules of the testis are connected to the rete testis space, which is echo-dense and visible by ultrasound. Images of rhesus macaque testes are shown in (**a**–**c**). A 25-gauge, 1.5 in. hypodermic needle (also visible on ultrasound) is inserted through the base of the scrotum and testicular parenchyma until the needle emerges into the rete testis space (**b**). Microbubbles are added to the donor testis cell suspension to allow tracking of injection progress. Infusion fills the rete testis space and then simultaneously fills all seminiferous tubules (**c**). The anatomy of human testes is similar to rhesus macaques. The ultrasound imaging clearly identifies the rete testis space in the testes of 16-years old and 17-years old patients, suggesting that the same ultrasound-guided rete testis injection approach should work in human patients (**d**, **e**). (Portions of this picture are reprinted with permission from Hermann et al., *Cell Stem Cell* 2012)

mones and there is a burst of Sertoli cell proliferation [127, 128], which is likely accompanied by an expansion of SSC niches. The rete testes of teenage boys should be accessible for SSC transplantation (Fig. 3d, e).

## Testicular Tissue Grafting

### History

Testicular tissue grafting and xenografting are established technologies in which pieces of immature testicular tissues, containing seminiferous tubules with SSCs, are grafted ectopically under the skin. The objective of this technique is not to regenerate normal spermatogenesis in the recipient seminiferous tubules. The objective is to promote the maturation of the grafted immature testicular tissues pieces and produce sperm that can be recovered for fertilization by intracytoplasmic sperm injection. Immature testicular tissues from mice, pigs, goats, rabbits, hamsters, dogs, cats, horses, cattle, and monkeys have been grafted under the back skin of immunodeficient nude mice and matured to produce sperm [66, 129]. Graft-derived sperm were competent to fertilize oocytes in mice, pigs, goats, and monkeys [62, 65, 130] with production of offspring in mice, pigs, and monkeys [61, 67, 130].

Therefore, it is theoretically possible to graft immature testicular tissue from a childhood cancer survivor into an animal host to produce sperm that can be used to achieve pregnancy with established assisted reproductive technologies. This approach may be particularly applicable in patients with leukemia or testicular cancer for whom it may be unsafe to transplant their tissues back into their own bodies or for transgender females who do not want to experience male puberty that would be required to mature testicular tissues. However, the possibility that viruses or other xenobiotics could be transmitted from the animal host to the patient needs to be carefully considered [131–133].

Homologous species immature testicular tissue grafting was pioneered in mice with the production of complete spermatogenesis and offspring [61–63]. Several groups have reported homologous and/or autologous testicular tissue grafting in nonhuman primates to establish safety and feasibility that may support translation to the human clinic [66, 134–136]. Luetjens and colleagues investigated graft success from immature tissues versus adult testicular tissues transplanted ectopically under the back skin of hemi-castrated monkeys (i.e., normal hormonal milieu). Adult tissues degenerated while immature tissues survived with spermatogenesis arrested at the level of spermatogonia. A second experiment compared graft location at ectopic (back skin) versus orthotopic (scrotum) sites in young, castrated recipients and whether cryopreservation affected graft outcomes. In that experiment, none of the cryopreserved grafts survived. Ectopic fresh grafts survived with spermatogenesis arrested in meiosis, while orthotopic fresh grafts developed complete spermatogenesis. In that experiment, cryopreserved grafts were only transplanted under the back skin, not in the scrotum, so it is not clear whether it was the cryopreservation, the ectopic graft site, or both that contributed to graft demise [135]. This question was answered in part by Jahnukainen and colleagues who transplanted cryopreserved prepubertal and pubertal testicular tissues to the orthotopic location in the scrotum. Similar to the results with adult tissues, pubertal grafts that already contained sperm at the time of grafting could not be recovered 5 months later. Prepubertal, cryopreserved grafts transplanted to the scrotum of castrated autologous recipients could be recovered. The graft recovery rate was low (5%), and complete spermatogenesis was observed in only 13% and 17% of seminiferous tubules in the two surviving grafts. Both studies transplanted small pieces (~0.5–1 $mm^3$) of testicular tissue to the graft site (4–6 pieces per graft site). Sperm function was not tested by fertilization or with production of offspring in either study [135, 137].

## Methodology and Outcomes

We recently repeated those experiments with slight modifications in a rhesus macaque model of cancer survivorship. Prepubertal animals with immature testicular tissues were hemi-castrated. The immature testicular tissue was cut into small pieces that were somewhat larger than previous studies (9–20 $\mu m^3$, Fig. 4a) and cryopreserved by controlled slow rate freezing in a medium containing 5% DMSO and 5% serum, as previously described [21]. Five to 7 months after hemi-castration, the remaining testis was removed and cut into small pieces (9–20 $\mu m^3$), some of which were designated for fresh tissue grafting. Immediately after removal of the second testis, fresh and previously cryopreserved tissues from the same animal were autologously grafted under the back skin (three sites fresh, three sites cryopreserved) or under the scrotal skin (one side fresh, one side cryopreserved) by individually suturing four pieces of testicular tissue to the subcutaneous aspect of a skin flap (Fig. 4b). This experimental design was repeated in five individual animals for a total of 40 graft sites (30 under the back skin and ten under the scrotal skin). Testicular tissues were recovered from 39 of the 40 graft sites; one graft was lost when the recipient animal opened the incision after surgery. Testosterone levels rose to the normal range for peripubertal rhesus macaques within 6–8 months after grafting. Testosterone could

