Spermatogonial Stem Cell Culture in Oncofertility

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KEYWORDS

• Spermatogonial stem cell culture • Fertility preservation • Male fertility

KEY POINTS

- Chemotherapy, radiation, and other medical treatments can cause permanent infertility. Sperm freezing is the standard of care method to preserve male fertility.
- Testicular tissue freezing is an experimental option to preserve the fertility of prepubertal boys and others who cannot produce sperm. Testicular tissues contain spermatogonial stem cells.
- Spermatogonial stem cell–based techniques that are currently in the research pipeline may be available in the male fertility clinic of the future.
- To facilitate clinical translation, methods are needed to isolate and enrich human spermatogonial stem cells as well as expand their numbers in culture.

INTRODUCTION

Advancements in cancer therapies over the past several decades have led to a rise in pediatric cancer survival rates to approximately 88%.[1] This increase in cancer survivorship has made it increasingly important to address factors that affect patient quality of life posttreatment, including treatment-induced gonadotoxicity and increased risk of infertility.[2,3] Most patients who are exposed to gonadotoxic therapies experience transient azoospermia and will recover normal levels of spermatogenesis within 1 to 5 years posttreatment[4]; however, approximately 24% of patients will be rendered permanently infertile by treatment for their primary disease.[5] The extent and permanence of azoospermia depends on a combination of several factors, including the primary disease diagnosis and the therapeutic regimen employed to treat the disease. Methods to predict the risk of infertility are imperfect,[6] but some guidance is available to predict treatment regimens that are associated with significant or high risk of infertility.[7,8] The prospect of having biological children is important to cancer survivors, and the risk of iatrogenic infertility causes psychosocial stress in these individuals.[9] Therefore, the American Society of Clinical Oncology, the American Society for Reproductive Medicine, and the American Academy of Pediatrics recommend that all patients with cancer and patients receiving cytotoxic treatments for hematologic conditions be counseled about the risk of infertility and about methods for fertility preservation before the onset of treatment.[10–13]

The standard of care approach to preserve fertility in adolescent and adult male patients is cryopreservation of spermatozoa that can be used at a later time to establish pregnancy through assisted reproductive technology.[14,15] This option is not available to prepubertal patients who do not produce sperm; however, several centers around the world are cryopreserving testicular tissues for young patients with anticipation that spermatogonial stem cells (SSCs) in those tissues might be used to restore fertility in the future.[16–23]

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SPERMATOGONIAL STEM CELL TRANSPLANTATION TO RESTORE FERTILITY

Several new technologies have emerged over the past 25 years that may allow patients to use their cryopreserved testicular tissues to produce sperm and have biological offspring, including SSC transplantation, de novo testicular morphogenesis, testicular tissue organ culture, testicular tissue grafting or xenografting, and derivation of germ cells from induced pluripotent stem cells.[24,25] SSC transplantation is a mature technology that may be ready for translation to the male fertility clinic. In fact, Radford and colleagues[26,27] reported a clinical trial in 1999 in which testicular cell suspensions were cryopreserved for 12 patients with Hodgkin disease. Seven of those patients returned to have their cryopreserved cells, including SSCs, transplanted back into their testes via injection into the rete testis space.[27] Although follow-up studies on the outcome of transplantation in those cases have not been reported, the study demonstrates patient willingness to undergo an experimental SSC-based therapy to have a biological child.

Like other tissue-specific stem cells, SSCs have the potential to colonize the testicular niche and regenerate spermatogenesis. Brinster and colleagues[28,29] first demonstrated this principle 25 years ago by showing that mouse testicular cell suspensions containing SSCs could be transplanted into the seminiferous tubules of an infertile mouse recipient to restore complete spermatogenesis and fertility. This method has since been replicated in a number of mammalian species, including rats, sheep, goats, pigs, bulls, dogs, and primates.[30–36] SSCs from all ages, newborn to adult, are competent to regenerate spermatogenesis, and spermatogenesis can be restored from testicular cells that have been cryopreserved for as long as 14 years.[33,37–41] Thus, it appears feasible to cryopreserve testicular tissues/cells containing SSCs for prepubertal patients and recover those cells years later for autologous transplantation and regeneration of spermatogenesis.

