Stem Cells and Cloning: Advances and Applications

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REVIEW

Role of stem cells in fertility preservation: current insights

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Abstract: While improvements made in the field of cancer therapy allow high survival rates, gonadotoxicity of chemo- and radiotherapy can lead to infertility in male and female pre- and postpubertal patients. Clinical options to preserve fertility before starting gonadotoxic therapies by cryopreserving sperm or oocytes for future use with assisted reproductive technology (ART) are now applied worldwide. Cryopreservation of pre- and postpubertal ovarian tissue containing primordial follicles, though still considered experimental, has already led to the birth of healthy babies after autotransplantation and is performed in an increasing number of centers. For prepubertal boys who do not produce gametes ready for fertilization, cryopreservation of immature testicular tissue (ITT) containing spermatogonial stem cells may be proposed as an experimental strategy with the aim of restoring fertility. Based on achievements in nonhuman primates, autotransplantation of ITT or testicular cell suspensions appears promising to restore fertility of young cancer survivors. So far, whether in two- or three-dimensional culture systems, in vitro maturation of immature male and female gonadal cells or tissue has not demonstrated a capacity to produce safe gametes for ART. Recently, primordial germ cells have been generated from embryonic and induced pluripotent stem cells, but further investigations regarding efficiency and safety are needed. Transplantation of mesenchymal stem cells to improve the vascularization of gonadal tissue grafts, increase the colonization of transplanted cells, and restore the damaged somatic compartment could overcome the current limitations encountered with transplantation.

Keywords: transplantation, fertility restoration, mesenchymal stem cells, germ-line stem cells, spermatogonial stem cells, in vitro maturation

Introduction

Some years ago, fertility preservation (FP) emerged as a treatment aiming to preserve future reproductive capacity of individuals facing therapies that could potentially affect their gonads, the majority being patients diagnosed with cancer.[1] Indeed, chemo- and radiotherapy are associated with gonadotoxicity in both males and females.[2] Other health conditions can motivate FP, such as genetic abnormalities or autoimmune diseases.[3,4] For adult men or adolescents, cryopreservation of ejaculated or surgically retrieved sperm is routinely proposed before gonadotoxic therapies, while for prepubertal boys, cryopreservation of a testicular biopsy containing spermatogonial stem cells (SSCs) is now ethically accepted as the only way to offer an FP strategy from the perspective of future developments allowing parenthood.[5] Several studies have broached the feasibility of cryopreservation of immature testicular tissue (ITT),[6–13] and some teams have developed protocols for its clinical implementation.[7,10,12,14] Although still at the research stage, autotransplantation and in vitro maturation (IVM) of ITT or SSCs have been considered

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to restore fertility from cryopreserved ITT. Restoration of the damaged SSC niche with mesenchymal stem cells (MSCs) was also recently proposed to enhance or restore endogenous spermatogenesis.[15]

For women, cryopreservation of oocytes or embryos is the most common way to preserve fertility.[16,17] However, while oocyte cryopreservation may also be proposed to adolescent girls, it cannot be proposed before puberty or to adult women requiring urgent therapy. Cryopreservation of ovarian tissue containing primordial follicles may be proposed with an aim to transplant it back to the patient after cure, a technique that has already proved its efficacy with births of healthy babies.[18] However, early postgrafting follicle loss has motivated researchers to improve the procedure, and potential neoplastic tissue contamination (making it unsafe for transplantation) increases the need to find alternative FP methods.

While SSCs, originating from differentiation of gonocytes after birth, continuously divide asymmetrically to give rise to new SSCs and differentiating germ cells,[19] embryonic oogonia enter a resting stage (prophase of meiosis I) and undergo final maturation only at the onset of puberty, thus constituting a fixed ovarian reserve that decreases during a lifetime.[20] This classical scheme was questioned during the last decade with the discovery of potential female germ-line stem cells (FGSCs) in the ovary, opening a debate that is not over yet.[21]

In this review, we present current FP approaches for male and female patients facing gonadotoxic therapies and methods that could be applied to improve their impaired fertility using cryostored gonadal material and other sources of stem cells (SCs) that may enhance in vitro and vivo germ-cell differentiation or develop into gametes.

Materials and methods

Methods

A search was performed on PubMed using the following combination of terms without time limitation: ([fertility] AND [restoration OR preservation]) AND (stem cell OR germline stem cell OR oogonia OR spermatogonial stem cell OR spermatogonia). Articles in languages other than English, guidelines, reviews, and scientific video protocols were excluded.

Results

Literature search

Figure 1 shows a flowchart describing the selection of papers. From the 458 results, 60 focusing on the main topic were selected and 136 added for their relevance to understanding and discussion.

SSCs to restore fertility in the male

SSCs are known as a subpopulation of spermatogonia localized at the basement membrane of seminiferous tubules (STs) and estimated to represent 0.03% of germ cells in the adult mouse.[22] These diploid SCs are able both to self-renew and give rise to differentiated haploid cells at the end of the spermatogenic process.[19]

Due to the smallness of testicular biopsies taken for cryopreservation in prepubertal boys, the scarcity of SSCs in the testes,[23] the low efficiency of the transplantation process observed in mice and nonhuman primates,[24,25] and the low proportion of human haploid germ cells generated with IVM,[26] amplification of SSCs is an essential step for fertility restoration.

SSC propagation

The development of SSC propagation–culture systems has mainly been achieved through studies in rodents. In 2003, Kanatsu-Shinohara et al reported the first long-term amplification of murine SSCs for >5 months in a specific medium containing glial cell line-derived neurotrophic factor (GDNF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and leukemia inhibitory factor (LIF), which were considered as essential for SSC culture.[27] Indeed, both in vivo and vitro studies brought evidence that GDNF plays a pivotal role in SSC self-renewal.[28,29] Moreover, bFGF was shown to potentiate the effect of GDNF, as addition of bFGF to culture media containing GDNF increased the number of SSC colonies compared to culture without bFGF,[28] while LIF and EGF were shown to act on SSC colony formation[30] and diameter,[31] respectively. Subsequently, several teams attempted to find a culture system of dissociated testicular cell suspensions (TCSs) able to propagate human SSCs in vitro (Table 1).[32–51] Sadri-Ardekani et al adapted the protocol developed by Kanatsu-Shinohara et al for human testicular cells (TCs). Briefly, this culture system relies on the capacity of somatic cells to adhere to the plate while the germ-cell fraction stays in suspension, allowing enrichment of SSCs after differential plating.[32,33] This technique led to an 18,450-fold enrichment of adult SSCs after 64 days and to a 9.6-fold enrichment of prepubertal SSCs after 11 days of culture using xenotransplantation as the gold standard to identify SSCs able to migrate along the basement membrane of the STs, colonize their niches,

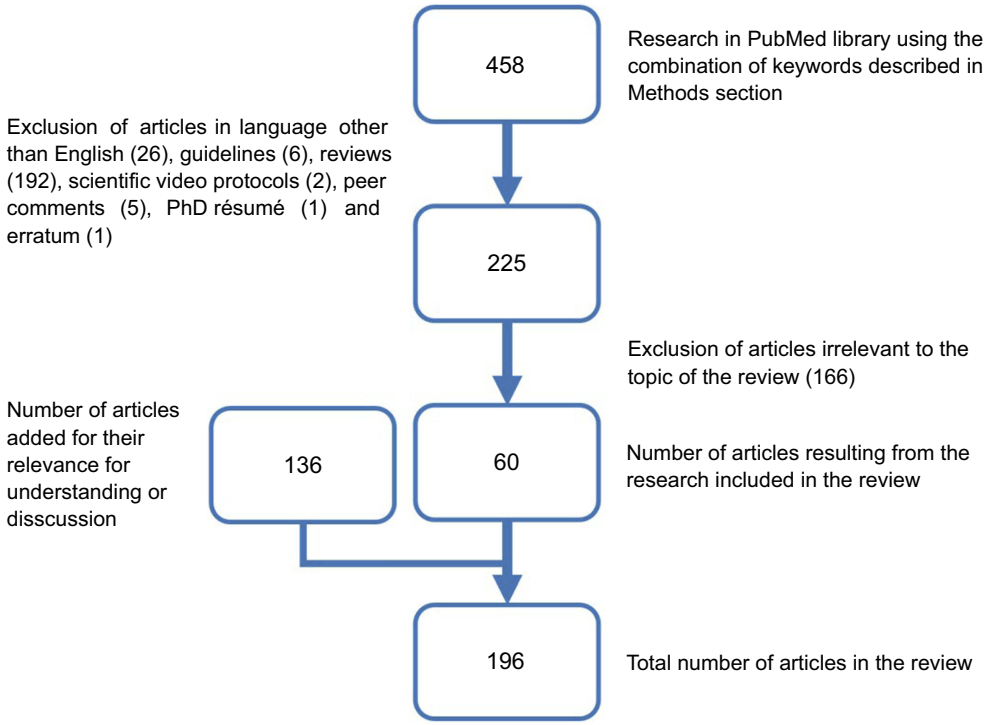

Figure 1 Flowchart of paper selection.