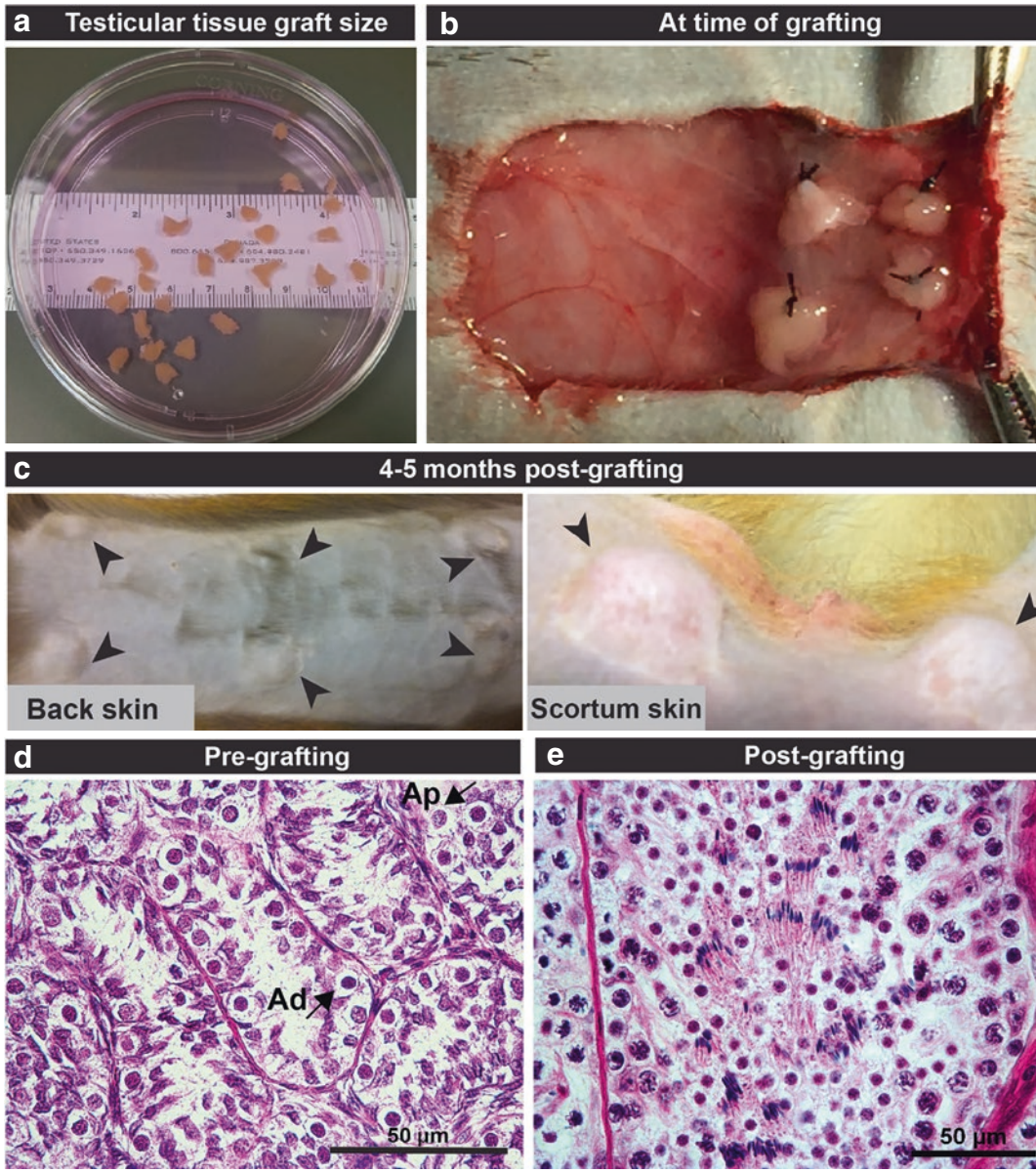


**Fig. 4** Testicular tissue grafting. Testicular tissues are collected by wedge resection biopsy. In the fertility preservation laboratory, biopsied tissues are cut into small pieces measuring 2–5 mm in diameter (estimated 9–20 $mm^3$) and cryopreserved by controlled slow rate freezing (**a**). After thawing, testicular tissue pieces are individually sutured to the underside of a skin flap (**b**). Grafted tissues grew continuously under the back skin or scrotal skin for 8–12 months after grafting (**c**). Testicular tissues were immature at the time of grafting, containing only undifferentiated $A_{dark}$ and $A_{pale}$ spermatogonia in the seminiferous tubules (**d**). When grafts were collected 8–12 months after transplantation, 70% of tubules contained complete spermatogenesis with fertilization competent sperm (**e**). (Images reprinted with permission from Fayomi et al., *SCIENCE* 2019)

only be from grafted tissues because recipient animals were castrated. Grafts grew continuously throughout the duration of the experiment (8–12 months) and were not impacted by graft location (Fig. 4c), cryopreservation, or addition of Matrigel to the graft site. Testicular tissues that were immature at the time of grafting (Fig. 4d) exhibited complete spermatogenesis

with spermatids or sperm in >70% of seminiferous tubules at the time of recovery (Fig. 4e). Sperm were recovered by manual dissection or enzymatic digestion and used to fertilize rhesus oocytes by ICSI in collaboration with the assisted reproductive technology core at the Oregon National Primate Research Center. A healthy graft-derived baby (Grady) was born on April 18, 2018 [66]. We speculated about factors that may explain the improved graft recovery and extent of spermatogenesis. First, the concentration of DMSO cryoprotectant in our study (5%, 0.7 M) was lower than previous studies (10%, 1.4 M). Second, testicular tissue pieces were larger in our study (9–20 $\mu m^3$) than previous studies (0.5–1 $mm^3$), which may increase the local concentration of autocrine or paracrine factors. Third, the larger pieces allowed us to individually suture each piece of tissue to the capillary-rich underside of the skin flap rather than depositing a slurry of small pieces into a subcutaneous pocket.

## Other Consideration

Similar to SSC transplantation, testicular tissue grafting and xenografting are established technologies that have been replicated in numerous mammalian species, including production of fertilization competent sperm and offspring in nonhuman primates [66, 67, 129]. In most species, cryopreserved grafts retained potential to regenerate complete spermatogenesis, an important consideration for adult survivors of childhood cancers. Immature testicular tissue grafting will not regenerate normal spermatogenesis in the endogenous tetses or natural fertility but can produce fertilization competent sperm that can be used to achieve pregnancy by intracytoplasmic sperm injection. In almost every report of immature testicular tissue grafting or xenografting, recipient animals were castrated, in theory to eliminate negative feedback from the endogenous testes on the hypothalamus and pituitary. Of course, our patient survivors will not be castrated, so it will be important to demonstrate in future studies that graft development can occur in patients with intact testes.

