Stem Cell and Tissue-Based Therapies for Male Infertility

by

Sherin David

B.Tech, Amity University, 2012

M.S., University at Buffalo, 2014

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This dissertation was presented

by

Sherin David

It was defended on

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and approved by

William H. Walker, Associate Professor, Cellular Biology and Physiology

Eric Lagasse, Associate Professor, Department of Pathology

Alejandro Soto-Gutierrez, Associate Professor, Department of Pathology

Michael Tsang, Associate Professor, Department of Developmental Biology

Dissertation Director: Kyle E. Orwig, Professor, Obstetrics, Gynecology and Reproductive Sciences

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Infertility due to exposure to chemotherapy and radiation has a detrimental effect of the patient's quality of life. A number of spermatogonial stem cell (SSC)-based methods are currently being developed for prepubertal male patients that cannot produce sperm for cryopreservation at the time of treatment. Spermatogonial stem cell culture and transplantation, and testicular tissue xenografting are technologies that have been proven to be successful in a number of mammalian species. Efforts to apply these technologies to human SSCs are currently underway. This dissertation describes work carried out to optimize methods for culturing human SSCs in vitro and to promote survival and maturation of cryopreserved and thawed prepubertal primate testicular xenografts in mice. We developed a multiparametric flow cytometry-based method to quantify human SSCs and used it to optimize the substrate and medium for culturing human SSCs. We also showed that human chorionic gonadotropin exposure promotes growth of both human and Rhesus xenografts and complete maturation of Rhesus xenografts. Additional studies need to be carried out to further develop both SSC culture and human xenografting technologies for application in the clinic for fertility preservation.

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1.0 Introduction: Gonadal dysfunction in cancer survivors

Radiation and chemotherapy have been shown to cause temporary or permanent loss of fertility in cancer survivors [1-3]. Recent studies have reported that cancer survivors are less likely than their siblings without cancer to sire a normal pregnancy [4, 5]. Given the survival rate of individuals diagnosed with cancer between the ages 0-19 has risen to about 88% in the last several decades, it has become increasingly important to address factors that may affect patient quality of life post-treatment [6, 7]. A number of surveys have indicated that psychosocial distress due to potential iatrogenic loss of fertility is common among cancer survivors [8-10]. However, many patients undergo gonadotoxic treatments without receiving information about their increased risk for infertility or about the various fertility preservation options available today [11, 12]. To improve the number of patients that can benefit from fertility preservation methods, the American Society of Clinical Oncology and the American Society for Reproductive Medicine have recommended counseling patients on the reproductive risks and available options at the earliest opportunity in the treatment process [13-15].

The extent of chemotherapy-associated azoospermia depends on the type and dosage of the chemotherapeutic agent used. Alkylating agents such as Busulfan, cyclophosphamide, chlorambucil, melphalan and procarbazine have been associated with significant long-term effect on sperm production (reviewed in [2]). The degree of azoospermia resulting from radiation therapy is determined by factors such as total dose, number of fractions and duration of exposure to radiation. Doses greater than 2Gy have been reported to cause permanent infertility [16]. Certain

forms of cancer, including testicular cancer and Hodgkin's disease have been associated with an increased risk for gonadal dysfunction even before the onset of treatment [17-19]. Therefore, the extent and permanence of azoospermia is the outcome of a combination of several factors, including the disease itself, the stress resulting from the disease and the therapeutic regimen used to treat the disease (reviewed in [20]). Although about 85% of cancer patients recover normal levels of spermatogenesis within 5 years post treatment, there are currently no mechanisms available to predict the risk of infertility prior to cancer therapy [21, 22], and no options to restore fertility in infertile adult survivors of childhood cancers.

1.1 Fertility preservation in adult males

For adult males, sperm cryopreservation is a well-established method of fertility preservation prior to cancer therapy [23-27]. Cryopreserved sperm can be thawed at a later date and used to achieve pregnancy through various procedures such as intrauterine insemination (IUI), *in vitro* fertilization (IVF) and IVF with intracytoplasmic sperm injection (ICSI) [28-32]. Intrauterine insemination, also known as artificial insemination, involves the direct introduction of sperm into the uterus thereby increasing the concentration of sperm at the site of fertilization (reviewed in [33]). Semen samples cryopreserved for as long as 40 years have been used successfully to generate live births using artificial insemination with a pregnancy rate per cycle of 14.8% [34-36]. In conventional IVF, oocytes are retrieved using transvaginal ultrasound (TVUS)-guided needle aspiration. Each oocyte is then incubated with about 50,000 sperm and fertilization is allowed to occur naturally [37]. IUI and IVF require a large number of motile sperm for

successful fertilization to occur and hence are less effective when semen parameters such as concentration, motility and morphology are below reference values [38]. In ICSI, 1 spermatozoan is selected to directly introduce into the ooplasm of the oocyte by passing through the zona pellucida [39]. ICSI has a higher pregnancy rate of 56.8% per retrieval and hence, is the preferred method of achieving pregnancy using cryopreserved sperm from cancer survivors [40]. Men who fail to preserve sperm samples prior to being treated for cancer may still be able to achieve pregnancy by undergoing testicular sperm extraction (TESE) [41]. In this technique testicular spermatozoa are directly retrieved from focal areas of spermatogenesis produced by rare SSCs that survived gonadotoxic treatment. Thus, even though there is no sperm in the ejaculate (azoospermia), it is sometimes possible to retrieve sperm from the testis. TESE followed by ICSI has a pregnancy rate of 50% in azoospermic cancer survivors [41]. Currently, there are no alternative options to treat infertility in patients who did not preserve sperm prior to treatment and who were not successful with TESE.

1.2 Fertility preservation in pediatric patients

All fertility preservation techniques currently available in the clinic rely on the isolation of mature sperm from male patients. While post-pubertal and adult males can benefit from these methods, there are no standard of care options available for pre-pubertal patients who do not make sperm at this stage. This problem affects a significant proportion of the population; about 10,590 individuals under the age of 15 were diagnosed with cancer in the United States in 2018 [6]. In addition, pre-pubertal males may undergo hematopoietic stem cell transplantation for a variety of

non-malignant disorders including severe aplastic anemia, Fanconi's anemia, B-Thalassemia major, congenital immunodeficiency disorders and inherited metabolic disorders [42-44]. Myeloablative conditioning performed as a preparative step to stem cell transplantation employs the use of radiotherapy and cytostatic drugs such as busulfan and cyclophosphamide, leading to a significant risk of irreversible azoospermia [45].

While pre-pubertal males do not produce sperm, they do have SSCs in their testes that are poised to initiate spermatogenesis at puberty. Several SSC-based fertility restoration methods, such as SSC transplantation, testicular tissue grafting/xenografting and *in vitro* derivation of germ cells, are currently in the research pipeline and have successfully been demonstrated to restore fertility in several mammalian model organisms (Figure 1). In anticipation of such techniques becoming available for fertility preservation in the future, several clinics around the world are cryopreserving testicular biopsies from pre-pubertal patients before the onset of cancer treatment [46-52].

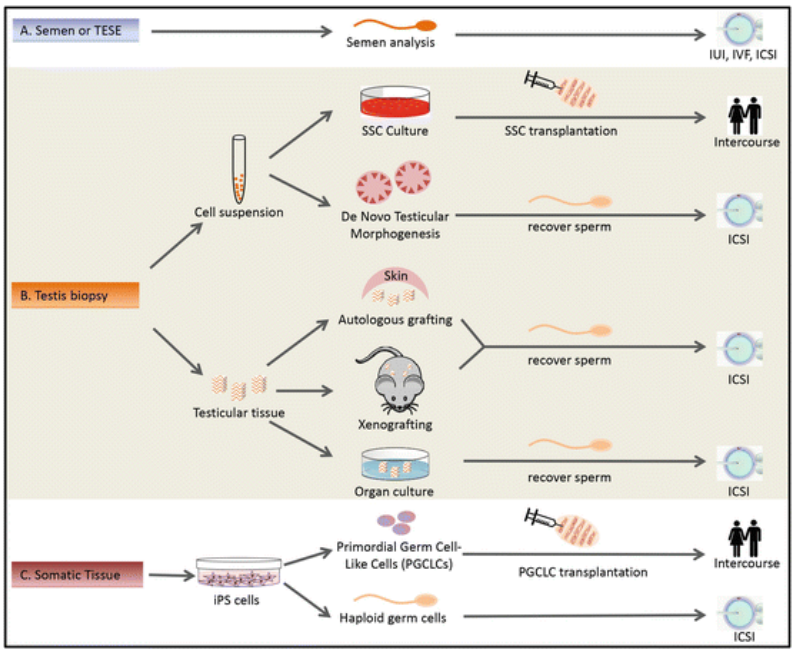

Figure 1: Methods for fertility restoration in prepuberation patients

Standard and experimental options to treat male infertility . (**a**) Sperm obtained from ejaculated semen, or by testicular sperm extraction (TESE) of infertile men, can be used to achieve pregnancy by intrauterine insemination (IUI), *in vitro* fertilization (IVF), or IVF with intracytoplasmic sperm injection (ICSI). (**b**) When it is not possible to obtain sperm, testicular tissue containing spermatogonial stem cells (SSCs) can be obtained by biopsy. Testicular tissue can be digested with enzymes to produce a cell suspension from which SSCs can be expanded in culture and/or transplanted into the testes of the patient. This method has the potential to regenerate spermatogenesis and possibly natural fertility. Heterogeneous testicular cell suspensions also have the potential undergo de novo testicular morphogenesis with seminiferous tubules and a polarized epithelium surrounded by a basement membrane with germ cells inside and interstitial cells outside the tubules. Sperm generated in the "rebuilt" testes can be used to fertilize eggs by ICSI. Intact testicular tissues from prepubertal animals can be grafted or xenografted under the skin or in the scrotum and produce mature sperm that can be used to fertilize eggs by ICSI. Sperm can also be generated when immature testicular tissues are maintained in organ culture and used to fertilize eggs by ICSI. (**c**) Patient-specific induced pluripotent stem (iPS) cells can be derived from patient somatic tissues (e.g., skin or blood) and differentiated into germline stem cells (GSCs) to be transplanted into patient testes. This method may have the potential to regenerate spermatogenesis and natural fertility. It may also be possible to differentiate iPS cells into sperm that can be used to fertilize eggs by ICSI.

1.2.1 Cryopreservation of testicular tissue and cell suspension

The majority of current fertility preservation strategies for cancer survivors rely on the ability to store biological samples for extended periods of time using cryopreservation methods.

Advances in reproductive biology and cryobiology have together led to development of fertility preservation methods for men and women. As described above, sperm cryopreservation is a well-established method to circumvent chemotherapy or radiation-associated loss of fertility in post-pubertal men [53, 54]. Post-pubertal women, on the other hand, have the option to cryopreserve oocytes or embryos prior to the onset of cancer treatment [55, 56]. For patients who are too young or otherwise unable to preserve sperm or eggs, there are experimental options to cryopreserve ovarian tissues and testicular tissues. Programed slow freezing of the ovarian cortex followed by thawing post-recovery for orthotopic or even heterotopic reimplantation has resulted in over 130 live births, including at from adult survivors of childhood cancers [56-60]. There are no live births from cryopreserved testicular tissues, so this method of fertility preservation remains experimental. Nonetheless, testicular tissue cryopreservation is being carried out in several centers around the world in anticipation that experimental procedures such as SSC transplantation, testicular tissue grafting or *in vitro* spermatogenesis may become available in the clinic in the future. The efficacy of these techniques depends on the number of SSCs recovered from cryopreserved biopsies or cell suspensions. Hence, it is necessary to show that cryopreserved testicular cells or tissues can retain their biological functions after thawing [61].

Cryopreservation of human testicular tissue was initially developed to preserve testis biopsies as a source of spermatozoa that could be used to fertilize oocytes *in vitro* by ICSI [62-64]. Effective cryopreservation of testis homogenates prevents the need for azoospermic patients to undergo multiple surgeries to extract spermatids or spermatozoa through TESE [65, 66]. SSCs present in cryopreserved mouse testis cells have been shown to effectively restore spermatogenesis when transplanted into seminiferous tubules of recipient mice [67]. This finding fueled the interest

in cryopreserving testicular biopsies and cell suspensions as a fertility preservation approach for pre-pubertal boys [68]. Since then various cryopreservation methods have been tested using cryoprotectants such as propanediol-sucrose, DMSO and glycerol along with rapid cooling or programmed slow-freezing protocols [69]. Glycerol has a lower penetrative capacity compared to other cryoprotectants. Therefore, while it is commonly used to cryopreserve adult testis tissue for the retrieval of spermatids and spermatozoa, it is not as effective in preserving spermatogonia [69]. Cryopreservation using DMSO with a slow-freezing protocol enables the preservation of spermatogonia and tissue morphology [67, 70, 71]. Vitrification is an alternative cryopreservation method, where formation of ice crystals that are detrimental to cellular integrity, is prevented by employing high concentrations of cryoprotectants and rapid cooling. This method has been shown to preserve spermatogonia and Sertoli cells along with cell-cell and cell-basement membrane associations [72].While comparative studies testing the spermatogenic potential of human testis samples cryopreserved using the various available methods are yet to be performed, a study using rhesus macaque as a model showed that fetal testicular biopsies cryopreserved with 1.4M DMSO at the rate of 0.5˚C/minute had a higher rate of survival and spermatogenesis compared to other methods tested after xenografting into immunodeficient mice [73]. Currently, programmed slow-freezing with DMSO with or without sucrose is most commonly used to cryopreserve human testis tissue. Uncontrolled slow-freezing using isopropanol containers with 1.5M DMSO and 0.1M sucrose has also shown successful preservation of spermatogonia in adult human testis biopsies [74].

1.2.2 Spermatogonial stem cell culture and transplantation

For the successful application of stem cell-based therapies, the ability to isolate and expand small populations of SSCs present in patient testis biopsies is of utmost importance. Currently, there is limited information on the identity of human SSCs and the cellular mechanisms that regulate proliferation, self-renewal or differentiation of these cells. However, rodent spermatogenesis has been extensively studied and well characterized. Spermatogonia in the rodent testes can be broadly classified into 3 distinct types including, type A, intermediate and type B spermatogonia (Figure 2). The type A spermatogonia can be further classified into A_{single} (A_s), A_{paired} (A_{pr}), $A_{aligned}$ (A_{al}) undifferentiated spermatogonia and A_1-A_4 differentiating spermatogonia [75, 76]. A_s spermatogonia have been described as the population containing SSCs. A_{pr} and A_{al} spermatogonia are considered to be transit amplifying spermatogonia committed to differentiation; these cells are morphologically distinct from the A_s subtype as they are clonally arranged on the basement membrane of the seminiferous tubules connected by intercellular cytoplasmic bridges [76, 77]. Some studies have shown that A_{pr} and A_{al} spermatogonia have the capacity to break off from the chain of interconnected cells to give rise to A_s type cells with potential SSC function [78].

In contrast, primate spermatogonia are classified into A_{dark} and A_{pale} undifferentiated spermatogonia (based on nuclear staining intensity with hematoxylin) and type B differentiating

spermatogonia (Figure 2). A_{dark} cells are considered to be reserve SSCs while the A_{pale} are generally considered the active SSCs that self-renew and differentiate to form type B spermatogonia [79]. While the different subtypes of type A spermatogonia can be defined morphologically in both humans and rodents, knowledge about unique SSC-specific molecular markers is limited, especially in humans. Rodent SSCs can only be defined retrospectively by testing their ability to produce and maintain long-term spermatogenesis in a SSC transplantation assay [80]. This method, along with genetic manipulation and lineage tracing experiments, has facilitated the identification of several markers of undifferentiated spermatogonia that are conserved from rodents to nonhuman primates and humans. These include UCHL1, GFRA1, PLZF, THY1, UTF1, ITGA6, LIN28, SALL4 and GPR125 [81-86]. Microarray analysis, single cell RNA sequencing and immunohistochemistry have been used to identify other markers of human spermatogonia such as exome component 10 (EXOSC10), fibroblast growth factor receptor 3 (FGFR3), desmoglein 2 (DSG2), Tetraspanin 33 (TSPAN 33) and Enolase 2 (ENO2) that have not been reported to be expressed in rodent spermatogonia [83, 87-90].

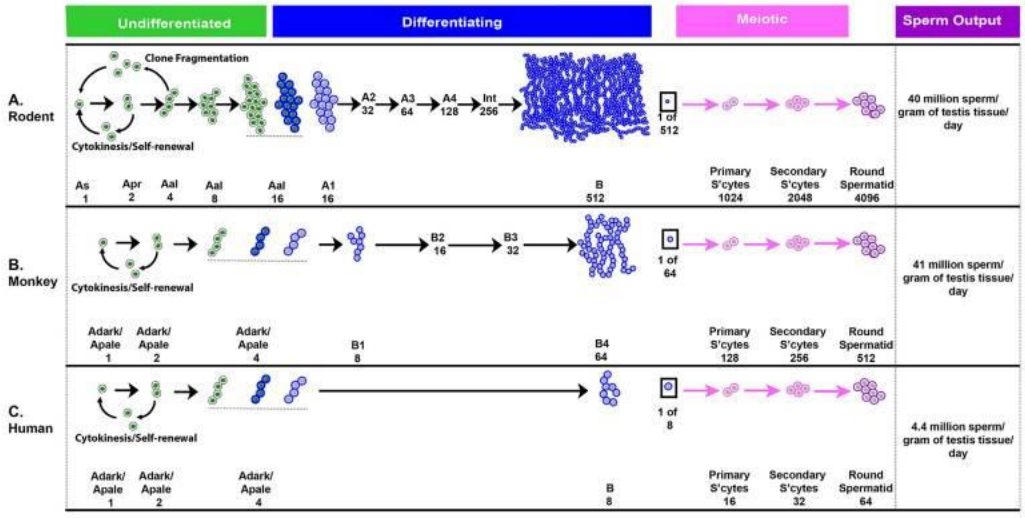

Figure 2: Spermatogenesis in mice, monkeys and humans

In rodents, undifferentiated spermatogonia are described as A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia. A_{single} spermatogonia undergo mitotic divisions to form chains of cells connected via intercytoplasmic bridges to form colonies of $A_{aligned}$ spermatogonia. The induction of the differentiation program within these cells caused them to differentiate into type A1-A4, Intermediate and type B spermatogonia, which then give rise to spermatocytes and spermatids upon completion of meiosis. These cells undergo spermiogenesis to produce sperm. In primates, SSCs reside within the A_{dark} and A_{pale} population of spermatogonia. Primate spermatogonia undergo fewer rounds of transit amplifications prior to initiating the differentiation program compared to rodent spermatogonia. Upon differentiation, type A spermatogonia form type B spermatogonia which undergo meiosis to form spermatocytes and spermatids, and eventually sperm upon the completion of spermiogenesis. This figure and legend are reproduced with permission and minor modification from Fayomi and Orwig, *Stem Cell Research*, 2018.

Determination of SSC fate is regulated by several components of the SSC niche. A stem cell niche is the microenvironment surrounding tissue-specific stem cells and is responsible for providing critical cues for the maintenance and differentiation of these cells. The stem cell niche for any type of tissue-specific stem cells is identified by transiently depleting the tissue of its stem cells, followed by their reintroduction to see if they localize to the niche and are maintained in a fully functional state [91]. In the testis, the SSCs reside on the basement membrane of the seminiferous tubules in association with Sertoli cells within the tubules and the adjacent interstitial

compartment comprised of Leydig cells, endothelial cells, peritubular myoid cells and various other cell types [92]. Sertoli cells play a critical role in spermatogenesis regulation as evidenced by the strong correlation between Sertoli cell content and spermatogenic capacity of the testis [93]. Glial cell-line derived neurotropic factor (GDNF), secreted by Sertoli cells, has been shown to play an important role in SSC self-renewal by the activation of AKT and Src family kinase (SFK) signaling [94, 95]. Fibroblast growth factor 2 (FGF2 or bFGF), also secreted by Sertoli cells, has been shown to promote self-renewal by the upregulation of *Bcl6b* and *Etv5* via mitogen-activated protein kinase (MAPK) activation [96-98]. Sertoli cells also secrete factors such as Bone morphogenetic protein 4 (BMP4) and Activin A that promote SSC differentiation [92, 99, 100]. Peritubular myoid cells secrete factors including leukemia inhibitory factor (LIF), GDNF and monocyte chemotactic protein 1 (MCP1) that contribute to the maintenance of spermatogenesis in the seminiferous tubules [101-103]. Leydig cells affect the activity of SSCs both directly, through factors such as the colony stimulating factor 1 (CSF1), which promotes SSC self-renewal, and indirectly through Sertoli cells, which are the only cells within the seminiferous tubules that express the androgen receptor (AR) for testosterone [104-106]. In fact, it has been observed in rodent testicular cross-sections that a higher number of SSCs are present in the areas of seminiferous tubules directly in contact with large patches of interstitial tissue, indicating the role played by interstitial cells on SSC function [107, 108].

In addition to growth factors, Sertoli cells and peritubular myoid cells secrete collagen α1(IV), α2(IV) and α3(IV) chains, which along with laminin, heparin sulfate proteoglycan and entactin form the basement membrane of the seminiferous tubule [109-112]. The ECM components of the basement membrane play an important role in maintaining spermatogenesis.

Abnormalities in the structure and components of the basement membrane have been associated with infertility [113-115]. In addition to providing structural support, the elaborate network of ECM proteins harbors a pool of growth factors and cytokines secreted by somatic and germ cells that are made available to the surrounding cells in a temporally controlled manner [116].

Studies on the characterization of the SSC niche in the rodent testis have aided the development of culture methods for maintaining and expanding rodent SSCs *in vitro* [117]. GDNF and soluble GFRA1 have been shown to promote SSC self-renewal in culture [118, 119]. Other factors that are commonly used include bFGF, epidermal growth factor (EGF), LIF and CSF1 [104, 120-123]. Previous studies have reported the use of serum being detrimental to SSC culture due to an increase in apoptosis and differentiation of cells [124, 125]. Hence, culture systems with defined media containing bovine serum albumin (BSA) or knockout serum replacement are used to grow SSCs on feeder cells such as SIM mouse embryo-derived thioguanine and ouabain-resistant (STO) cells, mouse embryonic fibroblasts (MEFs) and somatic testicular cells or on feeder-free systems, such as laminin [122, 125-128]. The ability to culture SSCs is essential in order to further elucidate the molecular and functional characteristics of these cells and to expand their numbers.

Initial methods for culturing human SSCs were derived from recent advances in rodent models. In one of the first studies on human SSC culture, Wu et al. compared prepubertal human spermatogonia and mouse gonocytes to show a significant level of conservation between the two cell populations. This finding prompted them to use mouse feeder cell lines, STO and C166 with medium supplemented with GDNF and GFRA1 to culture prepubertal human SSCs [129]. While these conditions did not optimally support the maintenance of human SSCs, this study effectively

proved that the role of GDNF in stimulating self-renewal is conserved from rodents to humans. Subsequent studies employed the use of feeder-based conditions with feeder cells such as human Sertoli cells, human embryonic stem cells-derived fibroblasts and THY1+ testicular somatic cells, and feeder-free methods such as human laminin-coated plates [130-133]. **Table 1** summarizes reports on human SSC culture along with the method of enrichment, cell culture conditions and experimental endpoints used for evaluation. While these results are promising, certain challenges including lack of human SSC-specific markers and functional assays to test the spermatogenic potential of cultured human SSCs make it difficult to critically evaluate these findings. Further progress in the field requires the development of *in vivo* or *in vitro* assays to induce differentiation of human SSCs. Methods such as xenografting and organ culture have been shown to induce complete spermatogenesis from SSCs in several organisms (discussed below). These methods do not require transplantation to achieve spermatogenesis and hence have a great potential for use in human studies.

SSCs, like other tissue-specific stem cells, have the potential to colonize their niche and undergo an indefinite number of divisions to produce cells that will differentiate while maintaining a steady pool of stem cells. Using this principle, Brinster et al. demonstrated that donor mouse SSCs, when introduced into the seminiferous tubules of sterile mice, have the ability to populate the recipient niche and regenerate spermatogenesis leading to the production of viable offspring through normal breeding [80]. Subsequently, Clouthier et al. showed in 1996 that rat SSCs transplanted into sterile immunodeficient mouse testes produced normal rat spermatogenesis, thereby indicating a remarkable conservation of the SSC niche between mouse and rat despite the fact that they diverged at least 20 million years ago [134-136]. A different study demonstrated

hamster spermatogenesis in immunodeficient mouse testes upon xenotransplantation of donor hamster testis cells. While donor cells underwent normal spermatogonial proliferation, differentiation and meiosis, they failed to undergo normal spermiogenesis [137]. These findings were not entirely surprising given the evolutionary distance between hamster and mouse is 1.6 times the distance between rat and mouse [136]. Xenotransplantation experiments have been carried out using species of increasing phylogenetic distance from mouse including rabbits, dogs, pigs, bulls, sheep and monkeys [138-141]. In these experiments, donor germ cells formed colonies on the basement membrane of the recipient seminiferous tubules but donor-derived spermatogenesis did not occur. These studies demonstrate the crucial role played by the components of the stem cell niche in regulating SSC function.

The transplantation assay is currently used as the experimental gold standard to functionally identify and quantify SSCs. The development of this assay has provided a means to elucidate markers uniquely expressed by undifferentiated spermatogonia and characterize the mechanisms governing the regulation of SSC function [83, 142-146]. Being able to accurately identify SSCs catalyzed the development of mouse SSC culture conditions, which allowed propagation of SSCs *in vitro* for several months followed by transplantation and induction of spermatogenesis [119, 147]. Restoration of spermatogenesis through SSC allotransplantation has been successfully demonstrated in various large animal models such as goats, boars, dogs, sheep and bulls [148-151].

SSCs derived from donors of all ages have the same potential for regeneration of spermatogenesis [152, 153]. This finding in addition to the ability to cryopreserve murine testicular cells and transplant them on a later date to achieve complete spermatogenesis showed that this

technique may have clinical application in preserving SSCs of cancer patients for future fertility restoration [154, 155]. Radford et al. initiated the first clinical study to freeze testicular tissues in Manchester, UK in 1999 [156]. Testicular biopsies from 12 non-Hodgkin's lymphoma patients were cryopreserved as cell suspensions prior to the initiation of chemotherapy. After treatment, 7 patients returned to the clinic to have their cryopreserved samples injected back into their testes through the rete testis [157]. While the study lacks follow-up reports on the fertility status of patients that received transplants, this report demonstrates that patients are willing to try stem cell-based methods to preserve their fertility [158, 159].

