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Review

Male fertility preservation and restoration strategies for patients undergoing gonadotoxic therapies†

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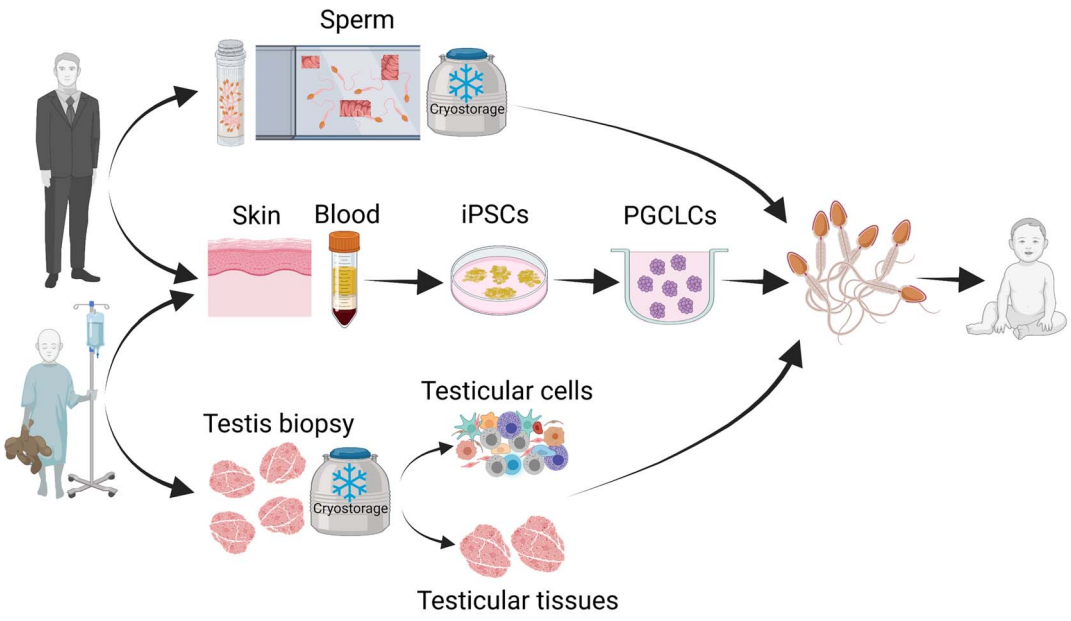
Abstract

Medical treatments for cancers or other conditions can lead to permanent infertility. Infertility is an insidious disease that impacts not only the ability to have a biological child but also the emotional well-being of the infertile individuals, relationships, finances, and overall health. Therefore, all patients should be educated about the effects of their medical treatments on future fertility and about fertility preservation options. The standard fertility preservation option for adolescent and adult men is sperm cryopreservation. Sperms can be frozen and stored for a long period, thawed at a later date, and used to achieve pregnancy with existing assisted reproductive technologies. However, sperm cryopreservation is not applicable for prepubertal patients who do not yet produce sperm. The only fertility preservation option available to prepubertal boys is testicular tissue cryopreservation. Next-generation technologies are being developed to mature those testicular cells or tissues to produce fertilization-competent sperms. When sperm and testicular tissues are not available for fertility preservation, inducing pluripotent stem cells derived from somatic cells, such as blood or skin, may provide an alternative path to produce sperms through a process call in vitro gametogenesis. This review describes standard and experimental options to preserve male fertility as well as the experimental options to produce functional spermatids or sperms from immature cryopreserved testicular tissues or somatic cells.

Summary Sentence

This review describes standard-of-care and experimental approaches to preserve male fertility and treat male infertility. New stem cell- and tissue-based fertility therapies are on the horizon.

Graphical Abstract



Keywords: fertility preservation, male infertility, spermatogonial stem cells, spermatogonial stem cell culture, testicular tissue organ culture, de novo testicular morphogenesis, induced pluripotent stem cells, primordial germ cell-like cells, transplantation, grafting, in vitro gametogenesis

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Introduction

Over the past few decades, childhood cancer survival rates have improved dramatically, up to 88% as recorded in 2018 [1]. Each year in the United States, around 10 000 children between the ages 0 and 14 develop cancers that require them to undergo gonadotoxic treatments, such as chemotherapy and radiation [1]. Patients with nonmalignant conditions (e.g., blood and immune deficiencies and autoimmune disorders) often receive myeloablative conditioning prior to bone marrow transplantation, which is also gonadotoxic [2]. Alkylating chemotherapeutic agents, total body irradiation [3, 4], and gonadal radiation [5] put patients at a significant risk of infertility [5–8]. Studies show that adult survivors of childhood cancers desire to have children [9–13]. Therefore, it is important to discuss the reproductive side effects before the initiation of gonadotoxic therapies.

To develop fertility preservation strategies, we first have to understand the process of sperm production, called spermatogenesis. The seminiferous tubules (ST) are the factory of spermatogenesis within the testes (Figure 1). Spermatogonial stem cells (SSCs) are located on the basement membrane of the ST and are the foundation of spermatogenesis. SSCs balance self-renewing and differentiating divisions to maintain continuous sperm production after puberty. When SSCs differentiate, they undergo several transit amplifying mitotic divisions, giving rise sequentially to undifferentiated type A progenitor spermatogonia, differentiating type A spermatogonia, differentiated type B spermatogonia, and spermatocytes. Primary spermatocytes migrate off the seminiferous tubule basement membrane, across the blood–testis barrier to the adluminal compartment of the ST, and complete meiosis to produce secondary spermatocytes and round spermatids. Round spermatids undergo spermiogenesis to produce terminally differentiated spermatozoa that are released into the lumen of the ST. Sertoli cells are the only somatic cell type inside the ST; they are in direct contact with all germ cells in the testis and regulate every step of the spermatogenic lineage development—from stem cells to sperm [14]. On the outer side of the basement membrane, peritubular myoid cells contain abundant actin filaments to support the structural integrity of the tubule [15]. They also possess contractile function to transport spermatozoa and testicular fluid in the tubule as well as secrete growth factors that are important for the regulation of spermatogenesis [15–17]. The interstitial space is where Leydig cells, endothelial cells, and macrophages are located. Leydig cells are the source of intratesticular testosterone that is required for spermatogenesis [18]. A clear understanding of testicular structure as well as distinct functions of germ cells and somatic cells can help us develop new fertility restoration therapies.

Prior to gonadotoxic treatments, adult and adolescent males have the option to cryopreserve sperms, which can be used to achieve pregnancy in the future using established assisted reproductive technologies (ARTs), including intrauterine insemination [19], in vitro fertilization (IVF) [20], and intracytoplasmic sperm injection (ICSI) [21]. Unfortunately, sperm cryopreservation is not an option for prepubertal patients who are not yet producing sperm. However, prepubertal testicular tissues do have prospermatogonia or SSCs in their testes that are poised to initiate spermatogenesis at puberty [22–24]. Cryopreservation of immature testicular tissues, containing SSCs, may preserve their reproductive potential. We will review testicular cell- and tissue-based methods that are in the research pipeline and may be available in the future to produce sperms from cryopreserved immature testicular tissues. At the end of the article, we will describe a potential alternative, nontesticular source of spermatids or sperms produced from patient-derived induced pluripotent stem cells (iPSCs).

Several cell-based and tissue-based technologies utilizing stem cell potential to restore spermatogenesis are in development. Those technologies include SSC transplantation [23–32], de novo testicular morphogenesis [33, 34], autologous testicular tissue grafting/xenografting [35–41], testicular tissue culture [42–45], and germ cells derived from iPSCs [46–50] (Figure 2). Centers in the United States and around the world are cryopreserving testicular tissue for prepubertal boys in anticipation that some or all of these technologies will be available for these patients in the future [22, 51–64] (Table 1). Therefore, all patients should be counseled about the effects of their medical treatment on fertility and about options (standard or experimental) to preserve fertility prior to their treatments.

Sperm cryopreservation for male adolescents and young adult cancer patients and worldwide fertility cryopreservation programs

Sperm cryopreservation

Sperm cryopreservation was introduced to the human fertility clinic in the 1950s by Bunge and Sherman as a fertility preservation method for adolescent boys and adult men who face a high risk of infertility due to their medical treatments [65]. Moreover, Szell et al. [66] reported that sperms cryopreserved for 40 years retained fertilization function after thawing to produce a healthy offspring. A study showed that 76% of adolescents and young adults (14–40 years old) expressed the desire to have children in the future [67]. The ability to have biological children is part of the recovery path to normalcy, health, and life fulfillment for childhood cancer survivors [9, 11, 13, 68]. However, a study in the United States reported that only 51% of young cancer patients (14–40 years) were offered sperm cryopreservation, and only 24% of them actually froze sperms prior to initiation of their gonadotoxic therapies [67]. An improvement was seen in a 2005 UK study of 13- to 21-year-old adolescents and young adults, in which they were all offered sperm banking; 67% of them successfully banked their sperms [69]. The most common reason cited for failing to preserve sperms was the lack of information among both patients and medical staff [67, 69]. A study in China showed that more than 70% of cancer patients did not know about the existence of sperm banks, and more than 80% of medical staff were not educated about fertility preservation [70]. Moreover, adolescent patients have lower semen volume and sperm motility compared with adult patients [71]. These differences may or may not hinder the success of ARTs in future fertility restoration. Therefore, education on fertility preservation regarding patients who face a significant infertility risk should be broadly provided to medical staff. This allows medical practitioners to thoroughly inform patients at an early stage of their treatments, such that an appropriate plan for preserving the chance of parenthood can be clearly established.