and generate germ-cell colonies. Among researchers who have xenotransplanted long-term cultured human SSCs,[32,33,39,40,42,43,51] only Sadri-Ardekani et al and Nickkholgh et al quantified SSCs in STs after transplantation and demonstrated SSC enrichment.[32,33,43] However, several other teams using the same protocol could not reproduce such results due to the complexity and skills needed to distinguish between SSCs and human embryonic stem cell-like (hESC-like) cells,[34] because of low germ-cell survival and overgrowth of remaining somatic cells.[35,47] Indeed, the importance of the germ- versus somatic-cell ratio in culture was demonstrated, showing an impact on SSC proliferation.[52,53] The influence of the medium was also pinpointed when Gat et al observed more germ-cell aggregate formation when using DMEM/F12 instead of StemPro-34.[54] Others also examined the efficiency of differential plating to select germ cells from TCSs, but did not find a difference in germ-cell numbers recovered from whole TCSs and differentially plated cells after 14 days of culture.[55] To improve SSC propagation, cell sorting prior to culture was further applied. Coculture of SSCs sorted by fluorescence-activated cell sorting based on their $HLA^{-}/EPCAM^{+}$ phenotype onto inactivated somatic feeder cells resulted in putative SSCs coexpressing DDX4 and UTF1, although their proliferation rate was poor and no survival was found after 4 weeks.[55]

Other phenotypic markers, ie, GFRα1, GPR125, SSEA-4, $KIT^{-}/ITG\beta1^{-}$, CD9, ITGα6, THY1, and FGFR3, have been used to select monkey or human SSCs,[38,43,44,48–50,56–65] but among 16 studies, only 5 cultured the sorted SSCs.[38,48–50,59] Lim et al succeeded in long-term culture of CD9-sorted spermatogonia onto laminin-coated plates, but reported a low proliferation rate (20,000–80,000 cells in 130 days).[49] However, when GPR125 was used to select spermatogonia from testicular tissue (TT) of patients diagnosed with obstructive azoospermia, a five fold enrichment was achieved in the first month when cultured onto hydrogel without a feeder layer.[38] While the authors claimed an advantage of their system over differential plating, as it avoided overgrowth of somatic cells, the SC potential was not evaluated. Human SSC sorting based on their SSEA-4 expression was performed by two teams with contradictory results, since one reported successful SSC amplification for 21 weeks onto Matrigel,[48] while the second achieved amplification only onto γ-irradiated feeder cells and observed an inability of SSCs to attach to Matrigel.[59] Coculture of $ITG\alpha6^{+}$ SSCs onto collagen-coated plates with Sertoli cells allowed a five fold increase in colony numbers.[50] Culturing unsorted cells prior to cell selection has also been attempted, showing that 50 days in the same culture conditions followed by isolation of $ITG\alpha6^{+}$ cells resulted in a seven fold enrichment of SSCs.[43]

Table 1 Studies including long-term culture (>1 month) of human SSCs

	Tissue origin and sample size	SSC-enrichment method	Culture (weeks)	SSC characterization	Enrichment evaluation after xenotransplantation
Sadri-Ardekani et al[32]	Patients undergoing bilateral orchiectomy (n=6) as part of prostate cancer treatment	Culture of TCSs onto uncoated plates for 1 night, followed by DP of floating cells onto uncoated plates; germ-line stem cell clusters subcultured onto placenta laminin–coated plates	28	Expression of PLZF, ITGα_6, and ITGβ_1 by RT-PCR; IF for PLZF; xenotransplantation assay	Fold enrichment in SSCs: 53-fold for cells cultured for 47 days compared to cells cultured for 28 days. 18,450-fold for cells cultured for 141 days compared to cells cultured for 77 days
Lim et al[49]	Patients with obstructive (n=18) or non-obstructive (n=19) azoospermia	Culture onto uncoated plates for 2 days followed by replating of unattached cells onto collagen-coated plates for 4 hrs; isolated CD9+ SSCs from floating cells cultured onto laminin-coated plates	26	Expression of ITGα6 by RT-PCR; FACS analysis for GFRα_1, ITGα_6, and ITGβ_1; telomerase activity during the entire culture period	No xenotransplantation; higher proportion of GFRα_1^+, ITGα_6^+, and ITGβ_1^+ cells at the end of culture compared to day 0; telomerase activity maintained in SSC culture until at least the 7th passage
Sadri-Ardekani et al[33]	Prepubertal boys aged 6.5 and 8 years (n=2)	Culture onto uncoated plates for 1 night, followed by DP and culture onto uncoated plates; germ-line stem-cell clusters subcultured onto placenta laminin–coated plates	15.5–20	Expression of PLZF, ITGα_6, ITGβ_1, CD9, GFRα_1, GPR125 and UCHL1 by RT-PCR; IHC for PLZF and UCHL1; xenotransplantation assay	Fold-enrichment in SSC numbers (days of culture): 9.6-fold (11 days) for 8-year-old boy. 6.2-fold (21 days) for 6.5-year-old boy
Kokkinaki et al[48]	Deceased organ donors (n=3)	Culture for 24 hrs on FBS-coated plates, followed by MACS isolation of SSEA-4+ spermatogonia in the floating cell fraction and culture on Matrigel	21	IF for SSEA-4, GPR125, and UCHL1; expression of EpCAM, GPR125, PLZF, OCT4 and SSEA-4 by RT-PCR	No xenotransplantation; higher expression of PLZF and GPR125 in SSEA-4 sorted cells compared to unsorted cells and GPR125 and ITGα_6 sorted cells
Mirzapour et al[42]	Patients aged between 28– 50 years with maturation arrest (n=8)	Culture for 3 hrs onto lectin-coated plates, followed by DP of SSCs; SSC culture with or without Sertoli cells as feeder layer	5	ALP reactivity; expression of OCT4, Stra8, Piwil2, and DDX4 by RT-PCR; xenotransplantation assay	Not evaluated
Koruji et al[39]	Patients aged 32–50 years old with incomplete or complete maturation arrest (n=20)	Comparison of culture on uncoated plates without growth factors (group 1), plates treated with growth factors (group 2) and laminin-coated plates supplemented with growth factors (group 3); DP performed depending of the somatic vs germ cell ratio	8	Expression of DAZL, PLZF, DDX4, ITGα_6, OCT4 and ITGβ_1 by RT-PCR; xenotransplantation assay	Larger diameters and numbers of SSC clusters in groups 2 and 3 compared to group 1 after 1 and 2 months of culture

(*Continued*)

Table 1 (Continued)

	Tissue origin and sample size	SSC-enrichment method	Culture (weeks)	SSC characterization	Enrichment evaluation after xenotransplantation
Mirzapour et al[41]	Patients aged 28– 50 years presenting a maturation arrest (n=8)	Adhesion of Sertoli cells onto lectin-coated plates; floating cells subcultured on fresh or frozen/thawed Sertoli cells	5	ALP activity; IHC for CDH1 and OCT4	No xenotransplantation; more SSC colonies when frozen/thawed SSCs were cocultured on fresh Sertoli cells compared to coculture on frozen/thawed Sertoli cells
Akhondi et al[34]	Brain-dead patient (n=1)	Culture onto uncoated plates for 14 days, followed by trypsinization and subculture of germ-cell clusters	6	Expression of PLZF by RT-PCR; IF for OCT4	Not evaluated
Piravar et al[45]	Patients with nonobstructive azoospermia (n=10)	Culture onto uncoated plates for 16 hrs; floating cells cultured onto uncoated dishes; germ-cell clusters isolated after 14 days and subcultured onto laminin	6	Expression of UCHL1 by RT-PCR	Not evaluated
Goharbakhsh et al,[37]	TESE from azoospermic patients (n=12)	Culture onto uncoated plates for 3 hrs followed by DP and culture onto laminin-coated plates	7–8	IHC for GPR125	No xenotransplantation; more cells positive for GPR125 in SSC culture compared to somatic cell culture
Conrad et al[36]	Patient undergoing orchiectomy as part of prostate cancer treatment (n=1); patients with sex-reassignment surgery after hormone therapy (n=6); patient with diagnostic testicular biopsy (n=1); patient with seminoma (n=1)	Isolated cells plated onto gelatin-coated plates; floating cells and cells bound to monolayers of adherent somatic cells recovered and plated onto collagen-coated plates for 4 hrs; unattached cells harvested and ITGα6+ cells isolated by MACS cultured onto MEF feeder cells	56	Microarray analysis on short- vs long-term cultured SSCs; expression of DDX4, DAZL, PLZF, LIN28, SOX2, and NANOG by RT-PCR	No xenotransplantation; higher expression of LIN28, SOX2, and NANOG in long-term cultured SSCs; decreased expression of DDX4, DAZL, and PLZF in long-term cultured SSCs
Nickkholgh et al[44]	Patients undergoing bilateral orchiectomy as part of prostate cancer treatment (n=2)	Culture for one night, followed by DP and subsequent cell culture for 50 days before MACS isolation of ITGα_6^+	7	IF for PLZF	Not evaluated
Nickkholgh et al[43]	Patients undergoing bilateral orchiectomy as part of prostate cancer treatment (n=2)	Culture for one night ,followed by DP and subsequent cell culture for 50 days before MACS isolation of ITGα_6^+, HLA-/ITGα6+, GPR125+ and HLA-/GPR125+ fractions	7	Xenotransplantation assay; expression of ITGα_6, ID4, GPR125, PLZF, and UCHL1 by RT-PCR	7-folds 8 weeks after transplantation of ITGα_6^+ sorted fraction compared to unsorted