Testicular cells are typically transplanted into the recipient testis through the rete testis space that is contiguous with all seminiferous tubules.[33,42–44] SSCs migrate from the lumen of the seminiferous tubules, through the blood-testis barrier (BTB), to the basement membrane. Rac1 and β1 Integrin have been shown to be critical in SSC transmigration through the BTB and attachment to the basement membrane, respectively, in mice.[45,46] Despite innate properties allowing SSCs to penetrate the BTB, most transplanted cells are eliminated through phagocytosis by Sertoli cells, which may be one factor that reduces overall efficiency of the method.[47] Nagano and colleagues[48] evaluated the kinetics of SSC engraftment in the mouse testis and deduced that transplantation with 1 million testicular cells led to colonization and spermatogenesis by 19 SSCs. Thus, methods to isolate and enrich SSCs and expand their numbers in culture are needed to ensure robust engraftment and regeneration of spermatogenesis.

In fertility preservation centers that provide testicular tissue cryopreservation services, approximately 20% of testicular volume from one testis is typically biopsied, although some centers allow for collection of larger volumes and/or biopsy of both testes.[22,23,49] Hence, the number of SSCs obtained from small biopsies of prepubertal testes could be a limiting factor in the successful application SSC transplantation in the clinic. One way to overcome this limitation is to isolate and enrich SSCs from the testicular biopsy and expand their numbers in vitro before transplantation. These approaches might also be used to assess and eliminate malignant contamination, as described in the following section.

SORTING METHODS TO ISOLATE AND ENRICH SPERMATOGONIAL STEM CELLS AS WELL AS ELIMINATE MALIGNANT CONTAMINATION

Using SSC transplantation as a functional assay, several cell surface markers have been identified that are conserved between murine and human spermatogonia. Murine SSCs have been shown to exhibit the phenotype GPR125^{+} (G-protein coupled receptor 125), EpCAM^{low} (epithelial cell adhesion molecule), ITGA6^{+} (α6 integrin), ITGB1^{+} (β1 integrin), CD9^{+}, THY1^{+} (CD90), GFRα1^{+} (GDNF family receptor alpha 1), MCAM^{+} (melanoma cell adhesion molecule 1), ITGAV^{-} (αV integrin), cKIT^{-} (CD117 or stem cell growth factor), MHC-I^{-} (major histocompatibility complex class I), SCA-1^{-} (stem cells antigen 1).[50–58] Characterization of human spermatogonia has identified GPR125, EpCAM, ITGA6, and GFRA1, as well as FGFR3 (fibroblast growth factor receptor 3), SSEA4 (stage specific embryonic antigen 4), TSPAN33 (tetraspanin 33), as cell surface markers of human SSCs.[59–61] In addition to its application in basic research, the ability to identify and enrich SSCs is important for clinical translation of SSC transplantation as a method to restore fertility. These methods could be especially valuable for patients with malignancies that may contaminate testicular cells, posing a risk of reintroducing cancer cells into patient survivors. A

study using a rat model showed that transplanting a testicular cell suspension with as few as 20 leukemic cells could cause the disease to recur in the recipient.[62] Some studies have reported the use of multiparametric flow cytometry methods to negatively select spermatogonia from cancer cells.[58,63] Other reports used markers for both spermatogonia and cancer cells for a more stringent segregation of the 2 populations but produced conflicting results.[64–66] In addition, these reports were based on the use of cancer cell lines, and the efficacy of these methods in eliminating heterogeneous populations of malignant cells needs to be determined.