## Concluding Remarks

Testicular tissues have already been cryopreserved for over 1000 patients worldwide [30], and some of those patients may be ready to use those tissues for reproduction. Spermatogonial stem cell transplantation and testicular tissue grafting are mature technologies that have been replicated in numerous labs and across numerous mammalian species over the past two decades. Translation to nonhuman primates provided critical safety and feasibility data that may justify translation to the human fertility clinic. Specifically, the demonstrations that cryopreserved, prepubertal testicular cells or tissues could produce spermatogenesis highlight the potential application in adolescent or adult survivors of childhood cancers or bone marrow transplantation for benign diseases. Autologous transplantation approaches may not be appropriate for leukemia or testicular cancer patients where there is a risk of reintroducing malignant cells to a patient survivor. For those patients methods to screen and/or remove malignant cells may be required [138]. Alternatively, it may be possible to mature testicular tissues ex vivo [69, 139]. However, the majority (>60%) of our patients who cryopreserved testicular tissues had solid tumors (sarcomas, neuroblastomas) that do not metastasize to the testes or nonmalignant diseases (e.g., sickle cell disease, β-thalassemia) (Fig. 1). Those patients may be ideal candidates for first autologous testicular cell or tissue transplantation trials.

There are no human live births from frozen/thawed immature testicular tissues or cells, and therefore, testicular tissue cryopreservation remains experimental in the United States. In contrast, ASRM recommended that the experimental label could be removed from ovarian tissue cryopreservation [39] based on reports of over 130 births after transplantation of frozen and thawed ovarian tissues [38]. This

helps reduce a significant barrier in access to fertility preservation care because it opens the door for some patients to get insurance coverage. It is important to note however that ovarian tissues have been cryopreserved for both prepubertal and adult patients and most documented births are from women who were already adult at the time of ovarian tissue cryopreservation [35]. Immature testicular tissue cryopreservation has been used almost exclusively in prepubertal patients. That means it could be years before the first males return to use their cryopreserved testicular tissues. How many more years and how many births will be required to remove the experimental label from testicular tissue cryopreservation? Furthermore, if a man produces sperm and/or offspring after autologous transplantation of spermatogonial stem cells, how will we know whether sperm were from transplanted or endogenous cells? That question can be addressed in part with the testicular tissue grafting option because those tissues can be removed and dissected to release sperm that are unequivocally from the frozen and thawed immature testicular tissues. Live births from those tissues will still be many years away because most of those patients are still young. The Danish experience may be instructive. In 1990, the Danish Minister of Health concluded that there were no restrictions in freezing ovarian tissues or testicular tissues if only autologous transplantation was considered. This ruling placed gonadal tissue cryopreservation in the context of normal medical practice. Perhaps this perspective along with published reports indicating few adverse outcomes associated with testicular tissue biopsies and cryopreservation could be adequate to justify removing the experimental label from testicular tissue cryopreservation [19, 22, 29, 30], one author's opinion.

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## References

1. Schover LR. Patient attitudes toward fertility preservation. Pediatr Blood Cancer. 2009;53:281–4.
2. Loren AW, Mangu PB, Beck LN, Brennan L, Magdalinski AJ, Partridge AH, Quinn G, Wallace WH, Oktay K, American Society of Clinical O. Fertility preservation for patients with cancer: American Society of Clinical Oncology clinical practice guideline update. J Clin Oncol. 2013;31:2500–10.
3. Practice Committee of American Society for Reproductive M. Fertility preservation in patients undergoing gonadotoxic therapy or gonadectomy: a committee opinion. Fertil Steril. 2013;100:1214–23.
4. Martinez F, Preservation. ISfF, Group. E-AEW. Update on fertility preservation from The Barcelona International Society For Fertility Preservation-ESHRE-ASRM 2015 expert meeting: indications, results and future perspectives. Fertil Steril. 2017;108:407–415.e411.
5. Agarwal A, Allamaneni SR. Artificial insemination. In: Falcone T, Hurd W, editors. Clinical reproductive medicine and surgery. Philadelphia, PA: Elsevier; 2007. p. 539–48.
6. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet. 1978;2:366.
7. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 1992;340:17–8.
8. Noone AM, Howlader N, Krapcho M, Miller D, Brest A, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA. Seer cancer statistics review, 1975-2015. Bethesda, MD: National Cancer Institute; 2018.
9. Nieman CL, Kinahan KE, Yount SE, Rosenbloom SK, Yost KJ, Hahn EA, Volpe T, Dilley KJ, Zoloth L, Woodruff TK. Fertility preservation and adolescent cancer patients: lessons from adult survivors of childhood cancer and their parents. Cancer Treat Res. 2007;138:201–17.
10. Zebrack BJ, Casillas J, Nohr L, Adams H, Zeltzer LK. Fertility issues for young adult survivors of childhood cancer. Psycho-Oncology. 2004;13:689–99.
11. Schover LR. Motivation for parenthood after cancer: a review. J Natl Cancer Inst Monogr. 2005;2005:2–5.
12. Kinahan KE, Didwania A, Nieman CL. Childhood cancer: fertility and psychosocial implications. Cancer Treat Res. 2007;138:191–200.