1.2.3 Sorting methods for the enrichment of spermatogonia and elimination of potential malignant contaminants

Several studies have reported successful enrichment of putative human SSCs by fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS) based on cell surface markers including GPR125, EpCAM, SSEA4, ITGA6 and CD9 [160-163]. In addition to its application in basic research, the ability to identify, isolate and enrich SSCs is important for clinical translation of SSC transplantation as a method to restore fertility. In cancer survivors, especially with a history of tumors in hematopoietic and lymphoid tissues, there is a significant risk of malignant contamination in the testicular tissues cryopreserved for fertility preservation. A previous study reported that transplantation of rat testicular cells with as few as 20 contaminating leukemia cells consistently transmitted the disease to recipients [164]. Fujita et al. established a sorting method based on cancer cell-specific surface antigens, MHC class I and CD45 to negatively

select germ cells. Mice transplanted with the cancer cell depleted fraction did not develop cancer whereas all mice transplanted with unsorted cells showed signs of terminal leukemia within 40 days [165]. In a subsequent study, the same group used the MHC class I and CD45-based sorting method to eliminate malignant contamination in a human model. They tested 8 leukemic cells line out of which only 1 cell line was present in the germ cell-enriched population [166]. Geens et al. used FACS to sort out leukemic cells from testicular cell suspensions based on HLA class I expression but were unable to obtain adequate results [167]. Another method employed CD49f MACS to enrich spermatogonia followed by selective adhesion using collagen I and laminin but also failed to deliver successful elimination of malignant contamination [168]. These initial studies show that negative selection based on leukemic markers is not sufficient to eliminate cancer cells from a human testicular cell suspension and that a multiparametric sort may provide more stringent selection. A combination of positive and negative selection using cell surface markers THY1 (CD90, expressed by spermatogonia) and CD45 followed by singlet discrimination effectively removed leukemic cells from nonhuman primate testicular cell suspensions [169]. Another study demonstrated through both *in vivo* and *in vitro* experiments that a multiparametric FACsort employing the use of antibodies against MOLT-4 leukemia cell line-specific markers, HLA-ABC andCD49e along with spermatogonia-specific marker EpCAM successfully eliminated MOLT-4 cells in the spermatogonia-enriched fraction [163]. These studies show that sorting methods can effectively be used to eliminate specific cancer cell lines from testicular cells; the efficacy of these methods in addressing the heterogeneous nature of cancer remains to be tested.

While these results are promising in light of application in the clinic, there is a significant need in the field to develop methods to test small amounts of patient tissue or cell suspensions for

malignant contaminants before pursuing autologous transplantation. Polymerase chain reaction (PCR) is now being used to evaluate minimal residual disease (MRD) in tissue prior to transplantation, which, often detects contamination even when less sensitive methods like immunohistochemistry fail to do so [170]. Currently, there is limited information of how low level contamination detected by PCR corresponds to tumor forming capacity and hence, the absolute risk for inducing relapse remains difficult to predict [171]. 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.

1.2.4 Testicular xenografting

Testicular tissue grafting was initially developed as a tool to study the somatic compartment of the testis and steroidogenesis [172-174]. Xenotransplantation of SSCs into the seminiferous tubules of recipient mice from donors of increased phylogenetic distances causes donor spermatogonia to arrest before undergoing differentiation. Honaramooz et al. recognized that this phenomenon is caused by SSC niche incompatibility and used testis tissue xenografting as a way of providing donor SSCs with their homologous niche [175]. They grafted fragments of testis tissue from neonatal mice, pigs and goats into immunodeficient recipient mice and observed complete spermatogenesis from all donor species. In a subsequent study, the same group grafted tissue pieces from sexually immature (13 month old) Rhesus monkeys into immunodeficient mice and observed spermatogenesis in 4% of all tubules within 7 months post-grafting [176]. Mature spermatozoa were isolated and used to successfully fertilize eggs via ICSI [175, 176]. This

technique has been replicated using testicular grafts from several species including dog, hamster, ferret, rabbit, domestic cattle, cat, etc. [177-185]. Arregui et al. showed that testicular xenografting may have application in the conservation of endangered species by grafting tissue fragments from Iberian Lynx, Cuvier's gazelle and Mohor Gazelle at various developmental stages. While complete spermatogenesis did not take place in these experiments, spermatocytes and round spermatids were observed in grafts from Cuvier's gazelle and Mohor gazelle, respectively [186].

Effective grafting is established by the formation of a vascular network between small capillaries made by the graft itself and larger subcutaneous blood vessels formed around the graft by the host [180]. Treatment of recipient tissue with vascular endothelial growth factor (VEGF) has been shown to improve grafting efficiency [187]. Vascularization is essential for the survival of the graft and for the establishment of a feedback loop between the donor endocrine cells and the murine hypothalamic-pituitary axis [174]. Leydig cells and Sertoli cells present in the graft respond to murine luteinizing hormone and follicle stimulating hormone, respectively. Androgens and inhibins secreted by the donor somatic compartment in response to murine gonadotropins, in turn, provide negative feedback to the murine hypothalamic-pituitary axis. A significant amount of variability has been observed in the spermatogenic potential of grafts between species. Grafts of porcine and ovine origin have been reported to have complete spermatogenesis in over 50% of seminiferous tubules [188]. In contrast, less than 10% of seminiferous tubules have been observed to have elongated spermatids in grafts from bull, equine and nonhuman primate testes [189-191]. These differences could be due to dissimilarities in the structure of gonadotropins between species leading to differences in the efficiency of interaction between donor gonadotropin receptors and murine gonadotropins [174]. In some studies using the primate model, supplementing recipient

mice with primate gonadotropins improved graft size and the extent of spermatogenesis [181, 191, 192].

The donor age at the time of xenografting plays an important role in the spermatogenic potential of the graft. Most studies using adult donor testicular grafts have reported graft degeneration, incomplete spermatogenesis and arrest at the spermatocyte stage or the presence of Sertoli cell only phenotype [183, 185, 193, 194]. The mechanism behind the differential spermatogenic potential and grafting efficiency with age is not completely understood, however, some hypotheses have been proposed. Immature testis tissue may have an increased resistance to transient hypoxia induced by the grafting process and may have a higher angiogenic capacity to facilitate grafting compared to adult tissue [194, 195]. Arregui et al. hypothesized that the degree of sperm production in donor tissue at the time of grafting negatively affects grafting efficiency due to high metabolic demands of cell division and differentiation, thereby making the tissue more susceptible to hypoxia. They showed that suppression of spermatogenesis in adult donor mice dramatically improved grafting efficiency with the complete recovery of spermatogenesis, while control grafts from mice with ongoing spermatogenesis degenerated and no spermatogenesis was observed [195]. Sexually immature testes at different donor ages also have been shown to have different grafting efficiencies, with prepubertal tissue having a higher spermatogenic potential compared to neonatal tissue [177, 185]. This occurrence could be due to the immaturity of the developing somatic compartment and their inability to respond to circulating gonadotropins [196].

Testicular tissue xenografting using human tissue has been performed in several studies. However, these studies failed to show complete spermatogenesis or the presence of haploid germ cells [197-199]. Unlike immature testicular tissue grafting from other species where complete

spermatogenesis was observed, grafts using human fetal or infant testis tissue showed maintenance of spermatogonia for extended periods of time [197, 198]. Orthotopic xenografts of prepubertal human tissue into the scrotum of immunodeficient mice also led to the maintenance of spermatogonia [200]. Interestingly, some studies have also reported maturation of germ cells up to the spermatocyte stage in grafts from infant, prepubertal and postpubertal donors [198, 200, 201]. Adult human donor-derived xenografts have been shown to regress over time with the presence of few spermatogonia in the tubules [199, 202]. While these findings are not as promising as the results demonstrated in other species, further studies on improving grafting efficiency may have a favorable outcome for human xenografts. Some approaches that have been proven to enhance grafting in other species, such as pretreatment of recipient tissue with VEGF or homologous gonadotropin supplementation, may be tested in context of human xenografting. Although this technique circumvents the need to reintroduce testicular tissue with potential malignant contamination back into the patient, there is a risk of zoonosis that may preclude clinical application.

The focus of my dissertation research was to develop methods of human SSC culture and testicular tissue xenografting. Chapter 2 describes my efforts to iteratively optimize cell culture components to promote the survival of human SSCs *in vitro*. I developed a multiparametric flow-cytometry-based quantitative method to evaluate the kinetics of cultured spermatogonia at various time points in culture. Additional experiments need to be carried out to further promote long-term survival and proliferation of cultured human SSCs *in vitro*. Chapters 3 and 4 describe the outcomes of xenografting cryopreserved and thawed Rhesus macaque and human testicular tissue into immunocompromised mice in response to exogenous administration of vascular endothelial

growth factor (VEGF), human chorionic gonadotropin (hCG) and follicle stimulating hormone (FSH). These experiments revealed that hCG administration was necessary and sufficient to drive the maturation and initiation of spermatogenesis in Rhesus xenografts. However, human xenografts, with or without prior cryopreservation, exhibited loss of germ cells with no evidence of differentiation over time. It is likely that the administered doses of FSH and hCG were insufficient to drive germ cell survival and differentiation. Additional experiments may be carried out in the future to test increased dosage of human gonadotropin and site of graft placement.

2.0 Human testis extracellular matrix enhances human spermatogonial stem cell survival *in vitro*

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Tissue Engineering Part A

"Human testis extracellular matrix enhances human spermatogonial stem cell survival *in vitro*"

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Mark H. Murdock (1)*; Sherin David (2)*; Ilea T. Swinehart (1); Janet E. Reing (1); Kien Tran (2); Kathrin Gassei (2); Kyle E. Orwig (2); Stephen F. Badylak (1)

1. McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA
2. Department of Obstetrics, Gynecology and Reproductive Sciences, Magee-Womens Research Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA

* These authors contributed equally to this work

2.1 Chapter Summary

Successful human spermatogonial stem cell (hSSC) culture could enable cell therapy for male infertility. Mammalian extracellular matrix (ECM) promotes mitogenesis, migration, and/or differentiation of various stem/progenitor cells and can plausibly facilitate hSSC survival in culture. Hydrogel forms of human testicular ECM (htECM), porcine testicular ECM (ptECM), porcine small intestinal submucosa ECM (SIS), and porcine urinary bladder ECM (UBM) were used to coat tissue culture plates for hSSC culture. In addition, hSSC were cultured on Sandos inbred mice (SIM) 6-thioguanine-resistance, ouabain-resistant (STO) mouse embryonic fibroblast feeder cells (control), murine laminin, or human laminin. Undifferentiated embryonic cell transcription factor 1-positive (UTF1+) human spermatogonia were quantified at days 0, 7, and 14 of culture. htECM was the only condition that retained a significantly higher number of UTF1+ cells than control STO feeder cell cultures (22% vs 3%). Overall, the number of hSSCs declined during the 14 day culture period under all conditions. A multiparameter flow cytometry analysis of cells cultured on htECM and ptECM revealed that Stage-specific embryonic antigen 4 (SSEA4)+ undifferentiated spermatogonia may be lost to differentiation (cKIT+ spermatogonia) and apoptosis (annexin V+ spermatogonia). Proliferation of undifferentiated human spermatogonia (Ki67+) was limited suggesting that hSSCs may have different growth factor requirements than mouse SSCs. ECM from the homologous species (human) and homologous tissue (testis) was the most effective substrate hSSCs and establishes a foundational feeder free, serum free condition for future iterative testing of culture conditions toward the long-term goal of stable hSSC cultures.

2.2 Introduction

Chemotherapy or radiation treatment for cancer or other conditions can cause permanent infertility [1, 2]. For men, the extent and persistence of treatment-related azoospermia is determined by a combination of factors including the disease itself, the drugs, their doses, and the treatment regimen [203-205]. Currently available fertility preservation methods for men rely on the isolation and cryopreservation of sperm from the ejaculate or testis that can be used for intrauterine insemination, *in vitro* fertilization (IVF), and IVF with intracytoplasmic sperm injection. These methods are available to adult and adolescent males but not to prepubertal boys who are not yet producing sperm. However, boys do have spermatogonial stem cells (SSC) in their testes that might be used to regenerate spermatogenesis [206, 207]. Brinster and colleagues showed that transplantation of frozen and thawed murine SSC into the seminiferous tubules of an infertile testis leads to complete regeneration of spermatogenesis in the recipient mouse [208, 209]. This finding, in turn, led to the conceptualization that SSC might be exploited to preserve and restore the fertility of prepubertal males wherein SSC obtained by testicular biopsy and cryopreserved prior to the onset of cancer treatment can be transplanted back into the patient's testes at a later time to restore complete spermatogenesis [50, 207, 210-213]. However, SSC are rare cells in the seminiferous tubule epithelium, and it is likely that a small testicular biopsy obtained from a prepubertal patient would contain only a small number of these cells [214]. The efficiency of SSC transplantation depends on the number of SSC introduced into the recipient niche [215, 216]. Therefore, it may be necessary to first expand patient SSC *in vitro* to achieve robust engraftment and regeneration of spermatogenesis.

Conditions for maintenance and expansion of rodent SSC in long-term culture are well established [217, 218]. However, these methods are ineffective in supporting proliferation and maintenance of human SSC (hSSC) [131]. Methods for long-term propagation of nonhuman primate and hSSC have been described in several recent reports [129, 131, 219-239], but tissue sources were variable; the analytical endpoints were varied (ranging from qRT-PCR to immunocytochemistry to xenotransplantation) and there is no consensus on best methods. However, review of the published hSSC culture work in **Table 1** does reveal some trends. Most studies have used some method of sorting or differential planting to enrich hSSC and/or deplete testicular somatic cells and included some concentration of glial cell line-derived neurotrophic factor (GDNF). There is a lack of consensus about the cell culture substrate with options ranging from plastic, laminin, Matrigel or gelatin to various feeder cell preparations.

Table 1: Literature review of reports of human SSC culture with methods used for culture and analysis of cells

Citation	Duration of culture	Sort/differential plating	Medium	Growth factors	Feeders or ECM	Passaging technique	End point	Type and age of donor
Sadri-Ardekani et al., JAMA, 2009[130]	15 weeks	Differential plating on plastic	MEM+10%FCS for differential plating followed by StemPro-34	20ng/ml EGF, 10ng/ml GDNF, 10ng/ml LIF, 10ng/ml bFGF	Human placental laminin	Passaged every 7-10 days using Trypsin EDTA and differential passaging if there was somatic cell overgrowth	Xenotransplants; ICC – PLZF; RT-PCR – PLZF, ITGA6, ITGB1;	Adult orchidectomy patients (n = 6)
Wu et al., PNAS, 2009[240]	1 week	Differential plating on Gelatin	rSFM	20ng/ml GDNF, 150ng/ml GFRA1 and 1ng/ml bFGF	C166	Not reported	ICC – UCHL1	Prepuberatal male aged 2-10 years diagnosed with cancer (n = 2)
Chen et al., Asian Journal of Andrology, 2009[219]	2 months	MACS for ITGA6	DMEM	10ng/ml GDNF, 4ng/ml bFGF and 1500 IU/ml LIF	Human embryonic stem cells derived fibroblasts (hdF)	Passaged every 4-5 days using Cell dissociation buffer or Trypsin	ICC – OCT4, SSEA1, ITGA6; RT-PCR – OCT4, STRA8, DAZL, NOTCH1, NGN3, SOX3, KIT	Fetal
Lim et al., Cell Proliferation, 2010[232]	>6 months	Percoll selection, differential plating on plastic and collagen followed by MACS for CD9	DMEM during enrichment followed by StemPro-34	10ng/ml GDNF, 10ng/ml bFGF, 20ng/ml EGF, 10^3 U/ml LIF	Laminin	Passaged very 2 weeks 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)

Table 1 continued

Kokkinaki et al., Journal of Stem Cell Research and Therapy, 2011[241]	4-5 months	Differential plating on FBS-coated dish, treatment with RBC Lysis Buffer and Dead Cell Removal Kit followed by SSEA4 MACS	StemPro-34	10ng/ml GDNF, 10ng/ml bFGF, 20ng/ml EGF, 10^3 U/ml LIF	Growth factor-reduced matrigel	Passaged manually at 1 month followed by digestion with dispase+collagenase every 10-15 days	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 yo organ donors
Sadri-Ardekani et al., JAMA, 2011[131]	15.5 and 10 weeks	Differential plating on plastic	StemPro-34	20ng/ml EGF, 10ng/ml GDNF, 10ng/ml LIF, 10ng/ml bFGF	Human placental laminin	Passaged every 7-10 days using Trypsin EDTA and differential passaging if there was somatic cell overgrowth	Xenotransplants and RT-PCR – PLZF, ITGA6, ITGB1, CD9, GFRA1, GPR125, UCHL1	Prepuberatal male Hodgkin lymphoma patients; 6.5 and 8 years old (n = 2)
Nowroozi et al., Urology, 2011[242]	18 days	Differential plating on lectin-coated plates	DMEM	Not Reported	Human Sertoli cells	Passaged every 7 days with Trypsin EDTA	ICC – OCT4, Vimentin; Alkaline phosphatase staining	Adults with non obstructuve azoospermia (n = 47)
Liu et al., Reproductive Biology and Endocrinology, 2011[243]	1 month	Percoll separation and differential plating on plastic	DMEM/F12	Not Reported	Human Sertoli cells	Not reported	ICC and Fc – OCT4, SSEA4	Fetal (n = 5)
Mirzapour et al., Andrologia, 2012[224]	5 weeks	Differential plating on lectin-coated plates	DMEM	Various concentrations of bFGF and LIF	Human Sertoli cells	Passaged every 7 days using Trypsin EDTA	Xenotransplants; Alkaline phosphatase staining;ICC – OCT4, Vimentin; RT-PCR – OCT4, NANOG, STRA8, PIWIL2, VASA	Adult males with NOA-maturation arrest (n = 20)
Koruji et al., 2012[244]	2 months	Differential plating on plastic	DMEM+5%FCS	20ng/ml GDNF, 10ng/ml BFGF, 10ng/ml LIF, 20ng/ml EGF	Laminin or plastic	Passaged every 5-7 days using Trypsin-EDTA	Morphology-number and diameter of colonies, RT-PCR for PLZF, DAZL, OCT4, VASA, ITGA6, ITGB1	Adult males with NOA

Table 1 continued

Goharbakhsh et al., Avicenna Journal of Medical Biotechnology, 2013[225]	52 days	Differential plating on plastic for cells>10^6, all cells were plated is number<10^6	DMEM-F12	10ng/ml GDNF, 10ng/ml bFGF, 20ng/ml EGF, 10^3 U/ml LIF	20ul/ml laminin or 0.2% gelatin	Passaged every 7-10 days, method wasn't mentioned	Morphological observation of EB-like colonies and ICC staining for GPR125	Azoospermic adult males (n = 12)
Piravar, Z et al., Journal of Reproduction and Infertility, 2013[245]	6 weeks	Differential plating on plastic	DMEM/F12 for 16 hours then StemPro-34	10ng/ml GDNF, 20ng/ml EGF, 10ng/ml LIF, bFGF	Uncoated plates for the first 14 days followed by laminin	Trypsinization every 2 weeks	qPCR for UCHL1 expression	non-obstructive azoospermic males (n = 10)
Akhondi, MM et al., Iranian Journal of Reproductive Medicine, 2013[227]	6 weeks	Enrichment was not performed	StemPro-34	10ng/ml GDNF, 20ng/ml EGF, 10ng/ml LIF, bFGF	Not reported	Trypsinization every 10 days	ICC for Oct4 expression and qPCR for PLZF expression	44 year old organ donor (n = 1)
Zheng et al., Human Reproduction, 2014[81]	2 weeks	Differential plating on plastic and collagen	DMEM during enrichment followed by StemPro-34	20ng/ml EGF, 10ng/ml GDNF, 10ng/ml LIF, 10ng/ml bFGF	Not reported	Passaged using Trypsin when confluent	Flow cytometry - SSEA4; RT-PCR - UTF1, FGFR3, SALL4, PLZF, DAZL, VIM, ACTA2, GATA4	Adult organ donors (n = 8)
Chikhovskaya et al., Molecular Human Reproduction, 2014[246]	2 weeks	Differential plating on plastic followed by MACS for ITGA6 and differential plating on Collagen I and Laminin	StemPro-34	20ng/ml EGF, 10ng/ml GDNF, 10ng/ml LIF, 10ng/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 cancer patients undergoing bilateral orchidectomy (n = 3)
Smith et al., Stem Cells Translational Medicine, 2014[228]	21 days	FACS – CD45-, THY1-, SSEA4+	StemPro-34	20ng/ml EGF, 10ng/ml GDNF, 10ng/ml LIF,	Adult human THY1+ cells	Not reported	ICC – SSEA4, VASA	Adults with normal spermatogenesis (n = 13)

Table 1 continued

				10ng/ml bFGF				
Guo et al., Experimental Biology and Medicine (Maywood, NJ), 2015[231]	1 month	Differential plating on plastic with DMEM-F12 followed by MACS for GPR125	StemPro-34	20ng/ml EGF, 10ng/ml bFGF, 10ng/ml LIF, 50ng/ml GDNF	Hydrogel Stem Easy	Not reported	Morphological observation, cell proliferation assay, ICC for GPR125, UCHL1 and THY1, PLZF and RT-PCR for GPR123, GFRa1, RET, PLZF, UCHL1, MAGEA4, SYCP3, PRM1 and TNP1 were done at 30 days	22-35 year old obstructive azoospermia patients (n = 40)
Baert et al., Fertility and Sterility, 2015[247]	2 months	Differential plating on plastic	StemPro-34	20ng/ml EGF, 10ng/ml GDNF, 10ng/ml LIF, 10ng/ml bFGF	no substrate	Not reported	ICC and RT-PCR - VASA, UCHL1	Vasectomy reversal patients and adult males who underwent bilateral orchidectomy due to prostate cancer (n = 6)
Abdul Wahab et al., Malaysian Journal of Medical Sciences, 2016[248]	49 days	Enrichment was not performed	DMEM	80µl bFGF	Plastic	Not reported	in-well staining for ITGA6, ITGB1, CD9 and GFRA1	non-obstructive azoospermic male (n = 1)
Medrano et al., Fertility and Sterility, 2016[233]	28 days	FACS for HLA-/EPCAM+	StemPro-34	20ng/ml EGF, 10ng/ml LIF, 10ng/ml bFGF, 10ng/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; ICC - VASA, UTF1, UCHL1	Adult males who underwent bilateral orchidectomy due to prostate cancer (n = 3)
Gat et al., Fertility and Sterility, 2017[249]	12 days	Differential plating on Gelatin	DMEM-F12 and StemPro-34	20ng/ml EGF, 10ng/ml GDNF, 10ng/ml LIF, 10ng/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

Table 1 continued

								who underwent microTESE due to NOA (n = 8)
Murdock et al., Tissue Engineering Part A, 2018[250]	14 days	MACS for ITGA6 followed by Differential plating on Collagen I	MEMα	20ng/ml GDNF and 1ng/ml bFGF[233, 250]	STO, mouse and human laminin, htECM, ptECM, SIS and UBM	Passaged using Trypsin at day 7	ICC for UTF1, Flow cytometry for SSEA4, cKIT, AnnexinV and Ki-67	Adult organ donors (n = 4)

Mammalian extracellular matrix (ECM) is produced by the resident cells of every tissue and organ, and contains numerous signaling molecules that promote mitogenesis, migration, and/or differentiation of various stem/progenitor cells [251-255], angiogenesis [256], and immune cell modulation [257-260]. Biologic scaffold materials composed of extracellular matrix have been widely used to facilitate the repair and reconstruction of diverse tissue types including esophagus [261, 262], skeletal muscle [255, 263], dura mater [264, 265], tendon [266, 267], breast tissue [268], and others [269]. The use of ECM hydrogels as substrates for *in vitro* cell culture, or the use of solubilized ECM as a supplement to culture media, can augment the proliferation and/or differentiation of selected cell types and therefore may be desirable for hSSC culture [270-272]. The development and use of testicular ECMs to culture testicular somatic and germ cells have been reported recently[273-275]. While these studies demonstrate the maintenance of the somatic compartment, the use of testis ECMs for maintenance and growth of SSCs in 2D culture systems is yet to be evaluated.

In the present study, a novel approach was used to isolate ECM from human and porcine testicular tissue. The objective was to establish serum-free, feeder-free conditions for maintaining human stem/progenitor spermatogonia in two-dimensional culture on ECM-coated plates. Herein, I tested the hypothesis that ECM from the homologous species (human) and/or homologous tissue (testis) will provide an optimal environment for maintaining or expanding human SSC in culture. Serum-free media supplemented with GDNF and bFGF was used to culture cells on various ECM substrates including human and porcine testis ECM (htECM and ptECM), porcine small intestinal submucosa (SIS-ECM), porcine urinary bladder matrix (UBM-ECM), and human laminin, an isolated component of ECM. Sandos inbred mice (SIM) 6-thioguanine-resistance, ouabain-

resistant (STO) feeder cells and mouse laminin that have been used to culture mouse SSC and were used as controls. Viable stem/progenitor spermatogonia were quantified at days 0, 7 and 14 of culture through the expression of undifferentiated embryonic cell transcription factor I (UTF1) and stage-specific embryonic antigen 4 (SSEA4).

SIS, UBM, ptECM and htECM were provided for these experiments by members of the laboratory of Stephen Badylak at the McGowen Institute for Regenerative Medicine at the University of Pittsburgh. Mark Murdock optimized methods for generating testicular ECMs and performed experiments to characterize the biochemical composition of the ECMs as described in the following sections.

2.3 Materials and Methods

2.3.1 Procurement and processing of human testis tissue

Deidentified human testes were obtained through the Center for Organ Recovery and Education and the University of Pittsburgh Health Sciences Tissue Bank under University of Pittsburgh CORID no. 686. Tissue was obtained from postpubertal male organ donors (**Appendix A**) and transported on ice in Lactated Ringer's solution following procurement. Seminiferous tubules were removed from the tunica albuginea. A single cell suspension of human testicular parenchyma was prepared by sequential enzymatic digestion with 2 mg/ml collagenase (Cat# LS004188, Worthington Biochemical Corporation), followed by 0.25% trypsin (Cat# 25200-14,

Invitrogen) plus 7mg/ml DNase I (Cat# DN25, Sigma-Aldrich) in Hank's Balanced Salt Solution (HBSS, Cat# 24020-117, Invitrogen), as described previously [276]. Cells were cryopreserved at a final concentration 20 x 10_6 cells/ml in cryoprotectant medium containing 15% FBS and 10% dimethyl sulfoxide (DMSO, Cat# D8418, Sigma) in minimum essential media (MEMα, Cat# 12561-056, Invitrogen). Cryovials were frozen at a rate of -1˚C/minute in controlled-rate freezing containers (Cat# 03-337-7D, Nalgene-Nuc International) in a -80 C freezer and transferred into liquid nitrogen the following day. For experiments, frozen testis cells were thawed rapidly, washed, and suspended in Dulbecco's phosphate buffered saline (DPBS) with 1% FBS.

2.3.2 Magnetic activated cell sorting and differential plating

Frozen and thawed cells were incubated with rat anti-CD49f-PE antibody (10µl/10_6cells, BD Biosciences) in DPBS-based sorting medium supplemented with 1% FBS (**Appendix B**). Cells were then washed and incubated with anti-Phycoerythrin (PE) microbeads (2µl/10_6 cells, Cat# 130-048-801, Miltenyi Biotec) for 45 minutes, then sorted on a magnetic column; the positive fraction was collected in serum free medium (**Appendix C**) supplemented with 20ng/ml GDNF (Cat# 450-10, Peprotech) and 1ng/ml bFGF (Cat# CB-40060, Fisher), washed and cultured on Collagen I-coated plates (1µg/cm_2, Cat# 08-774-550, Corning) for 48 hours to remove adherent somatic cells. Cells that were floating after 24 hours were removed and re-plated on fresh Collagen I-coated wells. Cells that were still floating after an additional 24 hours were collected and plated in the experimental culture conditions described below.