Although sorting techniques to enrich SSCs and eliminate contaminating malignant cells are promising, there is a need to develop stringent methods to test and quantify residual malignant contamination before autologous transplantation. Polymerase chain reaction (PCR)-based methods to detect minimal residual disease may be used in addition to flow cytometry approaches to increase the sensitivity of selection. Currently, there is limited information about how low-level contamination detected by PCR corresponds to tumor-forming capacity and, hence, the absolute risk for inducing relapse remains difficult to predict.[67,68] Development of human SSC culture methods may enable clonal expansion of SSCs from an enriched population providing an extra level of stringency for decontamination of patient samples.[69]

Methods for enriching spermatogonia are routinely used to establish SSC culture (**Table 1**). Shortly after the discovery of the role of glial cell line–derived neurotropic factor (GDNF) on SSC self-renewal,[70] Kanatsu-Shinohara and colleagues[71] described a method for the long-term culture of mouse SSCs. In this report, they placed testicular cells on plates coated with gelatin; the testicular somatic cells selectively adhered to the plates, whereas germ cells remained floating and could be aspirated and plated onto secondary plates. This approach served the dual purpose of enriching SSCs and removing testicular somatic cells that can rapidly overwhelm the cultures. After 2 or more rounds of differential plating, floating cells were plated on mouse embryonic fibroblasts in low serum medium supplemented with epidermal growth factor (EGF), leukemia inhibitory factor (LIF), GDNF, and fibroblast growth factor 2 (FGF2). Subsequent studies used fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) for the cell surface marker, THY1, to enrich spermatogonia.[72–74] Spermatogonial stem cell transplantation provided the experimental evidence that functional rodent SSCs could be maintained with expansion in number during long-term culture.[71,73–76] Cultured SSCs not only regenerated spermatogenesis in infertile recipients, but also produced sperm that were competent to fertilize rodent oocytes and give rise to healthy offspring.[71,73]

HUMAN SPERMATOGONIAL STEM CELL CULTURE: COMPONENTS AND METHODS OF ANALYSIS

In 2009, Sadri-Ardekani and colleagues[18] reported the long-term culture of adult human SSCs following a protocol very similar to that described by Kanatsu-Shinohara and colleagues[71] in their first report on mouse SSC cultures. Specifically, differential plating was used to reduce the number of testicular somatic cells, which attached to the plate; floating germ cells were passaged onto plates coated with human placental laminin in StemPro medium supplemented with EGF, LIF, GDNF, and FGF2. Using this method, the investigators reported that human SSCs could be maintained for several months and expanded more than 18,000-fold.[18] The same group later reported similar success culturing SSCs from prepubertal human testes.[77]

There are now more than 20 reports on human SSC culture (see **Table 1**). Many have used differential plating on plastic, lectin, collagen, or gelatin as the sole means to enrich SSCs and/or reduce testicular somatic cells before culture.[18,77–86] Others have supplemented differential plating with Percoll gradient selection[87,88] and/or positive or negative selection for cell surface markers using FACS or MACS[87,89–93] or used FACS/MACS selection alone.[94,95] Positive selection markers used for human SSC culture have included ITGA6, CD9, GPR125, SSEA4, and EPCAM. Negative selection markers have included cKIT, CD45, and THY1 (see **Table 1**). Interestingly, although THY1 has been used as a positive selection marker before mouse SSC culture,[73] Smith and colleagues[95] used THY1 as a negative marker of human SSCs and in fact used irradiated THY1+ human testis cells as feeders for their human SSC cultures.

Human spermatogonia, like murine spermatogonia, have been shown to require an extracellular matrix (ECM) or feeder-cell–based substrates to promote the attachment, survival, and proliferation in vitro. Feeder cells that have been used to culture human SSCs include fibroblasts derived from human embryonic stem cells, human Sertoli cells, mouse embryonic fibroblasts, mouse endothelial cells, human testicular somatic cells, and THY1+ testicular cells (see **Table 1**).[78–80,88,93–96] ECM