Worldwide testicular tissue cryopreservation programs

Fertility centers around the world are cryopreserving testicular tissues for prepubertal boys with the anticipation

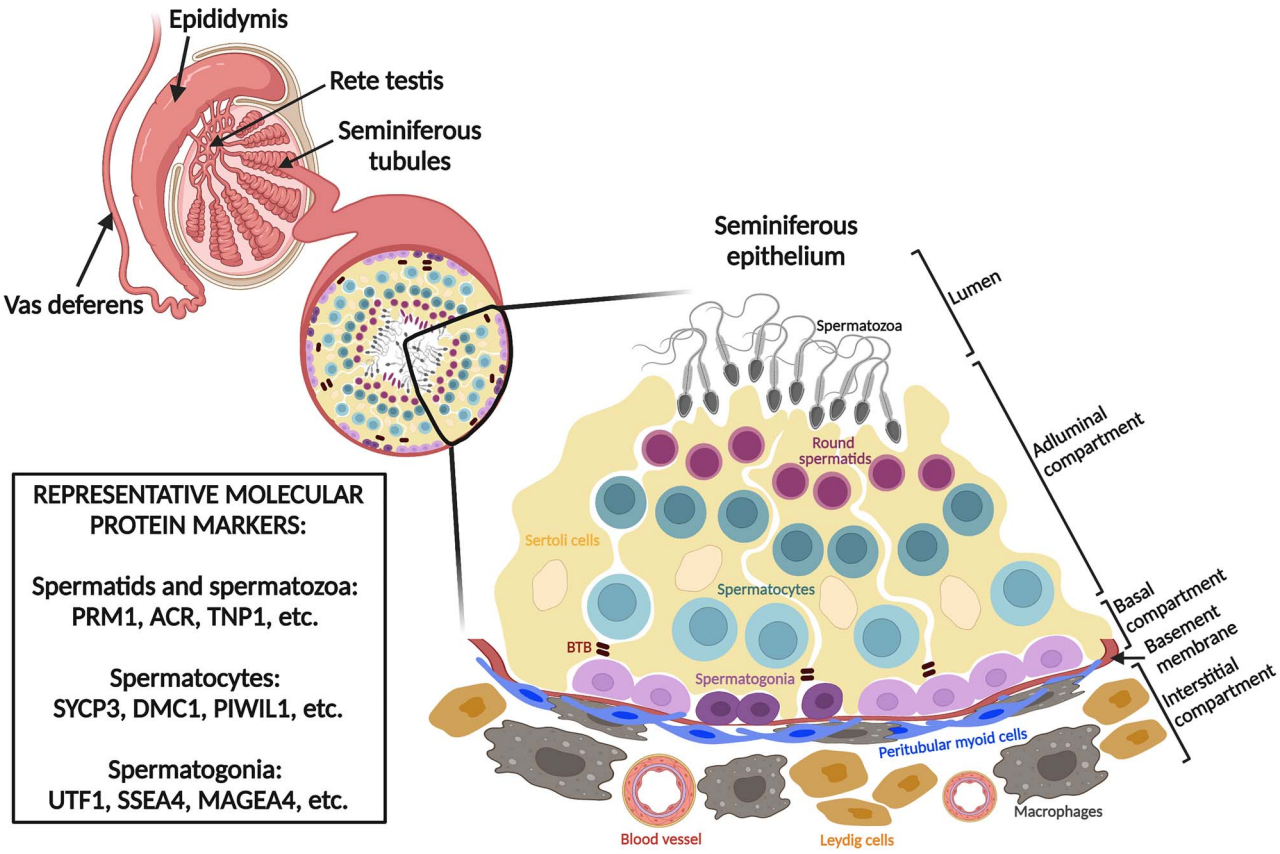

Figure 1. Spermatogenesis occurs within the ST of the testis that are connected to a common collecting reservoir in the testis space where sperms are deposited before flowing to the epididymis. The intratubular space within the ST includes the basal, adluminal, and lumen compartments. Sertoli cells (yellow cells) are the only somatic cell type that directly interacts with germ cells within the intratubular space. SSCs account for a small proportion of undifferentiated spermatogonia located on the basement membrane within the basal compartment. SSCs are responsible for self-renewing and differentiating divisions to maintain the stem cell pool and continuous sperm production process throughout a man's life. When SSCs differentiate, they undergo several transit amplifying mitotic divisions, giving rise sequentially to differentiating type A spermatogonia (dark purple cells) and differentiated type B spermatogonia (light purple cells). Differentiated type B spermatogonia then give rise to primary spermatocytes (light green cells). During meiosis I, primary spermatocytes lift off the basement membrane and pass through the blood–testis–barrier formed between Sertoli cells to enter the adluminal compartment to produce secondary spermatocytes (dark green cells). Secondary spermatocytes complete meiosis II to form round spermatids (magenta cells). Round spermatids undergo spermiogenesis to form elongating spermatids, elongated spermatids, and finally spermatozoa (gray cells). Spermatozoa are then released into the lumen. Peritubular myoid cells and peritubular macrophages are located on the outside of the basement membrane. The interstitial space is the area between STs where Leydig cells, blood vessels, and interstitial macrophages are located.

that new reproductive technologies will be available to them in the future [22, 51–64, 72, 73]. Table 1 summarizes the efforts of 15 centers that perform testicular tissue or cell cryopreservation for patients who were facing the risk of infertility due to their diseases, medical treatments or other circumstances. Three centers reported samples that included adult patients (older than 18 years old) [22, 52, 58]. The rest included patients with the age range between 0 and 18 years old with oncology as the most common diagnosis [53–56, 59, 60, 62, 74–78]. Eleven out of 15 centers reported using dimethyl sulfoxide (DMSO) as their choice of cryoprotectant [22, 54–56, 60, 62, 74–78] and controlled slow freezing as their preferred freezing method [22, 53–56, 59, 62, 74, 76–78]. Most sites in Table 1 are cryopreserving intact pieces of testicular tissue because this preserves the options for both cell- and tissue-based therapies in the future.

The University of Pittsburgh Medical Center (UPMC) Fertility Preservation Program and coordinated centers have cryopreserved testicular tissues for 517 patients between January 2011 and March 2022 (University of Pittsburgh IRB STUDY19020220; Coordinating Centers IRB STUDY19110083). These include patients at risk of infertility due to medical treatments for cancer, myeloablative conditioning prior to bone marrow transplantation for benign diseases (e.g., sickle cell disease, thalassemia, etc.), hormone modulating treatments for gender dysphoria, and gonadectomy for patients with differences in sexual development (Figure 3). The average age of patients was 7.6 years (standard error of the mean (SEM) = 0.22) and ranged from 3 months to 34 years old. The amount of tissue obtained by biopsy is at the discretion of the surgeons who typically recover as much tissue as possible (to maximize options for downstream use in reproduction) without compromising the function of the remaining testis (usually 10–20% of testicular volume). The average amount of tissue collected was 472.9 mg (SEM = 45.5) and ranged 10.1–9824.8 mg. Our center freezes intact pieces of testicular tissue (9–25 mm^3, ~15 mg) in a medium containing 5% DMSO and 5% serum substitute supplement (84% human serum albumin and 16% human globulins) using the method of controlled slow rate freezing described by Keros and colleagues [74]. We have found that the recovery

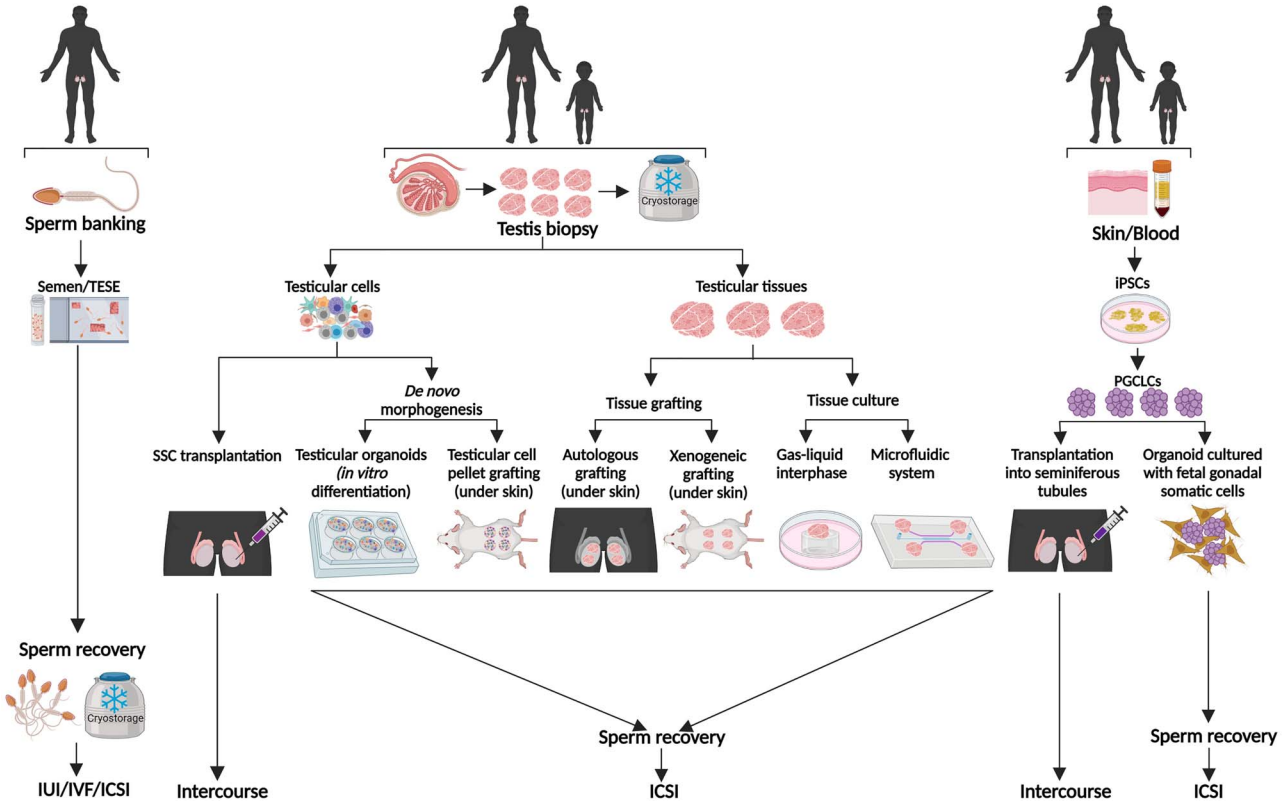

Figure 2. The schematic diagram of standard and experimental male fertility restoration technologies that have produced offspring in at least one mammalian species (Abbreviations: TESE, testicular sperm extraction; IUI, intrauterine insemination; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; SSC, spermatogonial stem cells; iPSCs, induced pluripotent stem cells; PGCLCs, primordial stem cell-like cells).

Table 1. Published reports of testicular tissue cryopreservation.