13. Ellis SJ, Wakefield CE, McLoone JK, Robertson EG, Cohn RJ. Fertility concerns among child and adolescent cancer survivors and their parents: a qualitative analysis. J Psychosoc Oncol. 2016;34:347–62.
14. Sadri-Ardekani H, Mizrak SC, van Daalen SK, Korver CM, Roepers-Gajadien HL, Koruji M, Hovingh S, de Reijke TM, de la Rosette JJ, van der Veen F, de Rooij DG, Repping S, van Pelt AM. Propagation of human spermatogonial stem cells in vitro. JAMA. 2009;302:2127–34.
15. Sadri-Ardekani H, Akhondi MA, van der Veen F, Repping S, van Pelt AM. In vitro propagation of human prepubertal spermatogonial stem cells. JAMA. 2011;305:2416–8.
16. Goossens E, Van Saen D, Tournaye H. Spermatogonial stem cell preservation and transplantation: from research to clinic. Hum Reprod. 2013;28:897–907.
17. Heckmann L, Langenstroth-Rower D, Pock T, Wistuba J, Stukenborg JB, Zitzmann M, Kliesch S, Schlatt S, Neuhaus N. A diagnostic germ cell score for immature testicular tissue at risk of germ cell loss. Hum Reprod. 2018;33:636–45.
18. Ho WLC, Bourne H, Gook D, Clarke G, Kemertzis M, Stern K, Agresta F, Heloury Y, Clark H, Orme L, Jayasinghe Y, Zacharin MR, The P, Adolescent Fertility Preservation Task Force M. A short report on current fertility preservation strategies for boys. Clin Endocrinol. 2017;87:279–85.
19. Wyns C, Curaba M, Petit S, Vanabelle B, Laurent P, Wese JF, Donnez J. Management of fertility preservation in prepubertal patients: 5 years' experience at the Catholic University of Louvain. Hum Reprod. 2011;26:737–47.
20. Ginsberg JP. New advances in fertility preservation for pediatric cancer patients. Curr Opin Pediatr. 2011;23:9–13.
21. Keros V, Hultenby K, Borgstrom B, Fridstrom M, Jahnukainen K, Hovatta O. Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. Hum Reprod. 2007;22:1384–95.
22. Picton HM, Wyns C, Anderson RA, Goossens E, Jahnukainen K, Kliesch S, Mitchell RT, Pennings G, Rives N, Tournaye H, van Pelt AM, Eichenlaub-Ritter U, Schlatt S, Diseases ETFOFPIS. A European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys. Hum Reprod. 2015;30:2463–75.
23. Onofre J, Baert Y, Faes K, Goossens E. Cryopreservation of testicular tissue or testicular cell suspensions: a pivotal step in fertility preservation. Hum Reprod Update. 2016;22:744–61.
24. Pietzak EJ III, Tasian GE, Tasian SK, Brinster RL, Carlson C, Ginsberg JP, Kolon TF. Histology of testicular biopsies obtained for experimental fertility preservation protocol in boys with cancer. J Urol. 2015;194:1420–4.
25. Uijldert M, Meißner A, de Melker AA, van Pelt AMM, van de Wetering MD, van Rijn RR, van Wely M, van der Veen F, Repping S. Development of the testis in pre-pubertal boys with cancer after biopsy for fertility preservation. Hum Reprod. 2017;32:2366–72.
26. Stukenborg JB, Alves-Lopes JP, Kurek M, Albalushi H, Reda A, Keros V, Töhönen V, Bjarnason R, Romerius P, Sundin M, Norén Nyström U, Langenskiöld C, Vogt H, Henningsohn L, Mitchell RT, Söder O, Petersen C, Jahnukainen K. Spermatogonial quantity in human prepubertal testicular tissue collected for fertility preservation prior to potentially sterilizing therapy. Hum Reprod. 2018;33:1677–83.
27. Poganitsch-Korhonen M, Masliukaite I, Nurmio M, Lähteenmäki P, van Wely M, van Pelt AMM, Jahnukainen K, Stukenborg JB. Decreased spermatogonial quantity in prepubertal boys with leukaemia treated with alkylating agents. Leukemia. 2017;31:1460.
28. Bahadur G, Chatterjee R, Ralph D. Testicular tissue cryopreservation in boys. Ethical and legal issues: case report. Hum Reprod. 2000;15:1416–20.
29. Valli-Pulaski H, Peters KA, Gassei K, Steimer SR, Sukhwani M, Hermann BP, Dwomor L, David S, Fayomi AP, Munyoki SK, Chu T, Chaudhry R, Cannon GM, Fox PJ, Jaffe TM, Sanfilippo JS, Menke MN, Lunenfeld E, Abofoul-Azab M, Sender LS, Messina J, Klimpel LM, Gosiengfiao Y, Rowell EE, Hsieh MH, Granberg CF, Reddy PP, Sandlow JI, Huleihel M, Orwig KE. Testicular tissue cryopreservation: 8 years of experience from a coordinated network of academic centers. Hum Reprod. 2019;34:966–77.
30. Goossens E, Jahnukainen K, Mitchell RT, van Pelt A, Pennings G, Rives N, Poels J, Wyns C, Lane S, Rodriguez-Wallberg KA, Rives A, Valli-Pulaski H, Steimer S, Kliesch S, Braye A, Andres MM, Medrano J, Ramos L, Kristensen SG, Andersen CY, Bjarnason R, Orwig KE, Neuhaus N, Stukenborg JB. Fertility preservation in boys: recent developments and new insights. Hum Reprod Open. 2020;2020:hoaa016.
31. Smith BM, Duncan FE, Ataman L, Smith K, Quinn GP, Chang RJ, Finlayson C, Orwig K, Valli-Pulaski H, Moravek MB, Zelinski MB, Irene Su H, Vitek W, Smith JF, Jeruss JS, Gracia C, Coutifaris C, Shah D, Nahata L, Gomez-Lobo V, Appiah LC, Brannigan RE, Gillis V, Gradishar W, Javed A, Rhoton-Vlasak AS, Kondapalli LA, Neuber E, Ginsberg JP, Muller CH, Hirshfeld-Cytron J, Kutteh WH, Lindheim SR, Cherven B, Meacham LR, Rao P, Torno L, Sender LS, Vadaparampil ST, Skiles JL, Schafer-Kalkhoff T, Frias OJ, Byrne J, Westphal LM, Schust DJ, Klosky JL, McCracken KA, Ting A, Khan Z, Granberg C, Lockart B, Scoccia B, Laronda MM, Mersereau JE, Marsh C, Pavone ME, Woodruff TK. The national physicians cooperative: transforming fertility management in the cancer setting and beyond. Future Oncol. 2018;14:3059–72.
32. von Wolff M, Donnez J, Hovatta O, Keros V, Maltaris T, Montag M, Salle B, Sonmezer M, Andersen CY. Cryopreservation and autotransplan-