Table 1
Literature review of reports on human SSC culture

Citation	Duration of Culture	Sort/ Differential Plating	Medium	Growth Factors	Feeders or ECM	Passaging Technique	End Point	Type and Age of Donor	Claim
Sadri-Ardekani et al,[18] 2009	15 wk	Differential plating on plastic	MEM+10% FCS for differential plating followed by StemPro-34	20 ng/mL EGF, 10 ng/mL GDNF, 10 ng/mL LIF, 10 ng/mL bFGF	Human placental laminin	Passaged every 7–10 d using Trypsin EDTA and differential passaging if there was somatic cell overgrowth	Xeno transplants; ICC – PLZF; RT-PCR – PLZF, ITGA6, ITGB1;	Adult orchidectomy patients (n = 6)	18,000-fold increase in xeno transplant colonizing activity over 64 d in culture
Wu et al,[78] 2009	1 wk	Differential plating on gelatin	MEMα	20 ng/mL GDNF, 150 ng/mL GFRA1, 1 ng/mL bFGF	C166 mouse endothelial cells	Not reported	ICC – UCHL1	Prepubertal male aged 2–10 y diagnosed with cancer (n = 2)	UCHL1+ spermatogonia can be maintained at least 19 d. No quantification. GDNF required.
Chen et al,[94] 2009	2 mo	MACS for ITGA6	DMEM	10 ng/ml GDNF, 4 ng/ml bFGF, 1500 IU/mL LIF	Human embryonic stem cells derived fibroblasts (hdF)	Passaged every 4–5 d using cell dissociation buffer or trypsin	ICC – OCT4, SSEA1, ITGA6; RT-PCR – OCT4, STRA8, DAZL, NOTCH1, NGN3, SOX3, KIT	Fetal	Colonies maintained over 10 passages. No quantification.

Lim et al,[87] 2010	>6 mo	Percoll selection, differential plating on plastic and collagen followed by MACS for CD9	DMEM during enrichment followed by StemPro-34	10 ng/mL GDNF, 10 ng/mL bFGF, 20 ng/mL EGF, 10,000 U/mL LIF	Laminin	Passaged very 2 wk using Trypsin	RT-PCR - OCT4, ITGA6, ITGB1, cKIT, TH2B, SYCP3, TP-1; MTT; TUNEL; ICC - GFRA1, CD-9, ITGA6; Alkaline phosphatase staining	Males with obstructive and non obstructive azoospermia (n = 37)	Clumps maintained and continued proliferating over 12 passages (>26 wk). Total cells quantified.
He et al,[89] 2010	14 d	Differential plating on plastic and MACS for GFR125	DMEM/F12 during enrichment followed StemPro-34	100 ng/mL GDNF, 300 ng/mL GFRA1-Fc, 10 ng/mL NUDT6, 10 ng/mL LIF, 20 ng/mL EGF, 30 ng/mL TGFB, 100 ng/mL Nodal	0.1% gelatin	Not reported	ICC – GPR125, ITGA6, GFRA1, THY1	Adult organ donors (n = 5)	GPR125+ cells proliferated during 2 wk in culture, but were not quantified.

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Citation	Duration of Culture	Sort/ Differential Plating	Medium	Growth Factors	Feeders or ECM	Passaging Technique	End Point	Type and Age of Donor	Claim
Kokkinaki et al,[90] 2011	4–5 mo	Differential plating on FBS-coated dish, treatment with RBC Lysis Buffer and Dead Cell Removal Kit followed by SSEA4 MACS	StemPro-34	10 ng/mL GDNF, 10 ng/mL bFGF, 20 ng/mL EGF, 10,000 U/mL LIF	Growth factor-reduced matrigel	Passaged manually at 1 mo followed by digestion with dispase + collagenase every 10–15 d	Morphology, number of colonies and cells/colony, RT-PCR for SSC markers (PLZF, GPR125, SSEA4) and pluripotency markers (KLF4, OCT4, LIN28, SOX2, NANOG)	14, 34, and 45-year-old organ donors (n = 3)	Number of colonies and number of cells per colony increased during 5 months in culture.
Sadri-Ardekani et al,[77] 2011	15.5 and 10 wk	Differential plating on plastic	StemPro-34	20 ng/mL EGF, 10 ng/mL GDNF, 10 ng/mL LIF, 10 ng/mL bFGF	Human placental laminin	Passaged every 7–10 d using Trypsin EDTA and differential passaging if there was somatic cell overgrowth	Xeno transplants; RT-PCR – PLZF, ITGA6, ITGB1, CD9, GFRA1, GPR125, UCHL1	Prepuberatal male patients with Hodgkin lymphoma; 6.5 and 8.0 years old (n = 2)	5.6-fold increase in xeno transplant colonizing activity over 14 d in culture and 6.2-fold increase over 21 d in culture.
Nowroozi et al,[79] 2011	18 d	Differential plating on lectin-coated plates	DMEM	Not reported	Human Sertoli cells	Passaged every 7 d with Trypsin EDTA	ICC – OCT4, Vimentin; Alkaline phosphatase staining	Adults with non obstructive azoospermia (n = 47)	Colonies were observed over 18 d in culture. No quantification.