Study	Left location	Number of patients	Indication (Biopsy)	Previous Gonado-toxic treatment (Yes/No)	Patients' age range	Frozen material	Freezing method	Cryoprotectant
Bahadur et al. [51]	United Kingdom (LDN)	2	Oncology	Yes and No	8 and 13	Tissue	Liquid nitrogen	Glycerol or 1,2-propanediol
Radford et al. [52]	United Kingdom (MAN)	12	Hodgkin's disease	No	Adults	Cells	CRF	DMSO, ethylene glycol, glycerol, and 1,2-propanediol
Kvist et al. [53]	Denmark (CPH)	8	Cryptorchid	No	1–5	Tissue	CRF	Ethylene glycol
Ginsberg et al. [54]	United States (PA)	48	Oncology	No	0–12	Tissue	CRF	DMSO
Sadri-Ardekani et al. [55]	United States (NC)	23	Oncology/cryptorchid	No	0.7–16 oncology 1.4–11 cryptorchid	Tissue	CRF	DMSO and glycerol
Uijldert et al. [56]	The Netherlands (AMS)	78	Oncology	No	0–15	Tissue	CRF	DMSO
Ho et al. [57]	Australia (MEL)	44	Hematology/Oncology	Yes and No	0.3–16.8	Tissue	CRF	DMSO
Heckman et al. [58]	Germany (Munster)	39	Oncology/Klinefelter	Yes and No	2–20	Tissue	Not indicated	DMSO
Valli-Pulaski et al. [22]	United States (PA)	189	Oncology/Orchiectomy /Cryptorchid/Klinefel-ter	Yes and No	0–39	Tissue	CRF	DMSO
Hildorf et al. [59]	Denmark (CPH)	37	Cryptorchid	No	0–3	Tissue	CRF	Ethylene glycol
Braye et al. [60]	Belgium (BXL)	112	Oncology/Cryptorchid/ Klinefelter	No	0–18	Tissue	Mr. Frosty in −80 °C	DMSO
Borgstrom et al. [61]	Sweden (STHLM)	20	Oncology	No	1.5–14.5	Tissue	CRF and vitrification	DMSO
Kanbar et al. [62]	Belgium (BXL)	139	Hematology/Oncology	Yes and No	0–16	Tissue	CRF	DMSO
Rives-Feraille et al. [63]	France	87	Oncology	Yes and No	0–16	Tissue	CRF	DMSO
Braye et al. [64]	Belgium (BXL)	22	Klinefelter	N/A	4.8–18.4	Tissue	Mr. Frosty in −80 °C	DMSO

Abbreviation: CRF, controlled rate freezing; DMSO, dimethyl sulfoxide; N/A, not available.

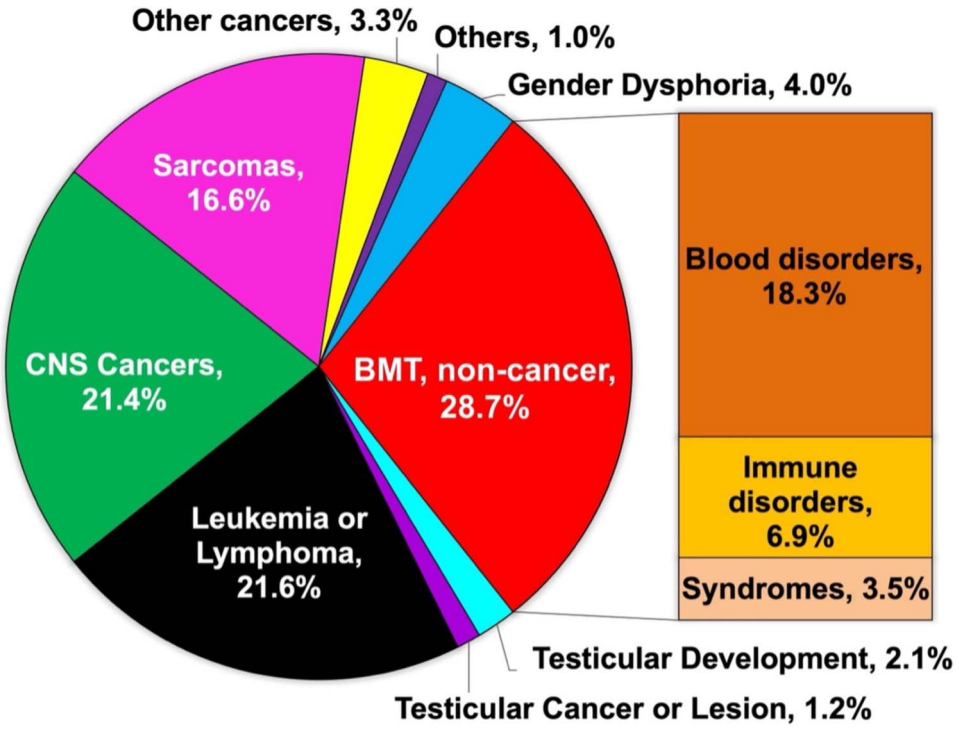

Figure 3. Testicular tissue cryopreservation of patients at the UPMC Fertility Preservation Program (March 2011–March 2022) (Abbreviation: CNS, central nervous system; BMT, bone marrow transplantation).

of transplantable stem cells from cryopreserved testicular tissue pieces is better than the recovery of transplantable stem cells from cryopreserved testicular cell suspensions (data not shown). Moreover, tissue pieces of this size (9–25 mm^3) are large enough to suture under the skin for testicular tissue grafting, as previously described [40].

Counseling young patients and their families about the reproductive side effects of medical treatments and options for fertility preservation can be challenging and requires a multidisciplinary effort. However, the effort is justified because adult survivors of childhood cancers report that fertility is important to them and patients are willing to undergo experimental therapies to preserve the possibility of having a biological child [9, 12, 13, 79–82].

Cell-based therapies for male fertility restoration

Spermatogonial stem cell transplantation

Mouse SSC transplantation was first reported by Brinster and colleagues in 1994 [30, 83]. These studies demonstrated that mouse SSCs were able to regenerate complete spermatogenesis when transplanted into the testes of infertile males [30, 83]. The efficiency of the regenerated spermatogenesis depends on the physiology of the recipient testes. Engraftment and initiation of spermatogenesis in neonatal testes is more robust than in adult testes [23]. Ablation of endogenous spermatogenesis by gonadotoxic treatment [25] or genetic mutation [23] reduces competition from endogenous stem cells, thus enhancing donor-derived spermatogenesis.

In addition, functional SSCs can be recovered from frozen and thawed mouse testicular cell suspension after 14 years of storage [84]. Indeed, transplantable spermatogonia have been recovered from frozen and thawed cell suspensions of several species [23–30, 83, 85–91]. Therefore, it is reasonable that testicular tissue frozen from prepubertal patients prior to gonadotoxic therapies can be thawed in the future to obtain functional SSCs for transplantation to regenerate spermatogenesis. These studies have built a strong foundation for translational medicine, so infertile adult survivors of childhood cancers who have cryopreserved testicular biopsies may have a chance to obtain biological children in the future. Autologous and homologous testicular cell transplantation was originally described over 25 years ago in mice [30, 83]. This is a robust technology that has been replicated in many mammalian species, including rats [26, 27], pigs [85, 86], goats [28, 87], bulls [88], sheep [29], dogs [89], and monkeys [24, 90–92] (Table 2). Embryos or offspring born from this technology were reported in mice [23, 25, 30, 93], rats [27], goats [28], and monkeys [24, 92] (Table 2).

Radford and colleagues reported the first autologous testicular cell transplantation in human patients in 1999 [94] and in 2003 [52]. Briefly, testicular cell suspensions 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 testicular cell transplantations in 1999. The technique has now been replicated in numerous mammalian species, demonstrating safety and feasibility that may provide stronger support for translation to the human clinic (Table 2). Human studies must proceed with caution and be performed with regulatory approval in experimental human trials.

Table 2. Stem cell- and tissue-based fertility therapies currently in the research pipeline.

Testicular cell suspension				
Technology	**Species**	**Study**	**Method**	**Result**
SSC transplantation	Mouse	Zambrowicz et al. [233], Brinster et al.[30], Ogawa et al. [26], Shinohara et al. [23], Brinster et al. [25], Kanatsu-Shinohara et al. [234], Azizi et al. [31], Morimoto et al. [32]	Autologous transplantation [30, 234] Allogenic transplantation [23, 25, 30–32, 233] Xenotransplantation [26]	• Spermatozoa [26, 31, 233] • Offspring [23, 25, 30, 32, 234]
	Rat	Clouthier et al. [235], Ogawa et al. [26], Hamra et al. [27]	Allogenic transplantation [26, 27] Xenotransplantation [235]	• Colonization [26] Spermatozoa [235] Offspring [27]
	Hamster	Ogawa et al. [236]	Xenotransplantation [236]	• Normal round spermatids, abnormal spermatozoa [236]
	Cat	Kim et al. [237]	Xenotransplantation [237]	• Colonization [237]
	Rabbit	Dobrinski et al. [238]	Xenotransplantation [238]	• Colonization and SSC proliferation [238]
	Dog	Dobrinski et al. [238] Kim et al. [89]	Allogenic transplantation [89] Xenotransplantation [238]	• Colonization and SSC proliferation [238] • Spermatozoa [89]
	Pig	Dobrinski et al. [93] Honaramooz et al. [85] Mikkola et al. [86]	Allogenic transplantation [85, 86] Xenotransplantation [93]	• Colonization and SSC proliferation [85, 93] • Spermatozoa [86]
	Goat	Honaramooz et al. [87] Honaramooz et al. [28]	Autologous transplantation [87] Allogenic transplantation [28, 87]	• Spermatogenesis initiation [87] • Offspring [28]
	Bull	Dobrinski et al. [93] Oatley et al. [239] Izadyar et al. [88]	Autologous transplantation [88] Xenotransplantation [93, 239]	• Colonization and SSC proliferation [93, 239] • Spermatozoa [88]
	Sheep	Herrid et al. [29]	Allogenic transplantation [29]	• Offspring [29]
	Horse	Dobrinski et al. [93]	Xenotransplantation [93]	• Colonization and SSC proliferation [93]
	Monkey	Nagano et al. [240] Schlatt et al. [241] Jahnukainen et al. [90] Hermann et al. [24] Shetty et al. [91] Shetty et al. [92]	Autologous transplantation [24, 90, 91, 241] Allogenic transplantation [24, 90, 92] Xenotransplantation [240]	• Colonization and SSC proliferation [240] • Elongated spermatids [241] • Spermatozoa [90, 91] • Embryos [24, 92]
	Human	Radford et al. [94] Brook et al. [242] Nagano et al. [129]	Autologous transplantation [94, 242] Xenotransplantation [129]	• Colonization and SSC proliferation [129] • No reported results [94, 242]
De novo testicular morphogenesis	Mouse	Kita et al. [33] Yokonishi et al. [150] Zhang et al. [151] Zhang et al. [34] Baert et al. [152] Sakib et al. [149]	Fresh fetal testicular cell aggregates + Matrigel → grafted under mouse dorsal skin [33] Fresh neonatal testicular cell aggregates + Matrigel → grafted under mouse dorsal skin [33, 34] Fresh neonatal testicular cell aggregates → cultured on agarose gel stand [150] Fresh neonatal testicular cell aggregates → cultured on collagen [151] Fresh neonatal testicular cells → seeded on alginate scaffold [152] Fresh neonatal testicular cell aggregates → cultured on Matrigel [149]	• Inverted organization of spermatogonia and somatic cells [149] • Spermatocytes [151] • Round spermatids [150] • Elongated spermatids [152] • Offspring [33, 34]
	Rat	Zenzes et al. [148] Gassei et al. [155] Kita et al. [33] Gassei et al. [156] Gassei et al. [138] Alves-Lopes et al. [139]	Fresh neonatal, prepubertal, pubertal, and adult testicular cells → cultured in a rotation system [148] Fresh neonatal cell aggregates + Matrigel → grafted under mouse dorsal skin [33, 138, 155, 156] Fresh neonatal, prepubertal, and adult testicular cells → cultured in three-layer or hanging-drop systems [139]	• Spermatogonia [138, 139, 148, 156] • Tubule formation and vascularization [155] • Offspring [33]

(Continued)

Table 2. Continued.