2.3.3 Production of porcine SIS-ECM and UBM-ECM

Extracellular matrix was isolated from porcine small intestine and urinary bladder as previously described [277, 278]. Briefly, tissue was harvested from market weight pigs, the tunica submucosa, muscularis mucosa, and stratum compactum were mechanically isolated from the intestines. The basement membrane and lamina propria were mechanically isolated from the bladders. Tissues were further decellularized and disinfected by agitation in 0.1% peracetic acid with 4% ethanol before rinsing, lyophilization, and milling into powder. ECM was solubilized by digestion with 1mg/mL pepsin at pH 2.5 for 24 hours.

2.3.4 Production of htECM and ptECM

Several detergents were evaluated to identify a preferred decellularization protocol for native human testis (htNative). Frozen testes were thawed, decapsulated, sliced to 4mm, rinsed in type I water which was replaced every 20-60 minutes until water remained colorless, and agitated in a 0.02% trypsin and 0.05% EGTA solution at 37°C for 2.5 hours. Tissue was agitated at room temperature in one of four detergent solutions for 24 hours with a single solution change at 12 hours: 4% sodium deoxycholate (SDC), 3% Triton X-100 (TX-100), 0.075% sodium dodecyl sulfate (SDS), or a mixture of 0.25% SDC and 0.25% TX-100. Material from each group was washed for 2 additional hours in a fresh solution of the same detergent type except the material in SDS which was washed in 1% TX-100 to help remove residual SDS. Material was washed in 0.1% peracetic acid (PAA) and 4% ethanol (EtOH) for 2 hours and PBS/water as described above. Decellularization efficiency was determined by histology including staining with hematoxylin and

eosin (H&E) and 4',6-diamidino-2-phenylindole (DAPI). PicoGreen assay (Quant-IT PicoGreen dsDNA reagent, Invitrogen) was used to quantify remnant double-stranded DNA (dsDNA) and gel electrophoresis with a 1.5% agarose gel was used to determine base pair size of any remnant DNA. Once validated as an effective method for decellularization of htECM, the same protocol was used on native porcine testis (ptNative) to produce ptECM. htECM and ptECM were lyophilized, milled into powder, and digested in 0.1% pepsin for 24 hours prior to use in cell culture.

2.3.5 Biochemical composition of htNative, htECM, ptNative, ptECM

Retention of sulfated glycosaminoglycans (sGAG) after decellularization was qualitatively determined by histology through Alcian Blue staining. sGAG concentrations in the pepsin-digested samples were quantitatively measured with the Blyscan Sulfated Glycosaminoglycan Assay Kit (Blyscan #B1000, Biocolor Ltd., Carrickfergus, Co Antrim, United Kingdom). Three independent biological testis samples of each of the four groups (htNative, htECM, ptNative, and ptECM) were pooled for urea-heparin extraction to assay total protein content (BCA protein assay kit, Pierce, Rockford, IL) and bFGF content (Human FGF basic Quantikine ELISA Kit, R&D Systems DFB50). All kits were used according to manufacturer's instructions.

2.3.6 Substrate preparation and cell culture

For feeder cell-based culture, previously mitomycin-treated and cryopreserved STO feeder cells (ATCC SCRC 1049) were rapidly thawed and washed in STO media (**Appendix D**) and plated on 24-well culture wells coated with 0.1% gelatin. Cells were allowed to attach to the

substrate for 24-48 hours. For culture on laminin, 24-well plates were coated with mouse laminin (Cat# L2020, Sigma) at 2µg/cm$_2$ as previously reported[127]. The concentration of Human Laminin from Fibroblasts (Cat# L4544, Sigma) used for coating plates was optimized by culturing human spermatogonia on plates coated with human laminin at the concentration used for mouse laminin (2µg/cm$_2$) and a lower concentration of 0.5µg/cm$_2$. Both concentrations led to a similar recovery of UTF1+ cells. Hence, the lower concentration of 0.5µg/cm$_2$ was selected as the working concentration of human laminin for the subsequent experiments. Plates were incubated at 37°C for 2-4 hours according to manufacturer's instructions. For culture on tissue-derived ECM, ECM digests were neutralized to pH 7.6 with 0.1N NaOH and diluted in PBS to a final concentration of 300µg/ml, and then added to 24-well plates and incubated for 2-4 hours at room temperature. At the time of initiation of culture, media from wells with STO feeder cells and residual ECM from the ECM-coated wells were aspirated. Any adherent molecular species within the solubilized ECM remained as a substrate for the SSC but no visible disc of ECM was present. The wells were then rinsed with HBSS prior to introduction of cells for culture. Floating and weakly adherent cells from Collagen I-coated plates used for differential plating were removed, washed by centrifugation at 600 x g, re-suspended in mouse serum free medium summarized in Supplementary Table 2 [118, 129]. Medium was supplemented with GDNF (20 ng/ml) and bFGF (1 ng/ml) and plated on substrates at a concentration of 50-70 x 10$_3$ cells/cm$_2$. Fresh medium was supplemented into culture wells every 3-4 days and cells were passaged 2:1 at day 7 using 0.05% Trypsin-EDTA (Cat# 25-300-120, Life Technologies).

2.3.7 Immunolabeling

Cells were spotted on Superfrost slides (Cat# 12-550-15, Fisherbrand) at a concentration of 5 x 10_6 cells/ml, fixed with chilled 100% acetone and allowed to dry. Fixed cells were rehydrated with DPBS, blocked for 1 hour with a blocking buffer containing 3% bovine serum albumin (Cat# ICN810661, MP Biochemicals) and 5% normal donkey serum (Cat# 017-000-121, Jackson ImmunoResearch). Mouse monoclonal anti-UTF1 antibody (1:200, Cat# MAB4337, Millipore) was added to cells and incubated for 90 minutes. Isotype-matched normal IgG monoclonal antibody (BD Pharmingen) was used as negative control. Slides were washed with DPBS containing 0.1% Tween20 (Cat# P227, Sigma) and incubated with donkey anti-mouse secondary antibody conjugated with AlexaFluor488 (1:200, Cat# BDB563636, ThermoFisher) for 45 minutes. Slides were washed and coverslips were mounted using Vectashield mounting medium containing DAPI (Cat# H-1200, Vector Laboratories). Cells were observed under the Nikon Eclipse 90i microscope and were analyzed using the NIS Elements Advanced Research software.

2.3.8 Flow cytometry

Cells were washed with Azide-free and serum-free PBS and incubate with viability dye, GhostDye Red780 (Cat# 12-0865-T100, Tonbo Biosciences) according to manufacturer's instructions. Cells were washed and incubated in buffer containing 2% FBS and 0.09% sodium azide in PBS with AlexaFluor488 mouse anti-SSEA4 antibody (2.5ul/10_6 cells, Cat# BDB560308, BD biosciences) and APC mouse anti-human CD117 (5ul/10_6 cells cKIT) antibody (Cat# BDB550412, BD Biosciences). Cells were then washed and stained with BV421 Annexin V

(5ul/10^6 cells, Cat# BDB563973, BD Biosciences) in AnnexinV binding buffer composed of 100mM Hepes (Cat# 11-330-032, ThermoFisher), 140mM NaCl and 25mM $CaCl_2$. Following AnnexinV staining, cells were fixed in a 1:1 solution of AnnexinV buffer and Cytofix/Cytoperm solution (Cat# BDB554722, BD Biosciences) and washed in Perm/Wash solution (Cat# BDB554723, BD Biosciences) containing AnnexinV buffer, mixed 1:1. Cells were then stained with BUV396 mouse anti-KI-67 antibody in Perm/Wash/AnnexinV buffer supplemented with Brilliant Stain Buffer (Cat# BDB563794, BD Biosciences) according to manufacturer's instructions, washed and analyzed using the BD FACSAria II flow cytometer. Isotype matched IgG monoclonal antibodies were used as negative control. Compensation controls were prepared using OneComp eBeads compensation beads (Cat# 01-1111-41, ThermoFisher).

2.3.9 Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the recovery of UTF1+ cells in the various cell culture conditions at days 7 and 14 using the GraphPad Prism software. One-way analysis of variance (ANOVA) and two-tailed t-tests were used for the statistical analysis of flow cytometry results. P values <0.05 were considered statistically significant.

2.4 Results

2.4.1 Decellularization protocol establishment

Detergents commonly used to decellularize tissue were compared for their ability to decellularize human testicular tissue (**Figure 3A**). Following exposure to the various decellularization protocols, remnant dsDNA in the ECM was quantified with PicoGreen reagent and compared to dsDNA amounts in native tissue (**Figure 3B**). ECM made with any of the detergents contained significantly less dsDNA ($p<0.001$) than found in the native tissue (29,900 ± 2,250 ng of dsDNA per mg of dry weight tissue). Remnant dsDNA in ECM made with 4% SDC (9,790 ± 1,610 ng/mg) was comparable with that of the 3% TX-100 (7,860 ± 544 ng/mg) and both contained significantly more dsDNA ($p<0.0001$) than ECM made with 0.25% SDC/TX-100 (1,010 ± 124 ng/mg). ECM made with 0.075% SDS contained the least dsDNA, ($p<0.001$ compared to the 0.25% SDC/TX-100 mixture) having 208 ± 23 ng/mg. The SDS protocol was used for both htECM and ptECM for the remainder of the study.

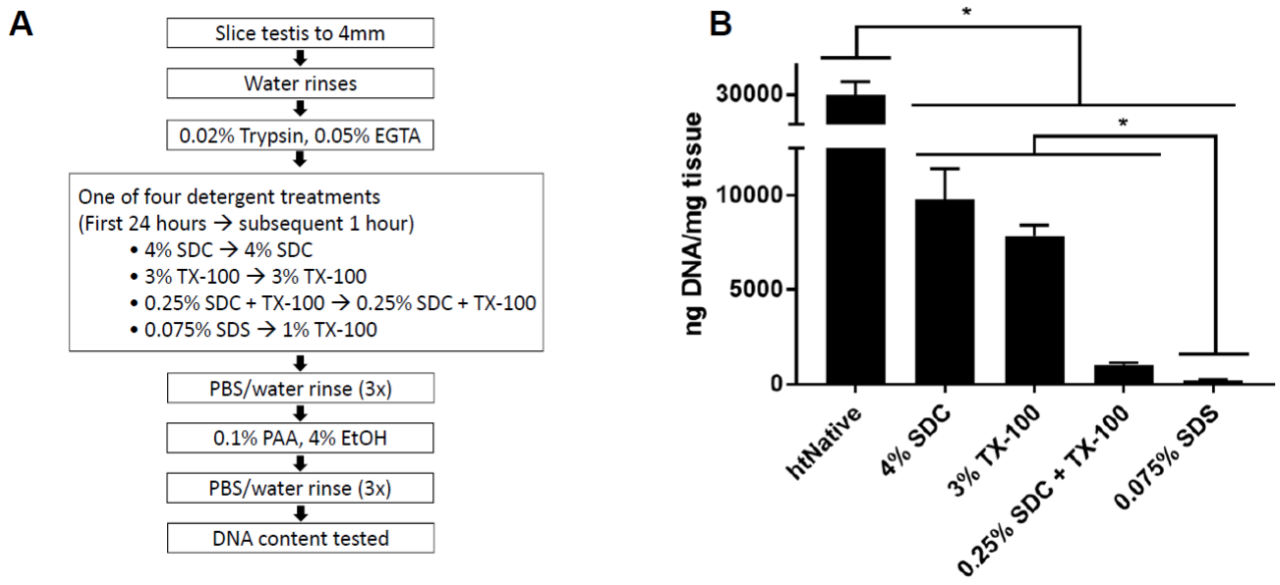

Figure 3: Overview of human testis decellularization strategy.

(A) Frozen human testes were thawed, decapsulated, sliced, then agitated in trypsin/EGTA. Tissue was agitated in various detergents commonly used for decellularization to identify a preferred decellularization protocol. Differentially processed htECM was then rinsed thoroughly, disinfected by PAA, and extent of DNA removal compared with native tissue was assessed. **(B)** PicoGreen assay was used to quantitatively measure remnant dsDNA and compare the decellularization efficacy between detergents. Each detergent treatment removed the majority of the DNA from the native tissue, and 0.075% SDS removed the most DNA compared with other detergent treatments (*$p < 0.05$). Data shown as mean ± SD of technical triplicate. dsDNA, double-stranded DNA; htECM, human testicular extracellular matrix; PAA, peracetic acid; SD, standard deviation; SDS, sodium dodecyl sulfate.

2.4.2 Decellularization efficacy

The degree to which htECM and ptECM were free of cells and cell remnants was assessed using previously established guidelines [279]. For htECM, no intact nuclei were visible by H&E or DAPI staining (**Figure 4A, 4C**). For ptECM, no intact nuclei were visible by H&E but rare clusters of nuclei were observed with DAPI staining (**Figure 4B, 4D**).

dsDNA was quantified from three biological replicates before and after decellularization. Concentration of dsDNA in native tissue between the two species was similar; htNative #1: 31,100

± 1,070 ng/mg, htNative #2: 28,100 ± 325 ng/mg, htNative #3: 26,000 ± 4,720ng/mg, ptNative #1: 46,500 ± 242 ng/mg, ptNative #2: 44,000 ± 217 ng/mg, ptNative #3: 38,800 ± 827ng/mg. htECM contained three orders of magnitude less dsDNA than htNative, and ptECM contained one order of magnitude less dsDNA than ptNative; htECM #1: 26 ± 1 ng/mg, htECM #2: 169 ± 14 ng/mg, htECM #3: 45±8 ng/mg, ptECM #1: 4,320 ± 75 ng/mg, ptECM #2: 3,340 ± 28 ng/mg, ptECM #3: 3,660 ± 48 ng/mg (**Figure 4E, 4F**).

Gel electrophoresis showed each native sample contained high amounts of genomic DNA. Two htECM samples contained no visible DNA, although the third showed a smear above 200 base pairs (**Figure 4G**). All three ptECM samples showed a faint smear of DNA at all sizes (**Figure 4H**).

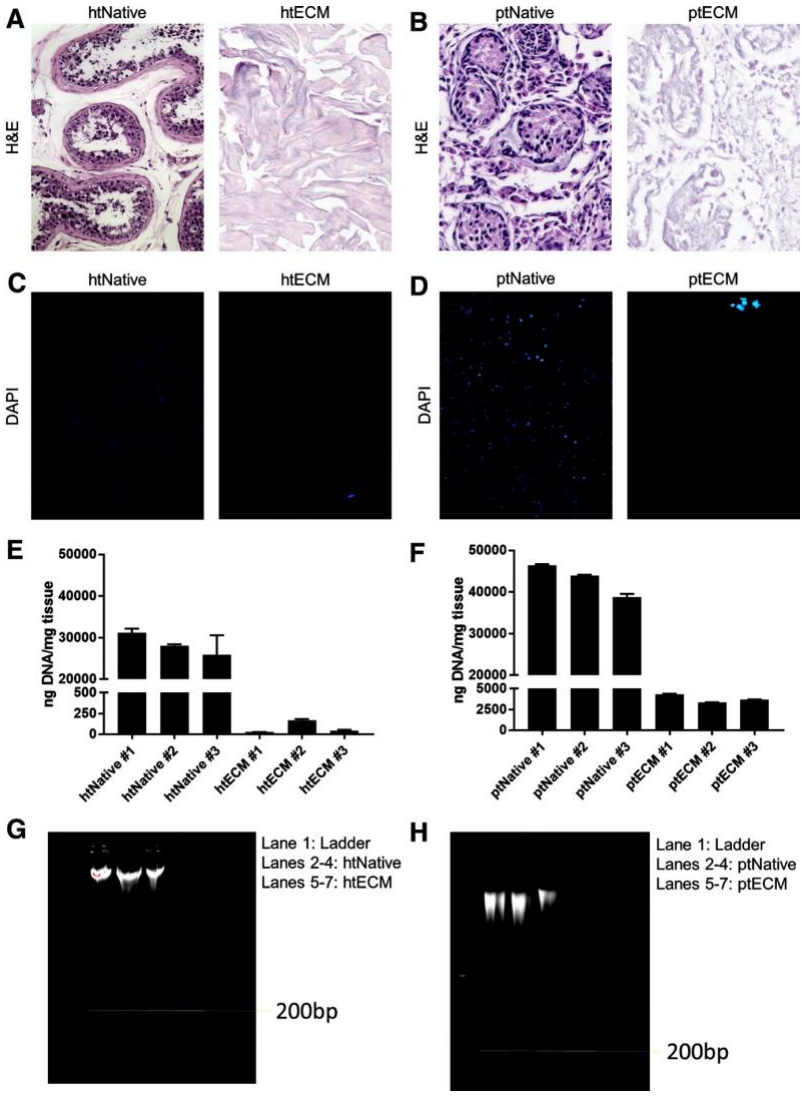

Figure 4: Efficacy of decellularization protocol.

Three biological replicates of human and porcine testis tissue were evaluated for DNA content before and after decellularization. Images in **(A–D)** are representative of each biological replicate. **(A, B)** For both species, H&E staining shows that native tissues contained many nuclei, whereas no intact nuclei are visible after decellularization. **(C, D)** For both species, DAPI staining shows that native tissue contained many nuclei, whereas no intact nuclei are visible after decellularization, though there seems to be some remnant DNA. **(E, F)** PicoGreen assay compared dsDNA between native samples and ECM. For both species, native samples contained high amounts of DNA, which was reduced by two to three orders of magnitude for htECM and one order of magnitude for ptECM after decellularization (note the axes). Data shown as mean ± SD of technical triplicate. **(G, H)** For both species, gel electrophoresis showed high amounts of genomic DNA in native samples. Appreciable amounts of DNA <200 base pairs are present in the htNative samples. Two htECM samples contained almost no DNA, and one sample showed a smear of DNA >200 base pairs. ptECM samples show light smears of DNA. DAPI, 4′,6-diamidino-2-phenylindole; H&E, hematoxylin and eosin; ptECM, porcine testicular ECM.

2.4.3 Biochemical composition

The amount of sulfated glycosaminoglycans (sGAG), total protein, and bFGF in the native tissue and ECM for both species was quantified. Alcian blue staining revealed that many of the sGAG that were present in the htNativetissue were preserved in the htECM throughout the decellularization process (**Figure 5A**). However, the porcine native tissue did not stain strongly for sGAG and no sGAG staining was visible in the ptECM histology (**Figure 5B**). These qualitative results were verified by the sGAG quantification. sGAG content in htECM was quantified in technical triplicate for both the human native and htECM samples of the three biologically distinct donors aforementioned (**Figure 5C**). Due to low tissue supply, the porcine native and ptECM samples were pooled into one sample for sGAG quantification (**Figure 5D**). htNative #1 contained 3.25 ± 0.01 ug of sGAG per mg of tissue (mean ± SD) and htECM #1 contained 0.80 ± 0.06 ug/mg. htNative #2 contained 2.23 ± 0.06 ug/mg of sGAG per mg of tissue and htECM #2 contained 1.38 ± 0.01 ug/mg. htNative #3 contained 1.63 ± 0.01 ug/mg of sGAG per mg of tissue and htECM #3 contained 0.69 ± 0.02 ug/mg (**Figure 5C**). The pooled ptNative contained 0.05 ug of sGAG per mg of tissue and no sGAG were detected in the pooled ptECM. To quantify total protein before and after decellularization, a urea-heparin extraction was performed on pooled samples. htNative yielded 111.8 ± 1.9 mg of protein per gram of tissue and htECM contained 10.0 ± 0.3 mg/g (**Figure 5E**). htPorcine yielded 142.9 ± 5.5 mg of protein per gram of tissue and ptECM contained 18.7 ± 0.5 mg/g (**Figure 5F**). Total bFGF was measured by ELISA for the urea-heparin extracts of each material. htNative contained 1,050 ± 35 ng of bFGF

per gram of tissue, and htECM contained 5.0 ± 0.3 ng/g (**Figure 5G**). ptNative contained 917 ± 86 ng of bFGF per gram of tissue, and ptECM contained 28.5 ± 0.2 ng/g (**Figure 5H**).

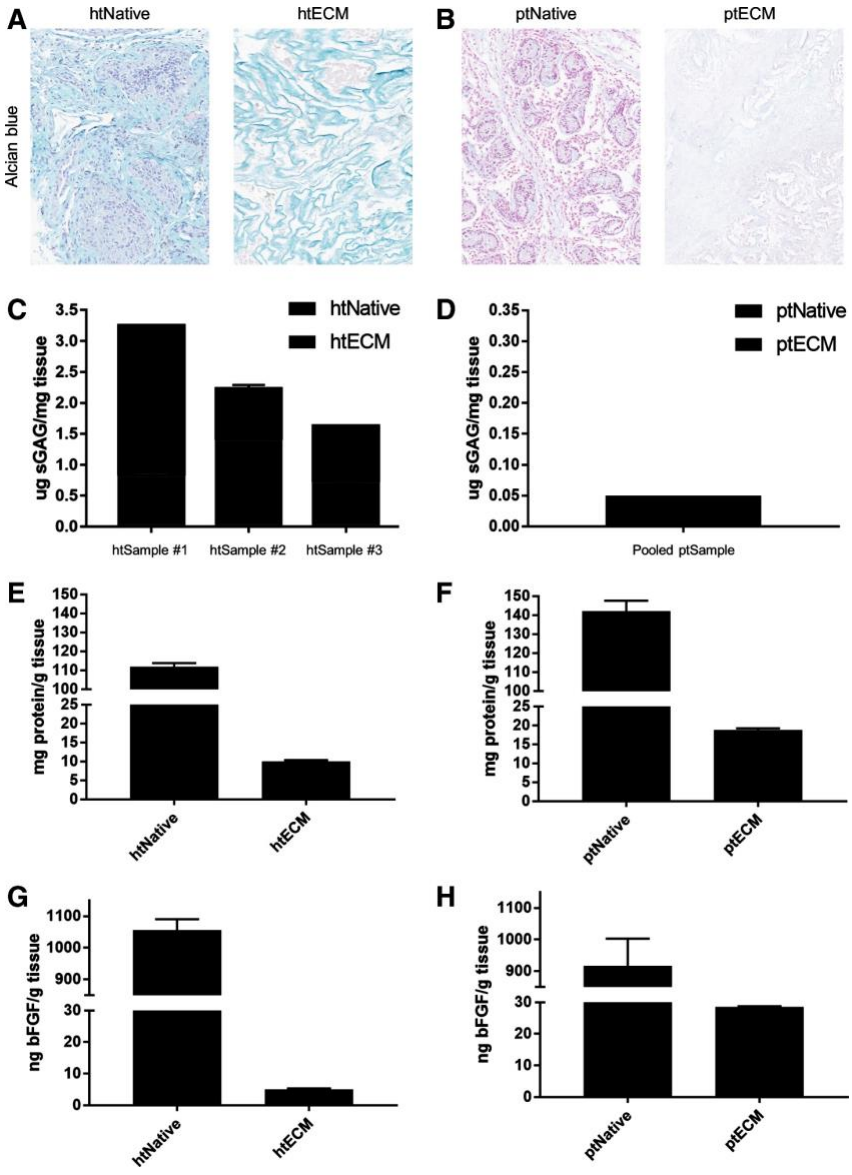

Figure 5: Biochemical composition of ECM.

(A) Alcian Blue staining indicates sGAG in htNative as well as in htECM (*light blue*). **(B)** Faint Alcian *Blue* staining is shown in ptNative, with no appreciable sGAG staining in ptECM. **(C)** Three biological replicates of human testis tissue were evaluated for sGAG content before and after decellularization. sGAG retention varies between replicates, perhaps due to biological variability between tissue donors. **(D)** A pooled sample of porcine testis tissue is evaluated for sGAG content; very little is measured in ptNative and none is detected in ptECM (note the axes). **(E, F)** Proteins from a pooled sample of human and porcine testis tissue were isolated through urea–heparin extraction, and total protein was measured by BCA assay. **(G, H)** bFGF was measured from the protein extractions by commercially available ELISA. Data shown as mean ± SD of technical triplicate. bFGF, basic fibroblast growth factor; sGAG, sulfated glycosaminoglycans.

2.4.4 Rheological properties of htECM hydrogel

Rheological properties of tissue-derived ECMs are concentration-dependent and can affect interactions with cells *in vitro*, especially in 3-dimensional culture models. The rheological properties of htECM hydrogel were determined for concentrations of 10mg/mL and 20mg/mL and were shown to be concentration dependent (**Figure 6**). A time sweep test showed that both concentrations of hydrogel had storage (G') and loss (G") moduli approximately one order of magnitude apart from each other, indicating the material to be a true hydrogel. Rheological testing indicated the viscosity of the pre-gel, forming gel, and formed gel of the 20 mg/mL material to be higher than that of the 10 mg/mL material. Interestingly, the time to reach 50% gelation for both materials was approximately 39 seconds but time to full gelation was about 35 minutes for the 20 mg/mL gel and about 49 minutes for the 10 mg/mL gel.

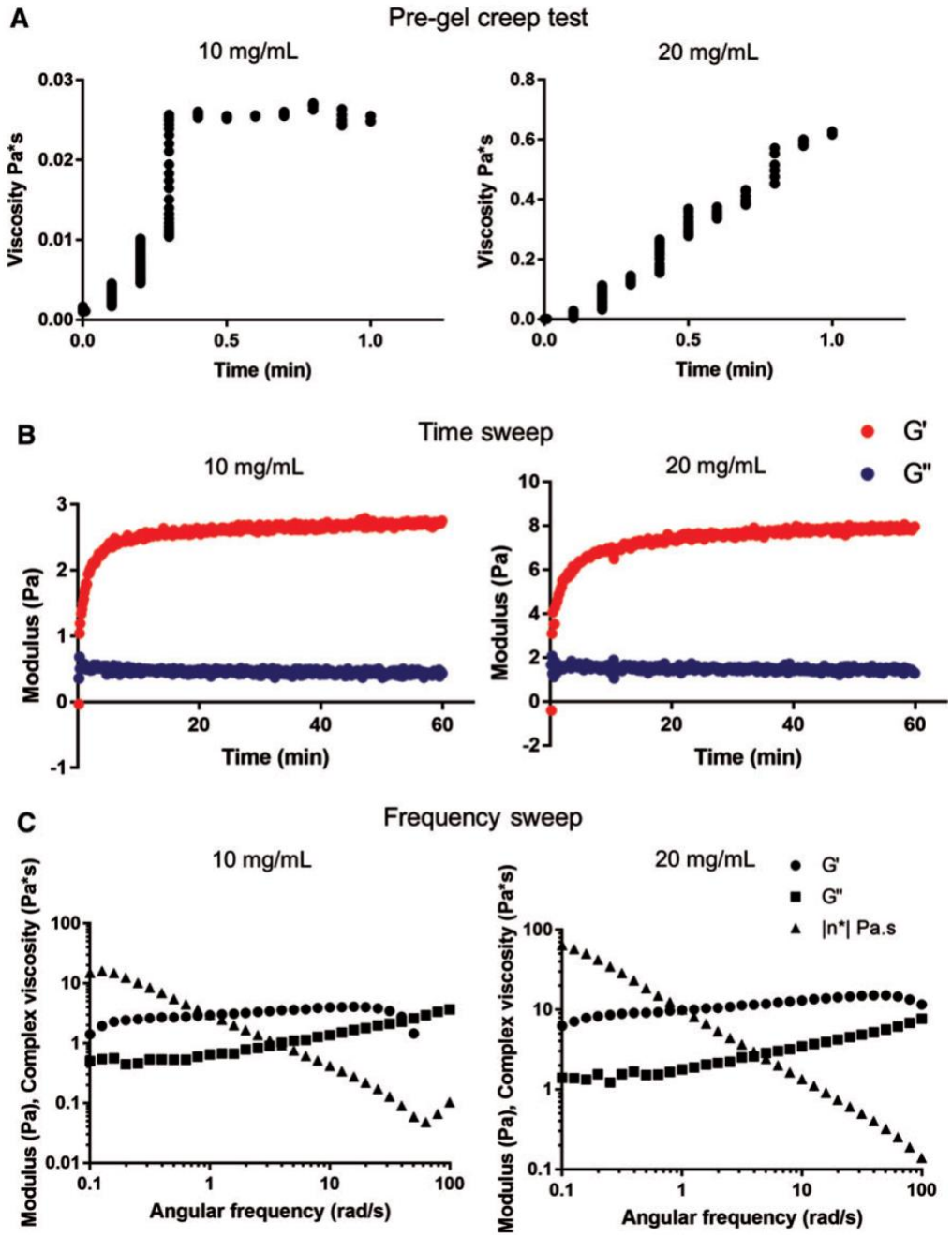

Figure 6: Rheological properties of htECM hydrogel.