Liu et al,[88] 2011	1 mo	Percoll separation and differential plating on plastic	DMEM/F12	Not reported	Human Sertoli cells	Not reported	ICC – OCT4, SSEA4; Flow cytometry – OCT4	Fetal (n = 5)	OCT4+ cells observed; timeframe uncertain. No quantification.
Mirzapour et al,[80] 2012	5 wk	Differential plating on lectin-coated plates	DMEM	Various concent-rations of bFGF and LIF	Human Sertoli cells	Passaged every 7 d using Trypsin EDTA	Xeno transplants; alkaline phosphatase staining; ICC – OCT4, Vimentin; RT-PCR – OCT4, NANOG, STRA8, PIWIL2, VASA	Adult males with NOA-maturation arrest (n = 20)	Tested bFGF and LIF concentrations. Colony number increased in some conditions over 30 d in culture.
Koruji et al,[86] 2012	2 mo	Differential plating on plastic	DMEM+ 5%FCS	20 ng/mL GDNF, 10 ng/mL bFGF, 10 ng/mL LIF, 20 ng/mL EGF	Laminin or plastic	Passaged every 5–7 d using Trypsin EDTA	Morphology-number and diameter of colonies; RT-PCR – PLZF, DAZL, OCT4, VASA, ITGA6, ITGB1	Adult males with NOA	Clusters present after 2 mo. Xeno transplant colonizing activity and expression of spermatogonial markers reported. No quantification.

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Citation	Duration of Culture	Sort/ Differential Plating	Medium	Growth Factors	Feeders or ECM	Passaging Technique	End Point	Type and Age of Donor	Claim
Goharbakhsh et al,[81] 2013	52 d	Differential plating on plastic for cells >10^6, all cells were plated is number<10^6	DMEM-F12	10 ng/mL GDNF, 10 ng/mL bFGF, 20 ng/mL EGF, 10,000 U/mL LIF	20 μL/mL laminin or 0.2% gelatin	Passaged every 7–10 d, method was not mentioned	Morphologic observation of EB-like colonies and ICC staining for GPR125	Azoospermic adult males (n = 12)	Clusters observed over several passages during 52 d in culture. GPR125+ cells observed at end of culture. No quantification of clusters or GPR125 cells.
Piravar et al,[82] 2013	6 wk	Differential plating on plastic	DMEM/F12 for 16 h then StemPro-34	10 ng/mL GDNF, 20 ng/mL EGF, 10 ng/mL LIF	Uncoated plates for the first 14 d followed by laminin	Trypsinization every 2 wk	qPCR for UCHL1	Non obstructive azoospermic males (n = 10)	Clusters number increased over 6-wk of culture. UCHL1 expression observed by RT-PCR.
Akhondi, MM et al,[112] 2013	6 wk	Enrichment was not performed	StemPro-34	10 ng/mL GDNF, 20 ng/mL EGF, 10 ng/mL LIF	Not reported	Trypsinization every 10 d	ICC for Oct4; qPCR for PLZF	44-year-old organ donor (n = 1)	Cluster number increased during 6-wk culture. OCT4 observed by ICC at end of culture. PLZF expression observed by RT-PCR