Testicular cell suspension				
Technology	**Species**	**Study**	**Method**	**Result**
	Pig	Dufour et al. [140] Kita et al. [33] Honaramooz et al. [141] Dores et al. [142] Sakib et al. [149]	Fresh neonatal Sertoli cell aggregates → grafted under mouse renal subcapsule [140] Fresh neonatal testicular cells aggregates + Matrigel → grafted under mouse dorsal skin [33, 141, 142] Fresh neonatal testicular cell aggregates → cultured on Matrigel [149]	• Inverted organization of germ cells and somatic cells [149] • Spermatogonia [33, 142] • Tubule formation and vascularization [140] • Round spermatids [141]
	Sheep	Arregui et al. [143]	Fresh neonatal testicular cell aggregates → grafted under mouse dorsal skin [143]	• Elongated spermatids [143]
	Monkey	Aeckerle et al. [144] Huleihel et al. [153] Sakib et al. [149]	Fresh neonatal, juvenile, adult testicular cells aggregates + Matrigel → grafted under mouse dorsal skin [144] Fresh juvenile testicular cell aggregates → cultured on agarose gel or methycellulose [153] Cryopreserved juvenile testicular cell aggregates → cultured on Matrigel [149]	• Inverted organization of spermatogonia and somatic cells [149] • Spermatogonia [144] • Round spermatids [153]
	Human	Baert et al. [145] Pendergraft et al. [146] Baert et al. [147] Sakib et al. [149] Oliver et al. [154]	Fresh fetal testicular cells → cultured on 3-LGS [154] Fresh and cryopreserved adult testicular cells aggregates → cultured on human testis ECM [145, 146] Fresh pubertal and adult testicular cells → cultured on human testis scaffold-based or scaffold-free transwells [147] Cryopreserved pre-pubertal testicular cell aggregates → cultured on Matrigel [149]	• Maturation of Sertoli cells and Leydig cells, decrease in prospermatogonia number [154] • Inverted organization of spermatogonia and somatic cells [149] • Spermatogonia clusters, normal spatial-temporal somatic cell arrangements and functions [145] [147] • Elongated spermatids [146]
Testicular tissue				
Technology	**Species**	**Study**	**Method**	**Result**
Autologous/Allogenic transplantation	Mouse	Honaramooz et al. [36] Schlatt et al. [157] Shinohara et al. [37] Schlatt et al. [35] Geens et al. [158] Yu et al. [159] Goossens et al. [160]	Neonatal tissues Fresh—dorsal skin [35, 36, 157–160] Cryopreserved—dorsal skin [36, 157, 160]; scrotum [37]	• Spermatids: [157] • Spermatozoa: [158–160] • Embryos: [36] • Offspring: [35, 37]
	Monkey	Luetjens et al. [161] Fayomi et al. [40]	Prepubertal tissues Fresh—dorsal skin [40, 161]; scrotum [40, 161] Cryopreserved—dorsal skin [40, 161]; scrotum [40, 161] Adult tissues Fresh—dorsal skin [161]; scrotum [161] Cryopreserved—dorsal skin [161]	• Degenerated tubules: fresh adult grafts under dorsal skin [53] • Spermatogonia: fresh prepubertal grafts under dorsal skin [53] • Spermatocytes: fresh and cryopreserved prepubertal grafts under dorsal skin [53] • Spermatozoa: fresh prepubertal grafts under dorsal skin and in the scrotum [161]; all grafts [40] • Offspring [40]

(Continued)

Table 2. Continued.

Testicular tissue				
Technology	**Species**	**Study**	**Method**	**Result**
Xenotransplantation into SCID/nude mice	Hamster	Schlatt et al. [157]	Neonatal tissues Fresh—dorsal skin [157] Cryopreserved—dorsal skin [157]	• Spermatozoa: [157]
	Cat	Snedaker et al. [162] Kim et al. [163] Arregui et al. [164]	Fetal tissues Cryopreserved—dorsal skin [164] Neonatal tissues Cryopreserved—dorsal skin [164] Prepubertal tissues Fresh—dorsal skin [162, 163] Adult tissues Fresh—dorsal skin [163, 164]	• Degenerated: ≥ 8-month-old tissues [163], cryopresereved perinatal grafts [164] • Spermatogonia: fresh adult grafts [164] • Elongating spermatids: 7-month-old grafts [163] • Spermatozoa: prepubertal grafts [162]; 8- to 16-week-old grafts [163]
	Rabbit	Shinohara et al. [37]	Prepubertal tissues Fresh—scrotum [37] Cryopreserved—scrotum [37]	• Offspring: [37]
	Dog	Abrishami et al. [165]	Prepubertal tissues Fresh—dorsal skin [165] Pubertal tissues Fresh—dorsal skin [165] Adult tissues Fresh—dorsal skin [165]	• Degenerated: adult grafts [165] • Elongated spermatids: pubertal grafts [165] • Spermatozoa: pre-pubertal grafts [165]
	Pig	Honaramooz et al. [36] Zeng et al. [166] Kaneko et al. [167] Arregui et al. [168] Nakai et al. [38] Abbasi et al. [169] Kaneko et al. [170]	Fetal tissues Cryopreserved—dorsal skin [170] Neonatal tissues Fresh—dorsal skin [38, 166, 167, 169] Prepubertal tissues Fresh—dorsal skin [36] Cryopreserved—dorsal skin [36] Adult tissues Fresh—dorsal skin [168]	• Degenerated: [168] • Elongated spermatids: [166] • Spermatozoa: [167, 169] • Embryos: [36, 170] • Offspring: [38]
	Goat	Honaramooz et al. [36] Arregui et al. [168]	Prepubertal tissues Fresh—dorsal skin [36] Cryopreserved—dorsal skin [36] Adult tissues Fresh—dorsal skin [168]	• Degenerated: [168] • Embryos: [36]
	Deer	Arregui et al. [164]	Fetal tissues Fresh—dorsal skin [66] Cryopreserved—dorsal skin [66] Adult tissues Fresh—dorsal skin [66] Cryopreserved—dorsal skin [66]	• Spermatocytes: fetal grafts [164] • Round spermatids: adult grafts [164]
	Bull	Oatley et al. [171] Oatley et al. [172] Rathi et al. [173] Zeng et al. [166] Huang et al. [174] Arregui et al. [168] Reddy et al. [175]	Neonatal tissues Fresh—dorsal skin [166, 172] Prepubertal tissues Fresh—dorsal skin [171, 173–175] Adult tissues Fresh—dorsal skin [168, 175]	• Degenerated: 28- to 32-week-old grafts [174] • Sertoli cell only: [168] • Round spermatids: 12- to 20-week-old grafts [174] • Elongated spermatids: [166, 171–173, 175]
	Donkey	Arregui et al. [168]	Adult tissues Fresh—dorsal skin [168]	• Sperm: [168]
	Horse	Rathi et al. [176] Arregui et al. [168]	Neonatal tissues Fresh—dorsal skin [176] Adult tissues Fresh—dorsal skin [168, 176]	• Spermatogonia: 2-week-old to 5-month-old grafts [176] • Spermatocytes: 5-month-old, 12-month-old, and 4-year-old grafts [176] • Elongated spermatids: all conditions [168]; 10-month-old grafts [176]
	Sheep	Zeng et al. [166] Arregui et al. [143]	Neonatal tissues Fresh—dorsal skin [143, 166]	• Elongated spermatids: [143, 166]

(Continued)

Table 2. Continued.

Testicular tissue				
Technology	**Species**	**Study**	**Method**	**Result**
	Monkey	Schlatt et al. [157] Honaramooz et al. [39] Rathi et al. [177] Arregui et al. [168] Lu et al. [41] Ehmcke et al. [178] Ntemou et al. [179]	Neonatal tissues Fresh—dorsal skin [177] Prepubertal tissues Fresh—dorsal [39, 41, 157, 168, 178, 179]; scrotum [179] Pubertal tissues Fresh—dorsal skin [168] Adult tissues Fresh—dorsal skin [168]	• Sertoli cell only: adult grafts [168] • Spermatocytes: [157]; pubertal grafts [168]; grafts under dorsal skin [179] • Elongated spermatids: [177]; prepubertal grafts [168] • Spermatozoa: all conditions [178]; grafts in scrotum [179] • Embryos: [39] • Offspring: [41]
	Human	Skakkebaek et al. [180] Geens et al. [158] Schlatt et al. [181] Yu et al. [159] Wyns et al. [182] Goossens et al. [183] Wyns et al. [184] Sato et al. [185] Van Saen et al. [75] Poels et al. [186] Poels et al. [187] Ntemou et al. [188]	Fetal tissues Fresh—dorsal skin [159, 180] Infant tissues Fresh tissues—dorsal skin [185] Prepubertal tissues Fresh tissues—dorsal skin [183, 188], scrotum [75, 186, 188]; Cryopreserved tissues—scrotum [75, 182, 184, 186, 187] Adult tissues Fresh tissues—dorsal skin [158, 181], scrotum [75] Cryopreserved tissues—scrotum [75]	• Degenerated tubules: [181] • Prospermatogonia: [180] • Spermatogonia: [158, 183]; prepubertal grafts [75]; [60] [182, 187] • Spermatocytes: adult grafts [75]; prepubertal grafts [184–186, 188]
Testicular tissue organ culture	Mouse	Livera et al. [191] Trautmann et al. [208] Gohbara et al. [192] Sato et al. [43] Sato et al. [193] Yokonishi et al. [44] Sato et al. [194] Arkoun et al. [195] Komeya et al. [42] Dumont et al. [196] Dumont et al. [197] Komeya et al. [199] Reda et al. [198] Yamanaka et al. [202] Pence et al. [200] Komeya et al. [201] Baert et al. [152]	Fetal tissues Fresh tissues—floating filter [191, 208] Neonatal tissues Fresh tissues—agarose gel stand [43, 44, 192, 193, 195–201]; microfluidic device [42, 199, 202] Cryopreserved tissues—agarose gel stand [43, 44, 193, 196, 197] Adult tissues Fresh tissues—agarose gel [194]	• Spermatogonia [191] • Spermatocytes [208] • Round spermatids [192, 198] • Elongating spermatids [194, 199, 201, 202] • Elongated spermatids [152] • Spermatozoa [193, 195–197, 200] • Offspring [42–44]
	Rat	Livera et al. [191] Reda et al. [203]	Fetal tissues Fresh tissues—floating filter [191] Neonatal tissues Fresh tissues—agarose gel stand [203]	• Spermatogonia [191] • Round spermatids [203]
	Goat	Patra et al. [243]	Prepubertal tissues Fresh tissues—agarose gel stand and hanging-drop system [243] Cryopreserved tissues—agarose gel stand and hanging-drop system [243]	• Spermatozoa [243]
	Monkey	Heckmann et al. [244] Sharma et al. [245]	Prepubertal tissues Fresh—agarose gel stand [244] Fresh—transwell [245]	• Spermatocytes [244] • Spermatocytes [245]

(Continued)

Table 2. Continued.