(*Continued*)

Table 1 (Continued)

	Tissue origin and sample size	SSC-enrichment method	Culture (weeks)	SSC characterization	Enrichment evaluation after xenotransplantation
Sadri-Ardekani et al[46]	Patient undergoing bilateral orchiectomy as part of prostate cancer treatment (n=1); prepubertal boys with Hodgkin's lymphoma who stored testis biopsy before chemotherapy (n=2)	Protocol used in 2009 and 2011	6.5–8.5	Expression of PLZF, UCHL1, and GPR125 by RT-PCR	Not evaluated
Zheng et al[47]	Organ donors aged 13–40 years (n=8)	Cells plated for one night onto uncoated plates; floating cells harvested before second DP onto collagen-coated plates and subsequent culture; somatic adherent cells maintained as control	6	Expression of UTF1, DAZL, FGFR3, and PLZF by RT-PCR; IF for DAZL, SALL4, and UTF1; FACS for SSEA-4	No xenotransplantation; higher expression of spermatogonial markers UTF1, DAZL, FGFR3, PLZF, and GPR125 in DP group compared to control
Mirzapour et al[40]	Patients with maturation arrest (n=8)	Culture of cells onto lectin-coated plates	5	Expression of DAZL and ITGα6 by RT-PCR; xenotransplantation assay	More colonized STs for low (27×10^6 cells/mL) and high (51×10^6 cells/mL) concentrations of SSCs compared to very low (10×10^6 cells/mL); no quantification of the number of colonies
Baert et al[35]	Patients undergoing reversal vasectomy or bilateral orchiectomy as part of prostate cancer treatment (n=6)	Cells cultured for one night onto plastic plates; floating cells recovered for subculture	8	Colocalization of DDX4 and UCHL1 by IF	Not evaluated
Guo et al[38]	Patients with obstructive azoospermia aged 22–35 years (n=40)	Cells obtained from 3–4 patients seeded onto culture plates for one night. $GPR125^+$ cells selected in the floating cell fraction and subcultured onto hydrogel	8	IHC for GPR125, PLZF, CD90, UCHL1 and MAGEA4; expression of GFRα1, GPR125, RET, PLZF, UCHL1, and MAGEA4 by RT-PCR	Not evaluated
Shiva et al[50]	Patients with nonobstructive azoospermia (n=NA)	Culture on DSA-coated plates for 1 hr, followed by FACS isolation of $ITG\alpha_6^+$ spermatogonia; isolated cells cocultured with Sertoli cells alone (group 1), Sertoli cells + growth factors (group 2), or Sertoli cells + growth factors in collagen-coated plates (group 3)	7	IF for OCT4; expression of OCT4, PLZF, DDX4 and $ITG\alpha_6$ by RT-PCR	No xenotransplantation; higher number and diameter of colonies in group 3 compared to groups 1 and 2

(*Continued*)

Table 1 (Continued)

	Tissue origin and sample size	SSC-enrichment method	Culture (weeks)	SSC characterization	Enrichment evaluation after xenotransplantation
Bhang et al[51]	Patients undergoing cryopreservation of ITT before starting gonadotoxic treatment (n=3)	Culture with bFGF and GDNF (group 1) or with iPS-ECs as feeder cells (group 2)	21	IF for SSEA-4 and ITGα_6	SSCs from group 1 died after 2 weeks in culture, while SSCs from group 2 proliferated for 150 days and were able to migrate along the basement membrane of STs following xenotransplantation to nude mice

Abbreviations: ALP, alkaline phosphatase; bFGF, basic fibroblast growth factor; CD, cluster of differentiation; CDH1, cadherin-1; DAZL, deleted in azoospermia like; DDX4, DEAD-box helicase 4; DP, differential plating; DSA, datura stramonium agglutinin; FACS, fluorescence-activated cell sorting; FGFR3, fibroblast growth factor receptor 3; GFRα1, GDNF family receptor alpha 1; GPR125, G-protein coupled receptor 125; GSCs, germ-line stem cell; ID4, inhibitor of DNA binding 4; IF, immunofluorescence; IHC, immunohistochemistry; ITGα6, integrin subunit alpha 6; ITGβ1, integrin subunit beta 1; iPS-EC, iPS-derived endothelial cell; ITT, immature testicular tissue; LIN28, LIN28 homolog A; MACS, magnetic activated cell sorting; MAGEA4, MAGE family member a4; OCT4, octamer-binding transcription factor 4; NA, not available; PIWIL2, Piwi like RNA-mediated gene silencing 2; PLZF, promyelocytic leukaemia zinc finger protein; RET, ret proto-oncogene; SALL4, sal-like protein 4; SSC, spermatogonial stem cell; SSEA-4, stage-specific embryonic antigen 4; STRA8, stimulated by retinoic acid 8; SOX2, SRY (sex-determining region Y)-box 2; TCS, testicular cell suspension; TESE, testicular sperm extraction; UCHL1, ubiquitin C-terminal hydrolase L1; UTF1, undifferentiated embryonic cell transcription factor 1.

Together, these results point to the need to identify the best method to propagate SSCs most efficiently. Recently, Bhang et al discovered that human endothelial TCs secreted GDNF, bFGF, stromal cell-derived factor-1 (SDF-1), macrophage inflammatory protein 2, and insulin-like growth factor-binding protein 2 and could support SSC growth for at least 150 days.[51] It also appeared that cells with MSC characteristics were able to support spermatogonia in vitro. Indeed, Smith et al showed that a $THY1^+$ fraction isolated from TCSs was of mesenchymal origin and could support SSEA-4^+ SSC growth, while mouse embryonic fibroblasts and human placental and fetal testicular stromal cells could not.[59] Interestingly, human umbilical perivascular cells (HUPVCs), which are also of mesenchymal origin and share common properties with somatic TCs (LIF, bFGF, and BMP4 secretion as well as expression of testicular extracellular matrix markers) also supported germ-cell proliferation and survival.[66]

SSC transplantation

Spermatogenesis restoration can be achieved both by injection of isolated SSCs into germ-cell-depleted testes and transplantation of an ITT piece where SSCs remain within their intact niche or original microenvironment.[67]

Transplantation of isolated SSCs

The first success using SSC transplantation to restore fertility was achieved in mice by Brinster and Avarbock who reported complete spermatogenesis and offspring after SSC injection into STs of busulfan-sterilized mice.[68] In order to evaluate the capacity of transplanted SSCs to colonize their niche, recipient mice were injected intraperitoneally with busulfan inducing germ-cell depletion and improving donor SSC colonization (Figure 2). Recently, a higher proportion of donor-derived offspring generation was reported when busulfan was injected directly into testes.[69] The power of the technique for FP was further demonstrated with offspring in several species, including rats, goats, chickens, and sheep, and embryo development in nonhuman primates.[70–74] The spermatogenic process has also been completed in bovines, pigs, and dogs, but sperm functionality was not evaluated.[75–77] In addition, cryopreservation of mouse, rat, rabbit, and baboon SSCs did not affect their viability neither their ability to colonize mouse STs,[78] and culture of thawed mouse[78-80] and rat[78] SSCs resulted in spermatogenesis after transplantation. The safety of the procedure was studied in mice, and although differences in histone acetylation of germ cells