Rheological measurements were taken for htECM hydrogel at both 10 and 20mg/mL. (A) Creep test showed that preformed hydrogel had a higher viscosity at 20mg/ mL than at 10mg/mL. (B) Time sweep test showed storage (G¢) and loss (G") moduli of forming gels approximately one order of magnitude apart from each other at both concentrations, indicating a true hydrogel. (C) Frequency sweep test of formed gel showed similar elastic behaviors between each concentration; the 20mg/mL gel had higher viscosity. htECM, human testicular extracellular matrix.

2.4.5 UTF1+ cell enrichment

Human spermatogonia were enriched from frozen and thawed testicular cell suspensions using magnetic activated cell sorting based on ITGA6 expression, as previously described [280]. Quantification of UTF1 expression by immunolabeling showed a nearly 3-fold enrichment of UTF1+ cells in the ITGA6+ fraction (13.78 ± 1.06%) compared to the unsorted cells (5.05 ± 1.07%) (p=0.004) (**Figure 7A**). The positive fraction of the sort contained a significant proportion of somatic cells that tended to over-grow the culture and therefore dilute the population of spermatogonia by day 14 (data not shown). Hence, we performed differential plating of the ITGA6+ positive fraction to further enrich UTF1+ cells and remove adherent somatic cells (**Figure 7B**). Somatic cells in the positive fraction adhered to the collagen-coated plates, while germ cells remained floating in the medium. Approximately 33.68 ± 6.18% of the floating cells expressed UTF1 and were used to initiate culture (**Figure 7B**).

2.4.6 Spermatogonial stem cell culture on ECM substrates

ITGA6+ cells after differential plating were introduced into culture wells coated with mouse laminin, human laminin, htECM, ptECM, SIS-ECM, UBM-ECM or STO feeder cells (controls) at a concentration of 50-70 x 10_3 cells/cm_2 (17-24 x 10_3 UTF1+ cells/cm_2). We used immunocytochemistry to determine the number of UTF1+ undifferentiated spermatogonia under each condition was compared with STO feeder cell controls on 0, 7 and 14 (**Figure 7A-C**). UTF1+ cells were present in all culture conditions at 14 days. Relative to the number of UTF1+ cells originally placed in culture (day 0), the number of UTF1+ cells remaining in culture were

significantly reduced by days 7 and 14 of culture (p=0.0001). However, there were differences between treatment groups. STO feeders retained 32.14±11.72% and 3.3±1.36% of UTF1+ cells on days 7 and 14 of culture respectively. Day 7 and 14 UTF1+ cell retention rates were: murine laminin (53.89±22.67% and 12.09±2.38%), human laminin (52.94±12.47% and 6.29±3.20%), htECM (54.18±12.94% and 21.90±5.50%), ptECM (37.95±4.82% and 16.22±4.00%), SIS (63.88±11.10% and 10.80±3.25%) and UBM (46.78±21.61% and 9.90±1.41%) (**Figure 7D, E**). While there was no significant difference in the number of UTF1+ cells between conditions at day 7, htECM retained the greatest number of UTF1+ cells by day 14 of culture. In addition, htECM was the only condition that retained a significantly greater number of UTF1+ cells than the control STO feeder cell-based condition at day 14 (p=0.0039). Flow cytometry results below provide additional insights about the cellular mechanisms that explain the loss of undifferentiated human spermatogonia on htECM and ptECM substrates during 14 days in culture.

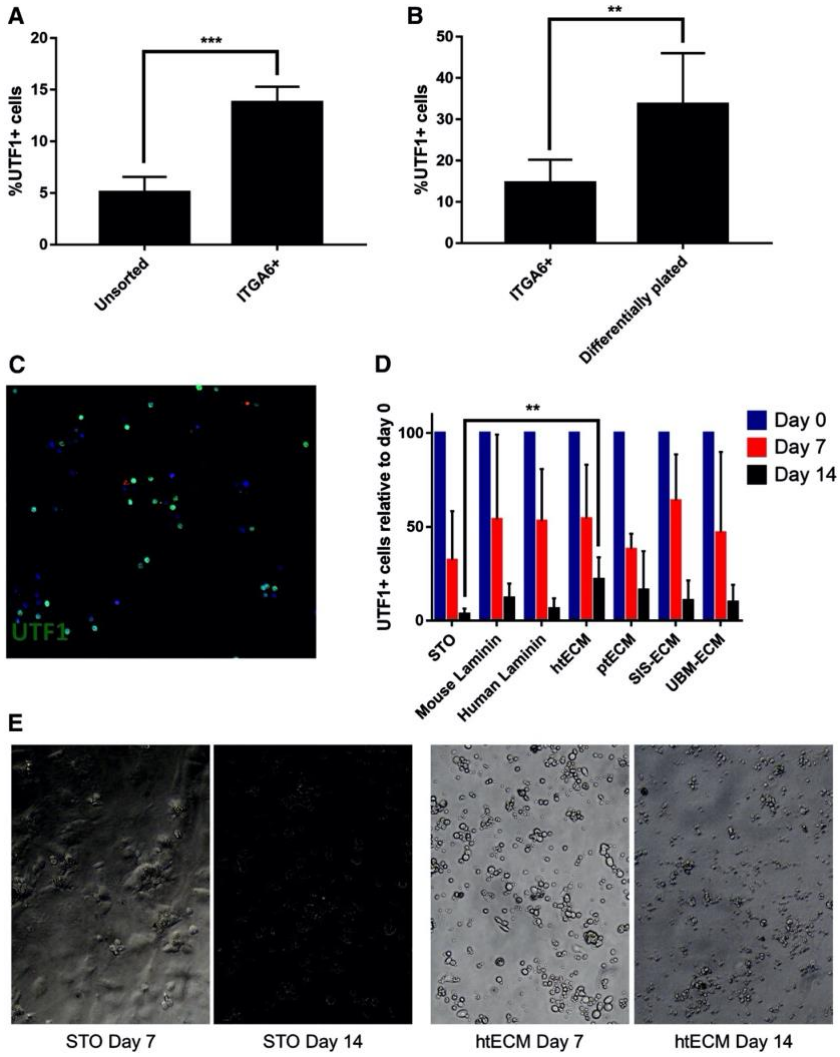

Figure 7: Enrichment and culture of undifferentiated spermatogonia on ECM substrates.

(A) MACS was used to enrich UTF1+ undifferentiated spermatogonia from human testicular cell suspensions based on the expression of ITGA6. Immunocytochemical analysis was then performed to show that UTF1+ cells were enriched in the positive fraction compared with unsorted cells ($p = 0.0042$). **(B)** To further enrich UTF1+ cells and contaminating somatic cells, MACS-sorted ITGA6+ cells were differentially plated on collagen I. Selected cells had a higher number of UTF1+ cells compared with ITGA6+ fraction of the sort ($p = 0.0153$). **(C)** Illustrative picture of immunostaining for UTF1 expression used to quantify enrichment of cells in **(A, B)** and maintenance of cells in culture. **(D)** Immunocytochemical analysis of UTF1 expression was performed on cells at the time of initiation of culture (day 0) and at days 7 and 14 of culture. UTF1+ cells were significantly depleted in all culture conditions by day 14 ($p = 0.0001$); however, htECM retained a significantly higher number of UTF1+ cells day 14 compared with the control STO feeder cell condition ($p = 0.039$). **(E)** Illustrative bright-field microscopy images of human spermatogonia cultured on STO feeder cells and htECM at days 7 and 14. Bar graphs are represented as mean ± SEM. $^{**}p<0.005$, $^{***}p<0.0005$. ITGA6, integrin alpha-6; MACS, magnetic-activated cell sorting; SEM, standard error of the mean; SIM, Sandos inbred mice; STO, SIM 6-thioguanine-resistance, ouabain-resistant; UTF1, undifferentiated embryonic cell transcription factor 1; UTF1+, undifferentiated embryonic cell transcription factor 1-positive.

2.4.7 Flow cytometry analysis of spermatogonial stem cells

We devised a multi-parameter flow cytometry strategy to determine the cellular mechanisms that explain the demise of undifferentiated spermatogonia during the 14 day culture period. Cultured cells were stained for SSEA4+ undifferentiated spermatogonia, cKIT (CD117)+ differentiated spermatogonia, annexin V+ apoptotic spermatogonia and Ki67+ proliferating spermatogonia (**Figure 8A-F**). SSEA4 is a robust and validated cell surface marker of human undifferentiated spermatogonia[162]. UTF1 immunostaining of fixed cells for flow cytometry did not work effectively in our hands. Therefore, SSEA4 was substituted for UTF1 as it has been effectively used to mark undifferentiated spermatogonia as reported by others [162, 228]. At day 0, 16.4±5.4% of live, ITGA6+, differentially plated cells were SSEA4+cKIT- , whereas 52.97±5.54% of the cells were SSEA4+cKIT+ and 20.23±7.98% of the cells were SSEA-cKIT+ (**figure 8A, quadrants A, B and D).** Similar to the results with UTF1 ICC, the number of SSEA4+/cKIT- undifferentiated spermatogonia declined significantly by days 7 and 14 of culture on htECM as well as ptECM ($p=0.0001$), with only 3.4±1.66% and 12.84±2.96% of the originally plated SSEA4+/cKIT- cells remaining by culture day 14, respectively (**Figure 8A-C & G**). In contrast to the total number of SSEA4+ undifferentiated spermatogonia, a greater proportion of differentiating SSEA4+/cKIT+ spermatogonia were retained on days 7 and 14 of culture, with only the ptECM number being significantly lower by day 14 ($p=0.01$; **Figure 8A-C & H**). These results suggest either that SSEA4+/cKIT+ differentiating spermatogonia survive better in culture or that SSEA4+/cKIT+ spermatogonia that are lost to apoptosis are replaced by differentiation of SSEA4+/cKIT- spermatogonia. Our data in **Figure 9** indicate a trend toward cKIT+ cells being

lost to apoptosis during the 14 day culture period ($p=0.056$ for day 14 htECM), which may favor the interpretation that SSEA4+/cKIT- spermatogonia are lost to differentiation and contribute to the maintenance of the population of SSEA4+/cKIT+ spermatogonia. Apoptosis clearly contributed to the demise of SSEA4+ total spermatogonia in culture as the number of SSEA4+/annexin V+ apoptotic spermatogonia increased significantly during the 14 days in culture on both htECM and ptECM ($p<0.05$; **Figure 8D-F & I).** Ki67 data in **Figure 8A-C** (green) and **J** indicate little or no proliferation of SSEA4+ cells on days 0 and 7 of culture (0-0.17%), but a significant increase on day 14 of culture on both htECM (39.28±4.42%; $p<0.0001$) and ptECM (37.19±11.60; $p<0.05$).

Together, these data indicate that SSEA4+/cKIT- undifferentiated human spermatogonia are lost to differentiation and apoptosis during the 14 day culture period and this loss was not compensated by proliferation of SSEA4+ cells. There were no significant differences in the proportions of SSEA4+ cells undergoing differentiation, apoptosis or proliferation between the 2 testicular ECMs.

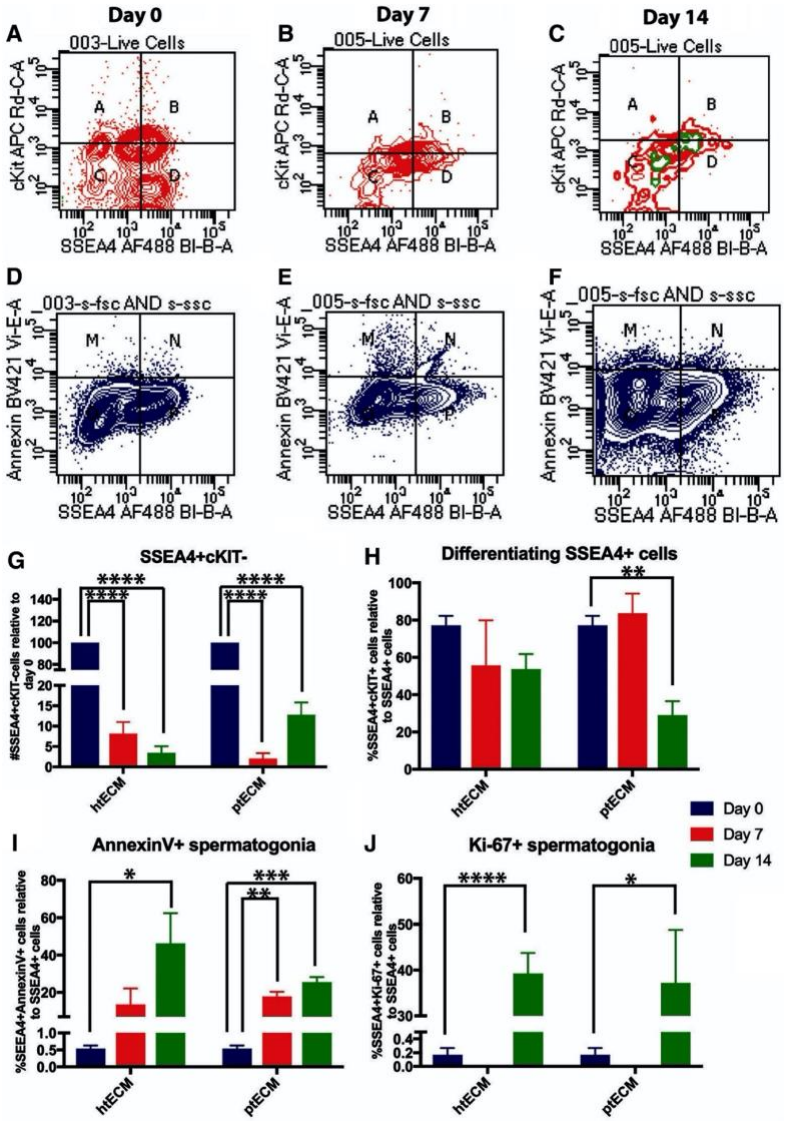

Figure 8:Evaluation of hSSC cultures on htECM and ptECM through flow cytometry.

(A–C) Flow cytometry was used to quantify the number of SSEA4+cKIT− (quadrant D) and SSEA4+cKIT+ (quadrant B) cells at the beginning of culture, day 7 and day 14. The population of cells expressing Ki-67 overlaid on *top* of the graph is indicated in *green*. **(D–F)** Plots show the populations of SSEA4+ cells that are annexin V− (quadrant P) and apoptotic SSEA4+ cells that bind annexin V (quadrant N). **(G)** SSEA4+cKIT− cells decline significantly over the duration of culture at days 7 and 14 on both ECMs relative to day 0. **(H)** The proportion of SSEA4+ cells expressing cKIT did not change significantly in htECM-based cultures over 2 weeks. ptECM-based cultures, however, had a lower number of SSEA4+ spermatogonia that were cKIT+ cells compared with day 0. **(I)** The proportion of SSEA4+ cells undergoing annexin V was significantly higher at day 14 of cultures on htECM and at days 7 and 14 of cultures on ptECM. **(J)** Quantification of Ki-67 expression in SSEA4+ cells showed a significant increase in proliferating cells within the SSEA4+ population at day 14 on htECM and ptECM. Bar graphs are represented as mean ± SEM. *Indicates $p < 0.05$, **indicates $p < 0.01$, ***indicates $p < 0.0005$, and ****indicates $p < 0.0001$. SSEA4, stage-specific embryonic antigen 4.

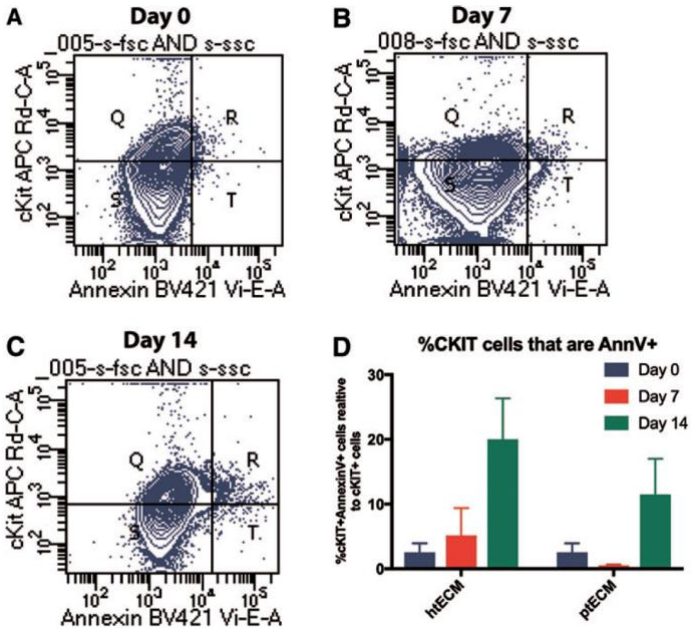

Figure 9: Proportion of cKIT+ cells undergoing apoptosis.

(A–C) Flow cytometry analysis was used to quantify cKIT+ annexin V- (quadrant Q) cKIT + annexin V+ (quadrant R) and cKIT-annexin V+ cells (quadrant T) on days 0, 7, and 14. (D) A trend toward increasing apoptotic cKIT+ cells was observed relative to total cKIT+ cells on both ECMs, although the difference was not statistically significant. Bar graphs are represented as mean–SEM. ECM, extracellular matrix; SEM, standard error of the mean.

2.4.8 Optimization of cell culture medium

My experiments in this study demonstrate that human testis ECM serves as a favorable substrate to culture human SSCs. In order to improve the retention of undifferentiated spermatogonia in culture, I compared the outcome of culturing enriched spermatogonia in mSFM as described above with 2 other media, StemPro-34 and IMDM-SFM, described previously [127, 228]. The compositions of both media are described in appendix E. Human spermatogonia were enriched using ITGA6-based MACsort and differential plating on type I collagen. Selected cells were then plated on human ECM and cultured on mSFM, StemPro-34 and IMDM-SFM for 14 days. Human spermatogonia did not attach to the substrate when cultured in IMDM-SFM (**Figure**

10C). This condition was hence eliminated, and quantitative comparisons were only made between mSFM and StemPro-34. Immunocytochemical analysis using antibody against UTF1 to quantify spermatogonia in culture at days 7 and 14 revealed loss of UTF1+ cells over time in StemPro-34 as is observed in mSFM (**Figure 10A, B and D**). However, at day 14, cultures with StemPro-34 had a significantly higher recovery of UTF1+ cells compared to mSFM ($p=0.007$). Flow cytometry analysis showed that cultures with StemPro-34 had significantly higher numbers of viable cells at days 7 and 14 compared to cultures in mSFM ($p<0.001$) (**Figure 10E and F**). Analysis of the expression of SSEA4 and cKIT to mark undifferentiated and differentiating spermatogonia showed a slightly higher recovery of SSEA4+cKIT-, SSEA4+cKIT+ and SSEA4-cKIT+ cells but the differences were not statistically significant (**Figure 10G-J**).

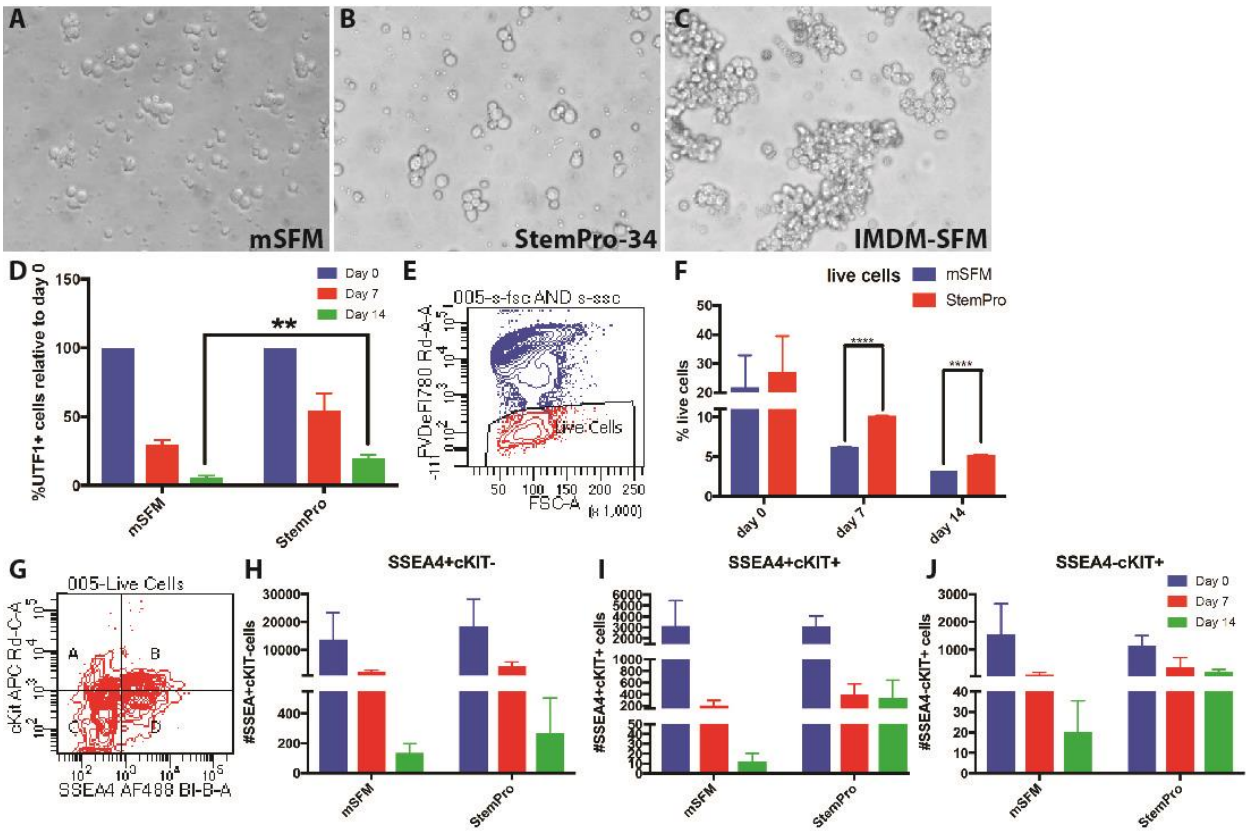

Figure 10: Comparing maintenance of undifferentiated spermatogonia in mSFM, IMDM and StemPro-34

(A-C) Spermatogonial stem cells were enriched and cultured in mSFM, IMDM-SFM and StemPro 34 for 14 days and analyzed at days 7 and 14. Spermatogonia did not adhere to human ECM when cultured in IMDM-SFM (C) and hence this condition was eliminated from further analysis. (D) Immunocytochemisty using an antibody against UTF1 was performed to quantify the recovery of undifferentiated spermatogonia in mSFM and StemPro-34-based cultured. Analysis revealed a significantly higher retention of UTF1+ cells in StemPro-34 at day 14. (E and F) Flow cytometry analysis was used to compared viability of cells cultured in mSFM and StemPro-34 and results show significantly increased recovery of cells cultured in StemPro-34 at days 7 and 14. (G-J) Quantification of cells expressing SSEA4 and cKIT using flow cytometry showed a trend towards increased SSEA4+cKIT-, SSEA4+cKIT+ and SSEA4-cKIT+ cells in StemPro-34 with no statistically significant differences between the 2 conditions. Data in D, F, H-J are mean±SEM. ** indicates $p<0.01$, **** indicates $p<0.001$.

2.5 Discussion

The present study identified methods to isolate extracellular matrix from human testis (htECM) and porcine testis (ptECM) and compared the ability of a solubilized form of this ECM to serve as a culture plate substrate and support the viability of hSSC vs. mouse laminin, human laminin, small intestinal submucosa (SIS-ECM), urinary bladder (UBM-ECM), and STO mouse embryonic fibroblast feeder cells. The htECM material was shown to meet established decellularization criteria [279] while retaining sGAG and growth factors although the same decellularization protocol produced ptECM with higher dsDNA content. This finding may be explained in part by differences in macromolecular content/density, cellularity, and biochemical distinctions between the testis microenvironment of the two species. Identical decellularization methods provided a head-to-head comparison of the two ECM environments although an alternative protocol may produce ptECM with lower DNA content.

Identification of a modified testis decellularization process involved the use of several commonly used detergents and subsequently comparing the cellularity, and the amount and integrity of remnant DNA within the testis ECM. This detergent-based approach was also described by Baert et al. [273] who compared 1% TX-100 to 1% SDS for both 24 and 48 hours and found SDS at 24 hours to be superior, due probably to its “harsh ionic nature”. The present study investigated the use of these same detergents but at different concentrations; 3% for the milder non-ionic TX-100 and 0.075% for SDS. Our collaborators at Stephen Badylak’s laboratory also evaluated the use of 4% SDC and a solution containing 0.25% of SDC and TX-100 each. Their results likewise indicated SDS to be a superior decellularization agent but required one order

of magnitude less detergent than Baert et al.'s protocol [275, 281]. Furthermore, they included a 2-hour wash step subsequent to the SDS exposure that has been shown to enhance the removal of residual SDS, which adversely affects the cytocompatibility of ECM scaffold materials [282]. They speculate that minimizing the SDS concentration during decellularization, and subsequently removing as much residual SDS as possible helped to preserve the biochemical composition and integrity of the resident structural and functional molecules.

Rheological studies of pepsin-solubilized htECM showed that upon polymerization at 37°C at both 10 mg/mL and 20 mg/mL concentrations, there was approximately a 10-fold difference between the storage (G') and loss (G'') moduli, indicating the biomaterial intrinsically behaves as a true hydrogel [283, 284]. The retention of UTF1+ cells on day 14 of culture was greatest on the htECM (21.9±5.5%) and ptECM (16.2±4.0%) substrates, but only htECM retained significantly more UTF1+ human spermatogonia than the STO feeder cell controls (3.3±1.36%). These observations may justify moving to an ECM-based, feeder-free, serum free system for downstream experiments to establish conditions for long-term maintenance and expansion of hSSC in culture. ptECM cultures retained the next highest number of UTF1+ spermatogonia after 14 days (16 ± 4% UTF1+ cells relative to day 0), but this was not statistically different than the STO feeder condition.