Zheng et al,[83] 2014	2 wk	Differential plating on plastic and collagen	DMEM during enrichment followed by StemPro-34	20 ng/mL EGF, 10 ng/mL GDNF, 10 ng/mL LIF, 10 ng/mL bFGF	Not reported	Passaged using Trypsin when confluent	Flow cytometry - SSEA4; qRT-PCR - UTF1, FGFR3, SALL4, PLZF, DAZL, VIM, ACTA2, GATA4	Adult organ donors (n = 8)	SSEA4+ spermatogonia decreased over time in culture. VIM+, ACTA2+ somatic cells were the main cell type present after 48 d in culture
Chikhovskaya et al,[91] 2014	2 wk	Differential plating on plastic followed by MACS for ITGA6 and differential plating on Collagen I and Laminin	StemPro-34	20 ng/mL EGF, 10 ng/mL GDNF, 10 ng/mL LIF, 10 ng/mL bFGF	MEFs or plastic	Not reported	qPCR for PLZF, MAGEA4, CD49f, DAZL, UTF1, DDX4, TM4SF1, ACTA2; flow cytometry for SSEA4, CD29, CD44, CD49f, CD73, CD90, CD105, HLAABC, HLADR, CD31, CD34, CD117, CD133	Adult patients with cancer undergoing bilateral orchidectomy (n = 3)	Mixed cultures: rapid proliferation of testicular somatic cells and rapid decrease in PLZF+ and MAGEA4+ germ cells. Isolated spermatogonia degenerated by 2 wk in culture.

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Citation	Duration of Culture	Sort/ Differential Plating	Medium	Growth Factors	Feeders or ECM	Passaging Technique	End Point	Type and Age of Donor	Claim
Smith et al,[95] 2014	21 d	FACS – CD45-, THY1-, SSEA4+	StemPro-34	20 ng/mL EGF, 10 ng/mL GDNF, 10 ng/mL LIF, 10 ng/mL bFGF	Adult human THY1+ cells	Not reported	ICC – SSEA4, VASA	Adults with normal spermato-genesis (n = 13)	Colonies expressing SSEA4 and VASA were present at 21 d. No quantification.
Guo et al,[92] 2015	2 mo	Differential plating on plastic with DMEM-F12 followed by MACS for GPR125	StemPro-34	20 ng/mL EGF, 10 ng/mL bFGF, 10 ng/mL LIF, 50 ng/mL GDNF	Hydrogel Stem Easy	Not reported	Morphologic observation; cell proliferation assay; ICC – GPR125, UCHL1, THY1 and PLZF; RT-PCR for GPR123, GFRa1, RET, PLZF, UCHL1, MAGEA4, SYCP3, PRM1 and TNP1 at 30 d	22–35-year-old patients with obstructive azoospermia (n = 40)	Colonies of grapelike cells observed at 14 d, 1 mo, and 2 mo. Colonies stained for GPR125, THY1, UCHL1 and MAGEA4. No quantification.

Baert et al,[84] 2015	2 mo	Differential plating on plastic	StemPro-34	20 ng/mL EGF, 10 ng/mL GDNF, 10 ng/mL LIF, 10 ng/mL bFGF	No substrate	Not reported	ICC and RT-PCR - VASA, UCHL1	Vasectomy reversal patients and adult male patients who underwent bilateral orchidectomy due to prostate cancer (n = 6)	Single or small groups of VASA+/ UCHL1+ cells detected in considerable amounts up to 1 mo but infrequently after 2 mo.
Abdul Wahab et al,[97] 2016	49 d	Enrichment was not performed	DMEM	80 μl bFGF	Plastic	Not reported	In-well staining for ITGA6, ITGB1, CD9 and GFRA1	Non obstructive azoospermic male (n = 1)	Clusters observed until 49 d in culture. Some ITGA6+ and CD9+ cells were observed. No quantification.
Medrano et al,[96] 2016	28 d	FACS for HLA-/ EPCAM+	StemPro-34	20 ng/mL EGF, 10 ng/mL LIF, 10 ng/mL bFGF, 10 ng/mL GDNF	Testicular somatic cells	Not reported	ICC - Ki67; TUNEL; RT-PCR - UTF1, DAZL, VASA, PLZF, FGFR3, UCHL1; Elecsys Testosterone II competitive immunoassay; ELISA - Inhibin B	Adult male patients who underwent bilateral orchidectomy due to prostate cancer (n = 3)	VASA+/UTF1+ cells observed after 2 wk but were rarely Ki67+ and disappeared by 4 wk

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Table 1 (*continued*)