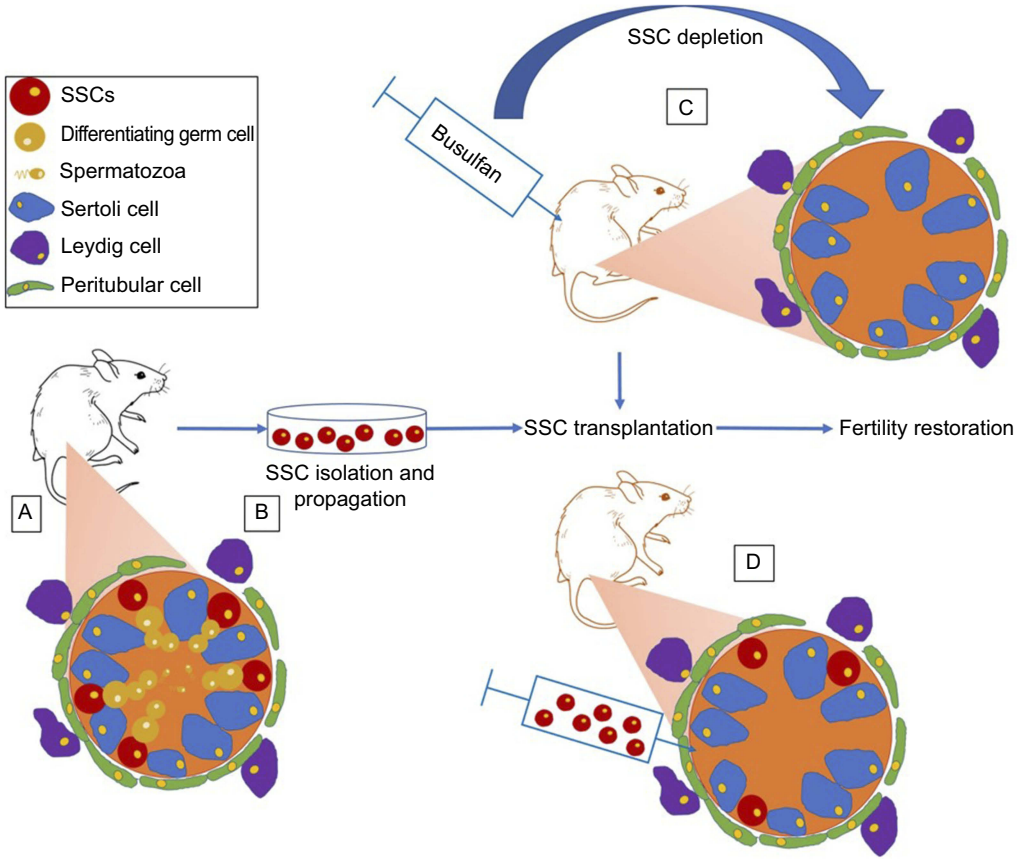

Figure 2 Classic mice model used for fertility restoration by SSC transplantation. (**A**) SSCs are located along the basement membrane of STs and surrounded by nursing Sertoli cells. Spermatogonia differentiate progressively into spermatozoa toward the lumina of STs. Myoid cells create a wall around the STs while Leydig cells reside in the testicular interstitium. (**B**) SSCs can be isolated and propagated in vitro. (**C**) Germ-cell depletion by busulfan treatment favors stem cell–niche colonization. (**D**) Transplantation of SSC to STs of germ cell-depleted mice to restore spermatogenesis.
Abbreviations: SSC, spermatogonial stem cell; ST, seminiferous tubule.

were observed,[81] no modifications in the genomes of offspring were found.[82] In addition, propagation of moue SSCs before transplantation did not increase the incidence of cancer or decrease the survival of mice that had undergone SSC transplantation.[83]

In view of these encouraging results, SSC transplantation is considered a potential fertility-restoration method for future clinical application (Figure 3). Using cadaver testes, ultrasound-guided injection in the rete testis has been determined as the best technique for cell transplantation in larger testes.[84,85]

So far, only one report has described autotransplantation of cryopreserved human TCSs in patients cured of non-Hodgkin's lymphoma, but no follow-up was published.[86] An important clinical concern is the risk of cancer-cell contamination of the TCSs to be transplanted, since transplantation of only 20 leukemic cells in rats has resulted in cancer relapse.[87] To address this issue, several teams searched for extracellular markers allowing separation of human SSCs from cancer cells but completely safe purification is not yet possible using cell-sorting techniques.[56,58,88–90] However, the culture protocol developed by Sadri-Ardekani et al allowed elimination of malignant cells added to the cell suspension, and may represent a good alternative to sorting approaches.[46]

Furthermore, long-term culture of human SSCs did not show increased chromosomal abnormalities in another study, but methylation assays demonstrated demethylation of three paternally imprinted genes and increased methylation of two maternally imprinted genes after 50 days.[44] The impact of such modifications on offspring are not known and difficult to predict. While it is possible that once transplanted, SSCs and generated spermatozoa could retrieve a normal methylation pattern, it was also hypothesized that cultured and transplanted human SSCs might be unable to enter meiosis or lead to embryos that will degenerate because of their inability to pass cellular checkpoints.[44]

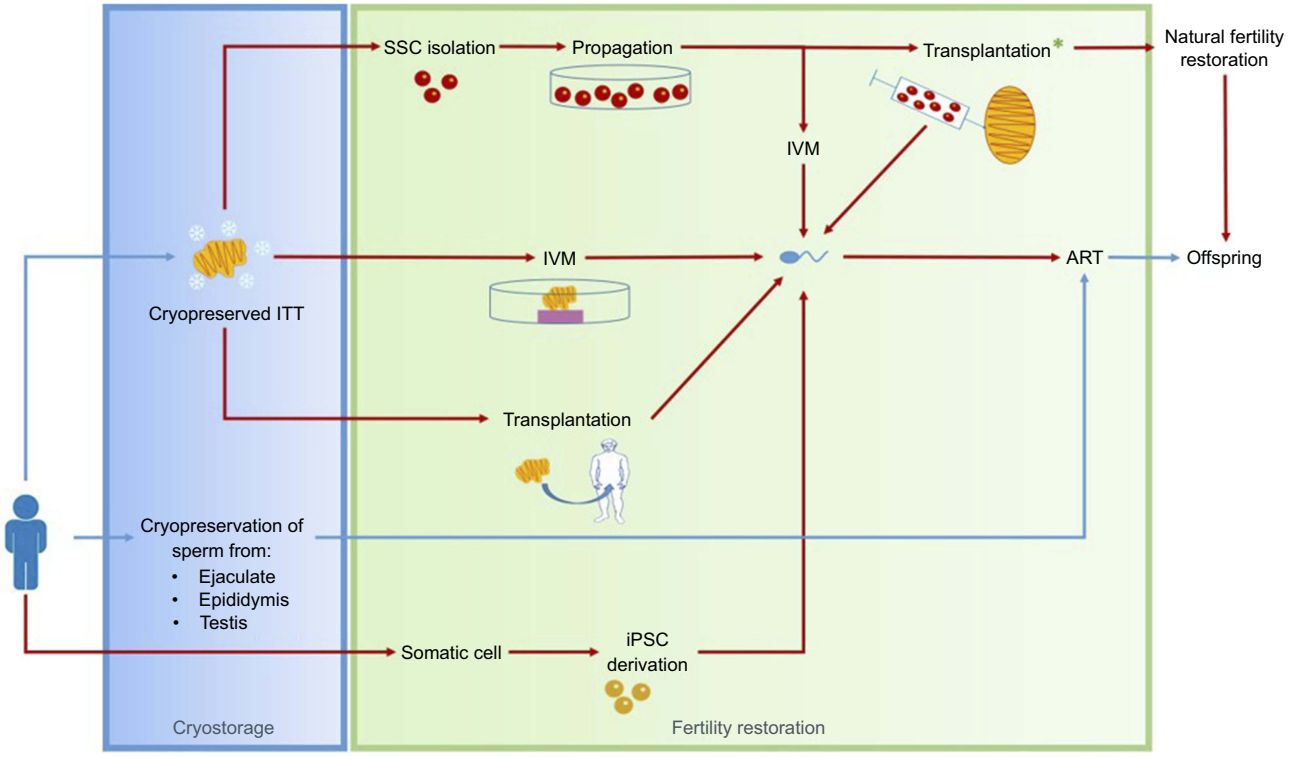

Figure 3 Fertility preservation in males.
Notes: As they do not produce sperm, prepubertal boys can benefit from cryopreservation of a testicular tissue biopsy that could be used in the future for: 1) SSC isolation and propagation, with a view to restoring fertility of the patient by transplantation into own STs or for IVM to produce competent sperm for ART; 2) IVM in organotypic or microfluidic culture systems, with the aim to obtain sperm usable in ART; and 3) transplantation back into the patient to induce maturation and generation of spermatozoa that can be recovered and used for ART. Alternatively, derivation of iPSCs from different sources of somatic cells could lead to generation of competent spermatozoa. *Processes that could be improved with use of MSCs. Red arrows represent techniques that are still considered experimental. Blue arrows indicate methods that are already implemented in clinical practice.
Abbreviations: ART, assisted reproductive technology; iPSCs, induced pluripotent stem cells; ITT, immature testicular tissue; IVM, in vitro maturation; MSCs, mesenchymal stem cells; SSC, spermatogonial stem cell.