tation of human ovarian tissue prior to cytotoxic therapy--a technique in its infancy but already successful in fertility preservation. Eur J Cancer. 2009;45:1547–53.
33. Meirow D, Ra'anani H, Biderman H. Ovarian tissue cryopreservation and transplantation: a realistic, effective technology for fertility preservation. Methods Mol Biol. 2014;1154:455–73.
34. Shapira M, Dolmans M-M, Silber S, Meirow D. Evaluation of ovarian tissue transplantation: results from three clinical centers. Fertil Steril. 2020;114:388–97.
35. Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B, Van Langendonckt A. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet. 2004;364:1405–10.
36. Donnez J, Silber S, Andersen CY, Demeestere I, Piver P, Meirow D, Pellicer A, Dolmans M-M. Children born after autotransplantation of cryopreserved ovarian tissue. A review of 13 live births. Ann Med. 2011;43:437–50.
37. Meirow D, Levron J, Eldar-Geva T, Hardan I, Fridman E, Zalel Y, Schiff E, Dor J. Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy. N Engl J Med. 2005;353:318–21.
38. Donnez J, Dolmans MM. Fertility preservation in women. N Engl J Med. 2017;377:1657–65.
39. Penzias A, Bendikson K, Falcone T, Gitlin S, Gracia C, Hansen K, Hill M, Hurd W, Jindal S, Kalra S, Mersereau J, Odem R, Racowsky C, Rebar R, Reindollar R, Rosen M, Sandlow J, Schlegel P, Steiner A, Tanrikut C, Stovall D. Fertility preservation in patients undergoing gonadotoxic therapy or gonadectomy: a committee opinion. Fertil Steril. 2019;112:1022–33.
40. Demeestere I, Simon P, Dedeken L, Moffa F, Tsépélidis S, Brachet C, Delbaere A, Devreker F, Ferster A. Live birth after autograft of ovarian tissue cryopreserved during childhood. Hum Reprod. 2015;30:2107–9.
41. Matthews SJ, Picton H, Ernst E, Andersen CY. Successful pregnancy in a woman previously suffering from β-thalassemia following transplantation of ovarian tissue cryopreserved before puberty. Minerva Ginecol. 2018;70:432–5.
42. Alliance for Fertility Preservation. https://www.allianceforfertilitypreservation.org/advocacy/state-legislation.
43. Fertility Preservation Program of Magee-Womens Hospital in Pittsburgh. http://fertilitypreservation-pittsburgh.org.
44. de Rooij DG, Grootegoed JA. Spermatogonial stem cells. Curr Opin Cell Biol. 1998;10:694–701.
45. Tegelenbosch RA, de Rooij DG. A quantitative study of spermatogonial multiplication and stem cell renewal in the c3h/101 f1 hybrid mouse. Mutat Res. 1993;290:193–200.
46. Phillips BT, Gassei K, Orwig KE. Spermatogonial stem cell regulation and spermatogenesis. Philos Trans R Soc Lond Ser B Biol Sci. 2010;365:1663–78.
47. Fayomi AP, Orwig KE. Spermatogonial stem cells and spermatogenesis in mice, monkeys and men. Stem Cell Res. 2018;29:207–14.
48. Paniagua R, Nistal M. Morphological and histometric study of human spermatogonia from birth to the onset of puberty. J Anat. 1984;139(Pt 3):535–52.
49. Clermont Y. Renewal of spermatogonia in man. Am J Anat. 1966;118:509–24.
50. Nistal M, Paniagua R, Gonzalez-Peramato P, Reyes-Múgica M. 4 - pubertal and adult testis. Pediatr Dev Pathol. 2015;18:187.
51. Gassei K, Orwig KE. Experimental methods to preserve male fertility and treat male factor infertility. Fertil Steril. 2016;105:256–66.
52. Brinster CJ, Ryu BY, Avarbock MR, Karagenc L, Brinster RL, Orwig KE. Restoration of fertility by germ cell transplantation requires effective recipient preparation. Biol Reprod. 2003;69:412–20.
53. Ogawa T, Dobrinski I, Brinster RL. Recipient preparation is critical for spermatogonial transplantation in the rat. Tissue Cell. 1999;31:461–72.
54. Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc Natl Acad Sci U S A. 2001;98:6186–91.
55. Hamra FK, Gatlin J, Chapman KM, Grellhesl DM, Garcia JV, Hammer RE, Garbers DL. Production of transgenic rats by lentiviral transduction of male germ-line stem cells. Proc Natl Acad Sci U S A. 2002;99:14931–6.
56. Honaramooz A, Behboodi E, Megee SO, Overton SA, Galantino-Homer H, Echelard Y, Dobrinski I. Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. Biol Reprod. 2003;69:1260–4.
57. Herrid M, Olejnik J, Jackson M, Suchowerska N, Stockwell S, Davey R, Hutton K, Hope S, Hill JR. Irradiation enhances the efficiency of testicular germ cell transplantation in sheep. Biol Reprod. 2009;81:898–905.
58. Hermann BP, Sukhwani M, Winkler F, Pascarella JN, Peters KA, Sheng Y, Valli H, Rodriguez M, Ezzelarab M, Dargo G, Peterson K, Masterson K, Ramsey C, Ward T, Lienesch M, Volk A, Cooper DK, Thomson AW, Kiss JE, Penedo MC, Schatten GP, Mitalipov S, Orwig KE. Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell. 2012;11:715–26.
59. Kita K, Watanabe T, Ohsaka K, Hayashi H, Kubota Y, Nagashima Y, Aoki I, Taniguchi H, Noce T, Inoue K, Miki H, Ogonuki N, Tanaka H, Ogura A, Ogawa T. Production of functional spermatids from mouse germline stem cells in ectopically reconstituted seminiferous tubules. Biol Reprod. 2007;76:211–7.