The ECM of the testis represents the natural *in vivo* niche for hSSC. The decellularization method reported herein preserves the biochemical composition of the testis ECM environment and these endogenous cues may contribute to hSSC growth/survival *in vitro*. The loss of sGAG, total protein, and bFGF following decellularization is not surprising as the bulk of these components lie in the cellular compartment of the tissue. The findings in the present study are consistent with those of other studies [285-290]. However, it is possible that the ECM proteins were also depleted

by the use of SDS in the protocol. The bioactivity of ECM bioscaffolds has been attributed to structural proteins [291-293], glycosaminoglycans [294], the ligand landscape including embedded growth factors and cytokines [295-298], the enzymatic creation of matricryptic peptides [299-302], and most recently, the inclusion of microRNA-containing matrix-bound nanovesicles [260]. The ECM is continuously degraded, remodeled, and synthesized by the resident cells of each tissue; therefore, ECM from different source tissues is biochemically distinct and can have tissue-specific effects on cells. Therefore, it makes intuitive sense that ECM from the homologous tissue (testis) and homologous species (human) might be most suitable for culturing hSSC although this is not always the case (see **Table 2**, [270, 287, 303-308]). Such tissue-specificity has been shown in studies involving ECM derived from skin and skeletal muscle [308], liver [270], and myocardium [309]. However, other studies show either no difference or a preference for heterologous tissue ECM (see **Table 2**). For example, neurites grew equally well in ECM hydrogels derived from CNS, spinal cord, or urinary bladder tissues [306], and in a clinical trial treating volumetric muscle loss no difference was found between ECM derived from small intestine, urinary bladder, or dermis [255]. ECM tissue-specificity has not previously been evaluated for expansion of hSSC in two-dimensional culture.

Table 2: Literature review of the response of cells and tissues exposed to homologous or heterologous ECM

Citation	ECM source tissue	ECM form	Study design	End point	Preferred source
Lin et al., Tissue Engineering, 2004	Liver Small intestine Urinary bladder	Scaffolds	In vitro- hepatocyte cell culture	Albumin secretion, urea synthesis	Heterologous (small intestine)
Sellaro et al., Tissue Engineering, 2007	Liver Small intestine Urinary bladder	Scaffolds	In vitro- sinusoidal endothelial cell & hepatocyte coculture	Sinusoidal endothelial cell differentiation	Homologous
Ellen P. Brennan, Doctoral dissertation, University of Pittsburgh, 2009	Dermis Liver	Hydrogel	In vitro- neonatal human epidermal keratinocyte, human fetal liver cell culture	Chemotaxis	No difference
	Liver Small intestine Urinary bladder	Hydrogel	In vitro- rat small intestinal epithelial cell culture	Chemotaxis	Homologous or heterologous (urinary bladder)
Zhang et al., Biomaterials, 2009	Skeletal muscle Dermis Liver	Hydrogel	In vitro- skeletal muscle, skin, liver cell culture	Proliferation, differentiation	Homologous
				Attachment	No difference
Wolf et al., Biomaterials, 2012	Skeletal muscle Small intestine	Scaffold	In vitro- myoblast, perivascular stem cell, fibroblast, endothelial cell, myotube cell culture	Attachment Survival Morphology	No difference
			In vitro- myoblast cell culture	Differentiation	No difference
			In vivo- abdominal wall defect	Remodeling	No difference
Crapo et al., Tissue Engineering Part A, 2013	Spinal cord Brain Urinary bladder	Hydrogel	In vitro- neural stem cell, perivascular stem cell culture	Viability	No difference
			In vitro- neural stem cell culture	Proliferation Chemotaxis	No difference
				Differentiation	Homologous
Medberry et al., Biomaterials, 2013	Spinal cord Brain Urinary bladder	Hydrogel	In vitro- neuroblast cell culture	Viability Neurite extension	No difference
Keane et al., Tissue Engineering Part A, 2015	Esophagus Small intestine Urinary bladder	Hydrogel	In vitro- esophageal stem cell culture	Migration Organoid formation	Homologous
				Proliferation	No difference
			In vivo- esophageal mucosa defect	Remodeling	No difference

While there have been several reports on the establishment of human SSC culture methods, the methods for validating cultures have been variable and there is no consensus on best methods [129, 131, 219-239]. One of the challenges for validating human SSC cultures is the absence of a

functional assay to test the spermatogenic potential of cultured cells. Human to nude mouse xenotransplantation has been used in some studies to quantify transplantable SSC in culture, but this approach is time consuming (2 months to analysis) and not compatible with iterative testing of culture conditions in early stages [131, 163, 224, 236, 276, 280]. Therefore, most studies rely on the expression of endogenous markers to characterize spermatogonia including GDNF family receptor alpha-1 (GFRα1), integrin alpha-6 (ITGA6), G-protein coupled receptor 125 (GPR125), thymocyte differentiation antigen 1 (THY1 or CD90), ubiquitin carboxyl-terminal hydrolase L1 (UCHL1), Sal-like 4 (SALL4), promyelocytic leukemia zinc finger (ZBTB16), undifferentiated embryonic cell transcription factor 1 (UTF1) and stage-specific embryonic antigen 4 (SSEA4). However, markers such as GPR125, UCHL1, SSEA4, ITGA6 and THY1 are also expressed by other cells in the testis [230, 233, 234, 310]. In contrast, the expression of UTF1 in adult mammals is restricted to the testes [311] and specifically the population of undifferentiated spermatogonia on the basement membrane of seminiferous tubules [233, 280, 312]. UTF1 is expressed by cells that also express the undifferentiated spermatogonial markers SALL4 and UCHL1 (PGP9.5) but does not overlap with the differentiated spermatogonial marker, cKIT [280].

In the present study, spermatogonia were enriched by sorting testicular cells based on ITGA6 expression, as previously described [280]. However, the ITGA6+ fraction retained a substantial proportion of somatic cells that tended to overgrow the culture by day 14. To deplete the somatic cell population, the ITGA6+ fraction was differentially plated on collagen I and the non-binding fraction was selected for cell culture, as has been reported previously [229, 237]. Results showed greater retention of UTF1+ cells on htECM substrate at 2 weeks compared with

cultures on STO feeder cells. However, UTF1+ cell numbers gradually declined over time on all substrates.

I developed a multi-parameter flow cytometry approach to understand the cellular mechanisms that explain the loss of undifferentiated human spermatogonia on htECM and ptECM substrates. My data indicated that the population of SSEA4+/cKIT- undifferentiated spermatogonia may be lost to differentiation (SSEA4+/cKIT+) and apoptosis (SSEA4+/annexin V+). These observations will help to focus future culture experiments to identify conditions that inhibit differentiation pathways and/or reduce apoptosis. I observed almost no proliferation of undifferentiated human spermatogonia (SSEA4+/Ki67+) on days 0 or 7 of culture, but a significant increase in proliferation on day 14. The increased proliferation of SSEA4+ human spermatogonia on day 14 could result from selection of a sub-population of spermatogonia that were compatible with the culture conditions. However, the data in **Figure 7C** suggest that the increased proliferation is associated with the cKIT+ differentiating portion of SSEA4+ cells. This result may simply reflect a higher rate of proliferation in differentiating spermatogonia compared with undifferentiated spermatogonia. Alternatively, the increased proliferation of SSEA4+ human spermatogonia on day 14 of culture could be an emergency stress response in which apoptosis in some cells causes compensatory proliferation in the neighboring cells to maintain homeostasis [313]. Culturing spermatogonia in StemPro-34 improved the viability of cells in culture in comparison to mSFM; however, both conditions demonstrated a similar pattern of loss of undifferentiated spermatogonia over time. Overall, these data suggest that GDNF and bFGF, growth factors that are required for survival and expansion of mouse SSCs in culture, are not

sufficient to support the survival or expansion of human SSCs in culture, at least at the concentrations tested in this study.

Results of the present study indicate that the homologous species and homologous tissue ECM represented the best substrate for maintaining human SSCs in culture. However, the overall significant decline of undifferentiated spermatogonia by day 14 indicates that more work is needed to achieve the long-term goal of expanding human SSC numbers in culture. Unlike the mouse, where SSC cultures could be validated with a functional transplantation assay that regenerates complete spermatogenesis, assessment of human SSC cultures has been largely limited to descriptive markers. We found that UTF1 and SSEA4 are robust markers of human spermatogonia that could be used in ICC and/or FACS analyses, consistent with previous reports [162, 228]. The flow cytometry approach enables high throughput, quantitative, simultaneously assessment of multiple endpoints, which will help to guide future experiments. The serum-free, feeder-free system described here should provide a simplified platform for iterative testing of various culture conditions.

3.0 Xenografting cryopreserved prepubertal non-human primate testicular tissue to induce spermatogenesis

This is an adaptation of the author's version of the work. The definitive version is being prepared for publication.

"**Xenografting cryopreserved prepubertal non-human primate testicular tissue to induce spermatogenesis"**

Sherin David[1,2], Meena Sukhwani[1], Karen Peters[1], Hanna Valli-Pulaski[1], Sarah Steimer[1], Kyle E. Orwig[1,2]

1) Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

2) Magee-Womens Research Institute, Pittsburgh, PA, USA

3) Molecular Genetics and Developmental Biology Program, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

3.1 Chapter Summary

Fertility preservation programs in the US and abroad are freezing testicular tissues for young patients who are at risk of infertility with anticipation that next generation reproductive technologies will be available to use those tissues in the future. Immature testicular tissues from several mammalian species can be induced to undergo maturation and produce sperm after xenografting under the back skin of immune-deficient nude mouse recipients. Previous studies have demonstrated that fresh non-human primate (nHP) testicular tissues can be matured in a mouse host to produce sperm and live offspring. This result has not been replicated with cryopreserved testicular tissue from primate donors, which is a critical aspect of the fertility preservation paradigm. We tested the maturation capacity of cryopreserved testicular biopsies obtained from prepubertal Rhesus macaques upon grafting into immune compromised mice. Additionally, we studied the effects of vascular endothelial growth factor (VEGF) and human chorionic gonadotropin (hCG) on graft survival and maturation. We observed complete spermatogenesis in grafts recovered from mice treated with hCG with or without VEGF. Immunostaining analysis revealed the presence of meiotic germ cells in 40.36±6.59% of the tubules and post-meiotic germ cells in 28.57±5.51% of the tubules in grafts retrieved from the hCG treatment group. In contrast, grafts from the VEGF treatment group had spermatogonia in 29.76±8.75% of the tubules with no evidence of differentiation. Sperm recovered from hCH+/-VEGF-treated grafts were used to fertilize Rhesus oocytes giving rise to a healthy male offspring. No grafts were recovered from mice that were left untreated.

3.2 Introduction

Immature testicular tissue from several species can undergo spontaneous maturation to generate spermatozoa when xenografted into immunocompromised recipient mice (reviewed in [314, 315]). Successful application of this method to human testicular tissue could serve as an approach for fertility restoration in prepubertal males who cryopreserved their testicular tissues prior to gonadotoxic therapies [316, 317]. In this method, the donor testicular tissue biopsy is typically grafted ectopically into a recipient mouse. Upon engraftment through the establishment of vascularization [318], a feedback loop between the recipient hypothalamic and pituitary glands and the donor somatic compartment is established, thereby triggering the onset of spermatogenesis in the graft. Xenografting has been successfully used to generate fertilization-competent spermatozoa in fresh prepubertal non-human primate testicular grafts [191, 319, 320]. However, application of this method to cryopreserved primate testicular tissue was unsuccessful [321]. This is a significant barrier to translation of this method to the clinic as cryopreservation is a critical component of the fertility preservation paradigm [52]. Here we show that human chorionic gonadotropin (hCG) treatment of recipient mice induced complete maturation of cryopreserved Rhesus testicular xenografts, while grafts from untreated or VEGF-treated mice showed no evidence of spermatogenesis. Sperm extracted from hCG-treated xenografts were used to fertilize rhesus macaque eggs by intracytoplasmic sperm injection (ICSI), establish a pregnancy and produce a healthy offspring. These results show that cryopreserved prepubertal primate testicular tissues retain spermatogenic potential upon exposure to hCG in the murine microenvironment. We envisage that in addition to extending the applicability of xenografting to cryopreserved primate

testicular tissue, this method also has the potential to serve as a functional *ex-vivo* assay to evaluate factors that affect germ and somatic cell function in the cryopreserved primate or human testis samples.

Procedures for collecting testicular samples from prepubertal macaques were performed in the animal housing facilities at Magee-Womens Research Institute and the Oregon Health and Science University. Intracytoplasmic Sperm Injection (ICSI) using sperm collected from xenografts were performed by Carol Hanna and Cathy Ramsey at the Oregon National Primate Research Center (ONPRC). Embryo transfer, ultrasound measurements and post parturition management were carried out by Brandy Dozier at ONPRC. Cesarean section and observations were led by Lauren Drew Martin; placental observations were performed by Victoria Roberts and behavioral and developmental assessments were carried out by Lisa Houser and Nicola Robertson at ONPRC.

3.3 Materials and Methods

3.3.1 Experimental animals

The use of severe combined immunodeficient (SCID) mice (Cat# ICRSC-M, Taconic Biosciences) and rhesus macaques in the study was approved by the Institutional Animal Care and Use Committees (IACUC) of Magee-Womens Research Institute (MWRI), the University of Pittsburgh and the Oregon Health and Science University. Experiments were performed in

accordance to the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. Recipient mice were obtained at 5 weeks of age and were allowed to acclimatize to the housing environment for one week prior to xenografting.

3.3.2 Testis collection, processing and cryopreservation

Prepubertal rhesus macaque testes were obtained either by hemicastration following sedation with Ketamine (10mg/kg)-Xylazine (0.7mg/kg) and anaesthesia with isoflurane or by castration of euthanized macaques. Testes were stored and transported in Lactated Ringers Solution (Cat# NC0957153, Fisher Scientific) at 4ºC and were processed by the removal of the tunica followed by dissecting of the organs into small pieces (9-20 mm^3) in a sterile laminar flow biosafety cabinet. Testicular tissue pieces were then cryopreserved in a cryoprotectant medium composed of 5% fetal bovine serum (FBS, Cat# 16141061, Thermo Fisher Scientific) and 5% dimethyl sulfoxide (DMSO, Cat# D8418, Sigma) in Minimum Essential Medium alpha (MEMα, Cat# 12561-056, Invitrogen). Testicular samples were cryopreserved using the controlled slow freezing protocol in the Freeze Control® temperature controller (Cat # CL-5500, CryoLogic), as described previously [49] with slight modifications. Briefly, cryovials were equilibrated to the cryoprotectant medium at 4ºC for 30 minutes. Samples were then cooled from 4ºC to 0ºC at a controlled rate of -1ºC/minute, then held at 0ºC for 5 minutes, followed by cooling to -8ºC at the rate of -0.5ºC/minute. Samples were manually seeded at -8ºC followed by cooling to -40ºC at the rate of -0.5ºC/minute. Samples were held that this temperature for 10 minutes followed by rapid

cooling to -80ºC at -1.5ºC/minute. At this point, the vials were plunged into liquid nitrogen, where they were stored until application for xenografting.

3.3.3 Thawing and xenografting

Cryovials containing testicular tissue pieces were retrieved and transferred to a water bath maintained at 37ºC until completely thawed. Samples were rinsed with and placed in Hanks Balanced Salt Solution (HBSS, Cat# 24-020-117, Fisher Scientific) on ice until grafting into mice. Recipient mice were anaesthetized with 2,2,2-tribromoethanol administered intraperitoneally (250mg/kg, Cat# T48402-100G, Sigma) and castrated by accessing the recipient testes through an incision made in the abdomen and the peritoneum. Testicular blood vessels were ligated prior to removal of the testes. Grafts were placed under the dorsal skin of the recipient mouse by making small incisions on each graft site. Mice in the VEGF treatment group received 1µg of recombinant human VEGF-165 reconstituted in Dulbecco's phosphate buffered saline (DPBSS, Cat# 14200166, Life Technologies) pipetted directly into to graft site prior to placement of the graft. Incisions were closed after insertion of the grafts by applying 1-2 simple interrupted stiches/site using absorbable undyed vicryl suture (Cat# 07-810-7022, Henry Schein). Mice in the hCG treatment group received 10U of hCG (Cat# CG10, Sigma) subcutaneously at the time of grafting and biweekly over the course of the study. All recipient mice were treated with Buprenorphine (0.1mg/kg) and provided with Medigel CPF (Clear H_2O) with Carprofen for pain management post-surgery.

3.3.4 Graft recovery and processing

Mice were euthanized by intraperitoneal administration of Avertin followed by cardiac puncture and cervical dislocation. Seminal vesicles and grafts were excised and weighed. Fragments of each graft and the seminal vesicles were fixed in 4% paraformaldehyde (PFA) overnight, washed in PBS and embedded in paraffin for histological analyses. The remaining graft tissue from each sample was mechanically dissected by placing the tissue in a sterile 35x10mm dish containing 200µl of HEPES-TALP buffer (Cat# IVL01-500ml, Caisson Labs) supplemented with 0.3% bovine serum albumin (BSA, Cat# 126575, Millipore) (TH medium). Spermatozoa released into the buffer were collected and pelleted in a centrifuge at 600xg for 10 minutes. The pellets were resuspended in HEPES-TALP buffer with 0.3% BSA and transferred to 2ml cryogenic tubes (Cat# 03-337-7C, Thermo Fisher Scientific). Some samples were shipped to the Oregon National Primate Research Center (ONPRC) at ambient temperature while others were cryopreserved for later use. For cryopreservation, freezing medium containing TEST yolk buffer (Cat #90128, Irvine Scientific) was added to the samples drop-wise until a 1:1 ratio of cell suspension and freezing medium was achieved. The samples were allowed to equilibrate at room temperature for 10 minutes then exposed to the vapor phase of liquid nitrogen for 30-60 minutes followed by transfer into liquid nitrogen for storage at -196ºC.

3.3.5 Hematoxylin and Eosin staining and immunohistochemistry

Prepubertal and adult testicular tissue along with xenograft tissue samples that were fixed in 4% PFA, washed and embedded in paraffin were sectioned into 5µm sections and mounted on

glass slides. For hematoxylin and eosin staining, sections were then deparaffinized and rehydrated by exposing sections to a graded ethanol series of decreasing ethanol concentrations with the final hydration in water. Sections were then stained in hematoxylin 560 (Cat# 3801575, Leica Biosystems Inc.) and Eosin (Cat# 3801606, Leica Biosystems Inc.) as described previously [322]. For immunohistochemistry, deparaffinized and rehydrated sections were placed in Tris buffer (pH 10) heated to 97ºC for 30 minutes for antigen retrieval. Slides were then cooled, rinsed with DPBST (0.1% Tween-20 in 1X DPBS) and incubated with blocking buffer composed of 3% BSA, 0.1% Triton X-100 and 5% normal donkey serum (Cat# 017-000-121, Jackson Immunoresearch Lab) in DPBS, for 1 hour. Sections were then incubated with primary antibodies: Goat anti-VASA (1:300, Cat#AF2030, R&D systems), Rabbit anti-SOX9 ((1:100, Cat# AB5535, Millipore Sigma), Mouse anti-PGP9.5 (1:100, Cat # 7863-1004, Bio-Rad), Goat anti-PIWIL1 (1:40, Cat# AF6548, R&D systems), Rabbit anti-NUMA (1:100, Cat# ab84680, Abcam) and Rabbit anti-TP1 (1:1000, Cat# ab73135, Abcam) for 90 minutes at room temperature or overnight at 4ºC. The sections were then rinsed in DPBST and incubated with fluorescently labeled secondary antibodies (1:200, Invitrogen) specific to the species each antibody was raised in for 45 minutes. Following staining with the secondary antibody, the slides were rinsed and mounted with DAPI-containing mounting medium, Vectashield (Cat# H-1200, Vector Labs). Sections were observed under the Nikon Eclipse 90i microscope and analyzed using the NIS Elements Advanced Research Software.

3.3.6 Sperm Processing and ICSI

Upon arrival at ONPRC, samples were observed under bright field and subjectively evaluated for quality based on morphology and concentration of sperm. One sample selected for intracytoplasmic sperm injection (ICSI) and was processed further by pelleting sample in a centrifuge at 300xg for 7 minutes. The supernatant was discarded, and the pellet was resuspended in TH medium. To this suspension, 300µl of TEST-yolk buffer (TYB) extender (Cat#90129, Irvine Scientific) was added dropwise and mixed gently. Spermatozoa were maintained in this medium during ICSI.

3.3.7 Ovarian follicle stimulation and oocyte recovery

Rhesus macaque oocytes were retrieved by laparoscopic aspiration of antral follicles following a 10-day controlled ovary stimulation (COS) protocol described previously [323, 324]. Briefly, within the first four days at the onset of menses, 15 IU of recombinant human follicle stimulating hormone (r-hFSH) was administered intramuscularly (IM) b.i.d. on days 1-8 with 30 IU of recombinant human luteinizing hormone (r-hLH) given IM b.i.d. on days 7-8. Estradiol and progesterone concentrations were evaluated daily in blood serum samples via electrochemiluminescent immune assay to verify a response to gonadotropin treatment. When serum estradiol exceeded 200 pg/mL, 1 mg/kg of a GnRH antagonist was administered subcutaneously to prevent an endogenous LH surge and premature ovulation. On day 8, 1000 IU human chorionic gonadotropin (hCG) was delivered IM. On day 10, 36 hours post hCG, antral follicles were aspirated via laparoscopy into TALP+Hepes (recipe; Ramsey and Hanna, 2019)

supplemented with 5 IU/mL of heparin at 37°C. Oocytes were isolated from follicular aspirate and washed through fresh TALP+Hepes. Surrounding cumulus granulosa cells were removed by repeated pipetting through a fine bore tip and stage of oocyte meiosis was evaluated based on nuclear configuration. Mature, metaphase II (MII) arrested ova with homogenous cytoplasm and an extruded polar body were washed three times through pre-equilibrated BO-IVC medium (IVF Bioscience) and maintained in 100 µL drops under oil at 37°C in 5% CO2 humidified air until use. Immature recovered oocytes were similarly treated and cultured for 22 hours in TALP to allow time for completion of meiosis to MII arrest.

3.3.8 Intracytoplasmic sperm injection and embryo culture

Sperm preparations for ICSI were made by pipetting 5 µL of sample suspension into a 5 µL drop of FBS in a 60 mm petri dish. Three successive 1:20 dilutions were made into adjacent drops of fresh TALP+Hepes warmed to 37°C. An additional 20 µL drop of TALP+Hepes was added to the dish to which MII ova were transferred before the plate was flooded with oil to cover all drops. Sperm injections were performed on a Zeiss Axiovert A1 inverted microscope using Narashigi 3-axis hydraulic manipulators. Suction was applied through a glass holding micropipette with a 120 µm outer diameter, 30 µm inner diameter, and a 30° angled bend to hold the ova in place. Sperm of normal morphology and with twitching flagellar movement were selected from the diluted sample drops and injected into the oocyte cytoplasm using a spiked glass pipette with a 5 µm inner diameter, 10 µm bevel, and a 30° angled bend. Following completion of injections,

oocytes were transferred to warmed BO-IVC and cultured at 37°C in 5% CO2 in humidified air. Subsequent embryos were then monitored daily and medium was changed every 48 hours.

3.3.9 Embryo transplant

The recipient rhesus macaque was positioned in dorsal recumbency followed by sterile preparation and draping of the abdomen. A linear 4 cm ventral midline laparotomy was utilized to enter the abdomen, followed by placement of a small Balfour retractor. The ovaries were mobilized with Debakeys and an ovulation site was identified on the right side. The ovary was suspended with Hartman forceps at the utero-ovarian ligament and vascular pedicle and .047" silastic tubing was inserted to a depth of approximately 1 cm in the oviduct via the fimbria, where 2 embryos were deposited. The ovarian exposure was reduced and the Balfour removed. The abdomen was closed with simple continuous 3-0 Vicryl in the rectus fascia and subcutis, with skin apposition by continuous intradermal 4-0 Monocryl. The recipient was allowed to recover on the O.R. table until extubation.

3.3.10 Delivery via C-section

The monkey was positioned in dorsal recumbency followed by sterile preparation and draping. The abdomen was entered via 10 cm linear ventral midline laparotomy, followed by delivery of and draping of the gravid uterus with moistened lap pads. The fetus was balloted to the fundic region where a fundic transverse hysterotomy was made and delivery of the infant was completed. The umbilical cord was clamped with Hartmans and transected. Finger

dissection of the placenta preceded release and delivery. The hysterotomy was then closed with two layers of 3-0 coated Vicryl with a cushing over simple continuous in an inverted pattern and hemostasis was noted. The rectus fascia was closed with continuous 3-0 coated Vicryl. Subcutis and skin apposition were with continuous intradermal 4-0 Monocryl.

3.3.11 Statistical Analyses

All statistical analyses were performed using Graphpad Prism. One-way ANOVA was used to compare the graft weights, seminal vesicle weights and to compare the percentages of tubules with germ cells at various stages of differentiation. Dunnett's test was used to perform multiple comparisons for analyzing the differences in graft weights, seminal vesicle weights and percent tubules with PGP9.5+ cells between the treatment groups and Tukey's test was used to perform multiple comparisons to analyze the differences percent tubules with germ cells, SCO phenotype, PIWIL1+ and TP1+ cells between the treatment groups. P values less than 0.05 were considered significant and marked * for <0.05, ** for <0.01, *** for <0.005 and **** for <0.001.

3.4 Results

3.4.1 Experimental design

Recipient mice were gonadectomized at the time of grafting to elevate gonadotropin secretion to mimic pubertal levels as reported previously [320]. While spermatogenesis occurs

spontaneously in fresh prepubertal primate xenografts [319, 320], cryopreserved primate testicular xenografts have been shown to exhibit complete loss of germ cells [321]. Other studies have demonstrated that treatment of recipient mice with hCG or vascular endothelial growth factor (VEGF) improved graft outcome in terms of extent of maturation [191], graft size [325] or number of intact seminiferous tubules [326]. I tested the efficacy of these treatments on cryopreserved Rhesus xenografts by dividing recipient mice into 4 treatment groups: the first group of mice was left untreated, the second group received 10 IU hCG biweekly, the third group received 100 ug of VEGF in the graft site at the time of grafting and the fourth group of recipients received both hCG and VEGF. At 6 months post-grafting, mice were euthanized and grafts were excised for analysis (**Figure 11**).

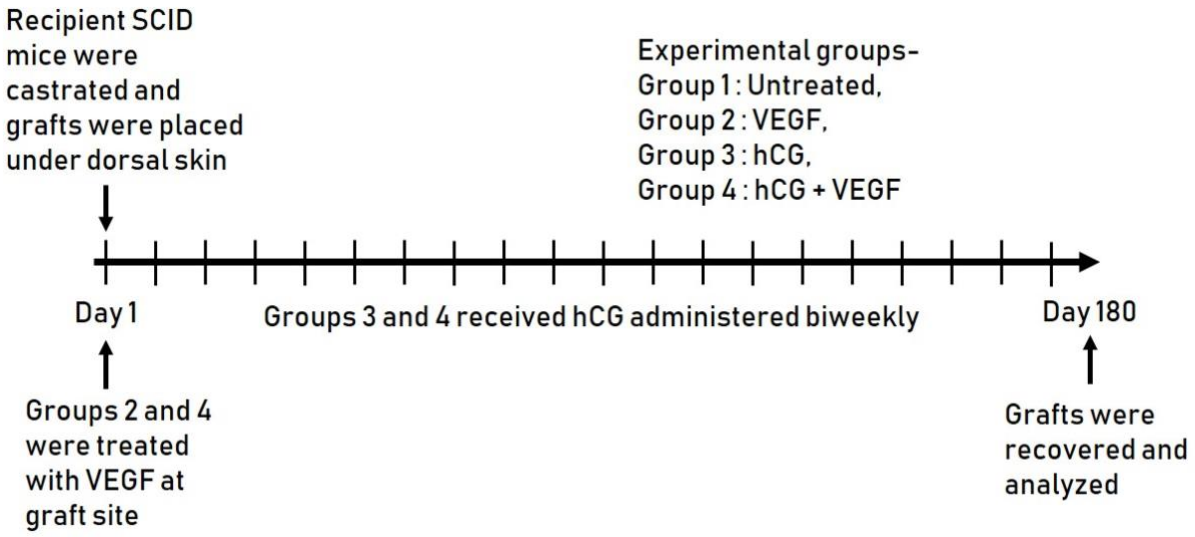

Figure 11: Schematic description of the study.