Testicular tissue				
Technology	**Species**	**Study**	**Method**	**Result**
	Human	Lambrot et al. [207] Jorgensen et al. [204] Jorgensen et al. [205] de Michele et al. [210] de Michele et al. [206] Medrano et al. [209] Yaun et al. [45]	Fetal tissues Fresh—floating membrane [207]; hanging-drop system [205]; agarose gel stand [45] Prepubertal tissues Cryopreserved—agarose gel stand [209]; transwell [206, 210] Adult tissues Fresh—hanging-drop system [204]	• Degenerated tubules [207] • Gonocytes [205] • Spermatogonia [204, 210] • Spermatocytes [209] • Round spermatids [206] • Embryos (ROSI) [45]
Embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs)				
Technology	**Species**	**Study**	**Method**	**Result**
ESCs/iPSCs-derived germline stem cells	Mouse	Hayashi et al. [46] Nakaki et al. [47] Ishikura et al. [49]	ESCs and/or iPSCs → adherent differentiation to EpiLCs on fibronectin → floating culture of PGCLC aggregates → transplantation into mouse pup testes → ICSI [46, 47] ESCs → adherent differentiation to EpiLCs on fibronectin → floating culture of PGCLC aggregates → mixed with fetal testicular somatic cells (FTSC) to generate GSCLCs → transplantation into adult mouse testes → ROSI + ICSI [49]	• Offspring [46, 47, 49]
	Monkey	Sosa et al. [218]	iPSCs → adherent differentiation into iMeLCs on fibronectin → floating PGCLC aggregates → transplantation into adult monkey and mouse testes [218]	• PGCLCs developed into VASA+ colonies of spermatogonia in both recipient species [218]
	Human	Park et al. [246] Durruthy et al. [228] Ramathal et al. [229] Sasaki et al. [224] Ramathal et al. [230] Irie et al. [225] Hwang et al. [215]	ESCs and iPSCs → adherent differentiation to PGCLCs on feeder cells derived from human fetal placenta and liver stroma [246] ESCs and iPSCs transplanted into adult mouse ST [228–230] iPSCs → adherent differentiation to iMeLCs on laminin → floating PGCLC aggregates [224] ESCs and iPSCs → adherent differentiation to EpiLCs on vitronectin/gelatin → floating PGCLC aggregates [225] iPSCs → adherent differentiation to iMeLCs on fibronectin → floating PGCLC aggregates → mixed with E12.5 mouse FTSCs into xrTestes in an air-liquid interphase system [215]	• PGCLCs [224, 225, 229, 246] • GCLCs located near the basement membrane [228–230] • Prospermatogonia [215]
ESCs/iPSCs-derived haploid germ cells	Mouse	Zhou et al. [48] Ishikura et al. [50]	ESCs → adherent differentiation to EpiLCs on fibronectin → culture as floating PGCLC aggregates →culture with testicular cells in 1:1 ratio to induce haploid cells → ICSI [48] ESCs → adherent differentiation to EpiLCs on fibronectin → floating culture of PGCLC aggregates → mixed with FTSCs to generate GSCLCs → transplantation into mouse pup testes → organ culture → ROSI [50]	• Offspring [48, 50]

(Continued)

Table 2. Continued.

Embryonic stem cells or induced pluripotent stem cells				
Technology	**Species**	**Study**	**Method**	**Result**
	Monkey	Teramura et al. [219] Yamauchi et al. [220]	ESCs → floating EB aggregates → adherent culture on gelatin [219] ESCs → floating EB aggregates [220]	• VASA+ prospermatogonia, and DMC1+/SCP3+ meiotic cells [219] • Increased *VASA*, *SCP1*, *PIWIL1* transcription [192]
	Human	Kee et al. [221] Panula et al. [222] Eguizabal et al. [231] Easley et al. [223]	ESCs → adherent differentiation on Matrigel [221] ESCs and iPSCs → adherent differentiation on Matrigel [222] iPSCs → adherent differentiation on feeder cells [231] ESCs and iPSCs → cultured in mouse SSC conditions [223]	• Acrosin+ spermatids and 1 N haploid cells [221–223, 231]

Abbreviations: ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; GCLCs, germ cell-like cells; GSCLCs, germline stem cell-like cells; EpiLCs, epiblast-like cells; PGCLCs, primordial germ cell-like cells; EBs, embryoid bodies; SSCs, spermatogonial stem cells; ROSI, round spermatid injection; ICSI, intracytoplasmic sperm injection; FACS, fluorescence-activated cell sorting; IHC, immunohistochemistry; xrTestis, xenogeneic reconstituted testis; VASA, DEAD-box helicase 4; DMC1, DNA meiotic recombinase 1; SCP1, synaptonemal complex protein 1; SCP3, synaptonemal complex protein 3; PIWIL1, Piwi-like RNA-mediated gene silencing 1.

Spermatogonial stem cell culture

SSC transplantation is a robust technology that may be ready for translation to the human fertility clinic (e.g., for patients who cryopreserved immature testicular tissues with SSCs when they were young). However, based on our experiences in Pittsburgh and coordinated centers and other published reports [22, 74, 95], the amount of tissue obtained by biopsy from prepubertal patients is small (30–400 mg) and may contain a limited number of SSCs. Therefore, in vitro SSC expansion may be needed prior to transplantation to achieve robust engraftment and regeneration of spermatogenesis.

In rodents, SSCs can be maintained in long-term culture with significant expansion in number, and these SSCs retain their potential to restore spermatogenesis and fertility upon transplantation [96–101]. The success of SSC culture in rodents required the development of methods to isolate and enrich SSCs while eliminating testicular somatic cells that could rapidly overwhelm the cultures. In their initial report on mouse SSC culture, Kanatsu-Shinohara and colleagues [99] plated heterogeneous testis cells from newborn mice on gelatin-coated plates. Testicular somatic cells rapidly adhered to the plates while germ cells remained floating and could be sequentially aspirated and replated onto secondary plates to gradually remove somatic cells. In contrast, Hamra and colleagues [96] used a positive selection approach with rat pup testis cells that were plated on laminin. SSCs rapidly adhered to the laminin-coated plates and floating testicular somatic cells could be removed by aspiration. Other studies used fluorescence activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) for the cell surface marker THY1 (CD90) to enrich SSCs and reduce contaminating somatic cells prior to culture [102]. Development of a serum-free, defined medium facilitated the discovery of specific growth factors that were required for SSC maintenance and proliferation in culture. While Kanatsu-Shinohara used glial cell-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2), epidermal growth factor (EGF), and leukemia inhibitory factor (LIF) [99], others have shown that GDNF is necessary and sufficient to support rodent SSC expansion in culture. The addition of FGF2 and the soluble GDNF family receptor (GFRα1) can increase the rate of SSC expansion [100, 103]. Finally, feeder cells, such as mouse embryonic fibroblasts are frequently needed to maintain SSCs in culture, although feeder-free culture conditions on substrates such as laminin have been described [104]. SSC transplantation provided a definitive bioassay to confirm the presence and number of functional SSCs in culture [105].

A number of laboratories around the world have reported culturing nonhuman primate and human SSCs (hSSCs) [106–128]. Langenstroth and colleagues [128] reported on the isolation, enrichment, and short-term 11-day culture of adult marmoset SSCs. Adult marmoset testis cells were placed in regular tissue culture plates with minimal essential media alpha (MEMα) supplemented with 10% fetal bovine serum (FBS) at 35 °C [128]. No feeder cells or other tissue culture substrates (e.g., laminin) were used and no growth factors were added [128]. Similar to the experience with mouse SSC cultures, they found that when heterogeneous testis cell suspensions were placed in culture, the somatic cells rapidly overwhelmed the culture [128]. Therefore, they established a separation culture system in which floating supernatant cells were aspirated after 24 h and placed in a secondary culture dish [128]. Supernatant cells and attached cells were then cultured separately for 11 days [128]. Then, supernatant cells were collected from the supernatant cultures and attached cells were collected from the attached cultures for colonization potential quantification by primate-to-nude-mouse xenotransplantation [128]. Monkey and hSSCs do not produce complete spermatogenesis after xenotransplantation to recipient mouse testes, but they do recapitulate several functions that are unique to SSCs. They migrate and engraft the basement membrane of recipient mouse ST, proliferate to produce chains and clusters of cells with typical spermatogonial appearance (high nuclear to cytoplasmic ratio and frequently connected by intracytoplasmic bridges), and survive long term [107, 129–134]. Using primate-to-nude-mouse xenotransplantation, Langenstroth and colleagues [128] found that the 11-day supernatant culture

retained about 60% of the number of colonizing spermatogonia that were originally placed in culture on day 0, and this was 8-fold higher than the colonizing activity from 11-day attached cultures.

More than 20 studies on hSSC culture methods have been published [106–126], including three with fetal or prepubertal testicular cells [76, 108, 109]. Many have used differential plating on plastic, lectin, collagen, or gelatin to enrich SSCs and reduce testicular somatic cell contamination. Others have used positive or negative FACS or MACS selection alone or in combination with differential plating. Positive selection markers have included integrin subunit alpha 6 (ITGA6), CD9, G-protein coupled receptor 125 (GPR125), expression of stage-specific embryonic antigen 4 (SSEA4), and epithelial cell adhesion molecule (EpCAM). Negative selection markers include KIT proto-oncogene (cKIT), protein tyrosine phosphatase receptor type C (PTPRC or CD45), and THY1 (reviewed in [135]). It is noteworthy that THY1 is a positive selection marker for mouse spermatogonia but a negative selection marker for transplantable human spermatogonia [102, 119, 135, 136].

Most hSSC culture studies used culture conditions similar to those originally described by Kanatsu-Shinohara and colleagues in mice, including StemPro-34 medium supplemented with various combinations of GDNF, FGF2, EGF and LIF. Some of those studies reported significant expansion of hSSC numbers in culture [76, 107, 137], while others reported a rapid decline in hSSC numbers using the same conditions [118, 120, 122, 125]. These disparate outcomes may be explained by differences in starting cell populations, culture conditions, and approaches that were employed to identify and quantify hSSCs in culture (ranging from counting total cells in culture to quantifying xenotransplantation colonizing events). Therefore, there is no consensus "best method" for culturing hSSCs that has been independently replicated in different laboratories and no consensus on best methods to identify and quantify bona fide hSSCs in culture. Nonetheless, a number of research groups have reported hSSC survival and expansion for periods ranging from 1 week to 6 months [106–126]. While the results for hSSC culture are promising, they are challenged by the inability to test the full spermatogenic potential of these cells in xenotransplantation; and autologous or homologous transplantation in humans is currently not feasible.