60. Zhang M, Zhou H, Zheng C, Xiao J, Zuo E, Liu W, Xie D, Shi Y, Wu C, Wang H, Li D, Li J. The roles of testicular c-kit positive cells in de novo morphogenesis of testis. Sci Rep. 2014;4:5936.
61. Schlatt S, Honaramooz A, Boiani M, Scholer HR, Dobrinski I. Progeny from sperm obtained after ectopic grafting of neonatal mouse testes. Biol Reprod. 2003;68:2331–5.
62. Honaramooz A, Snedaker A, Boiani M, Scholer H, Dobrinski I, Schlatt S. Sperm from neonatal mammalian testes grafted in mice. Nature. 2002;418:778–81.
63. Shinohara T, Inoue K, Ogonuki N, Kanatsu-Shinohara M, Miki H, Nakata K, Kurome M, Nagashima H, Toyokuni S, Kogishi K, Honjo T, Ogura A. Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro microinsemination. Hum Reprod. 2002;17:3039–45.
64. Nakai M, Kaneko H, Somfai T, Maedomari N, Ozawa M, Noguchi J, Ito J, Kashiwazaki N, Kikuchi K. Production of viable piglets for the first time using sperm derived from ectopic testicular xenografts. Reproduction. 2010;139:331–5.
65. Honaramooz A, Li MW, Penedo MCT, Meyers S, Dobrinski I. Accelerated maturation of primate testis by xenografting into mice. Biol Reprod. 2004;70:1500–3.
66. Fayomi AP, Peters K, Sukhwani M, Valli-Pulaski H, Shetty G, Meistrich ML, Houser L, Robertson N, Roberts V, Ramsey C, Hanna C, Hennebold JD, Dobrinski I, Orwig KE. Autologous grafting of cryopreserved prepubertal rhesus testis produces sperm and offspring. Science. 2019;363:1314–9.
67. Liu Z, Nie Y-H, Zhang C-C, Cai Y-J, Wang Y, Lu H-P, Li Y-Z, Cheng C, Qiu Z-L, Sun Q. Generation of macaques with sperm derived from juvenile monkey testicular xenografts. Cell Res. 2016;26:139–42.
68. Komeya M, Kimura H, Nakamura H, Yokonishi T, Sato T, Kojima K, Hayashi K, Katagiri K, Yamanaka H, Sanjo H, Yao M, Kamimura S, Inoue K, Ogonuki N, Ogura A, Fujii T, Ogawa T. Long-term ex vivo maintenance of testis tissues producing fertile sperm in a microfluidic device. Sci Rep. 2016;6:21472.
69. Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, Kubota Y, Ogawa T. In vitro production of functional sperm in cultured neonatal mouse testes. Nature. 2011;471:504–7.
70. Yokonishi T, Sato T, Komeya M, Katagiri K, Kubota Y, Nakabayashi K, Hata K, Inoue K, Ogonuki N, Ogura A, Ogawa T. Offspring production with sperm grown in vitro from cryopreserved testis tissues. Nat Commun. 2014;5:4320.
71. Yuan Y, Li L, Cheng Q, Diao F, Zeng Q, Yang X, Wu Y, Zhang H, Huang M, Chen J, Zhou Q, Zhu Y, Hua R, Tian J, Wang X, Zhou Z, Hao J, Yu J, Hua D, Liu J, Guo X, Zhou Q, Sha J. In vitro testicular organogenesis from human fetal gonads produces fertilization-competent spermatids. Cell Res. 2020;30:244.
72. Hayashi K, Ohta H, Kurimoto K, Aramaki S, Saitou M. Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. Cell. 2011;146:519–32.
73. Nakaki F, Hayashi K, Ohta H, Kurimoto K, Yabuta Y, Saitou M. Induction of mouse germ-cell fate by transcription factors in vitro. Nature. 2013;501:222–6.
74. Zhou Q, Wang M, Yuan Y, Wang X, Fu R, Wan H, Xie M, Liu M, Guo X, Zheng Y, Feng G, Shi Q, Zhao XY, Sha J, Zhou Q. Complete meiosis from embryonic stem cell-derived germ cells in vitro. Cell Stem Cell. 2016;18:330–40.
75. Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A. 1994;91:11298–302.
76. Brinster RL, Avarbock MR. Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A. 1994;91:11303–7.
77. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL. Transplantation of male germ line stem cells restores fertility in infertile mice. Nat Med. 2000;6:29–34.
78. Nagano M, Brinster CJ, Orwig KE, Ryu BY, Avarbock MR, Brinster RL. Transgenic mice produced by retroviral transduction of male germ-line stem cells. Proc Natl Acad Sci U S A. 2001;98:13090–5.
79. Mikkola M, Sironen A, Kopp C, Taponen J, Sukura A, Vilkki J, Katila T, Andersson M. Transplantation of normal boar testicular cells resulted in complete focal spermatogenesis in a boar affected by the immotile short-tail sperm defect. Reprod Domest Anim. 2006;41:124–8.
80. Kim Y, Turner D, Nelson J, Dobrinski I, McEntee M, Travis AJ. Production of donor-derived sperm after spermatogonial stem cell transplantation in the dog. Reproduction. 2008;136:823–31.
81. Izadyar F, Den Ouden K, Stout TA, Stout J, Coret J, Lankveld DP, Spoormakers TJ, Colenbrander B, Oldenbroek JK, Van der Ploeg KD, Woelders H, Kal HB, De Rooij DG. Autologous and homologous transplantation of bovine spermatogonial stem cells. Reproduction. 2003;126:765–74.
82. Schlatt S, Foppiani L, Rolf C, Weinbauer GF, Nieschlag E. Germ cell transplantation into x-irradiated monkey testes. Hum Reprod. 2002;17:55–62.
83. Jahnukainen K, Ehmcke J, Quader MA, Saiful Huq M, Epperly MW, Hergenrother S, Nurmio M, Schlatt S. Testicular recovery after irradiation differs in prepubertal and pubertal non-human primates, and can be enhanced by autologous germ cell transplantation. Hum Reprod. 2011;26:1945–54.
84. Ryu BY, Orwig KE, Avarbock MR, Brinster RL. Stem cell and niche development in the postnatal rat testis. Dev Biol. 2003;263:253–63.