Transplantation of ITT (SSCs within their niche)

The main aim of tissue transplantation rather than cell transplantation is that cellular interactions within the SC niche are preserved, which is important for germ-cell proliferation and maturation.[91] However, as grafting of thawed ITT contaminated by leukemic cells has resulted in development of generalized leukemia in rats,[92] this technique must be restricted to nonhematological or non-metastasizing cancers and to benign disorders requiring gonadotoxic therapies.

Xenotransplantation of mouse, rabbit, porcine, Japanese quail, and cynomolgus monkey ITT to nude mice leads to offspring generated with sperm retrieved from the in vivo matured grafts.[93–96] With regard to human ITT, experiments have shown a blockade of differentiation at the pachytene spermatocyte stage, probably due to the phylogenetic distance between the mice and humans.[11,97] Different grafting sites have been put forward. Intratesticular grafting was proposed as a grafting site, assuming that it could be advantageous to transplant the tissue into its natural environment with high testosterone levels and that breeches created in the parenchyma to insert the graft favor donor SSC colonization, although human germ-cell differentiation was still arrested at the spermatocyte stage.[98,99] For obvious microbiological reasons, xenotransplantation cannot be considered for clinical purposes. Autologous transplantation of ITT, however, suppresses such animal contamination risks (Figure 3). Initially, ectopic transplantation in monkeys showed meiotic arrest.[100,101] Importantly, Jahnukainen et al reported sperm maturation after autologous grafting of cryopreserved ITT into the scrota of busulfan-treated monkeys, suggesting that the technique could be translated to the clinic.[13] Very recently, this potential was further supported by successful production of sperm and generation of a healthy baby following autologous transplantation of rhesus macaque ITT. Interestingly, offspring were obtained with sperm recovered from a scrotal graft, but the authors

did not detect any differences in the percentage of STs displaying complete spermatogenesis between grafting sites (back skin and scrotum).[102]

In vitro maturation of SSCs

The aim of IVM is to promote in vitro differentiation of SSCs into spermatozoa able to fertilize an oocyte during an assistedreproductive technology (ART) procedure (Figure 3). This strategy presents an advantage over transplantation to avoid the risk of disease relapse in cases of tissue contamination with neoplastic cells.

IVM of dissociated TCs

In mammals, in vitro differentiation of germ cells seems to require a 3D rather than 2D environment considering promising results obtained in monkeys[103] and humans[104] using soft-agar and methylcellulose-culture systems. With regard to human SSCs, postmeiotic cells in 2 of 6 immature TCSs cultured in a methylcellulose system and spermatozoon-like cells (based on mitochondria localization) in 1 out of 6 cultured TCSs were obtained in one study.[104] In another, spermatozoon-like cells were also generated using chitosan cylinders to culture dissociated STs from adult transsexual patients after hormonal therapy.[105] However, whether differentiated germ cells originate from SSCs or spermatogonia already committed to differentiation remains unknown. Recently, the fertilization capacity of round spermatids obtained after IVM of human GPR125^{+} spermatogonia was demonstrated using mouse oocytes with subsequent 8-cell stage embryo development.[106]

IVM of intact ITT (SSCs within their niche)

Organotypic culture of ITT allows preservation of cell interactions inside the niche and leads to germ-cell differentiation up to the haploid stage in rodents, with generation of offspring in mice.[107,108] Recently, a long-term organotypic culture of human ITT able to preserve ST integrity and Leydig cell functionality and achieve Sertoli cell maturation with partial establishment of the blood–testicular barrier[109,110] eventually led to the generation of haploid germ cells.[26] As a decrease in spermatogonial numbers and only a few postmeiotic germ cells were observed, the next hurdles to overcome before clinical translation are enhancing the efficiency of the technique and demonstrating the fertilizing capacity and genetic integrity of in vitro matured cells. Recently, Ogawa developed a microfluidic culture system allowing growth of mice ITT for up to 6 months and resulting in higher spermatogenesis efficiency compared to standard organotypic culture, which could eventually address issues that have been encountered with human tissue.[111] In this well-designed system, a porous polydimethylsiloxane (PDMS) membrane separated mouse ITT from flowing medium, allowing physiological exchanges between the chamber and the media as secreted molecules were maintained for a longer period in the chamber compared to free diffusion occurring in the classical organotypic culture system. Moreover, diffusion of oxygen through the PDMS membrane resulted in a reduction in oxygen toxicity compared to direct exposure. Later, the same group modified their culture system by suppressing the need for a pump, making its use simpler.[112,113]

Using other SCs to restore male fertility

In vitro spermatogenesis from embryonic and induced pluripotent SCs

Different SC sources have been considered to generate haploid germ cells in vitro. In mice, while the first generation of spermatids derived from ESCs led to abnormal offspring,[114] viable offspring with normal karyotype and methylation status were achieved a decade later.[115] Differentiation of hESCs into germ cell-like cells was first reported in 2004.[116] However, ESCs are genetically unrelated to patients, and their procurement is complicated by ethical issues on embryo destruction. Researchers thus focused on human-induced pluripotent stem cells (hiPSCs) derived from skin and cord-blood cells that were also differentiated in haploid germ cells, though with incomplete imprinting reestablishment (Figure 3).[117] Other teams derived male germ cells from hESCs or hiPSCs, but most of the differentiated cells remained at early stages, suggesting low efficiency of the process.[118–124] Lower efficiency has been observed for differentiation of skin-derived iPSCs into haploid cells for patients with azoospermic factor C deletion.[125] One group suggested the existence of another source of SCs they called "very small embryonic stem cells (VSELs)" residing in the testes, where they undergo asymmetric divisions, giving rise to A (dark) spermatogonia that proliferate and differentiate into A (pale) and B spermatogonia.[126] In humans, the potential of these cells to differentiate in vitro has never been investigated, although based on the nuclear expression of OCT4 and cytoplasmic expression of SSEA-4 and STELLA, their presence was suggested in testes of childhood cancer survivors aged 23 to 35 years.[127] However, a large part of the scientific community is not convinced about the existence of VSELs, and researches refuted their SC

properties.[128,129] While researchers are currently actively working on these approaches, it is important to note that besides a high degree of uncertainty regarding functionality and safety, the fertilizing capacity of human in vitro differentiated ESCs and iPSCs has not been evaluated.

Using SCs to rescue damaged SSC niches

From the perspective of future clinical application, the question of whether SSC transplantation in a chemotherapy/radiotherapy-damaged niche may restore fertility arises, as Sertoli and Leydig cell defects have both been reported after gonadotoxic therapy.[130,131] As healthy Sertoli cells present in the TCSs were shown to enhance SSC engraftment and bring adequate signals to surviving endogenous SSCs,[132,133] the use of SCs as supporting cells was considered to improve SSC-transplantation outcomes (Figure 3). In this regard, MSCs can be considered deal candidates, since several studies have suggested that male fertility can be improved, thanks to their paracrine secretions (Table 2). Indeed, umbilical cord-derived MSCs secrete factors known to play an important role in spermatogenesis such as granulocyte-colony stimulating factor, vascular endothelial growth factor, and GDNF,[134] as well as enhanced expression of meiotic genes, when injected into busulfan-sterilized mice.[135] Also, SDF-1 is another MSC-secreted factor[136] involved in SSC migration and homing, as deletion of the CXCR4 in mouse germ cells reduces SSC homing, but not their proliferation or survival.[137] It can thus be hypothesized that cotransplantation of MSCs with SSCs could improve colonization efficiency, previously reported as low.[24] Moreover, in one study HUPVCs shared molecular properties with adult somatic TCs, notably secretion of LIF, bFGF, and BMP4, known as regulators of spermatogenesis, and their transplantation promoted ST regeneration after exposure to mono-2-ethylhexyl phthalate, while all STs were damaged in controls.[66] The authors assumed that the mesenchymal origin shared by Sertoli cells and HUPVCs explained the common properties of the two cell types and their ability to support SSCs. In the same way, adipose-derived stem cell (ASC) transplantation in efferent ducts of busulfan-sterilized hamsters allowed resumption of spermatogenesis.[138] Furthermore, in a rat model of testicular torsion, injection of MSCs from human fat orbital tissue into the testes of animals not only resulted in rescue of germ cells from apoptosis but also in higher levels of testosterone, suggesting that MSCs may also support Leydig cells.[139]