Recipient SCID mice were castrated and xenografted with prepubertal Rhesus macaque testicular tissue under the dorsal skin. Mice were divided into 4 groups- mice in the untreated group received no treatment (n=2), mice in the VEGF group received 1µg of rhVEGF-165 at the graft site at the time of grafting (n=2), mice in the hCG treatment group received 10U of hCG subcutaneously 2 times a week until the grafts were recovered at 6 months (n=2) and mice in the hCG+VEGF treatment groups received both treatments (n=2). Mice were euthanized at 6 months and grafts were recovered for further analyses.

3.4.2 Donor testicular tissue samples exhibit presence of undifferentiated spermatogonia but lack meiotic and post-meiotic germ cells at the time of grafting.

In this study, prepubertal primate testicular tissue pieces were grafted under the dorsal skin of severe combined immune deficient (SCID) mice. Absence of spermatogenesis in donor testicular tissue at the time of grafting was confirmed via hematoxylin and eosin (H&E) staining (**Figure 16A**) and immunohistochemistry for markers of germ cells at various stages of differentiation (**Figure 12**). At this time, seminiferous tubules exhibited the presence of SOX9+ Sertoli cells and VASA+ (Dead box helicase 4 or DDX4) germ cells that also expressed PGP9.5

(protein gene product 9.5 or UCHL1), a marker of undifferentiated spermatogonia, but lacked the expression of PIWIL1 (PIWI-like protein 1) and TP1 (Transition Protein 1), that mark spermatocytes and spermatids, respectively (**Figure 12A, C, E, G**). Adult testicular cross sections are included for comparison (**Figure 12B, D, F, H and Figure 16B**).

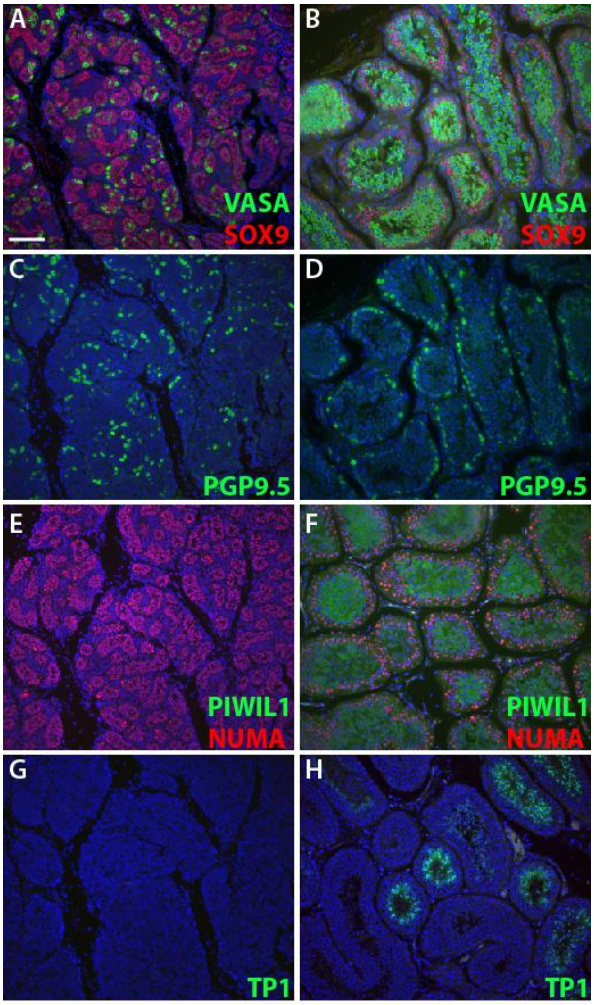

Figure 12: Immunofluorescence analysis of donor prepubertal testicular tissue at the time of grafting.

In contrast to the multiple layers of VASA+ germ cells observed in adult nHP testicular cross sections (B), immunostaining of prepubertal donor tissue cross-sections showed the presence of fewer VASA+ germs cells along with SOX9+ Sertoli cells (A), that is typical of the testis in that stage. PGP9.5+ spermatogonia were present in the prepubertal samples (C), while no evidence of PIWLI1+ spermatocytes (E) and TP1+ spermatids (G) were detected, confirming lack of spermatogenesis in the donor samples. Adult cross-sections stained with VASA (B), PGP9.5 (D), PIWIL1 (F) and TP1 (H) were used as controls. Scale bar=100µm.

3.4.3 hCG+/-VEGF treated mice had larger grafts and seminal vesicles at 6 months post-grafting

Grafts and recipient seminal vesicles were excised and analyzed 6-7 months post grafting. Mice from the hCG treatment groups had significantly larger grafts (p values: hCG=0.0001 and hCG+VEGF=0.0010, mean±SEM: Untreated- no grafts recovered, VEGF = 16.21±2.62 mg, hCG = 101.64±17.13 mg, hCG+VEGF = 62.58±9.79 mg) (**Figure 13 A-D**). No grafts were recovered from the untreated group at 6 months. Seminal vesicles in the hCG treatment groups also showed significant enlargement compared to gonadectomized control mice that did not received grafts, indicating testosterone production in the hCG±VEGF-treated grafts (p values: hCG=0.0005, hCG+VEGF=0.0014, mean±SEM: Untreated = 0.00±0.00, VEGF = 0.00±0.00, hCG = 217.08±46.25, hCG+VEGF = 227.5±0.00, castrated negative control = 24.72±2.24, uncastrated positive control = 305.41±12.79) (**Figure 14 A-E**).

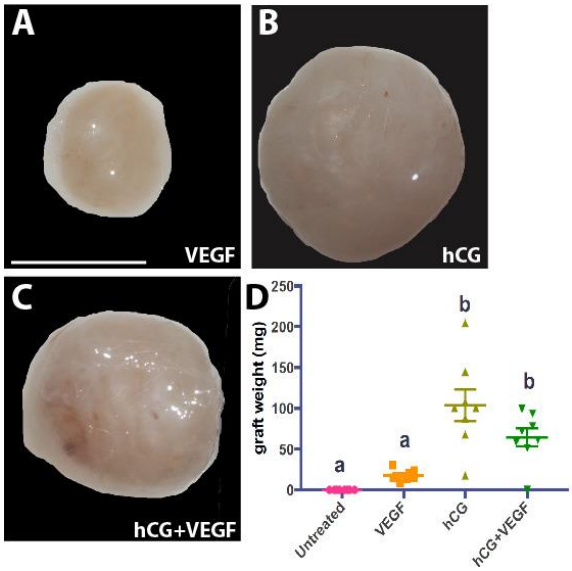

Figure 13:Treatment dependent recovery and weights of grafts

At 6 months post-grafting, no grafts were recovered from the untreated group. Grafts recovered from the hCG (B) and hCG+VEGF (C) treated groups were significantly larger than those recovered from the VEGF treated group (A) (n=8 grafts in each group). Scale bar = 0.5cm. Data in (D) are mean±SEM of graft weights (mg). Bars are labeled with letters a and b to indicate statistically significant difference in means. P values were determined using one-way ANOVA and Tukey's multiple comparisons test.

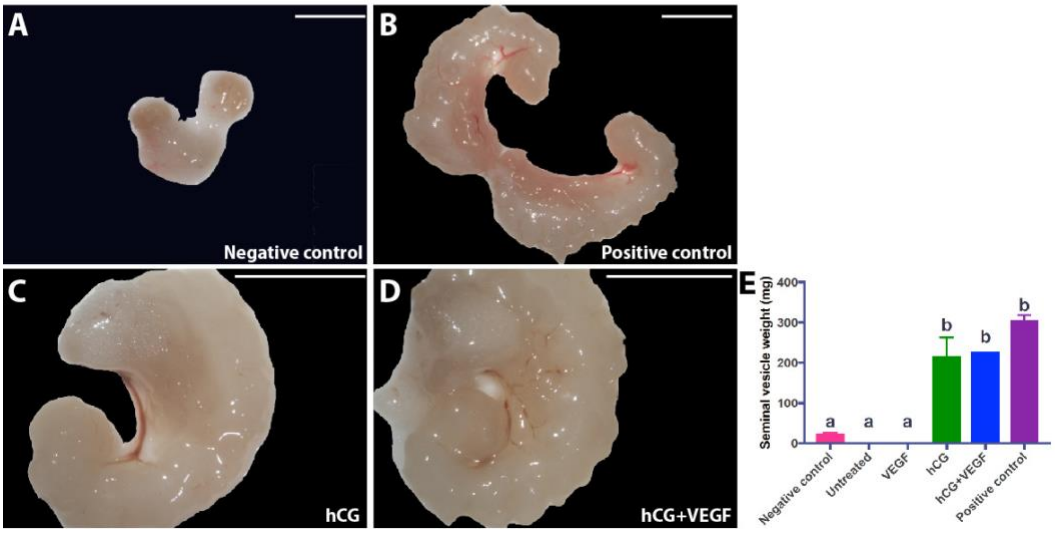

Figure 14: Treatment dependent recovery and weights of recipient mouse seminal vesicles.

Seminal vesicles were recovered from mice at 6 months post-grafting. Mice from the hCG **(C)** and hCG+VEGF **(D)** treatment groups (n=2,1) had significantly larger seminal vesicles compared to castrated negative controls **(A)** (n=3) (p=0.001, 0.003). There were no significant differences in the weights of seminal vesicles obtained from uncastrated positive control males **(B)** and those recovered from hCG±VEGF-treated mice. No seminal vesicle were recovered from untreated and VEGF-treated mice. Scale bars = 0.5cm. The data on (E) are mean±SEM. Bars are labeled with letters a and b to indicate statistically significant difference in means. P values were determined using one-way ANOVA and Tukey's multiple comparisons test.

3.4.4 Immunohistological analysis confirms complete spermatogenesis in hCG-treated grafts

To assess the extent of maturation in the recovered grafts, we analyzed cross-sections of grafts by immunostaining for VASA, SOX9, PGP9.5, PIWIL1 and TP1. VASA+ germ cells and SOX9+ Sertoli cells were present in grafts from all treatment groups (VEGF, hCG, hCG + VEGF). No grafts were recovered from untreated recipients. Grafts recovered from the VEGF only treatment group retained VASA+ germ cells and PGP9.5+ undifferentiated spermatogonia (**Figure**

15A-B) but PIWIL1+ spermatocytes and TP1+ spermatids were not detected (**Figure 15B-D**). hCG+VEGF treated grafts had a significantly increased number of tubules with VASA+ germ cells compared to VEGF only grafts (p=0.006) (**Figure 15 M**) and correspondingly, a significantly lower number of tubules with Sertoli cell only (SCO) phenotype (p=0.0023). Pgp9.5+ undifferentiated spermatogonia were detected in 52.48±16.78%, 49.24±7.59% and 55.31±10.00% of tubules in VEGF, hCG and hCG+VEGF treated grafts, respectively. PIWIL1+ spermatocytes were not detected in grafts from VEGF-treated recipients, but were detected in 40.36±6.59% of all tubules in grafts from hCG only treated recipients and in 24.30±4.12% of all tubules in hCG+VEGF treated recipients (**Figure 15G, K and O**). TP1+ spermatids were not detected in grafts from VEGF-only treated recipients, but were detected in 28.57±5.51% and 14.88±3.26% of all tubules in grafts from hCG only and hCG+VEGF treated recipients, respectively (**Figure 15H, L and P**). Sections were stained with primate-specific NUMA (Nuclear mitotic apparatus protein 1) to confirm that all analyzed cells in the grafts were derived from the rhesus donor (**Figure 15C, G and K**). Spermatozoa were observed in all hCG±VEGF-treated grafts when cross-sections were stained with H&E (**Figure16D, E**).

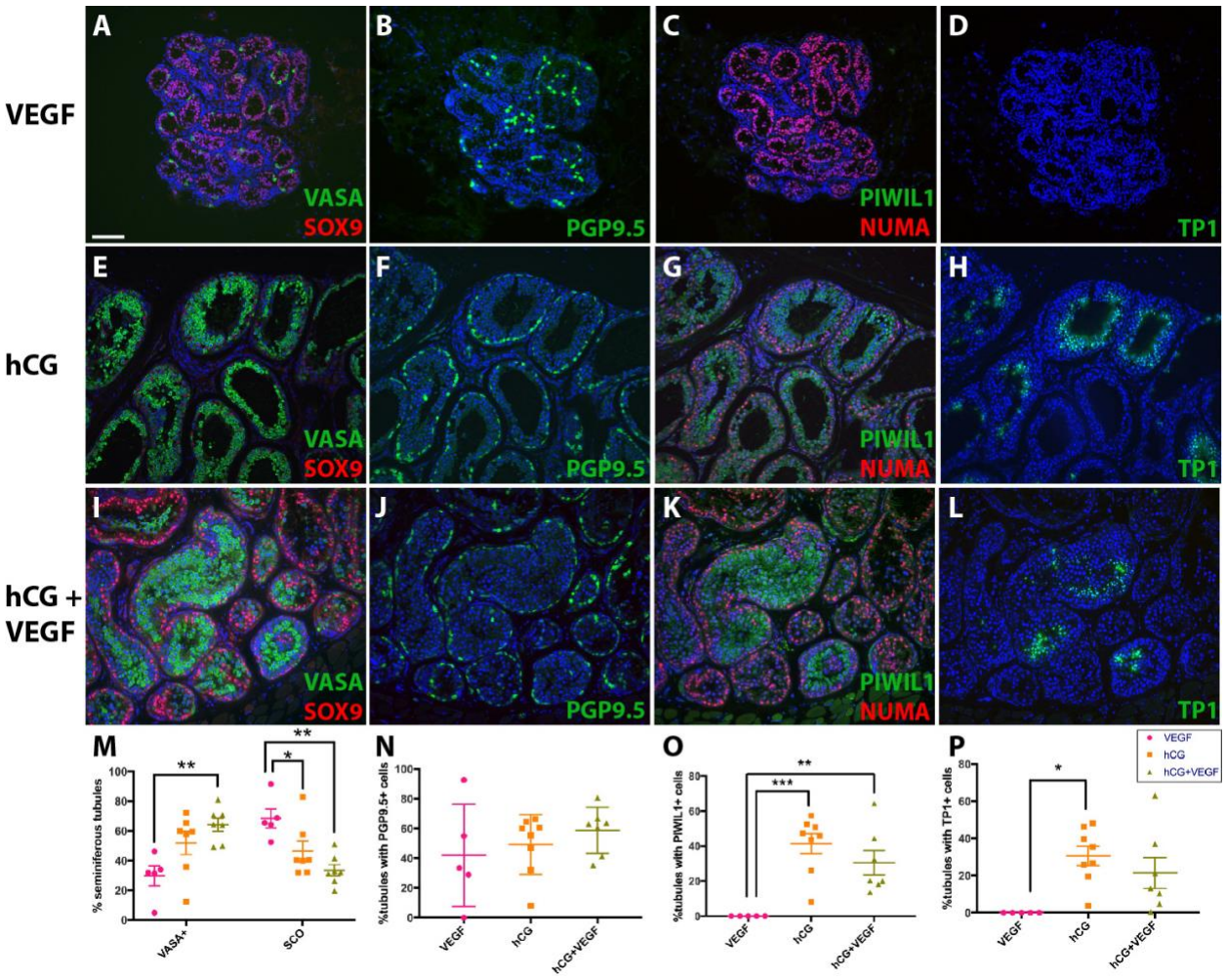

Figure 15: Immunohistological evaluation of spermatogenic development in grafts

Cross-sections of grafts were stained with VASA and SOX9 **(A, E, I)**, PGP9.5 **(B, F, J)**, PIWIL1 and NUMA **(C, G, K)** and TP1 **(D, H, L)**. Quantification of the occurrence of the markers in grafts from 3 treatment group revealed that VEGF treated grafts had a significantly lower number of tubules with VASA+ cells compared to hCG+VEGF treated grafts (n= 5 VEGF treated grafts and 7 hCG+VEGF treated grafts, p = 0.0045) and a significantly higher number of tubules with both hCG (n = 8, p = 0.03) and hCG+VEGF (p = 0.002) treated grafts **(M)**. There were no significant differences in the retention of PGP9.5+ spermatogonia in the 3 treatment groups **(N)**. PIWIL1+ spermatocytes were not detected in any VEGF-treated grafts and were found in 40.36±6.69% and 24.30±4.12% of tubules in the hCG and hCG+VEGF treated groups and were significantly higher than the VEGF treated grafts (p = 0.0005 and 0.0081, respectively) **(O)**. hCG treated grafts had TP1+ spermatids in 28.57±5.51% of tubules, significantly higher compared to VEGF treated grafts where no spermatids were detected (p = 0.01). hCG+VEGF treated grafts had spermatids in 14.88±3.26% of tubules and was not significantly different compared to VEGF treated grafts (p = 0.08). Scale bar = 100µm. Data in **M, N, O and P** are mean±SEM. P values were determined using one-way ANOVA and Tukey's multiple comparisons test.

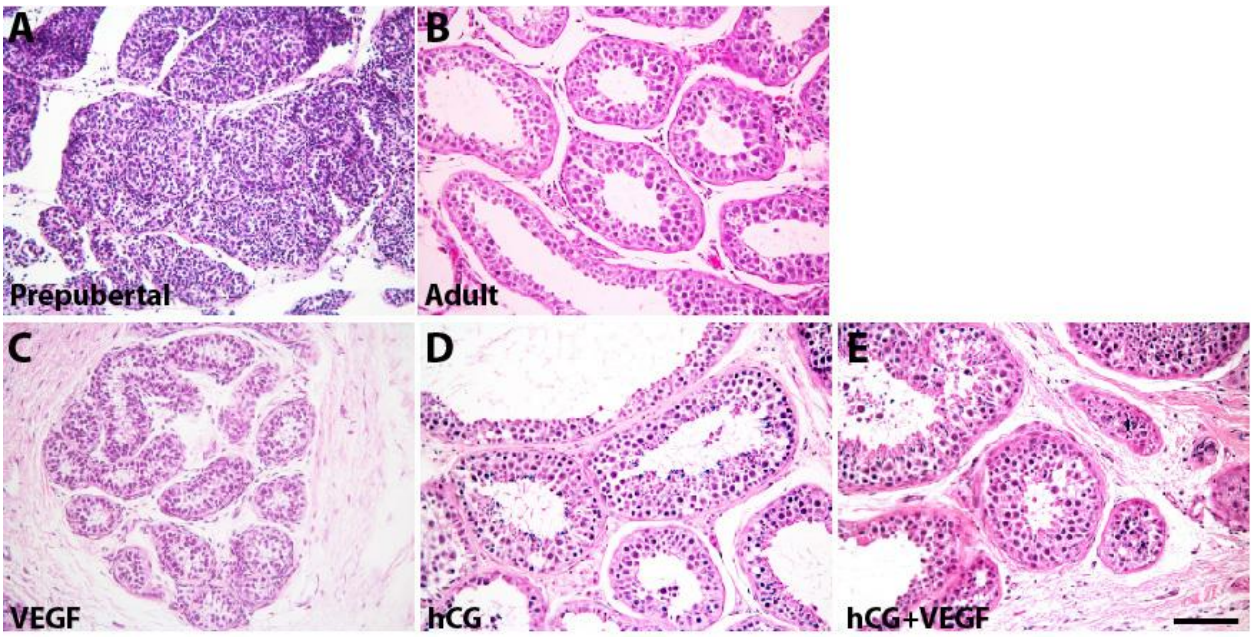

Figure 16:Histology of grafts compared to prepubertal and adult Rhesus testicular cross-sections.

Grafts recovered at 6 months were fixed and stained with hematoxylin and eosin. Cross-sections of VEGF-treated grafts **(C)** were similar in morphology to prepubertal testicular tissue **(A)** with intact tubules containing Sertoli cells and some germ cells. In contrast, cross-sections of hCG- and hCG+VEGF-treated grafts **(D, E)** resembled adult Rhesus testicular tissue **(B)** with the presence of multiple layers of germ cells and enlarged lumen. Spermatozoa were observed in the lumen of these cross-sections, indicating the occurrence of complete spermatogenesis in these grafts. Scale bar = 100μm.

3.4.5 Effect of biopsy size on outcome of grafting

Next, I tested the effect of biopsy size on the outcome of xenografting. Fertility preservation centers around the world excise up to 20% of the patient's testicular volume for cryopreservation [51, 52]. This sample is then dissected further for qualitative and quantitative analyses, while the rest is cryopreserved. Therefore, depending on the age of the donor, the size of patient testicular tissue that can be used for xenografting can be as small as $1mm^3$. Cryopreservation of human testicular tissue has been shown to lead to a significant loss of viable cells [327, 328].The process of xenografting itself may further decrease the number of germ cells

due to exposure to hypoxia prior to onset of vascularization. Human ovarian xenografts in nude mice were shown to be exposed to hypoxic conditions for 5 days before a gradual increase in oxygenation was observed [329]. I, therefore, asked if reduced input graft size would have an effect on the extent of germ cell maturation- on one hand smaller grafts are likely to be perfused more rapidly than larger grafts while on the other hand they retain a smaller number of germ cells that could potentially lower the probability of spermatogenic events. To test the effect of graft size, I grafted cryopreserved rhesus testicular tissue pieces that were small (1-4mm^3) in size and compared the extent of maturation with larger grafts (9-20mm^3) that successfully produced sperm in the previous experiments (**Figure 17A**). All recipients were treated with hCG biweekly, as this treatment group has the highest percentage of tubules with spermatids in the previous experiment (**Figure 15P**). Immunohistological analysis of grafts upon recovery after 6 months showed the occurrence of spermatogenesis in all grafts irrespective of graft size (**Figure 17A-G**). In addition, there were no statistically significant differences between percent tubules with VASA+, PGP9.5+, PIWIL1+ and TP1+ germ cells between the 2 groups (**Figure 17F**). These results indicate that limitations in obtaining a significant amount of input testicular tissue from the donor is not a barrier to the successful application of this method in the clinic.

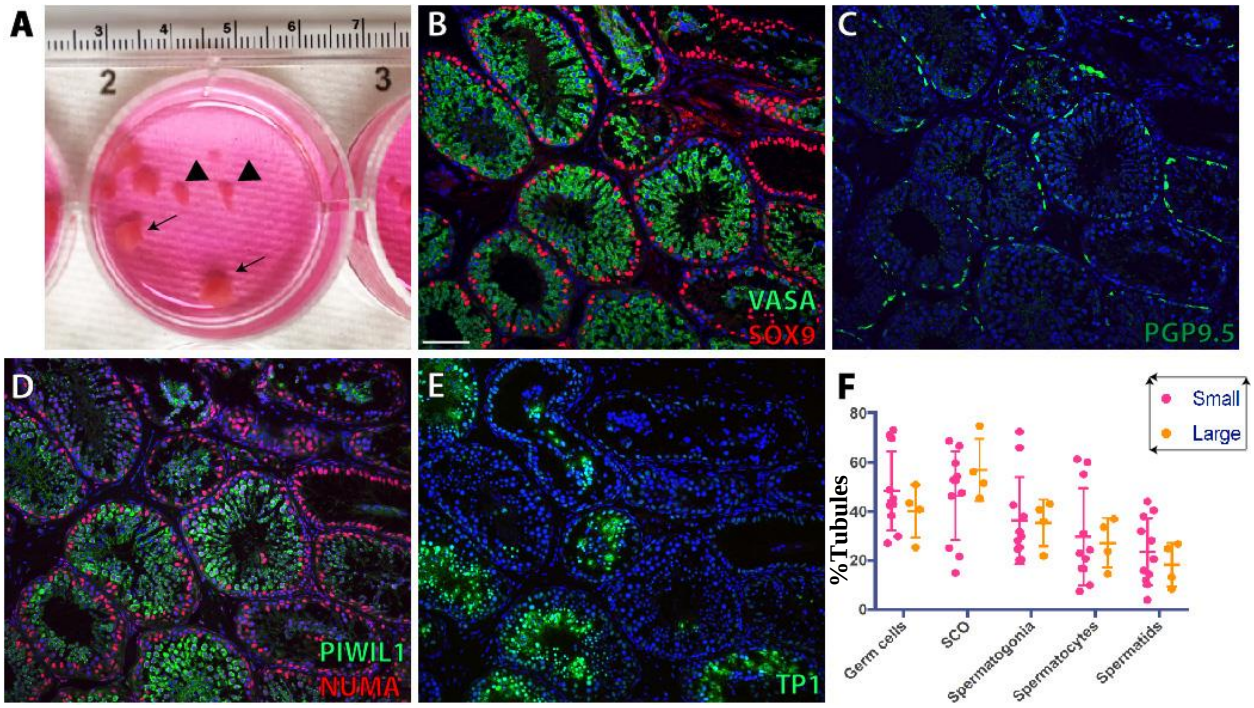

Figure 17: Comparing large and small cryopreserved testicular tissue

Prepubertal testicular tissue cut into 1-4mm3 fragments, indicated with arrow heads (A) were xenografted into mice and the outcome of grafting was compared with that of grafting larger pieces (9-20mm3), indicated with arrows in **(A)**. All recipients with treated with hCG. Immunohistological staining with VASA and SOX9 **(B)**, PGP9.5 **(C)**, PIWIL1 **(D)** and TP1 **(E)** and quantification showed that all grafts had differentiated germ cells up to the spermatid stage, irrespective of graft size, and there were no significant differences in %tubules with germ cells, Sertoli cell-only phenotype, spermatocytes and spermatids between large (n=4) and small grafts (n=11) **(F)**. Scale bar = 100µm. Data in **(F)** are in mean±SEM. Unpaired t-tests were used to determine if the differences in the occurrence of cells expressing the markers used in the experiment were statistically significant.

3.4.6 Sperm recovered from hCG-treated grafts were used to fertilize rhesus oocytes

To test the fertilization potential of spermatozoa observed in the hCG+/-VEGF grafts, we mechanically dissected grafts to release spermatozoa (**Figure 18A**). Aliquots of spermatozoa were shipped to the Oregon National Primate Research Center at ambient temperature for Intracytoplasmic Sperm Injection (ICSI) and were found to be fully formed and with normal morphology following shipment. Less than 5% displayed flagellar twitching without forward

progression, similar to reports of samples obtained from testicular sperm extractions (TESE) recovered from men for use in ICSI [330]. Spermatozoa from 1 graft were selected based on abundance and morphology and were used to fertilize oocytes retrieved from 2 female donors (**Figure 18B**). ICSI was performed on 87 metaphase II-stage oocytes, 27 of these oocytes cleaved into 2-cell stage embryos (fertilization rate = 39.0%±0.3%) (**Table 3**). Embryos were monitored for progression into the morula stage (Figure 16C). Two out of the 27 cleaved embryos developed further to form blastocysts (blast rate = 7.4%) (**Figure 18D**). Both blastocysts were transferred into 1 recipient female monkey on January 31st, 2019 and pregnancy with 1 offspring was confirmed by evaluating serum estrogen and progesterone levels at 2.5- and 3.5-weeks post-embryo transfer, both of which were within the positive range for pregnancy. Routine ultrasound evaluations were conducted to ensure normal fetal development (**Figure 18E**).