85. Dobrinski I, Avarbock MR, Brinster RL. Transplantation of germ cells from rabbits and dogs into mouse testes. Biol Reprod. 1999;61:1331–9.
86. Dobrinski I, Avarbock MR, Brinster RL. Germ cell transplantation from large domestic animals into mouse testes. Mol Reprod Dev. 2000;57:270–9.
87. Wu X, Goodyear SM, Abramowitz LK, Bartolomei MS, Tobias JW, Avarbock MR, Brinster RL. Fertile offspring derived from mouse spermatogonial stem cells cryopreserved for more than 14 years. Hum Reprod. 2012;27:1249–59.
88. Radford JA, Shalet SM, Lieberman BA. Fertility after treatment for cancer. BMJ. 1999;319:935–6.
89. Radford J. Restoration of fertility after treatment for cancer. Horm Res. 2003;59(Suppl 1):21–3.
90. Ogawa T, Arechaga JM, Avarbock MR, Brinster RL. Transplantation of testis germinal cells into mouse seminiferous tubules. Int J Dev Biol. 1997;41:111–22.
91. Schlatt S, Rosiepen G, Weinbauer GF, Rolf C, Brook PF, Nieschlag E. Germ cell transfer into rat, bovine, monkey and human testes. Hum Reprod. 1999;14:144–50.
92. Shetty G, Mitchell JM, Meyer JM, Wu Z, Lam TNA, Phan TT, Zhang J, Hill L, Tailor RC, Peters KA, Penedo MC, Hanna C, Orwig KE, Meistrich ML. Restoration of functional sperm production in irradiated pubertal rhesus monkeys by spermatogonial stem cell transplantation. Andrology. 2020;8:1428–41.
93. Kubota H, Avarbock MR, Brinster RL. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A. 2004;101:16489–94.
94. Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, Shinohara T. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod. 2003;69:612–6.
95. Hamra FK, Chapman KM, Nguyen DM, Williams-Stephens AA, Hammer RE, Garbers DL. Self-renewal, expansion, and transfection of rat spermatogonial stem cells in culture. Proc Natl Acad Sci U S A. 2005;102:17430–5.
96. Ryu BY, Kubota H, Avarbock MR, Brinster RL. Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. Proc Natl Acad Sci U S A. 2005;102:14302–7.
97. Kanatsu-Shinohara M, Takehashi M, Takashima S, Lee J, Morimoto H, Chuma S, Raducanu A, Nakatsuji N, Fassler R, Shinohara T. Homing of mouse spermatogonial stem cells to germline niche depends on beta1-integrin. Cell Stem Cell. 2008;3:533–42.
98. Kanatsu-Shinohara M, Ogonuki N, Iwano T, Lee J, Kazuki Y, Inoue K, Miki H, Takehashi M, Toyokuni S, Shinkai Y, Oshimura M, Ishino F, Ogura A, Shinohara T. Genetic and epigenetic properties of mouse male germline stem cells during long-term culture. Development. 2005;132:4155–63.
99. Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A, Shinohara T. Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biol Reprod. 2005;72:985–91.
100. Kanatsu-Shinohara M, Ogonuki N, Matoba S, Morimoto H, Ogura A, Shinohara T. Improved serum- and feeder-free culture of mouse germline stem cells. Biol Reprod. 2014;91:88.
101. Kanatsu-Shinohara M, Muneto T, Lee J, Takenaka M, Chuma S, Nakatsuji N, Horiuchi T, Shinohara T. Long-term culture of male germline stem cells from hamster testes. Biol Reprod. 2008;78:611–7.
102. Kubota H, Wu X, Goodyear SM, Avarbock MR, Brinster RL. Glial cell line-derived neurotrophic factor and endothelial cells promote self-renewal of rabbit germ cells with spermatogonial stem cell properties. FASEB J. 2011;25:2604–14.
103. Kakiuchi K, Taniguchi K, Kubota H. Conserved and non-conserved characteristics of porcine glial cell line-derived neurotrophic factor expressed in the testis. Sci Rep. 2018;8:7656.
104. Langenstroth D, Kossack N, Westernströer B, Wistuba J, Behr R, Gromoll J, Schlatt S. Separation of somatic and germ cells is required to establish primate spermatogonial cultures. Hum Reprod. 2014;29:2018–31.
105. Wu X, Schmidt JA, Avarbock MR, Tobias JW, Carlson CA, Kolon TF, Ginsberg JP, Brinster RL. Prepubertal human spermatogonia and mouse gonocytes share conserved gene expression of germline stem cell regulatory molecules. Proc Natl Acad Sci U S A. 2009;106:21672–7.
106. Mirzapour T, Movahedin M, Ibrahim TAT, Koruji M, Haron AW, Nowroozi MR, Rafieian SH. Effects of basic fibroblast growth factor and leukaemia inhibitory factor on proliferation and short-term culture of human spermatogonial stem cells. Andrologia. 2012;44:41–55.
107. He ZP, Kokkinaki M, Jiang JJ, Dobrinski I, Dym M. Isolation, characterization, and culture of human spermatogonia. Biol Reprod. 2010;82:363–72.
108. Liu SX, Tang ZW, Xiong T, Tang W. Isolation and characterization of human spermatogonial stem cells. Reprod Biol Endocrinol. 2011;9:141.
109. Akhondi MM, Mohazzab A, Jeddi-Tehrani M, Sadeghi MR, Eidi A, Khodadadi A, Piravar Z. Propagation of human germ stem cells in long-term culture. Iran J Reprod Med. 2013;11:551–8.
110. Goharbakhsh L, Mohazzab A, Salehkhou S, Heidari M, Zarnani AH, Parivar K, Akhondi MM. Isolation and culture of human spermatogonial stem cells derived from testis biopsy. Avicenna J Med Biotechnol. 2013;5:54–61.
111. Guo Y, Liu L, Sun M, Hai Y, Li Z, He Z. Expansion and long-term culture of human spermatogonial stem cells via the activation of smad3 and akt pathways. Exp Biol Med (Maywood). 2015;240:1112–22.
112. Piravar Z, Jeddi-Tehrani M, Sadeghi MR, Mohazzab A, Eidi A, Akhondi MM. In vitro culture of human testicular stem cells on feeder-free condition. J Reprod Infert. 2013;14:17–22.