Moreover, pure MSCs ($CD45^{-}Sca1^{+}Lin^{-}$) isolated from bone marrow of GFP^{+} mice injected into testes of busulfan-treated GFP^{-} mice resulted in more STs presenting spermatogenesis (70%) compared to injection of hematopoietic SCs ($CD45^{+}Sca1^{+}Lin^{-}$) (18%) or DMEM (19%).[15] Pretreatment of MSCs before transplantation was also evaluated with the objective of improving SSC-transplantation efficiency. Interestingly, while cotransplantation of SSCs with or without TGFβ1-treated MSCs in sterilized mice testes resulted in an equivalent resumption of endogenous spermatogenesis, a higher proportion of STs containing donor-derived spermatogenesis was observed when TGFβ1-treated MSCs were cotransplanted with SSCs. This observation could be explained by the lower expression of genes involved in inflammation and cell migration in TGFβ1-treated MSCs, resulting in reduced lymphatic migration toward other organs.[140]

SCs to restore fertility in the female

Current evidence of SCs in the ovary

The conventional view that mammalian ovaries do not produce oocytes after birth has been challenged in recent decades with the discovery of FGSCs in ovaries of juvenile and adult mice.[21] Mathematical calculations demonstrated that the rate of follicular atresia did not coincide with the age at which mice exhausted their follicular reserve, suggesting that neo-oogenesis occurred in ovarian tissue to reestablish the follicle pool and ensure reproductive potential during adulthood.[21] Indeed, it was demonstrated that FGSCs isolated from mice ovaries maintained proliferative activity in vitro and led to offspring after transplantation to sterile mice.[21,141–145] Their presence was also demonstrated in prepubertal rat[146] and pig[147] and adult pig[148] and human[149] ovaries. Indeed, when FGSCs isolated from adult minipig ovaries were infected by an EGFP lentivirus and injected into human ovarian cortex pieces, $EGFP^{+}$ oocytes were observed after 3 weeks in ovarian cortical xenografts.[148] In addition, FGSCs isolated from human cortical tissue (based on DDX4 expression) and transduced with a GFP-expression vector were shown to reform structures resembling follicles in culture with dispersed adult ovarian cells and to differentiate into oocytes when injected into human cortical tissue before xenotransplantation to nude mice.[149] In that study, the authors attributed FGSCs not being detected earlier by other teams to their smallness size (5–8 μm) and proportion (0.014±0.002%) of total ovarian cells. Ding et al also reported oocyte differentiation of FGSCs obtained from

Table 2 Studies that attempted to restore male fertility using stem cells of mesenchymal origin

	Type of stem cells	Transplantation method and cell numbers	Outcome
Yang et al[135]	HUC-MSCs	Injection of 10^5 HUC-MSCs, 10^5 HEK293 cells or saline solution under the tunicae albuginae of busulphan-treated mice	Higher expression of 10 meiosis-associated genes and higher protein levels of Miwi, DDX4, and SCP3 compared to controlateral uninjected testis; no difference between injected and uninjected testes in saline and HEK293 control groups
Hsiao et al[139]	OFSCs from human orbital fat tissue	Injection of 3×10^4 OFSCs or PBS 30 mins before detorsion of testis	Higher Johnsen score in testes injected with OFSCs than those injected with PBS; reduced oxidative stress and apoptosis in OFSC-injected testes compared to controls
Maghen et al[66]	HUCPVCs from human umbilical cord	Intratesticular injection of 5×10^4 HUCPVCs or saline solution in mice presenting mono-2-ethylhexyl phthalate-induced ST damage	Increased proportion of intact STs (2%– 22% from week 1 to 3) compared to absence of intact STs in controls; DAZL- and ACR-positive cells detected after 3 weeks only in HUCPVC-injected group
Anand et al[133]	BMSCs isolated from GFP^+ mice	Injection of 10^4–10^5 BMSCs or Sertoli cells expressing GFP or vehicle into testicular interstitia of busulfan-sterilized mice	GFP^+-transplanted cells detected only in the interstitia; spermatogenesis recovery in all groups; more STs showing spermatogenesis, PCNA, and MVH expressions in BMSC-transplanted mice
Kadam et al[15]	MSCs enriched from bone marrow or hematopoietic stem cells (HSCs), both isolated from GFP^+ mice	Injection of 1×10^5 MSCs enriched by bone marrow ($CD45^-Sca1^+Lin^-$) or HSCs ($CD45^+Sca1^+Lin^-$) or DMEM into the rete testis of busulfan-treated GFP^- mice	Higher percentage of STs with spermatogenesis in MSC-injected group (70%) compared to HSCs (18%) and DMEM (19%); detection of cells coexpressing GFP with Leydig (StAR) and Sertoli (WT1) cell markers but not the germ (MVH)-cell marker
Kadam et al[140]	MSCs isolated from mice bone marrow and transfected to express RFP. SSCs isolated from GFP^+ mice	Injection of 2×10^5 SSCs, MSCs, SSCs + MSCs or SSCs + TGFβ1-treated MSCs into the rete testes of mice sterilized with busulfan and $CdCl_2$	Higher percentage of STs (TFI) with endogenous spermatogenesis in all transplanted testes; cotransplantation of MSCs or TGFβ1-treated MSCs with SSCs did not result in better TFI than transplantation of SSCs alone for endogenous spermatogenesis; improved TFI of donor-derived SSCs for cotransplantation of SSCs with TGFβ1-treated MSCs compared to other groups
Karimaghai et al[138]	ASCs derived from hamster adipose tissue	Injection 1×10^6 ASCs or PBS into efferent ducts of busulfan-sterilized hamsters	Presence of spermatozoa in STs of mice from the ASC-transplanted group, but not in controls

Abbreviations: ACR, acrosin; ASC, adipose tissue-derived stem cell; $CdCl_2$, cadmium chlorure; DAZL, deleted in azoospermia like; DDX4, DEAD-box helicase 4; FSH-R, follicle stimulating hormone receptor; GFP, green fluorescent protein; HEK293, human embryonic kidney 293 cells; HUC-MSC, human umbilical cord mesenchymal stem cell; HUCPVC, first trimester human umbilical cord perivascular cell; MIWI, Piwi-like protein 1; MVH, mouse vasa homolog; P450scc, cytochrome P450 side-chain cleavage enzyme; PCNA, proliferating cell nuclear antigen; RFP, red fluorescent protein; ST, seminiferous tubule, SCP1, synaptonemal complex protein 1; SCP3, synaptonemal complex protein 3; StAR, steroidogenic acute regulatory protein; TFI, testicular fertility index; WT1, Wilms tumor protein 1.

small cortical tissue fragments present in IVF patients' follicular aspirates.[150] However, the existence of FGSCs is not accepted universally. Even more controversy on the subject arose when Johnson et al published a study suggesting an extragonadal source from bone marrow and peripheral blood.[151] Eventually, with transplantation and parabiotic mouse models, the hypothesis that circulatory bone-marrow cells can generate ovulated oocytes both

in the steady state and after induced damage was discredited by several teams.[152,153] Later, Lei and Spradling concluded that FGSCs could be dedifferentiated cells able to become germ cells under specific conditions as they did not detect these cells in mouse ovaries using a cell lineage–labeling system and demonstrated that the pool of primordial follicles generated during fetal life is sufficient to sustain adult oogenesis without a source of renewal.[154] Subsequently, other studies corroborated this hypothesis, as different teams were not able to detect FGSCs in mouse and human ovarian tissue using DDX4 lineage tracing, RT-PCR, or immunohistochemistry.[155–157] Reizel et al carried out an interesting study in which somatic mutations accumulated in microsatellites were used to reconstruct cell-lineage trees, which gave information on lineage relationships among different cell types. Reconstructed cell trees showed that oocytes formed clusters distinct from bone-marrow cells in both young and adult mice, suggesting that the two cell types belong to separate lineages. A second interesting observation was that oocyte depth increased with mouse age. In other words, oocytes of older mice had undergone more mitotic divisions than those of younger mice, which could be explained by either depth-guided selection of oocytes for ovulation or postnatal renewal.[158]

Use of SCs to treat ovarian reproductive failure

MSCs have been shown to act on the somatic compartment of the ovary, leading to reactivation and differentiation of "dormant" SCs (Figure 4). Notably, transplanted menstrual blood-derived endometrial MSCs (MenSCs) are able to migrate to the ovarian cortex and differentiate to granulosa cells, which improves FGSC renewal and restores fertility of sterilized mice.[159,160] Other studies have demonstrated fertility restoration of sterilized mice or rats using SCs isolated from bone marrow,[161–166] adipose tissue,[167,168] amniotic fluid,[169] amnion,[170] and chorion (Table 3).[171] Moreover, repeated bone marrow–derived MSCs (BMSCs) infusions through the tail vein not only postpone age-related ovarian failure in mice but improve the survival rate of offspring, suggesting a potential effect on egg quality.[172] With regard to humans, one team investigated transplantation of BMSCs into ovaries of 10 women diagnosed with premature ovarian failure and reported recovery of menstruation in two cases and one pregnancy with delivery of a healthy baby.[173] Even if promising, these results should be further confirmed and viewed with caution, since risks of transformation and tumorigenicity in MSC-based therapies are still debatable.[174]