Citation	Duration of Culture	Sort/ Differential Plating	Medium	Growth Factors	Feeders or ECM	Passaging Technique	End Point	Type and Age of Donor	Claim
Gat et al,[85] 2017	12 d	Differential plating on Gelatin	DMEM-F12 and StemPro-34	20 ng/mL EGF, 10 ng/mL GDNF, 10 ng/mL LIF, 10 ng/mL bFGF	Laminin and testicular somatic cells	Passaged using Trypsin when cells were 80%–90% confluent	SSC-like aggregates and targeted RNA seq for DAZL, ITGA6 and SYCP3	Adult orchidectomy patients (4 for testicular malignancies and 3 for testicular pain) and 1 adult who underwent microTESE due to NOA (n = 8)	Germ cell aggregates observed. Number impacted by medium and ratio of somatic cells to germ cells. No quantification over time.
Murdock et al,[93] 2018	14 d	MACS for ITGA6 followed by differential plating on Collagen I	MEMα	20 ng/mL GDNF, 1 ng/mL bFGF	STO, mouse and human laminin, htECM, ptECM, SIS, and UBM	Passaged using Trypsin at day 7	ICC – UTF1; flow cytometry – SSEA4, cKIT, AnnexinV and Ki-67	Adult organ donors (n = 4)	Aggregates observed. Number of UTF1+ cell declined over 14 d in culture.

Abbreviations: bFBF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EGF, epidermal growth factor; FACS, fluorescenceactivated cell sorting; FBS, fetal bovine serum; FCS, fetal calf serum; GDNF, glial cell line–derived neurotropic factor; htECM, human testis extracellular matrix; ITGA6, Integrin a6; ICC, immunocytochemistry; LIF, leukemia inhibitory factor; MACS, magnetic-activated cell sorting; MEM, minimum essential medium; MEF, mouse embryonic fibroblasts; NOA, nonobstructive azoospermia; PLZF, promyelocytic leukemia zinc finger; ptECM, porcine testis extracellular matrix; qPCR, quantitative polymerase chain reaction; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; RBC, red blood cell; RT-PCR, reverse-transcriptase polymerase chain reaction; SIS, small intestine submucosa; SSC, spermatogonial stem cell; STO, SIM mouse embryonic fibroblasts; TGFB, transforming gorwoth factor beta; UBM, urinary bladder matrix.

Data from Refs.[18,77–97,112]

substrates that have been used for human SSC culture include human laminin, gelatin, Matrigel, Hydrogen Stem Easy, human and porcine testicular ECM, porcine small intestinal submucosa ECM, and urinary bladder ECM.[18,77,81,82,87,90,92,93] Most human SSC culture studies summarized in **Table 1** used culture conditions similar to what was originally described by Kanatsu-Shinohara and colleagues[71] in mouse and Sadri-Ardekani and colleagues[18] in human, including StemPro-34 medium supplemented with GDNF, basic fibroblast growth factor (bFGF), EGF, and LIF. Some of those studies reported significant expansion of spermatogonia in culture, suggesting an evolutionary conservation of factors required for SSC maintenance and proliferation,[18,77,90] although few studies have formally tested the requirement for those factors in human SSC culture.[78,80] Others reported a rapid decline in the number of human spermatogonia using those conditions.[83,84,91,96] The discrepancy in results can be explained in part by differences in starting cell populations and in part by different approaches to analysis of culture outcomes. Some studies reported the presence of clusters or colonies of putative spermatogonia with no attempt to quantify.[79,85,94] Some studies observed spermatogonial markers or xenotransplant colonizing activity in culture, but did not quantify.[78,81,86,88,89,92,95,97] Some quantified the number of clusters/colonies or total cells in culture but did not specifically quantify spermatogonia using markers or transplantation.[77,80,82,87,90] Finally, some studies quantified the number of cells with spermatogonial markers or xenotransplant colonizing activity over time in culture.[18,77,83,84,91,93,96]