113. Smith JF, Yango P, Altman E, Choudhry S, Poelzl A, Zamah AM, Rosen M, Klatsky PC, Tran ND. Testicular niche required for human spermatogonial stem cell expansion. Stem Cells Transl Med. 2014;3:1043–54.
114. Zheng Y, Thomas A, Schmidt CM, Dann CT. Quantitative detection of human spermatogonia for optimization of spermatogonial stem cell culture. Hum Reprod. 2014;29:2497–511.
115. Abdul Wahab AY, Md Isa ML, Ramli R. Spermatogonial stem cells protein identification in in vitro culture from non-obstructive azoospermia patient. Malay J Med Sci. 2016;23:40–8.
116. Medrano JV, Rombaut C, Simon C, Pellicer A, Goossens E. Human spermatogonial stem cells display limited proliferation in vitro under mouse spermatogonial stem cell culture conditions. Fertil Steril. 2016;106:1539.
117. Chen B, Wang YB, Zhang ZL, Xia WL, Wang HX, Xiang ZQ, Hu K, Han YF, Wang YX, Huang YR, Wang Z. Xeno-free culture of human spermatogonial stem cells supported by human embryonic stem cell-derived fibroblast-like cells. Asian J Androl. 2009;11:557–65.
118. Kokkinaki M, Djourabtchi A, Golestaneh N. Long-term culture of human ssea-4 positive spermatogonial stem cells (SSCS). J Stem Cell Res Ther. 2011;S2:003.
119. Nowroozi MR, Ahmadi H, Rafiian S, Mirzapour T, Movahedin M. In vitro colonization of human spermatogonia stem cells: effect of patient's clinical characteristics and testicular histologic findings. Urology. 2011;78:1075–81.
120. Gat I, Maghen L, Filice M, Wyse B, Zohni K, Jarvi K, Lo KC, Gauthier Fisher A, Librach C. Optimal culture conditions are critical for efficient expansion of human testicular somatic and germ cells in vitro. Fertil Steril. 2017;107:595–605.e597.
121. Murdock MH, David S, Swinehart IT, Reing JE, Tran K, Gassei K, Orwig KE, Badylak SF. Human testis extracellular matrix enhances human spermatogonial stem cell survival in vitro. Tissue Eng A. 2018;25:663.
122. Chikhovskaya JV, van Daalen SKM, Korver CM, Repping S, van Pelt AMM. Mesenchymal origin of multipotent human testis-derived stem cells in human testicular cell cultures. MHR: Basic Sci Reprod Med. 2014;20:155–67.
123. David S, Orwig KE. Spermatogonial stem cell culture in oncofertility. Urol Clin North Am. 2020;47:227–44.
124. McCarrey JR. Development of the germ cell. In: Desjardins C, Ewing LL, editors. Cell and molecular biology of the testis. New York, NY: Oxford University Press; 1993. p. 58–89.
125. Bellve AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M. Spermatogenic cells of the prepubertal mouse. Isolation and morphological characterization. J Cell Biol. 1977;74:68–85.
126. Busada JT, Velte EK, Serra ND, Cook KK, Niedenberger BA, Willis WD, Goulding EH, Eddy EM, Geyer CB. Rhox13 is required for a quantitatively normal first wave of spermatogenesis in mice. Reproduction. 2016;152:379.
127. Koskenniemi JJ, Virtanen HE, Toppari J. Testicular growth and development in puberty. Curr Opin Endocrinol Diab Obes. 2017;24:215.
128. Simorangkir DR, Ramaswamy S, Marshall GR, Roslund R, Plant TM. Sertoli cell differentiation in rhesus monkey (macaca mulatta) is an early event in puberty and precedes attainment of the adult complement of undifferentiated spermatogonia. Reproduction. 2012;143:513–22.
129. Arregui L, Dobrinski I. Xenografting of testicular tissue pieces: 12 years of an in vivo spermatogenesis system. Reproduction. 2014;148:R71–84.
130. Kaneko H, Kikuchi K, Nakai M, Somfai T, Noguchi J, Tanihara F, Ito J, Kashiwazaki N. Generation of live piglets for the first time using sperm retrieved from immature testicular tissue cryopreserved and grafted into nude mice. PLoS One. 2013;8:e70989.
131. Weiss RA. The discovery of endogenous retroviruses. Retrovirology. 2006;3:67.
132. Kimsa MC, Strzalka-Mrozik B, Kimsa MW, Gola J, Nicholson P, Lopata K, Mazurek U. Porcine endogenous retroviruses in xenotransplantation--molecular aspects. Viruses. 2014;6:2062–83.
133. CDC. Diseases directly transmitted by rodents. Atlanta, GA: CDC; 2017. https://www.cdc.gov/rodents/diseases/direct.html.
134. Wistuba J, Luetjens CM, Wesselmann R, Nieschlag E, Simoni M, Schlatt S. Meiosis in autologous ectopic transplants of immature testicular tissue grafted to Callithrix jacchus. Biol Reprod. 2006;74:706–13.
135. Luetjens CM, Stukenborg J-B, Nieschlag E, Simoni M, Wistuba J. Complete spermatogenesis in orthotopic but not in ectopic transplants of autologously grafted marmoset testicular tissue. Endocrinology. 2008;149:1736–47.
136. Jahnukainen K, Ehmcke J, Nurmio M, Schlatt S. Autologous ectopic grafting of cryopreserved testicular tissue preserves the fertility of prepubescent monkeys which receive a sterilizing cytotoxic therapy. Cancer Res. 2012;72:5174–8.
137. Jahnukainen K, Ehmcke J, Nurmio M, Schlatt S. Autologous ectopic grafting of cryopreserved testicular tissue preserves the fertility of prepubescent monkeys that receive sterilizing cytotoxic therapy. Cancer Res. 2012;72:5174–8.
138. Dovey SL, Valli H, Hermann BP, Sukhwani M, Donohue J, Castro CA, Chu T, Sanfilippo JS, Orwig KE. Eliminating malignant contamination from therapeutic human spermatogonial stem cells. J Clin Invest. 2013;123:1833–43.
139. de Michele F, Poels J, Vermeulen M, Ambroise J, Gruson D, Guiot Y, Wyns C. Haploid germ cells generated in organotypic culture of testicular tissue from prepubertal boys. Front Physiol. 2018;9:1413.