Use of SCs to improve ovarian transplantation outcomes

Orthotopic autotransplantation of freeze–thawed pre- and postpubertal ovarian tissue already proved its efficacy, with more than 100 live births reported thus far[18,175–178] and a cumulative success rate of 57% (Figure 4).[179]

Although these results are encouraging, an important loss of primordial follicles has been reported after transplantation.[180] To overcome this issue, several types of SCs have been used to improve graft oxygenation and follicle survival (Figure 4). Aware that MSCs play an important role in angiogenesis and stabilization of the blood-vessel network, Xia et al cotransplanted MSCs and ovarian tissue, both encapsulated in Matrigel and demonstrated that MSCs promoted neoangiogenesis and prevented loss of primordial follicles in grafts.[181] Angiogenin, which plays a role in angiogenesis and endothelial cell proliferation, has been further identified as a key MSC-secreted factor involved in follicle survival and revascularization of xenografted human ovarian tissue.[182] ASCs as another source of MSCs, with the advantage of easier access compared to BMSCs, have also been evaluated. After encapsulation of human ovarian tissue using a mix of ASCs and fibrin, higher graft oxygenation and vascular density with improved survival of primordial follicles was achieved compared to tissue transplantation only.[183] These results highlight the potency of MSCs in promoting graft revascularization.

Use of stem cells to improve follicle IVM

As autotransplantation has the potential risk of reintroducing cancer cells, succeeding in IVM of primordial follicles recovered from cryopreserved ovarian tissue is of paramount importance.[184] IVM of preantral and antral follicles isolated from thawed human ovarian tissue until a competent oocyte stage has been achieved,[185–187] although with lower efficiency for prepubertal tissue,[188] which could be explained by the higher proportion of abnormal follicles before puberty.[189] In an attempt to improve follicle IVM, MSCs have been exploited (Figure 4). Experiments conducted in vitro demonstrated that conditioned medium from human umbilical cord MSCs increased microvessel density and decreased apoptosis of in vitro cultured cortical tissue compared to serum-free

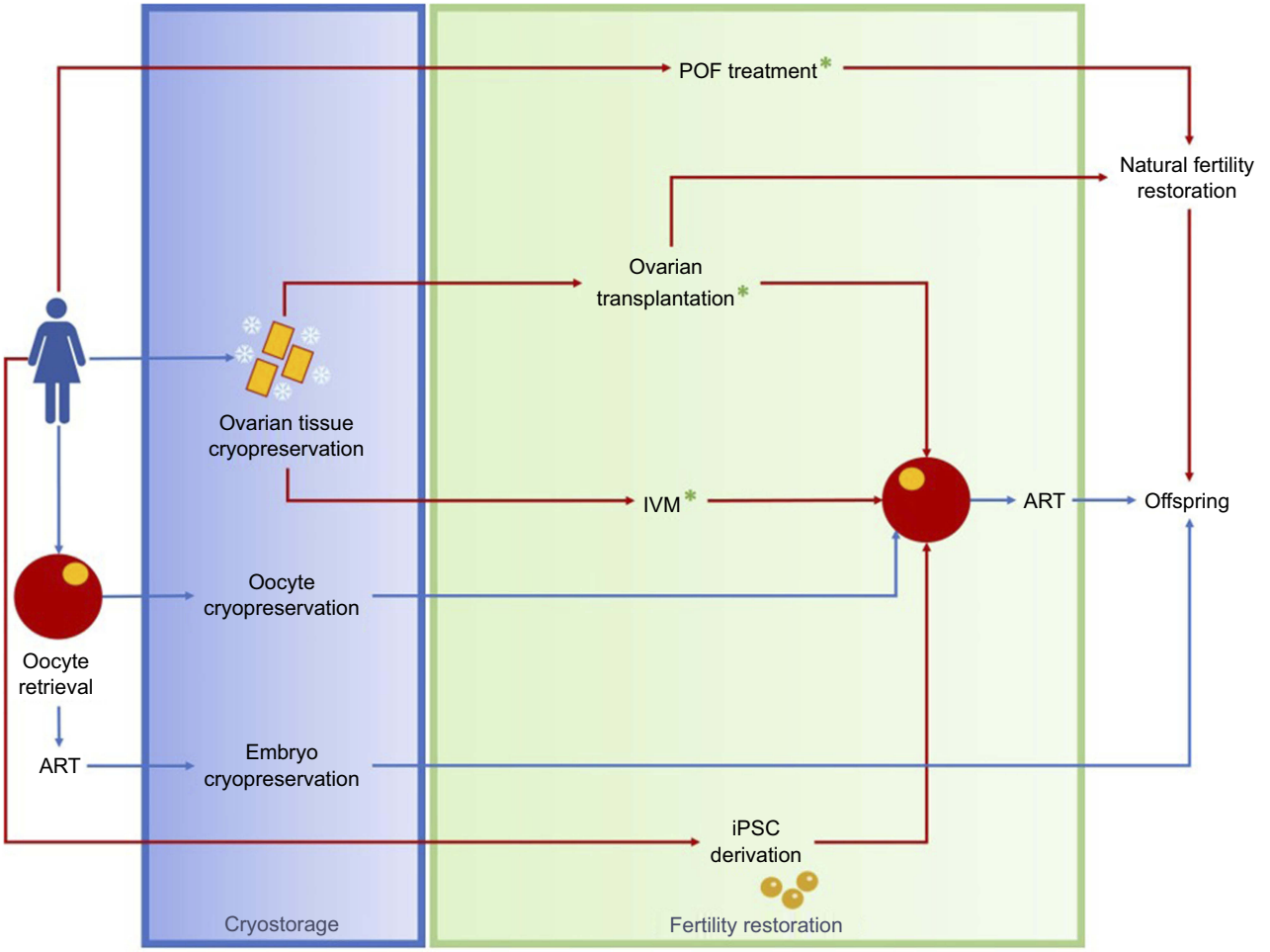

Figure 4 Fertility preservation in females.
Notes: Women at reproductive age can cryopreserve oocytes or embryos with aim of using it in the future. Women who have no time for ovarian stimulation and prepubertal girls can cryopreserve ovarian tissue, which can be transplanted back to the patient to restore her fertility or to obtain competent oocytes for ART. Generation of competent oocytes by IVM of follicles originating from the cryopreserved tissue could also be an option. Treatment of women who developed a POF due to cancer therapy could potentially restore their ovarian functions and fertility. Alternatively, derivation of iPSCs from different sources of somatic cells could lead to generation of competent oocytes. *Processes that could be improved with use of MSCs. Red arrows represent techniques that are still considered experimental. Blue arrows indicate methods that are already implemented in clinical practice.
Abbreviations: ART, assisted reproductive technology; iPSCs, induced pluripotent stem cells; IVM, in vitro maturation, MSCs, mesenchymal stem cells; POF, premature ovarian failure.

culture.[190] Human menstrual blood–derived endometrial MSCs increased follicular growth and IVM rates when cocultured with mouse alginate-encapsulated preantral follicles.[191] In the same way, coculture of BMSCs with human alginate-encapsulated follicles improved follicle growth and viability in a dose-dependent manner, suggesting that the number of MSCs influences culture outcomes.[192]

Generation of oocytes from embryonic and induced pluripotent stem cells

Hübner et al reported for the first time derivation of oocyte-like cells from mouse ESCs.[193] In 2012, Hayashi et al demonstrated that it was possible to differentiate female ESCs and iPSCs into primordial germ cell–like cells (PGCLCs) and that their aggregation with ovarian somatic cells allowed reconstitution of an ovarian structure in which the PGCLCs exhibited meiotic potential.[194] Moreover, transplantation of such reconstituted ovaries under the mouse ovarian bursa resulted in maturation of PGCLCs to vesicle-stage oocytes that were fertilized following IVM (Figure 4). Offspring were generated after in vitro fertilization of PGCLC-derived oocytes and embryo transfer to foster-mother mice, but epigenetic abnormalities were observed in half the generated eggs. The entire cycle of mouse oogenesis was later reproduced in vitro from ESCs

Table 3 Studies that attempted to improve female fertility using stem cells of mesenchymal origin