De novo testicular morphogenesis

De novo testicular morphogenesis is defined as the reconstruction of testicular architecture and spermatogenesis from a heterogenous testicular cell suspension to replicate the in vivo cell-to-cell interactions and functions of germ cells and somatic cells. De novo testicular morphogenesis can be performed entirely in culture or by subcutaneous xenotransplantation into animal recipients (Figure 2, Table 2). This approach can be used to dissect, manipulate, or modify the component parts of a complex tissue to learn the impact on testicular cell development or function. The approach may also be used to eliminate unwanted and unnecessary components, such as contaminating malignant cells, which could disrupt development or be unsafe for therapy. Cells from a single testis can be disaggregated to produce many organoids that can be used for high throughput toxicity testing or novel compound screening. De novo testicular morphogenesis has been described in rodents as well as farm animals, nonhuman primates, and humans [33, 138–149].

Methods for de novo testicular morphogenesis, in vitro, were first described in rodents (Figure 2, Table 2). Yokonishi and colleagues [150] pioneered the technique by producing organoids from neonatal mouse testis cells in V-bottomed 96-well plates. After 2 days, organoids were collected and cultured at the gas–liquid interface by placing on agarose gel islands half-soaked in culture medium that was supplemented with knockout serum replacement (KSR). Seminiferous tubule-like structures were apparent by two weeks in culture. Sertoli cells and germ cells were arranged inside ST and Leydig cells were located in the space between the de novo-formed tubules. Spermatocytes and spermatids were observed by 60 days in culture. Zhang and colleagues used a variation of the gas–liquid interface culture system; testicular cell/collagen aggregates were deposited on a nucleopore filter that was then floated on culture medium [151]. Seminiferous tubule-like structures contained spermatogonia and spermatocytes [151]. Sertoli cell differentiation occurred only when KSR was included in the medium. Baert and colleagues [152] seeded neonatal mouse testis cells on an alginate lattice scaffold alone or embedded with testicular interstitial cells. The testicular cells formed organoids within the lattice but did not self-organize into ST [152]. Nonetheless germ cell differentiation to the elongated spermatid stage was reported on both the cell-free and cell-laden scaffold [152]. Donor age is a factor. Alves-Lopes and colleagues [139] reported seminiferous tubule formation from 5- to 8-day-old and 20-day-old rat pup testis cells sandwiched in a three-layer Matrigel culture system but not from 60-day old rat testicular cells. To our knowledge, no offspring have been produced from any mouse in vitro de novo testicular morphogenesis approach.

Huleihel and coworkers [153] cultured heterogeneous testicular cell suspensions from prepubertal rhesus macaques in 3-dimensional (3D) methylcellulose or soft agar culture systems. Cells aggregated to produce colonies, including CAMP-responsive element modulator-positive (CREM)+ and acrosin (ACR) + spermatids, but did not reconstitute ST [153]. Sakib and colleagues [149] reported a microwell aggregation approach to producing 3D testicular organoids from neonatal or prepubertal testicular cell suspensions of mice, pigs, macaques, and humans. In all species, the organoids exhibited an inverted seminiferous tubule architecture with germ cells and Sertoli cells on the outside and other somatic cells on the inside of the tubules [149].

Two studies reported adult and pubertal human testicular organoids formation in human testicular extracellular matrix (htECM) [146, 147]. Pendergraft and colleagues [146] used a hanging-drop method to induce organoid formation from cultured adult human spermatogonia mixed with immortalized human Sertoli and Leydig cells suspended in a hydrogel of htECM. Baert and colleagues [147] seeded adult or pubertal heterogeneous human testis cell suspensions onto a 3-dimensional htECM scaffold that retained the tubular architecture. Both approaches led to the production of organoids including germ cells and somatic cells but neither approach produced seminiferous tubule-like structures [146, 147]. Furthermore, Baert and colleagues [147] reported that human testis cell organoids formed with or without the benefit of the scaffold. Similar to results in rodents, age appears to be a factor in the formation of seminiferous tubule-like structures. Oliver and colleagues [154] sandwiched fresh human fetal testicular cells in a three-layer Matrigel gradient system. Seminiferous cord-like structures appeared within 7 days with a clear basement membrane separating Sertoli cells on the inside

from peritubular myoid cells and Leydig cells on the outside [154]. However, although DEAD-box helicase 4 (DDX4 or VASA) + prospermatogonia were observed in the preculture 8-week-old fetal testicular tissues, they disappeared over 7 and 14 days of culture [154]. In summary, fetal or prepubertal testis cell suspensions (mouse, rat, or human) can self-organize to produce seminiferous cord- or seminiferous tubule-like structures that do not appear when pubertal or adult cells are used [154]. Meiotic or postmeiotic cells were occasionally observed, and this does not appear to depend on the formation of tubules [154]. To our knowledge, the function of haploid cells produced by in vitro de novo testicular morphogenesis approaches has not been tested [154].

The second de novo morphogenesis approach is to inject the heterogenous testicular cell suspension under the skin of a mouse host (Figure 2, Table 2). Pelleted testicular cells from mice [33, 34], rats [33, 138, 155, 156], pigs [33, 141, 142], sheep [143], and monkeys [144] have been transplanted into mouse hosts and self-organized in vivo to form seminiferous tubule-like structures (Table 2). Studies from Kita et al. [33] and Zhang et al. [151] confirmed that cells from different sources could be mixed to test their importance in tubule formation or spermatogenesis and that haploid mouse cells produced in de novo reconstructed tubules were competent to fertilize and produce live offspring. Similar seminiferous tubule-like structures containing spermatogenesis with haploid germ cells have been reported from pig [141, 142] and sheep [143] by transplanting neonatal testis cells under the skin of immune-compromised mice. Spermatogenic efficiency was improved by addition of Matrigel but not by introduction of an enriched germ cell fraction into a heterogeneous pig testis cell suspension [142]. The fertilization potential of pig and sheep spermatids in de novo-formed tubules was not tested in those studies [141–143]. Neonatal marmoset monkey testis cells self-organized into seminiferous cord-like structures without lumens after subcutaneous xenotransplantation to mouse hosts [144]. The cords contained Sertoli cells and germ cells, but complete spermatogenesis was not observed and there was no evidence of meiotic or postmeiotic cells [144]. Cords were not formed from juvenile or adult marmoset testis cells [144]. To our knowledge, in vivo de novo testicular morphogenesis has not been attempted with human cells. If human testis cells can produce results similar to those reported above for mice, pigs and sheep, de novo-derived haploid germ cells could theoretically be used for clinical applications. This may raise concerns about xenobiotics that could be circumvented if de novo testicular morphogenesis from humans can be achieved in organoid cultures described above. However, this has not occurred yet.

Testicular tissue-based therapies for male fertility restoration

Testicular tissue grafting

For testicular tissue grafting, SSCs are maintained within intact pieces of tissue, preserving the original architecture of the ST, extracellular matrix, and testicular somatic cells with associated paracrine signaling. Tissue grafting is performed by implanting testicular tissue pieces at an orthotopic (e.g., scrotum) or ectopic (e.g., skin) site in the recipient animals (autologous, allogenic, or xenogeneic recipients) with or without exogenous matrices or hormones [35–41, 75, 143, 157–188] (Table 2). The recipients are usually castrated to stimulate the gonadotropic hormone secretion to promote maturation of the grafted tissues [35–41, 75, 143, 157–188]. The overall goal is not to restore fertility since grafted testicular tissues are not connected to the excurrent duct system. The goal is to mature the grafted tissue to produce sperm that can be used to fertilize eggs by ICSI and produce offspring. Honaramooz and colleagues [85] reported that immature testicular tissues from mice, pigs, and goats could be transplanted under the back skin of immune-deficient nude mice and matured to produce sperm. Two groups later reported that sperms from fresh [35] or cryopreserved [37] immature mouse testicular tissue grafts were competent to fertilize mouse eggs and produce offspring. Immature testicular tissue grafting (autologous or allogeneic) or xenografting is a robust technology that has been replicated in mice [35–37, 157, 158, 160], hamsters [157], rabbits [37], dogs [165], cats [162–164], horses [168, 176], pigs [36, 38, 166, 167, 169, 170], bulls [166, 171–175], goats [36], sheep [143, 166], and monkeys [39, 41, 168, 177–179] with the production of spermatids or spermatozoa (Table 2). Function of graft-derived sperm has been tested by fertilization, and pre-implantation embryo development up to blastocyst stage and/or production of viable offspring were reported in mice [35–37], rabbits [37], pigs [38], goats [36], and rhesus macaques [39–41] (Table 2). Only immature (fetal, neonatal, pre-pubertal) testicular tissues were able to survive and undergo complete spermatogenesis; whereas adult tissues gradually lost differentiated germ cells and degenerated [75, 158, 161, 163–165, 168, 175, 176] (Table 2).

In addition, the efficiency of inducing complete spermatogenesis varies widely among species. In studies of pigs, goats, sheep, and monkeys, at least 50% of tubules contained complete spermatogenesis post xenografting [36, 40, 166]. However, only less than 10% of tubules contained complete spermatogenesis in horse and bull xenografts [171, 173, 174, 176]. Graft retrieval time also greatly affects spermatogenesis efficiency. In mice, grafted tissues recovered after 120 days yielded about four times more tubules with complete spermatogenesis compared with grafts recovered before 120 days [158]. Another mouse study also showed increasing percentage of tubules containing complete spermatogenesis from ~20 to ~55% in grafts retrieved at later timepoints between 4–16 weeks post-grafting [36]. However, the number of surviving spermatogonia in pre-pubertal human testicular tissues xenografted to mice decreased over time and complete spermatogenesis was not observed [158]. Honaramooz et al. [189] reported that the fertilization potential of sperm produced in the pig-to-mouse xenografts decreased over time and suggested that this might be due to lack of excurrent ducts to remove older sperm.

Live birth outcomes from autologous, allogeneic, or xenograft experiments suggest that these approaches may have application for maturing prepubertal testicular tissues that were frozen for patients prior to gonadotoxic therapies (Table 2). Furthermore, xenografting of human tissues to mice or other animal hosts may circumvent the risk of reintroducing malignant cells to patient survivors. Spermatogonia survival from fresh and cryopreserved prepubertal grafts were reported at various timepoints, ranging from days to months [75, 158, 159, 182, 183, 187, 190]. The longest observation time was 9 months with orthotopic xenografts retaining spermatogonia survival better than the ectopic grafts [75, 183]. A few studies have reported the initiation of spermatogenesis up to early spermatocytes

from fresh or cryopreserved immature human orthotopic or ectopic xenografts [184–186, 188]. However, complete spermatogenesis from xenografting human testicular tissues into mice has not yet been achieved [75, 158, 159, 180–188]. Recipient choice may be a factor that should be tested in future studies, including porcine or nonhuman primate species, which are more closely related to humans.