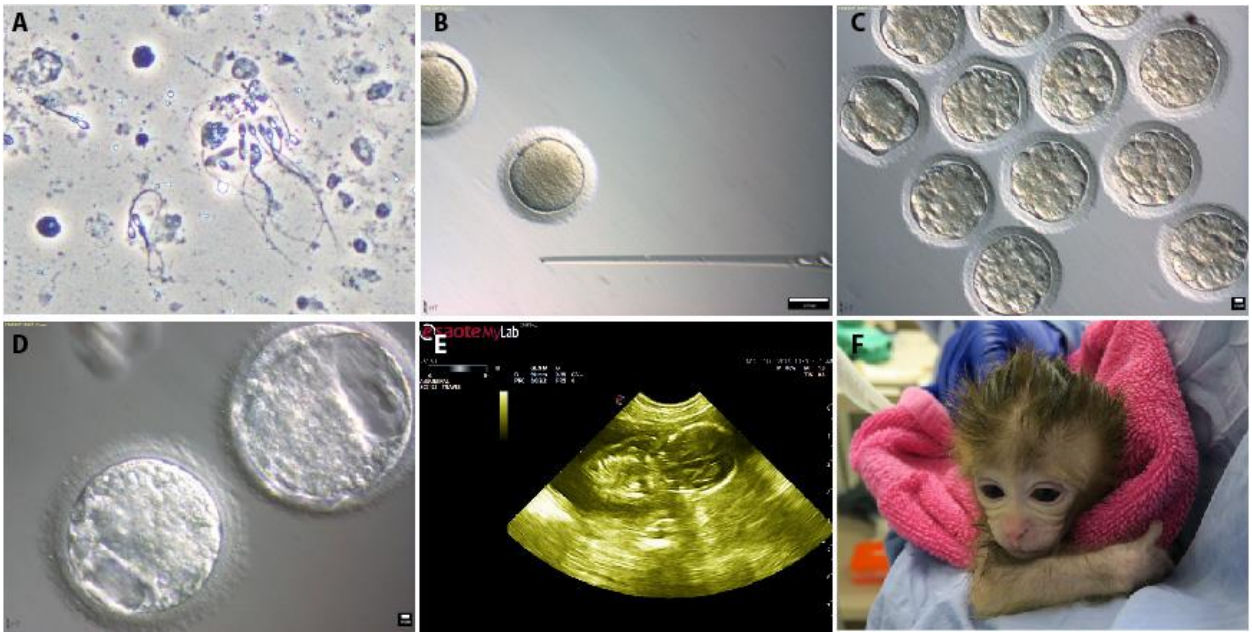

Figure 18: Sperm extracted from xenograft were used to fertilize oocytes and generate healthy offspring.

Spermatozoa were extracted from hCG±VEGF-treated grafts by mechanical dissection **(A)**. Spermatozoa that displayed normal morphology were selected and used to fertilize Rhesus oocytes by ICSI **(B)**. Morula stage and blastocyst stage embryos were observed at days 5 and 10 post-injection, respectively **(C, D).** Two blastocysts were transferred into a recipient female Rhesus macaque on January 31st, 2019 and pregnancy was monitored via ultrasonography at regular intervals **(E)**. Cryxus (offspring from cryopreserved xenografted testicular tissue) was born on July 7th, 2019 **(F)**.

Table 3: Oocyte retieval and fertization rate

Total # Oocytes	#GV	#MI	#MII	#Deg	# Cleaved	Fert Rate	# Blastocysts	# Transferred	Pregnancy?
69	24	4	40	5	22	55%	0	0	-
56	9	12	47	0	5	11%	2	2	PREGNANT!

3.4.7 Generation of live offspring from xenograft sperm

Infant Cryxus (offspring from cryopreserved xenografted testicular tissue) was born on July 7th, 2019 via c-section. C-section was scheduled due to evidence of mild vaginal spotting and suspected leakage of amniotic fluid from the pregnant dam. At 452g, the infant's weight at birth was lower than the reported average weight of male Rhesus infants (506±74g) [331]. The fetal/placental ratio was also observed to be lower than average (1.85 compared to 3.5-3.8). The placenta was enlarged (243.9g), with evidence of necrosis in the secondary lobe (**Figure 19**). Assessment of the infant using the Apgar test showed that the infant displayed adequate recovery within 30 minutes after surgical birth with Apgar scores of 5, 8 and 11 at 1 minute, 5 minutes and 30 minutes after birth. These scores are consistent with previous reports on surgically delivered macaque infants [322, 332]. Cryxus displayed normal growth and nursing/feeding habits with no further complications.

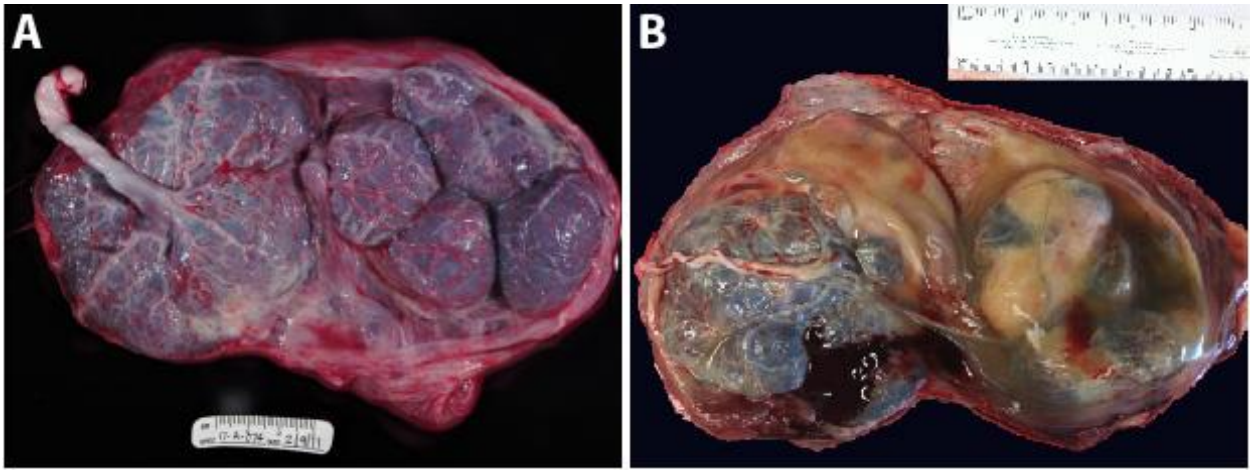

Figure 19: Morphology of the placenta at birth.

At 243.9g, the placenta recovered from this pregnancy (B) was found to be enlarged compared to normal Rhesus macaque placentas (A). Necrosis was observed in secondary lobe of the placenta.

3.5 Discussion

Autologous grafting and xenografting of testicular tissue have been proposed as methods for fertility preservation in pediatric patients exposed to cytotoxic chemotherapy or radiation [207]. Fayomi et al. recently demonstrated the efficacy of autologous grafting in a rhesus macaque model wherein cryopreserved donor testicular tissue pieces were thawed and grafted back into the donor to induce the production of sperm that were then used to generate a live offspring [322]. Autologous grafting, hence, is potentially ready for clinical translation especially in patients undergoing exposure to cytotoxic treatments due to non-malignant disorders such as sickle cell anemia, etc. However, application of this method to patients with malignant disorders such as leukemia, lymphoma or metastatic cancers may be hindered by the risk of reintroducing malignant cells back into the donor. In addition, successful onset of spermatogenesis with autologous grafting

has only been demonstrated when the recipients were castrated at the time of grafting [333]. This poses an additional barrier to translation of the method to the clinic. In testicular xenografting, donor testicular tissue is grafted into a recipient of a different species, thereby circumventing the aforementioned risks associated with autologous grafting.

Testicular xenografting has been demonstrated as a method to rescue fertility in over 18 donor species [315]. Previous reports on xenografting Rhesus testicular tissue demonstrated success when using fresh (non-cryopreserved) donor tissue [191, 320, 325], while cryopreserved xenografts exhibited loss of germ cells [321]. In the current study, I showed that exposure of recipient mice to exogenous hCG promotes spermatogenesis in cryopreserved Rhesus xenografts. Spermatozoa recovered from grafts were used to fertilize oocytes via ICSI. The fertilization rate of 33% observed in this was lower than the reported rates using ejaculated sperm (~77%) [334] but comparable to the fertilization rate reported using sperm from autologous grafts (28%) [322]. The low fertilization rate observed in the study may be indicative of poor sperm quality or because non-motile testicular sperm have lower fertilization potential than mature motile ejaculated sperm. Morphological assessment of sperm recovered from the grafts revealed that most sperm were tightly bound to cells and debris. This could be due to incomplete spermiation or due to the fact that each graft is a closed system with no outlet for the release of the sperm produced unlike the testis.

Pregnancy was established with 1 out of the 2 embryos transferred to a recipient. Monitoring of the fetus via ultrasonography showed normal development in utero. At birth, Cryxus had below average body weight and fetal to placental ratio with observable necrosis in the secondary lobe of the placenta. Necrosis and fetal growth restriction are commonly observed in

association with placental infarction or gestational metabolic disorders [335]. The cause in the context of the current study is unknown. Assessments carried out periodically after birth indicate normal growth and social development of the offspring.

In summary, through this study, I have demonstrated that cryopreserved prepubertal Rhesus testicular xenografts in mice can undergo complete spermatogenesis when exposed to exogenous hCG. This is a crucial advancement towards the translation of the testicular xenografting as a potential method for fertility restoration in prepubertal patients who have cryopreserved their testicular tissues. Further studies using human testicular tissue need to be carried out to demonstrate the efficacy of this method on human tissue and its potential for translation into the clinic.

4.0 Xenografting human testicular tissue into mice

This is an adaptation of the author's version of the work. The definitive version is being prepared for publication.

"**Effect of exogenous human chorionic gonadotropin administration on cryopreserved prepubertal human xenografts"**

Sherin David[1,2], Meena Sukhwani[1], Karen Peters[1], Hanna Valli-Pulaski[1], Sarah Steimer[1], Kyle E. Orwig[1,2]

1) Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

2) Magee-Womens Research Institute, Pittsburgh, PA, USA

3) Molecular genetics and developmental biology program, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA.

4.1 Chapter Summary

Testicular tissue xenografting has been studied extensively using various animal models as a method to rescue fertility in prepubertal patients at the risk of iatrogenic infertility. In addition, this method is currently being used as a bioassay to study the effects of exposure to environmental factors on testicular somatic cell function. While there have been several reports describing outcomes of xenografting human testicular tissue into immune compromised mice, successful induction of complete spermatogenesis in the grafts has not been reported. The current study described the effect of exposure of recipient mice exogenous human chorionic gonadotropin (hCG) and vascular endothelial growth factor (VEGF) on the development of cryopreserved prepubertal human testicular tissue xenografts. I found that at 6 months post-grafting, mice that received hCG had significantly larger grafts with enlarged tubules compared to mice that were untreated. However, immunohistological analyses revealed a dramatic loss of germ cells in all grafts, irrespective of the treatment. Further studies need to be carried out to further optimize this method for human testicular tissue.

4.2 Introduction

Human testicular tissue xenografting was first proposed as a bioassay to study germ and somatic cell function in the human testis [336]. Several reports demonstrated the use of the method to evaluate the effects of environmental factors and drugs such as bisphenol A, phthalates, ibuprofen, acetaminophen, etc. on steroidogenesis and germ cell density in fetal

testicular tissue [337-340]. Xenografting may also have an application in restoration in adult survivors of pediatric cancers who cryopreserved their testicular tissues prior to chemotherapy and radiation treatments of cancer or hematological disorders. In addition, there has been a growing recognition of the need for fertility preservation/restoration options in transgender patients [341-343]. Prepubertal and adult transgender females who do not go through male puberty will not be able to mature their testicular tissues to produce sperm. [344]. However, these patients may choose to cryopreserve a testicular tissue biopsy before initiating hormone suppression or cross-sex steroid treatments. Approaches to mature testicular tissue outside the patient's body may be required. Xenografting may have application in these cases since it involves the grafting of donor tissue into an animal host, thereby circumventing the need for trans women to have to discontinue gender confirming hormone therapy to initiate endogenous spermatogenesis.

Human testicular tissue xenografting in the context of fertility preservation has been documented in several recent reports, as summarized in **Table 4**. These studies have demonstrated the survival of prepubertal human grafts and the retention of spermatogonia for up to 1 year in the murine microenvironment. However, onset of spermatogenesis in donor testicular tissue has not been reported. The current study tests the effects of VEGF and hCG on the survival and maturation of germ cells in human testicular tissues that are xenografted into immune-deficient mouse hosts.

Table 4: Literature review of reports on human testicular tissue xenografting

Authors	Donor age	Fresh/frozen	Mouse strain	Castration +/-	Graft location	Treatment	Duration	Most mature germ cell stage	Markers used	Citation
Yu et al., 2006	20-26 week fetus	Fresh	Nu/Nu	Yes	dorsal skin	None	4-4.5 months	Spg	None	[197]
Schlatt et al., 2006	Adult	Fresh	Nu/Nu and SCID	Yes	dorsal skin	None	up to 19 weeks	Spg	None	[345]
Geens et al., 2006	Adult	Fresh	Nu/Nu and SCID	Yes	dorsal skin	None	up to 6.5 months	Spg	None	[346]
Wyns et al. 2007	2-12yo cryptorchid patients	Frozen	Nu/Nu	Yes	Scrotum	None	3 weeks	Spg	MAGEA4, Vimentin, Ki67	[347]
Goossens et al., 2008	10-11yo	Fresh	Nu/Nu	Not stated	dorsal skin	None	4 and 9mo	Spg	MAGE-A4, Vimentin	[348]
Wyns et al., 2008	7-14yo	Frozen	Nu/Nu	Yes	Scrotum	None	6 months	Spg	MAGE-A4, Ki67, Caspase3, LDH-C, ACE, 4D4, 3βHSD	[349]
Sato et al., 2010	3mo infant	Fresh	Nu/Nu	Yes	dorsal skin	None	1, 2, 4, 7mo and 1 year	Spc	BOULE, CDC25A, AR and 3βHSD	[198]
Van Saen et al., 2011	3, 5, 12 and 13yo	Fresh and Frozen	Nu/Nu	No	Testis	None	4 and 9mo	Spc in fresh older donor grafts and Spg in frozen grafts	MAGE-A4, Ki67, Caspase3, LDH-C, ACE, 4D4, 3βHSD, BOLL	[200]

Table 4 continued

Van Saen et al., 2013	2.5-12.5yo	Fresh and Frozen	Nu/Nu	No	Testis	FSH	9-12mo	Spc	Vimentin, MAGE-A4 and BOLL	[350]
Peols et al., 2013	2-12yo	Frozen	Nu/Nu	Yes	Scrotum	None	6mo	Spg	MAGE-A4, Ki67, 3βHSD	[351]
Ntemou et al., 2019	3, 8 and 9yo	Frozen	Nu/Nu	No	Testis	VEGF	4 and 9mo	Spc	MAGE-A4, BOLL, γH2AX	[326]

4.3 Materials and Methods

4.3.1 Experimental animals

The use of severe combined immunodeficient (SCID) mice (Cat# ICRSC-M, Taconic Biosciences) and Rhesus Macaques in the study was approved by the Institutional Animal Care and Use Committees (IACUC) of Magee-Womens Research Institute (MWRI), the University of Pittsburgh and the Oregon Health and Science University. Experiments were performed in accordance to the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals.* Recipient mice were obtained at 5 weeks of age and were allowed to acclimatize to the housing environment for a week prior to xenografting.

4.3.2 Testis collection, processing and cryopreservation

Deidentified patient testicular tissue biopsies were obtained through the Fertility Preservation Program of the University of Pittsburgh Medical Center and coordinated recruitment sites with approval of the University of Pittsburgh Institutional Review Board (STUDY19020220; STUDY19110083; STUDY19070264). Age and medical disposition of donors used in this study are summarized in table 5. Samples were stored and transported in Lactated Ringers Solution (Cat# NC0957153, Fisher Scientific) at 4ºC and were by dissecting of the tissue into small pieces in a sterile laminar flow biosafety cabinet. Testicular tissue pieces were then cryopreserved in a

cryoprotectant medium composed of 5% Serum Substitute Supplement (SSS, Cat# 99193, Irvine Scientific) and 5% dimethyl sulfoxide (DMSO, Cat# 67457-178-10, Mylan) in Quinn's Advantage Blastocyst Media (Cat# ART-1029, Origio). Testicular samples were cryopreserved using the controlled slow freezing protocol in the Freeze Control® temperature controller (Cat # CL-5500, CryoLogic), as described previously [49] with slight modifications. Briefly, cryovials were equilibrated to the cryoprotectant medium at 4ºC for 30 minutes. Samples were then cooled from 4ºC to 0C at a controlled rate of -1ºC/minute, then held at 0ºC for 5 minutes, followed by cooling to -8ºC at the rate of -0.5ºC/minute. Samples were manually seeded at -8ºC followed by cooling to -40ºC at the rate of -0.5ºC/minute. Samples were held that this temperature for 10 minutes followed by rapid cooling to -80ºC at -1.5ºC/minute. At this point, the vials were plunged into liquid nitrogen, where they were stored until application for xenografting.

Table 5: Age and diagnoses of donors

Patient ID	Age	Diagnosis	Patient ID	Age	Diagnosis
TTC-025	7	Ewing's Sarcoma	CN-014	12	Beta thalassemia major
CN-011	10	Ewing's Sarcoma	CN-019	8	Sickle cell disease
LCH-003	5mo	Embryonal rabdomyosarcoma	TTC-021	12	Sickle cell disease
TTC-028	2	Wiskott-Aldrich syndrome	Mayo-002	11	Acute lymphoblastic leukemia
LCH-001	11	Medulloblastoma	Mayo-006	10	Hodgkin's lymphoma
CN-008	5	Sickle cell disease	Mayo-011	6	Ewing's Sarcoma
TTC-019	9	Sickle cell disease	LCH-018	2	Medulloblastoma
CN-005	5	Farber Lipogranulomatosis	Mayo-008	4	Osteosarcoma
CN-004	7	Medulloblastoma	CN-026	8	Ewing's Sarcoma
Mayo-004	9	T-cell lymphoma	CCHMC-015	6	Beta thalassemia
Mayo-001	11	Ewing's Sarcoma	LCH-039	10	Medulloblastoma
			LCH-040	8	Medulloblastoma

4.3.3 Thawing and xenografting

Cryovials containing testicular tissue pieces were retrieved and transferred to a water bath maintained at 37ºC until completely thawed. Samples were rinsed with and placed in Hanks Balanced Salt Solution (HBSS, Cat# 24-020-117, Fisher Scientific) on ice until grafting into mice. Recipient mice were anaesthetized with 2,2,2-tribromoethanol administered intraperitoneally (250mg/kg, Cat# T48402-100G, Sigma) and castrated by accessing the recipient testes through an

incision made in the abdomen and the peritoneum. Testicular blood vessels were ligated prior to removal of the testes. Grafts were placed under the dorsal skin of the recipient mouse by making small incisions on each graft site. Mice in the VEGF treatment group received 1µg of recombinant human VEGF-165 reconstituted in Dulbecco's phosphate buffered saline (DPBSS, Cat# 14200166, Life Technologies) pipetted directly into to graft site prior to placement of the graft. Incisions were closed after insertion of the grafts by applying 1-2 simple interrupted stiches/site using absorbable undyed vicryl suture (Cat# 07-810-7022, Henry Schein). Mice in the hCG treatment group received 10U of hCG (Cat# CG10, Sigma) subcutaneously at the time of grafting and biweekly over the course of the study. All recipient mice were treated with Buprenorphine (0.1mg/kg) and provided with Medigel CPF (Clear H_2O) with Carprofen for pain management post-surgery.

4.3.4 Graft recovery and processing

Mice were euthanized by intraperitoneal administration of Avertin followed by cardiac puncture. Seminal vesicles and grafts were excised, weighed and fixed in 4% paraformaldehyde (PFA) overnight. Samples were then washed in PBS and embedded in paraffin for histological analyses.

4.3.5 Hematoxylin and Eosin staining and immunohistochemistry

Prepubertal and adult testicular tissue along with xenograft tissue samples that were fixed in 4% PFA, washed and embedded in paraffin were sectioned into 5µm sections and mounted on

glass slides. For hematoxylin and eosin staining, sections were then deparaffinized and rehydrated by exposing sections to a graded ethanol series of decreasing ethanol concentrations with the final hydration in water. Sections were then stained in hematoxylin 560 (Cat# 3801575, Leica Biosystems Inc.) and Eosin (Cat# 3801606, Leica Biosystems Inc.) as described previously [322]. For immunohistochemistry, deparaffinized and rehydrated sections were placed in Tris buffer (pH 10) heated to 97ºC for 30 minutes for antigen retrieval. Slides were then cooled, rinsed with DPBST (0.1% Tween-20 in 1X DPBS) and incubated with blocking buffer composed of 3% BSA, 0.1% Triton X-100 and 5% normal donkey serum (Cat# 017-000-121, Jackson Immunoresearch Lab) in DPBS, for 1 hour. Sections with then incubated with primary antibodies- Goat anti-VASA (1:300, Cat#AF2030, R&D systems), Rabbit anti-SOX9 ((1:100, Cat# AB5535, Millipore Sigma), Mouse anti-PGP9.5 (1:100, Cat # 7863-1004, Bio-Rad), Goat anti-PIWIL1 (1:40, Cat# AF6548, R&D systems), Rabbit anti-NUMA (1:100, Cat# ab84680, Abcam), Rabbit anti-TP1 (1:1000, Cat# ab73135, Abcam), and Mouse anti-Ki-67 (1:100, Cat# LS-C137260-100, LSBio) for 90 minutes at room temperature or overnight at 4ºC. The sections were then rinsed in DPBST and incubated with fluorescently labeled secondary antibodies (1:200, Invitrogen) specific to the species each antibody was raised in for 45 minutes. Following staining with the secondary antibody, the slides were rinsed and mounted with DAPI-containing mounting medium, Vectashield (Cat# H-1200, Vector Labs). Sections were observed under the Nikon Eclipse 90i microscope and analyzed using the NIS Elements Advanced Research Software.

4.3.6 Statistical analyses

All statistical analyses were performed using Graphpad Prism. One-way ANOVA was used to compare the graft weights, seminal vesicle weights and to compare the percentages of tubules with germ cells at various stages of differentiation. Dunnett's test was used to perform multiple comparisons for analyzing the differences in graft weights, seminal vesicle weights and percent tubules with PGP9.5+ cells between the treatment groups and Tukey's test was used to perform multiple comparisons to analyze the differences percent tubules with germ cells, SCO phenotype, PIWIL1+ and TP1+ cells between the treatment groups. P values less than 0.05 were considered significant and marked * for <0.05, ** for <0.01, *** for <0.005 and **** for <0.001.

4.4 Results

4.4.1 Donor testicular tissue samples exhibit presence of undifferentiated spermatogonia but lack meiotic and post-meiotic germ cells at the time of grafting.

In this study, prepubertal human testicular tissue pieces were grafted under the dorsal skin of severe combined immune deficient (SCID) mice. Absence of spermatogenesis in donor testicular tissue at the time of grafting was confirmed via immunohistochemistry for markers of germ cells at various stages of differentiation (**Figure 20**). At this time, seminiferous tubules exhibited the presence of SOX9+ Sertoli cells and VASA+ (Dead box helicase 4 or DDX4) germ cells that also expressed PGP9.5 (protein gene product 9.5 or UCHL1), a marker of

undifferentiated spermatogonia, but lacked the expression of PIWIL1 (PIWI-like protein 1) and TP1 (Transition Protein 1), that mark spermatocytes and spermatids, respectively (**Figure 20B, D, F, H**). Adult testicular cross sections are included for comparison (**Figure 20A, C, E, F**).

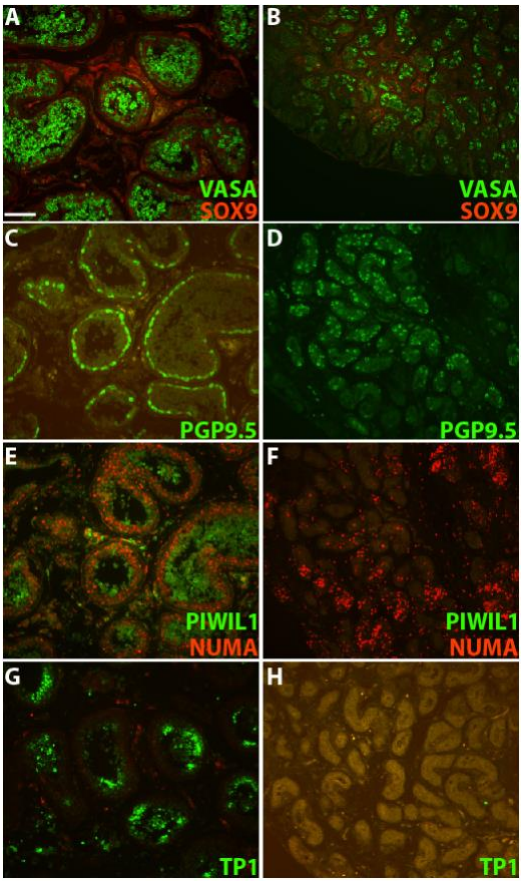

Figure 20: Immunofluorescence analyses of donor testicular tissue at the time of grafting

In contrast to the multiple layers of VASA+ germ cells observed in adult human testicular cross sections (A), immunostaining of prepubertal donor tissue cross-sections showed the presence of fewer VASA+ germs cells along with SOX9+ Sertoli cells (B), that is typical of the testis in this stage. PGP9.5+ spermatogonia were present in the prepubertal samples (D), while no evidence of PIWLI1+ spermatocytes (F) and TP1+ spermatids (H) were detected, confirming lack of spermatogenesis in the donor samples. Adult cross-sections stained with PGP9.5, PIWIL1 and TP1 were used as controls (E and G). Scale bar=100µm.

4.4.2 Grafts and seminal vesicles demonstrated treatment-dependent growth at 6 months post-grafting

Grafts and recipient seminal vesicles were excised and weighed 6 months post grafting. Mice from the hCG treatment groups had significantly larger grafts (p values: hCG=0.05 and hCG+VEGF=0.003) (**Figure 21 A-E**). Seminal vesicles in the hCG+VEGF treatment group also showed significant enlargement compared to gonadectomized control mice that did not receive grafts, indicating testosterone production in the hCG+VEGF-treated grafts (p value = 0.04) (**Figure 22A-F**).