CHALLENGES, OPPORTUNITIES, AND FUTURE DIRECTIONS

Variations in the methods for (1) selection of spermatogonia before culture, (2) culture conditions, and (3) analytical endpoints have made it difficult to compare studies or reach a consensus about optimal human SSC culture conditions. There are several cell surface markers that can be used to isolate and enrich human SSCs, but none of those can produce a pure population of SSCs. Therefore, any method used to sort before culture will produce a heterogeneous population of cells that is likely to include testicular germ cells and somatic cells. Quantification of colony or cluster number in culture is valuable but not sufficient as a single endpoint because it is possible to produce colonies of mesenchymal cells from human testes.[83,91]

Spermatogonial stem cell transplantation was the "gold-standard" assay that validated success expanding functional rodent SSCs in culture. Mouse and rat SSCs can colonize infertile mouse recipient testes and regenerate complete spermatogenesis.[28,29,98] Spermatogonial stem cell transplantation in humans is not possible as a routine biological assay, but human to nude mouse xenotransplantation has emerged as a powerful tool to quantify human spermatogonia with transplantation potential. Human cells do not produce complete spermatogenesis in mouse testes, but they do migrate to the basement membrane of seminiferous tubules, proliferate to produce characteristic chains and clusters of spermatogonia, and survive long-term.[64,99,100]

It is recognized that not all laboratories will have the expertise or infrastructure for human to nude mouse xenotransplantation. There has been significant progress in the last few years identifying protein markers of undifferentiated human stem/progenitor spermatogonia (eg, UTF1, PIWIL4, UCHL1, PLZF, SALL4, GFRA1, LPPR3, TCF3, TSPAN33, and others).[100–103] These markers can be detected and quantified at a single-cell level using immunocytochemistry or flow cytometry. Reverse-transcriptase PCR (RT-PCR) is a complementary and sensitive method to confirm the presence of spermatogonial transcripts but does not reveal protein expression or provide information about spermatogonial quantity. Similarly, markers that are expressed by spermatogonia and other somatic cell types in the testis can be misleading (eg, ITGA6, THY1, UCHL1).[84,91,95,96] Multiparameter staining (eg, VASA+/UCHL1+) may help to resolve these issues.[84,96] Spermatogonial marker–positive cells should be monitored and quantified throughout the culture period and not just the end because this will help to understand spermatogonial proliferation dynamics over time. Complementing markers of undifferentiated spermatogonia with markers of differentiation (eg, cKIT), apoptosis (eg, annexinV, Caspase 3), and proliferation (eg, ki67) will help elucidate the fate of spermatogonia once they are placed in culture.

The growth requirements to maintain or expand human SSCs in culture are not known. Many studies have started with factors that were used in mouse SSC culture (any combination of GDNF, bFGF, EGF, and LIF), but few have formally tested the requirement for those factors.[78,80] Characterization of human germ and testicular somatic cells through single-cell RNA sequencing may help shed light on additional factors or signaling pathways in human SSCs that might be manipulated in culture to promote SSC survival and/or proliferation or new markers that can be used to isolate and enrich human SSCs.[101,102,104]

Mouse and rat SSCs divide once every 3 to 11 days in culture, similar to their in vivo proliferation dynamics. The in vivo cell cycle time of undifferentiated human A_{dark} and A_{pale} spermatogonia ranges from 1.5 to 8.0 months. If, like rodents, human SSC proliferation dynamics in culture are similar to the in vivo situation, it raises questions about whether it will ever be possible to expand human SSC numbers in culture. An emerging alternative approach could be to expand patient-derived induced pluripotent stem cells (iPSCs) in culture and then differentiate them into primordial germ cell–like cells (PGCLCs).[105–109] PGCLCs can potentially be transplanted into the testes to regenerate spermatogenesis[110] or differentiated to sperm in vitro,[111] outcomes that have been reported in mice, but not yet for any other species.

Despite the challenges outlined in this article, culture of human male germline stem cells, including SSCs or iPSC-derived PGCLCs, will have important applications for fundamental investigations of human germ lineage development and spermatogenesis. This is important because our current understanding of human SSC function and spermatogenesis is based primarily on data generated in mice. Knowledge generated from human germline stem cell culture could have important implications for development of next-generation reproductive technologies using stem cells.

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