Testicular tissue organ culture

Testicular tissue organ culture (TTOC) is an ex vivo system that utilizes small testicular tissue fragments in culture initiation to preserve the original 3D organization of germ cells and somatic cells. TTOC aims to induce in vitro maturation of germ cell and somatic cell compartments. This approach may help circumvent the risks of malignant contamination associated with autologous cell/tissue transplantation and xenobiotic transmission in xenogeneic transplantation. Several TTOC systems have been described in the past two decades culturing immature testicular tissues of mice, rats, monkeys, and humans (Figure 2, Table 2). In 2006, Livera and colleagues [191] cultured fetal mouse or rat testes on floating filter papers, but could not achieve in vitro maturation of prospermatogonia. Since 2010, several studies have reported spermatid or spermatozoa production from cultures of fresh and/or cryopreserved neonatal mouse testicular tissues in either the gas–liquid interphase systems [43, 44, 192–201] or microfluidic systems [42, 199, 202] (Figure 2, Table 2). In the conventional gas–liquid interphase system, 0.75–3 mm^3 pieces of tissue are cultured on an agarose gel island (0.25–1.5% w/v) half submerged in medium. Neonatal mouse tissues yielded higher in vitro spermatogenesis efficiency compared with adult testes [194]. When starting with immature mouse testicular tissues, the in vitro spermatogenesis timeline was very approximal to in vivo timeline. Round spermatids emerged by day 21 and spermatozoa were observed by day 35 [42–44, 192–202]. Spermatids or spermatozoa isolated from dissociated cultured tissues were used for fertilization via ICSI from which healthy offspring were reported [42–44]. Reda and colleagues [203] reported round spermatids but no spermatozoa in cultures of neonatal rat testicular tissue using the agarose gel system, and the fertilization potential of the round spermatids was not tested.

The testicular tissues cultured on agarose gel tend to mound up after a few days leading to necrosis in the central part of the tissue mound, and spermatogenesis as well as tissue integrity declined after 35 days in culture [34]. Komeya and colleagues invented several microfluidic systems that confine tissues to a small space to prevent mounding of tubules and ensure that all tubules have direct access to the air interface on one side and the medium interface on the other side for exchange of nutrients and waste with medium that flows past the tissue at controlled rates [42, 199, 202]. The pump-driven microfluidic device could maintain continuous spermatogenesis to produce functional spermatozoa for up to six months in culture with more than 90% of tubules containing haploid cells. Healthy offspring were produced from both round spermatid injection (ROSI) and ICSI from spermatozoa [42]. Similarly, pumpless microfluidic devices maintained tubules with spermatogenesis up to the round spermatid stage for at least four months [199, 202]. However, spermatozoa were not observed with the pumpless microfluidics devices, so some optimization of media flow rates or other conditions may be needed.

There are only a few studies describing human TTOC. Jorgensen and colleges were able to preserve the architecture of human fetal gonads and adult testis tissues for up to 2 weeks using a hanging-drop culture system [204, 205]. They reported that fetal germ cells proliferated for at least 2 weeks in culture, and apoptosis was not increased during this time [205]. Differentiated germ cells in adult testicular tissues did not survive past 7–10 days in culture, and proliferation of germ cells also significantly reduced [204]. Yuan et al. cultured fresh gonadal tissue pieces, obtained from 12–19-week-old male fetuses, on agarose gel islands; they reported the presence of round spermatids in 1-month cultures [45]. These in-vitro-derived round spermatids were extracted and used to fertilize human oocytes via ROSI, from which they obtained human blastocysts with normal karyotype and maternal-paternal genetic materials [45]. Moreover, de Michele and colleagues [206] cultured frozen-and-thawed pre-pubertal human testicular tissue pieces in a transwell system and observed round spermatids (1–2 haploid cells per seminiferous tubule cross-section) from 16 to 139 days in culture.

Some of the key medium supplements, including retinoic acid (RA) and follicle stimulating hormone (FSH), were analyzed more in depth in a few studies. RA plays a crucial role in initiating spermatogenesis, but its effects may be age dependent. RA supplement enhanced in vitro spermatogenesis in neonatal mouse testis tissues [195, 197] but was found to be detrimental to fetal human [45, 207] or mouse [195, 208] testicular tissues in culture. FSH and luteinizing hormone (LH) play important roles in maturation of Sertoli cells and Leydig cells, respectively, which are required for germ cell development. Medrano and colleagues [209] reported that FSH promoted Sertoli cell differentiation ((androgen receptor (AR) expression) in prepubertal human testicular tissue cultures. Indeed, FSH and LH promoted in vitro germ cell survival and differentiation in both mouse [195] and human testis tissues [209]. Medrano et al. [209] also reported that replacing fetal bovine serum with KSR improved Sertoli and Leydig cell differentiation as well as the number of undifferentiated embryonic cell transcription factor 1 (UTF1)+ undifferentiated spermatogonia and synaptonemal complex protein 3 (SYCP3)+ premeiotic spermatogonia. Furthermore, Medrano et al. [209] found that sex determining region (SRY)-box transcription factor 9 (SOX9)+ Sertoli and undifferentiated embryonic cell transcription factor 1 (UTF1)+ undifferentiated spermatogonia numbers were higher in cultures maintained a 34 °C (approximating the temperature in the scrotum) than cultures maintained at 37 °C. In a mouse TTOC study, 34 °C cultures yielded higher spermatogenesis efficiency than 32 °C or 37 °C cultures (40.9% at 32 °C, 82.6% at 34 °C, and 0% at 37 °C) [192]. In 2017, de Michele and colleagues [210] reported that when cryopreserved pre-pubertal human testicular tissues were cultured for 139 days in medium supplemented with 50 IU/L FSH, somatic cell maturation, including Sertoli cells and Leydig cells, was observed. Spermatogonia were still present but significantly declined in number after two weeks in culture, and spermatogonia differentiation was not observed in their study [210]. In 2018, the same group reported that a lower concentration of FSH (5 IU/L) supported Sertoli cell and Leydig cell maturation as well as germ cell differentiation to round spermatids [206]. The number of round spermatids was quite low, (1 spermatid/tubule),

which precluded further genetic/epigenetic or functional characterization. Nonetheless, this was the most advanced germ cell stage obtained from cultures of frozen-and-thawed pre-pubertal human testicular tissues. As de Michelle and colleagues [206] still encountered a significant decrease of spermatogonia as soon as 2 weeks after culture initiation, further investigations are guaranteed to enhance the survival and self-renewal of spermatogonia to avoid stem cell exhaustion to differentiation.

Induced pluripotent stem cell-based methods for male fertility restoration

For patients who did not cryopreserve sperm or testicular tissues prior to gonadotoxic treatments, it may be possible in the future to generate germ cells from iPSCs derived from any cell type in the body (e.g., skin fibroblasts, blood lymphocytes). The proof in concept studies were performed using pluripotent mouse embryonic stem cells (ESCs) and mouse iPSCs. The process of in vitro gametogenesis (ESCs or iPSCs → primordial germ cell-like cells (PGCLCs) → spermatogonia and/or sperm) recapitulates germ cell development in vivo. During in vivo development, around day 7–8 in mouse embryonic development or week 2–3 in human embryonic development, primordial germ cells (PGCs) are specified in the epiblast stage embryo and segregate to the yolk sac endoderm [211, 212]. PGCs then proliferate and migrate into the embryo with the hindgut endoderm around mouse embryonic day 10.5 or human embryonic week 4 [211, 212]. On mouse embryonic day 12.5 or human embryonic week 5, PGCs arrive at the genital ridges where they continue proliferation in the indifferent gonads [191, 192]. Sex determination is driven by differentiation of gonadal somatic cells at mouse embryonic day 15.5 or human embryonic week 5–6, forming the embryonic testes or embryonic ovaries [211, 212]. In vivo germ cell development provides a blueprint for how to produce germ cells and gametes in vitro, using the appropriate developmental milestones including progression through meiosis to produce haploid germ cells, evidence of recombination, epigenetic reprogramming, euploid chromosome content and production of healthy euploid progeny [213].

Previous studies presented several approaches to differentiate ESCs and iPSCs, derived from mice, monkeys, or humans, into the germ cell lineage (Figure 4, Table 2). In mouse studies, ESCs or iPSCs are differentiated over two days into epiblast stem cells (EpiSCs) or epiblast-like cells (EpiLCs), respectively [46–50]. These EpiSCs/EpiLCs can be further differentiated over 3–4 days into PGCLCs as floating aggregates [46–50]. Putative mouse PGCLCs can be FACS sorted based on expression of *PR/SET domain 1 (Blimp1)-Venus/Stella-Cyan* transgenes or cell surface markers SSEA1 and INTEGRINβ3 for transplantation into mouse pup testes to regenerate spermatogenesis and produce offspring [46, 47]. Alternatively, PGCLCs can be further differentiated in vitro to more advanced germ cell stages, including germline stem cell-like cells (GSCLCs) and/or spermatids that are competent to fertilize and produce offspring [48–50] (Figure 4, Table 2). PGCLCs showed global transcription profiles that were similar to mouse PGCs on embryonic day 9.5 [47]. PGCLCs could only regenerate complete spermatogenesis to produce functional spermatozoa when transplanted into infertile neonatal mouse recipients, but not adult recipients. The authors proposed that the neonatal testes provided that necessary developmental cues to support the further development of PGCLCs and that these were lacking in the adult testis [46, 47].

If it is true that PGCLCs can only regenerate spermatogenesis when transplanted into neonatal recipients, the application in the human clinic is not obvious. This limitation was partially addressed by Ishikura and colleagues, who developed a method to further differentiate PGCLCs into GSCLCs in a reconstituted testis (rTestis) by mixing PGCLCs with fetal testicular somatic cells (FTSCs) in a transwell plate [49]. The resulting GSCLCs could be maintained in adherent cultures, similar to mouse SSC cultures [102, 214]. GSCLCs could also be transplanted to regenerate complete spermatogenesis in adult recipients, including sperm that were competent to fertilize and produce offspring [50]. A similar rTestis approach recently described mixing human PGCLCs (hPGCLCs) with fetal mouse gonadal cells (xenogeneic, reconstituted (xr) Testis) that resulted in the appearance of DDX4+, piwi-like RNA-mediated gene silencing 4 (PIWIL4)+ prospermatogonia [215]. Zhou and colleagues [48] reported a transplantation-free approach by mixing PGCLCs with FTSCs to form aggregates and continue to culture for 2 more weeks with RA, bone morphogenetic proteins (BMPs), Activin A, FSH, bovine pituitary extract (BPE), and testosterone (Figure 4). Spermatids were present in these cultured aggregates and isolated for ROSI that led to production of healthy, fertile offspring [48]. These studies may have important implications for the human fertility clinic, but they are early stage and need to be independently replicated in other laboratories and in other species. These approaches are further complicated be the requirement for FTSCs, which would be difficult or impossible to obtain for human application. An alternative approach may be the in vitro derivation of the necessary fetal gonadal somatic cell types from pluripotent stem cells, as described by Hayashi and colleagues [216] for the derivation of fetal ovarian somatic-like cells; or direct differentiation of patient somatic cells (e.g., skin fibroblasts) into FTSCs such as Sertoli cells, as described by Buganim and colleagues [217].