	Type of stem cells	Transplantation method and cell numbers	Outcome
Lee et al[161]	BMSCs isolated from mice femurs and tibiae	Injection of 2–3×10^7 cells into tail vein of busulphan- and cyclophosphamide-sterilized mice	More pregnancies in mice of the transplanted group compared to mice injected with only busulphan and cyclophosphamide
Fu et al[162]	BMSCs isolated from rat femurs and tibiae	Injection of 2×10^6 MSCs or saline solution into both ovaries of cyclophosphamide-treated rats	Decreased granulosa-cell apoptosis 2 weeks after transplantation; improved ovarian function in MSC-treated rats demonstrated by restoration of the estrous cycle; increased estradiol level and follicle numbers 4 weeks after transplantation
Selesniemi et al[172]	BMSCs isolated from mice femurs and tibiae	Injection of 1.5–3×10^7 BMSCs or PBS every 4 weeks via tail vein of mice	Extended fertility demonstrated by more pregnancies at age of 14.5–17.5 months and higher survival of offspring than controls
Santiquet et al[164]	BMSCs isolated from GFP^+ mice femurs	Injection of 10^7 BMSCs in the blood circulation or 2×10^4 into ovaries of mice treated with cyclophosphamide and busulphan	Higher fertility (based on average number of pups per litter) after injection of BMSCs into the blood circulation compared to non-injected group; no improvement of fertility for BMSC injection into the ovary
Lai et al[169]	HAFSCs isolated from human amniotic fluid recovered during amniocentesis	Injection of 2–5×10^3 HAFSCs with MSC-like properties or culture medium (control group) into both ovaries of busulphan- and cyclophosphamide-sterilized mice	Presence of follicles at all stages at histology in transplanted mice, but not in control group; differentiation of HAFSCs-derived MSCs into granulosa cells, supporting oocyte maturation; restoration of AMH expression in ovaries of mice grafted with HAFSCs-derived MSCs, but not in controls
Wang et al[170]	HAECs isolated from human placenta	Injection of 2×10^6 GFP^+ HAECs or culture medium into the tail vein of busulphan- and cyclophosphamide-sterilized mice.	Follicles at all stages in the transplanted group but not in controls; transplanted cells expressing GFP migrated to the ovary and differentiated in granulosa cells; partial restoration of ovarian function indicated by AMH expression in primary follicles of mice of the transplanted group
Takehara et al[167]	ASCs recovered from rat adipose tissue	Injection of 2×10^6 ASCs or saline solution into the ovary of cyclophosphamide-sterilized rats	Induction of angiogenesis and increased corpus lutea, follicles, StAR expression, and number of litters in the transplanted compared to control group
Liu et al[163]	BMSCs from rat tibiae	Injection of 4×10^6 EGFP-labelled BMSCs in the tail vein of cisplatin-sterilized rats	More antral follicles and E_2 level in transplanted compared to non-transplanted rats
Liu et al[159]	MenSCs derived from human menstrual blood	Injection of 10^4 MenSCs labelled with DiO or PBS into ovaries of mice sterilized with cyclophosphamide	Higher expression of AMH, FSHR, and Ki67 and increase over time of ovarian weight, E_2 levels, and follicle number in the transplanted compared to control group
Lai et al[160]	MenSCs derived from human menstrual blood	Injection of 2×10^6 MenSCs or culture medium in the tail vein of busulphan- and cyclophosphamide-sterilized mice	Recovered estrous cyclicity and fertility in transplanted mice; transplanted cells migrated to the ovarian stroma, differentiated in granulosa cells, and reduced depletion of germ-line stem cells caused by chemotherapy
Edessy et al[173]	Human iliac-crest-derived BMSCs	Injection of autologous BMSCs into ovaries of 10 women diagnosed with POF	2 women recovered menstruation and one of them get pregnant

(*Continued*)

Table 3 (Continued)

	Type of stem cells	Transplantation method and cell numbers	Outcome
Su et al[168]	ASCs recovered from rat adipose tissue	Injection of 2×10^6 GFP^+ ASCs with or without collagen or PBS into ovaries of rats with fertility impaired by *Tripterygium glycosides*	GFP signal was higher in the ASC+collagen group, suggesting better retention of ASCs in the tissue compared to ASCs without collagen; improved E_2 levels and higher pregnancy rate with transplantation of ASCs+collagen compared to PBS; higher number and proliferation rate of antral follicles in ovaries of rats transplanted with ASCs and ASC +collagen compared to PBS
Herraiz et al[165]	BMSCs and PBMNCs recovered from blood circulation of women	Injection of PBS, 10^6 PBMNCs, or 10^6 BMSCs (both labeled with MIRB) via tail vein of busulphan- and cyclophosphamide-treated mice	Mice transplanted with BMSCs recovered cyclicity by exhibiting proestrous and estrous phases; more apoptotic and pyknoctic bodies in ovaries of control and PBMNC-transplanted mice. BMSCs were localized within the theca cells of follicles, while only three PBMNCs were found in 1 of 16 samples; more antral and preovulatory follicles after BMSCs transplantation
Li et al[171]	CP-MSCs derived from human chorionic plate of placenta	Injection of 2×10^6 CP-MSCs or saline solution in the tail veins of cyclophosphamide-sterilized mice	Recovery of normal serum concentrations of FSH and E_2 and more follicles, estrous cycles, and ovulated oocytes compared to controls
Mohamed et al[166]	Human iliac crest–derived BMSCs	Injection of 5×10^5 BMSCs or PBS into both ovaries of cyclophosphamide- and busulphan-treated mice	Distribution of BMSCs mostly around growing follicles; higher E_2 and AMH levels in blood circulation; more follicles and AMH and inhibin expression into ovaries; more pregnancies

Abbreviations: AMH, anti-Mullerian hormone; ASC, adipose tissue-derived stem cell; BMSC, bone marrow-derived MSC; CP-MSC, chorionic plate-derived MSC; DiO, 3, 3′-dioctadecyloxacarbocyanine perchlorate; E_2, estrogen; EGFP, enhanced green fluorescent protein; FSHR, follicle stimulating hormone receptor; GFP, green fluorescent protein; HAEC, human amniotic epithelial cell; HAFSC, human amniotic fluid stem cell; MenSC, menstrual blood-derived MSC; MIRB, molday ion rhodamine b; MSC, mesenchymal stem cell; PBMNCs, peripheral blood mononuclear cells; StAR, steroidogenic acute regulatory protein; WT, wild type.

and iPSCs, although a low success rate of full-term development was reported for ESC-derived embryos.[195] However, with regard to hESCs, development of structures resembling primary ovarian follicles was the most advanced stage of differentiation that could be reached.[196]

Conclusion

Development of methods to preserve and restore fertility of patients subjected to gonadotoxic therapies has become an urgent matter in these last few decades. On the male side, SSCs constitute a pool of SCs able to differentiate into spermatozoa. Restoration of male fertility with SSCs is still at the research stage, but experiments in animals suggest that autotransplantation of propagated and selected SSCs into the rete testis or autografting of ITT will be possible in future. In vitro differentiation of human spermatozoa with the aim of using in vitro matured sperm in ART can also be an option, especially when there is a risk of malignant contamination of ITT but needs further development with regard to efficiency of haploid-cell generation, completion of spermatogenesis and safety issues. The classical scheme that the female germ-cell pool is fixed after birth is under debate. Several studies lean toward the existence of SCs, but it cannot be excluded that FGSCs derive from dedifferentiated cells. Development of germ cells from other sources of SCs such as ESCs and iPSCs has also been proposed to restore fertility in both males and females, but the genetic stability of the cells and capacity to generate healthy offspring is uncertain. Finally, the use of MSCs to act against follicular loss in grafts or restore the damaged male or female somatic germ-cell environment has shown promising results, but long-term risks associated with MSC transplantation or culture still need to be evaluated.

Abbreviation list

ART, assisted reproductive technology; ASC, adipose-derived stem cell; bFGF, basic fibroblast growth factor;

BMP4, bone morphogenic protein 4; BMSC, bone marrow-derived stem cell; ESC, embryonic stem cell; FACS, fluorescence-activated cell sorting; FGSC, female germline stem cell; FP, fertility preservation; hiPSC, human-induced pluripotent stem cell; HLA, human leukocyte antigen; HUPVC, human umbilical perivascular cell; HUPVC, human umbilical perivascular mesenchymal stem cell; ITT, immature testicular tissue; IVF, in vitro fertilization; IVM, In vitro maturation; LIF, leukemia inhibitory factor; Lin, lineage; MEF, mouse embryonic fibroblast; MenSC, menstrual blood-derived endometrial mesenchymal stem cell; MSC, mesenchymal stem cell; PDMS, polydimethylsiloxane; PGCLC, primordial germ cell-like cell; POF, premature ovarian failure; SSC, spermatogonial stem cell; SSEA-4, stage-specific embryonic antigen-4; ST, seminiferous tubule; TCS, testicular cell suspension; UC-MSC, umbilical cord-derived mesenchymal stem cell.

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Author contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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