Multiple researchers attempted to differentiate nonhuman primate ESCs and iPSCs into the germ cell lineage in vitro [218–220] (Figure 4, Table 2). Cynomolgus monkey ESCs were established and used for in vitro differentiation into embryoid bodies (EBs) [219]. EBs started expressing VASA at day 4 post differentiation, suggesting the presence of prospermatogonia [219]. At day 16, *synaptonemal complex protein 3 (SCP3)* and *DNA meiotic recombinase 1 (DMC1)* transcriptions were detected, suggesting the presence of meiotic cells [219]. Yamauchi and colleagues [220] reported that addition of bone morphogenetic protein 4 (BMP4) to differentiation culture of Cynomolgus ESCs promoted earlier gene transcription of *synaptonemal complex protein 1 (SYP1)* in the EBs. In a separate study, Sosa and colleagues [218] examined the capacity of Rhesus iPSC to differentiate into incipient mesoderm-like cells (iMeLCs) and then rPGCLCs in culture (Figure 4). rPGCLCs were then transplanted into adult mouse or Rhesus testes to determine if the in vivo somatic environment could support germ cell engraftment and spermatogenesis [218]. Transplanted rPGCLCs into the adult gonadal niche of either recipient species differentiated into colonies of MAGE family member A4 (MAGEA4)+/Enolase 2 (ENO2)-/VASA+ germ cells and did not differentiate to produce sperm in adult mouse or monkey recipient testes

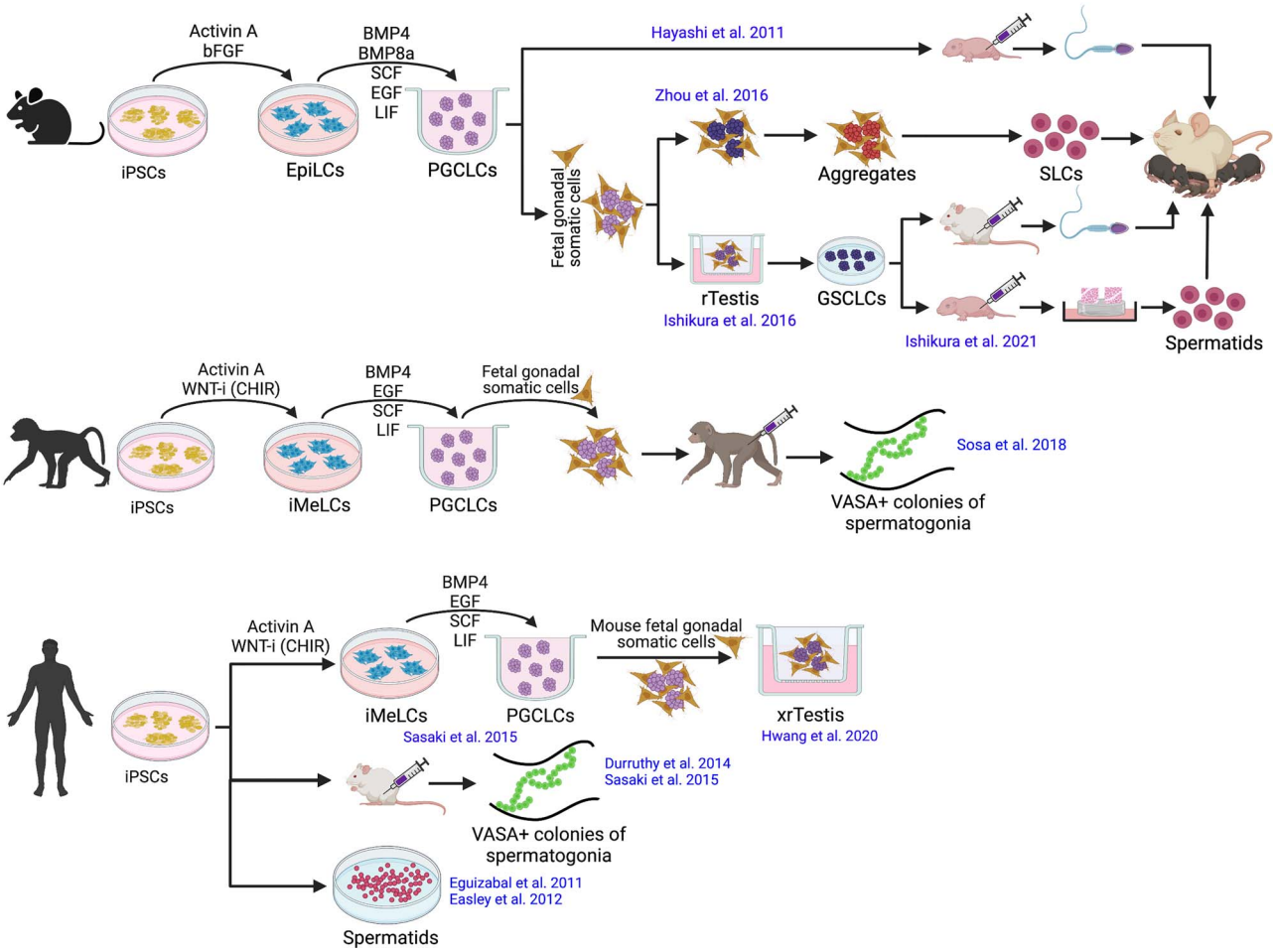

Figure 4. Illustration of various in vitro gametogenesis approaches using iPSCs of mice, monkeys, and humans. (Abbreviation: iPSCs, induced pluripotent stem cells; iMeLCs, incipient mesoderm-like cells; PGCLCs, primordial stem cell-like cells; rTestis, reconstituted testis; GSCLCs, germline stem cell-like cells; SLCs, spermatid-like cells; xrTestis, xenogeneic reconstituted testis).

[218]. These results indicated that rPGCLCs acquired transplantation potential (engraftment, proliferation, and survival for long-term upon transplantation) but not spermatogenic potential [218]. Adult Rhesus testicular environment did not provide proper biological cues for to support spermatogenesis from transplanted rPGCLCs [218]. Like previous mouse studies, transplantation into neonatal Rhesus could provide the necessary environment to support spermatogenesis from transplanted PGCLCs. However, those experiments will be expensive and complex, and the justification is questionable since transplantation to neonates in the human clinic is not likely.

A number of laboratories have applied various differentiation conditions (matrices and medium compositions) to differentiate human ESCs (hESCs) and/or human iPSCs (hiPSCs) into hPGCLCs [221–225] (Table 2). Clark and colleagues [226] initially reported that hESCs could spontaneously differentiate into VASA+ germ cells in EBs, but the number of VASA+ cells was low. The same group later showed that BMP4 increased the expression of germ cell-specific markers (VASA, SYCP3) during differentiation of hESCs in EBs compared with EB cultures without BMP4 supplement. BMP7 and BMP8b had modest additive effects [222, 227]. Two groups reported a stepwise differentiation of hiPSCs into iMeLCs as an intermediate stage to enhance the efficiency of hPGCLC derivation [215, 224]. EPCAM and INTEGRINα6 were used as surface markers to identify and isolate hPGCLCs [224]. In human PGC specification, sex determining region (SRY)-box transcription factor 17 (SOX17) regulates BLIMP1 to suppress the expression of endoderm and mesoderm genes as well as acting as a key regulator in hPGCLC specification [225].

In a series of studies, Durruthy and colleagues [228] tested the hypothesis that the in vivo environment of mouse ST could direct the differentiation of human pluripotent stem cells into the germ cell lineage. They found that ESCs and iPSCs reprogrammed with the typical Yamanaka OSKM (Oct4, Sox2, Klf4, and c-Myc) factors produced large masses similar to embryonal carcinoma cells and yolk sac tumors. In contrast when VASA was added to the reprogramming mix (OSKMV) (Oct4, Sox2, Klf4, c-Myc, and Vasa), the resulting iPSCs did not form large masses and left the mouse testis structure intact. The authors observed clusters of deleted in azoospermia-like (DAZL)+/VASA+/UTF1+/GFRa1+ germ cell-like cells in the ST [228–230]. Several groups have reported in vitro differentiation of hESCs and hiPSCs into haploid cells, indicated by Acrosin expression and a 1 N (haploid) peak observed by Hoechst or propidium iodide (PI) staining followed by flow cytometry [221–223, 231]. Functional testing of human haploid cells is important but challenged by federal and state

regulations and the need to secure funding from non-federal sources.

Conclusions

Cancer survivors are concerned about the side effects of medical treatments on their future fertility, and many of them have expressed their desire to have children later in life [232]. Prior to gonadotoxic treatments, adolescent and adult men can cryopreserve semen samples containing sperm that can be used to achieve pregnancies with their partners using existing ARTs (Figure 2). However, this is not an option for prepubertal male patients who are not yet producing mature gametes. Many clinics around the world are cryopreserving testicular tissues for these prepubertal patients (Table 1) in anticipation that next-generation technologies will be fully developed in the future to mature those tissues and produce sperm, in vivo or in vitro (Figure 2 and Table 2). This review has described in detail on the progress of each technology being developed. Every technology has its strengths and drawbacks; therefore, it is reasonable to simultaneously invest efforts into several technologies to accommodate various patient circumstances. Some of these techniques, including autologous testicular cell transplantation and testicular tissue grafting, are mature and may be ready for translation to the human clinic (Table 2). This should be done only with approval of an institutional review board. Other technologies reviewed in this chapter (e.g., de novo testicular morphogenesis, TTOC, and iPSC-derived germ cells) are in earlier stages of development (Figures 2 and 4, Table 2). Louise Brown was born over 40 years ago as the world's first baby who was conceived by IVF [20]. Louise was possible because her parents were able to produce eggs and sperms that were combined outside the body to achieve fertilization. Advanced reproductive technologies are in the research pipeline that may allow men/boys without sperms or women/girls without eggs to have biologically related children.

Authors' contributions

K.T.D.T. prepared the manuscript. K.E.O. reviewed and edited the manuscript. H.V.-P. and A.C. provided patient data of the Fertility Preservation Program in Pittsburgh.

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Conflict of interest

There are no conflicts of interest to disclose for any of the authors.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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