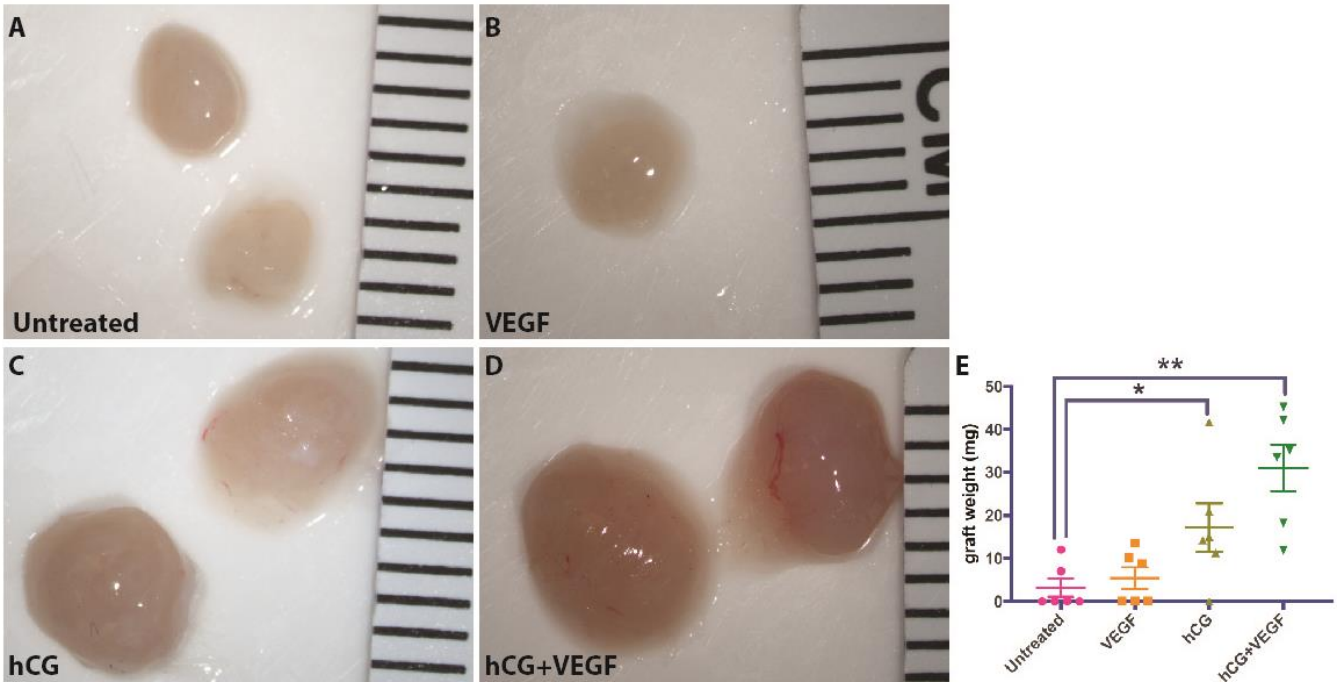

Figure 21: Human xenograft weights at 6 months post-grafting

Human xenografts were recovered at 6 months post-grafting. There were no significant differences in the average weights of grafts in the untreated and VEGF-treated groups (A and B). hCG and hCG+VEGF-treated grafts were significantly larger than grafts from the untreated group (C, D and E). Data in E are mean±SEM. P values were determined using one-way ANOVA and Dunnett's multiple comparisons test.

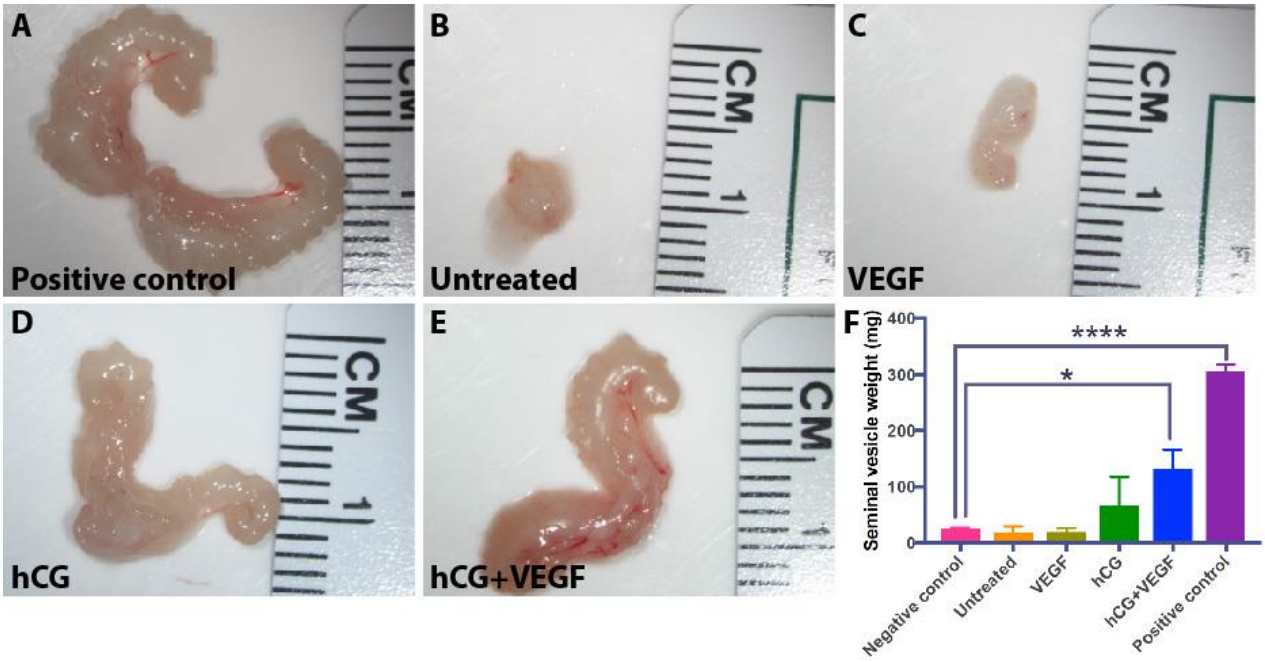

Figure 22: Recipient seminal vesicle weights 6 months post-grafting

Seminal vesicles from recipient mice were weighed to evaluate testosterone production in grafts from the various treatment groups. Castrated mice that didn't receive grafts served as negative control and mice that were left intact with no grafts served as positive control (A). Seminal vesicles from untreated (B) and VEGF-treated mice were similar in size to those recovered from castrated negative control mice (F). hCG-treated mice had slightly larger seminal vesicles but the difference was not statistically significant (D,F). hCG+VEGF-treated mice had significantly larger seminal vesicles compared to castrated control mice (E,F). Data in F are mean±SEM. P values were determined using one-way ANOVA and Dunnett's multiple comparisons test.

Next, we evaluated treatment-dependent growth of seminal vesicles and grafts over time by excising and evaluating sample weights at 2, 4 and 6 months. Since the previous experiments showed no differences between the untreated and VEGF-treated groups, we performed the next set of experiments with untreated, 10UhCG+VEGF and 25UhCG+VEGF. Our analyses showed that there were no significant differences in seminal vesicle and graft weights within groups over time. However, hCG+VEGF treatment promoted growth of grafts over time and at 6 months hCG+VEGF treated grafts were significantly larger than untreated grafts (p values:

10UhCG+VEGF = 0.02, 25UhCG+VEGF = 0.008) (**Figure 23 B**). In addition, seminal vesicle in the hCG-treatment groups showed significant enlargement at 4 and 6 months indicating significant testosterone production in those grafts (p values: 4 months- 25UhCG+VEGF = 0.03, 6 months- 10UhCVG+VEGF = 0.05) (**Figure 23A**).

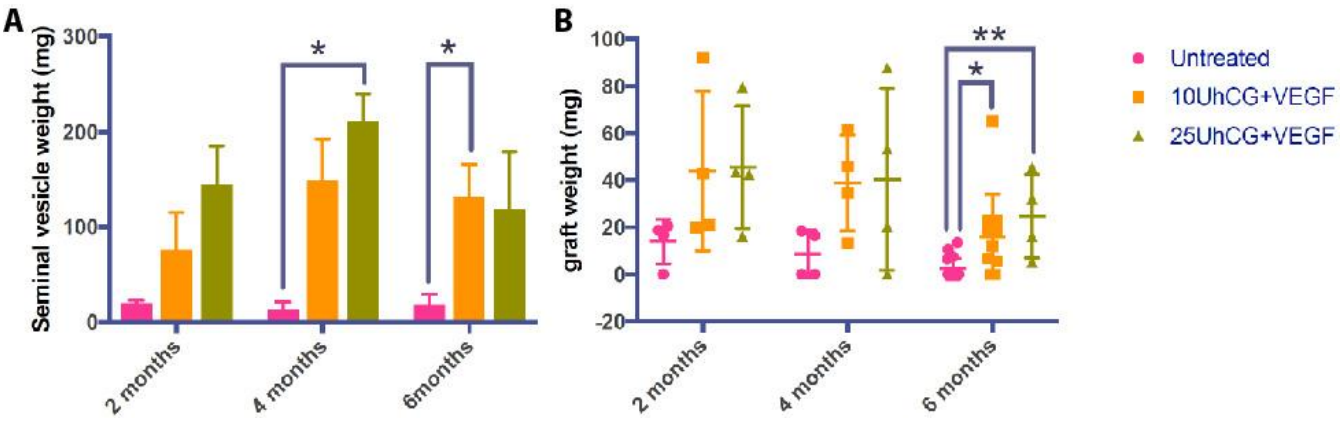

Figure 23: Seminal vesicle and graft weights over time

Evaluation of seminal vesicle and graft weights at 2, 4, and 6 months post-grafting showed significant enlargement of seminal vesicles, indicative of testosterone production in 25U hCG+VEGF-treated grafts at 4 months and by 10U hCG+VEGF-treated grafts at 6 months (A). There were no significant differences in graft or seminal vesicle weights within treatment groups over time. At 6 months hCG-treated grafts were significantly larger than untreated grafts (B). Data in A and B are mean±SEM. P values were determined using one-way ANOVA and Dunnett's multiple comparisons test.

4.4.3 Immunohistological analyses of grafts at 6 months shows loss of germ cells in all treatment groups

Grafts were stained with SOX9, VASA and Ki67 to evaluate the presence of Sertoli and germ cells, and to quantify proliferation events in these cell types at 2-, 4- and 6-months post-grafting. Grafts from all treatment groups exhibited loss of germ cells at all time points (**Figure 24A, B, D, E and J**). Despite the loss of germ cells, hCG±VEGF-treated grafts demonstrated

significant tubular enlargement compared to untreated grafts at 6 months (mean±SD values : Untreated = 3791.39±1260.79, VEGF = 4745.64±1513.17, hCG = 9014.70±3685.55, hCG+VEGF = 11498.99±4647.33) (**Figure 24C**). While there was no significant difference in the average number of Sertoli cells per tubular cross section between treatment groups and over time, grafts from the untreated group demonstrated a decreasing trend in the number of SOX9+ cells over time (**Figure 24F**). Quantification of the expression of Ki67 in germ cells (**Figure 24G**) revealed rare proliferating germ cells in untreated and 25U hCG+VEGF-treated grafts (**Figure 24K**). Grafts from all treatment groups exhibited a trend towards decreasing SOX9+Ki67+ cells over time with grafts from the 10U hCG+VEGF demonstrating a significant decrease in proliferating Sertoli cells at 6 months compared to 2 months post-grafting (p=0.04) (**Figure 24H and I**).

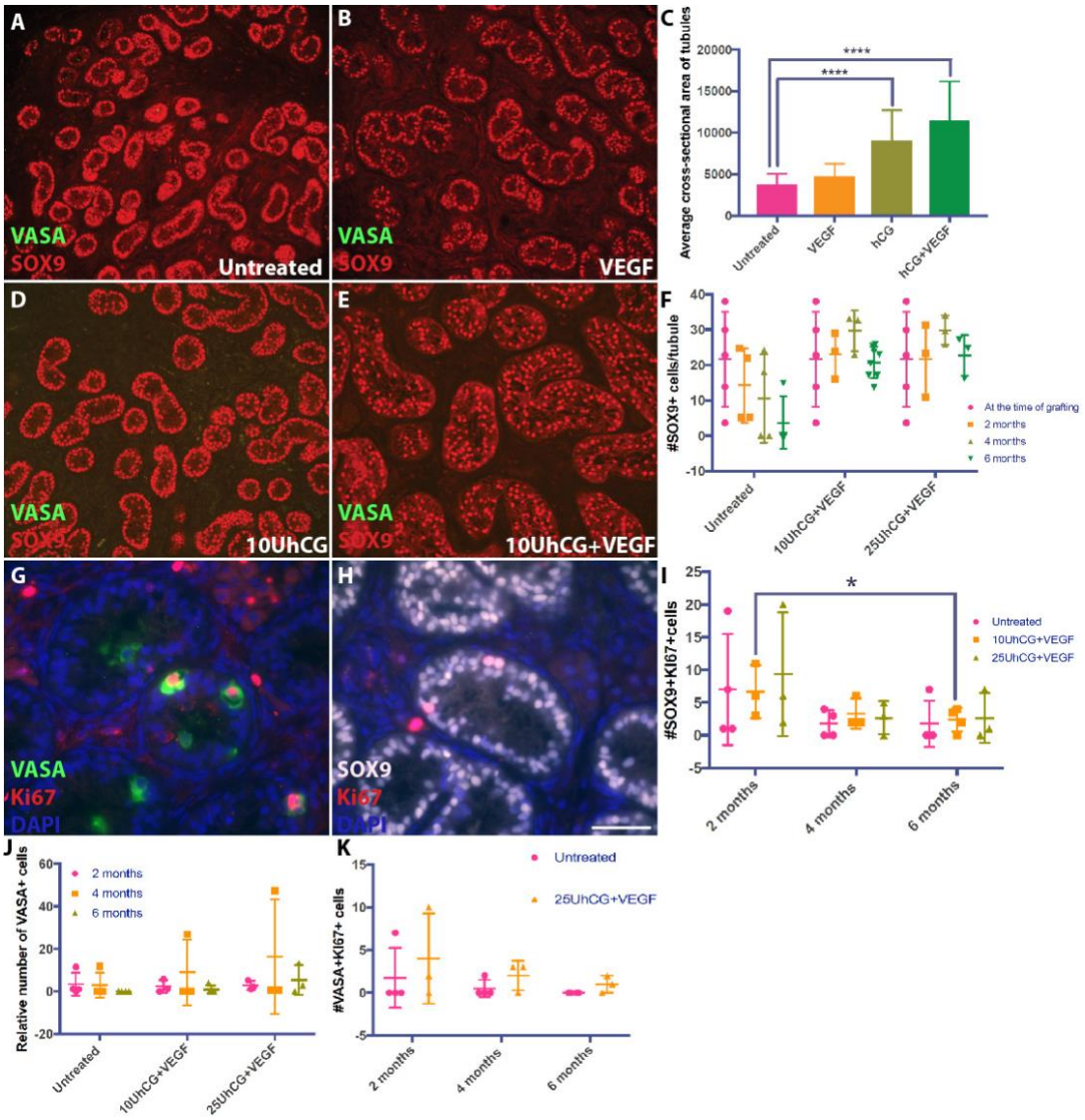

Figure 24: Analysis of grafts over time demonstrates loss of germ cells with the retention of Sertoli cells

Immunostaining of cross-sections of grafts with VASA and SOX9 to mark germ cells and Sertoli cells, respectively, reveals loss of germ cells by 6 months post-grafting in untreated (A) VEGF (B) 10U hCG (D) and 10U hCG+VEGF-treated grafts (E). hCG-treated grafts demonstrated significant tubular expansion compared to untreated grafts (C). Quantification of the number of Sertoli cells per tubular cross-section revealed a decreasing trend in SOX9+ cells in untreated grafts, however, there were no significant differences in the average number of Sertoli cells over time and between treatment groups (F). Quantification of the expression of Ki67 by VASA+ germ cells revealed rare proliferating germ cells in untreated and 25U hCG+VEGF-treated grafts (K) and loss of germ cells across all time points and treatments groups at 2-, 4-, and 6-months post-grafting (J). Grafts from the 10UhCG+VEGF treatment group demonstrated a significant decrease in proliferating Sertoli cells at 6 months compared to 2 months post-grafting (I). P values were determined by one-way ANOVA and Dunnett's multiple comparisons test. Data in C, F, I, J and K are mean±SD.

4.5 Discussion

This study evaluated the effect of hCG and VEGF exposure on the outcome of xenografting prepubertal human testicular tissue into SCID mice. Our observations indicated that treatment of graft site with VEGF had no effect on graft size, seminal vesicle weight (indicative of testosterone production) or retention of germ cells in the donor tissue. These observations are consistent with those reported by Ntemou et al. where the group showed that when compared to untreated controls, VEGF pretreatment of donor tissue did not significantly improve maintenance or differentiation of spermatogonia [326].

hCG-treatment has been shown to promote growth and extent of maturation in non-human primate xenografts [191, 325]. Exogenous gonadotropin administration in juvenile macaques has also been shown to cause an increase in the diameter of seminiferous tubules [352]. In this study, we observed that hCG administration in recipient mice promoted recovery and growth of human xenografts, with significant cross-sectional enlargement of the seminiferous tubules in the grafts. In addition, hCG+VEGF-treated grafts had a significant increase in seminal vesicle weights compared to mice that were left untreated, indicating increased testosterone production in these grafts. However, all grafts irrespective of the treatment group, exhibited loss of germ cells as early as 2 months post-grafting. Loss of germ cells in human xenografts has been reported by some studies [347, 348], while others have reported maintenance and differentiation of spermatogonia to the pachytene spermatocyte stage [326, 350].

The cause of the drastic loss of germ cells observed in this study is unknown. However, a number of factors may have played a role including the strain of recipient mice used in the study,

dosage of gonadotropins used or the site of grafting. As reviewed in **Table 4**, the majority of studies on human xenografting have used nude (Nu/Nu) mice, whereas in the current study we use SCID mice. Nude mice are athymic and hence lack T-lymphocytes while SCID mice are deficient in both B- and T-lymphocytes [353, 354]. It is reasonable to expect that the added severity of immunodeficiency in SCID mice might promote long-term survival and hence recovery of xenografts. On the other hand, the absence of hair in Nude mice might be associated with a lower subcutaneous temperature that might in turn support spermatogenesis in the grafts. A systematic comparison of the outcome of grafting porcine testicular tissue in Nude and SCID mice showed a higher recovery of grafts and tubules with germ cells in SCID recipients compared to Nude recipients but no differences in the extent of maturation [355]. Studies comparing the 2 recipient strains in the context of primate xenografting have not been reported.

hCG dosage may have an effect on the survival and differentiation of spermatogonia. Intratesticular testosterone levels in the human testis tend to be over 10-fold higher than serum testosterone levels [356]. Lowering intratesticular testosterone levels to serum levels has been shown to be insufficient to support normal spermatogenesis [357]. Therefore, increasing the amount of hCG administered to cause a corresponding increase in testosterone levels within the grafts might promote germ cell differentiation. In addition, the site of graft placement might affect testosterone bioavailability within the grafts. Studies systematically comparing the outcome of grafting human testicular tissue into the testis and under the dorsal skin of recipient mice need to be tested. In addition, there is also a possibility of absence or incompatibility of factors essential for human spermatogenesis in the murine microenvironment. Therefore, testing other recipient species might lead to interesting and more promising results.

5.0 Summary and Conclusion

Advancements in cancer therapies over the last several decades have led to a rise in pediatric cancer survival rates to about 88% [7]. This increase in cancer survivorship has made it increasingly important to address factors that affect patient quality of life post-treatment, including treatment-induced gonadotoxicity and increased risk of infertility [358, 359]. The majority of patients that are exposed to chemotherapy or radiation experience transient azoospermia and will recover normal levels of spermatogenesis within 1-5 years post-treatment [21]. However, about 24% of patients will be rendered permanently infertile by treatment for their primary disease [360].

Currently, the only standard of care fertility preservation method available in the clinic is cryopreservation of spermatozoa that can be used at a later time to establish pregnancy through assisted reproductive technology (ART) [31, 32]. Because prepubertal patients do not have ongoing spermatogenesis in their seminiferous epithelium, the option of cryopreserving sperm is not available to them. However, several research and clinical centers around the world are developing spermatogonial stem cell (SSC)-based methods for fertility preservation and are recruiting patients to cryopreserve testicular tissue biopsies in anticipation of these methods becoming clinically available in the future [48, 49, 51, 52, 130, 361-363].

The focus of this dissertation research was to develop 2 SSC-based fertility preservation technologies- SSC culture and testicular tissue xenografting for application in the clinic. SSC culture experiments were performed using adult donor testicular samples obtained from Center for Organ Recovery and Education and the University of Pittsburgh Health Sciences Tissue Bank,

whereas prepubertal testicular tissue samples obtained from pediatric patients enrolled in fertility preservation program of University of Pittsburgh Medical Center were used to conduct the xenografting experiments.

In the SSC culture study, I tested protein-based substrates including mouse and human laminin, SIS, UBM, human and porcine testis ECMs and compared the recovery of undifferentiated spermatogonia in these culture systems against a mouse embryonic fibroblast feeder cell line-based system that has been used previously to culture spermatogonia [147]. I enriched spermatogonia using MACS against the expression of ITGA6 followed by differential plating as described by Chikhovskaya et al. [246]. The presence of undifferentiated spermatogonia was quantified at days 7 and 14 in culture using immunostaining for UTF1. I also developed a multiparametric flow cytometry method using markers for undifferentiated spermatogonia (SSEA4), differentiating germ cells (cKIT), proliferating cells (KI67) and apoptosis (Annexin V) to gain a better understanding of the kinetics of spermatogonia in culture. My experiments showed a decline of spermatogonia in culture to differentiation and apoptosis over time which is consistent with other reports that performed similar iterative quantifications [81, 233, 247, 250]. Immunocytochemical analysis showed that human testis ECM retained the highest number of UTF1+ cells compared to STO feeder-based system at day 14 and hence was used as substrate for the subsequent experiments. To further enhance the recovery of the spermatogonia, I compared 3 different culture media described in reports used to culture mouse and human SSCs- mSFM (modified from [240], described in Appendix C), StemPro-34-(modified from [228]) and IMDM-based media [127] described in Appendix E and found that StemPro-34-based serum-free medium had a significantly higher recovery of live cells and spermatogonia at day 14.

We have yet to develop a method for culturing and expanding populations of human SSCs, however, in this study, I established a robust quantitative flow cytometry-based tool that can be used to perform iterative experiments to further optimize components of SSC culture, including growth factors, temperature etc. While there is experimental evidence that indicates that factors involved in the self-renewal and proliferation of SSCs are conserved between mice and rats, little is known about the factors that determine human SSCs fate and kinetics *in vivo*. Characterization of human germ and testicular somatic cells through single cell RNA sequencing may help shed light on additional factors that can potentially be used to promote maintenance of human SSCs in culture [364, 365].

Xenografting prepubertal testicular tissue into mice to induce complete spermatogenesis has been carried out in several mammalian species [177-185, 320]. Previous reports demonstrated that fresh prepubertal non-human primate xenografts can undergo complete spermatogenesis in the murine microenvironment, either spontaneously or upon exposure to hCG [191, 319, 320] whereas frozen and thawed tissue failed to mature upon grafting [321]. There have been no reports on the successful induction of complete spermatogenesis in human xenografts. In our xenografting studies I grafted cryopreserved and thawed prepubertal Rhesus macaque and human testicular tissue into recipient mice. I tested the effect of exposure of grafts to hCG and/or VEGF. I found that treatment of recipient mice with hCG promoted the maturation of Rhesus xenografts while no spermatogenesis was observed in untreated grafts. Sperm isolated from hCG-treated grafts were used to fertilize oocytes to generate a healthy offspring.

In the human xenografting experiments, I observed growth of grafts with tubular expansion in the hCG-treatment groups. However, contrary to the Rhesus xenografts, human grafts displayed

loss of germ cells over time. I performed additional experiments to study the kinetics of germ and Sertoli cells in the xenografts over time and observed that the decline of germ cells occurs within 2 months post-grafting while Sertoli cells are maintained over time, especially in the hCG-treatment groups. The loss of germ cells was also observed in xenografts derived from fresh prepubertal testicular tissue. This indicated that cryopreservation is not a cause of the decline of germ cells. The poor retention of germ cells in the grafts could be an outcome of several contributing factors. Firstly, the dosages of hCG and FSH administered in the study might not have been optimal supporting maintenance and differentiation of human spermatogonia. Elevated intratesticular testosterone levels have been shown to be critical for spermatogenesis in the human testis. Therefore, higher hCG doses may be tested to further increase the production of testosterone in the grafts. Higher intratesticular testosterone levels may also be achieved by placing the grafts within the testis of the recipient in addition to exogenous hCG supplementation. And finally, the loss of germ cells could also be a result of incompatibility based on unknown factors between the murine microenvironment and the donor tissue. This hypothesis can be tested by employing the use of recipients of a difference species, such as pigs, that are closer in evolutionary distance to humans compared to mice [366]. Several immune-compromised porcine models have been developed over the last decade that lack T and B lymphocytes that can serve as ideal substitutes for immune-compromised mice in these experiments [367].

Appendix A

Appendix table 1: Description of sources of testicular tissue used for SSC culture

Donor ID	Age range	Race	Cell viability prior to cryopreservation (%)	Cell viabilty post thawing (%)
14070709	40-45	White	87.5	66.67
13022001	45-55	White	98.36	67.7
14100106	25-35	White	71	84.2
15010101	15-20	White	85.5	86.6

Appendix B

Appendix table 2: Composition of medium used during MACSort

DPBS-S medium composition		
Item	**Source**	**Concentration**
Dulbecco's PBS	Life Technologies	1X
FBS	Life Technologies	1%v/v
HEPES	Invitrogen	1mM
Glucose	Sigma	1mg/ml
Pen/Strep	Invitrogen	1%v/v

Appendix C

Appendix table 3:Composition of mouse serum-free medium used during SSC culture

mSFM medium composition		
Item	**Source**	**Concentration**
MEMα	Invitrogen	
Pen/Strep	Invitrogen	1X
BSA	MP Biochemicals	0.2% w/v
Transferrin	Sigma	10µg/ml
FFA Mix	100mM stock made from Palmitic acid (2.36uM, Sigma), Palmitoleic acid (0.21uM, Sigma), Stearic acid (0.88uM, Sigma), Oleic acid (1.02uM, Sigma), Linoleic acid (2.71uM, Sigma), Linolenic acid (0.43uM, Sigma)	7.6µg/L
Sodium Selenite (Na2SeO3)	Sigma	3x10-8M
L-Glutamine	Invitrogen	2mM
BME (β-Mercaptoethanol)	Sigma	50µM
Insulin	Sigma	5µg/ml
HEPES	Invitrogen	10mM
Putrescine	Sigma	60µM
Recombinant human Glial cell line derived neurotropic factor (GDNF)	Peprotech	20ng/ml
Basic fibroblast growth factor (bFGF)	Fisher	1ng/ml

Appendix D

Appendix table 4: Composition of STO medium used to culture STO feeder cells

STO medium composition		
Component	**Source**	**Concentration**
DMEM with L-Glutamine	Fisher Scientific	
Pen/Strep	Invitrogen	1X
Fetal Bovine Serum	Life Technologies	7%
BME (β-Mercaptoethanol)	Sigma	100µM

Appendix E

Appendix table 5: Composition of StemPro-34 medium used to culture human SSCs

StemPro SSC culture medium			
Item	Final Concentration	Stock concentration	500ml
StemPro medium	-	-	500ml
StemPro supplement	-	-	13ml
L-Glutamine	2mM	200mM	5.1 ml
Pen/Strep	1%	-	5.1ml
Knockout serum replacement	-	-	5.2ml
Insulin	10mg/ml	5ug/ml	263ul
Sodium selenite	3X10^-4M	.3nM	52.3ul
Transferrin	100mg/ml	10ug/ml	52.4ml

Appendix table 6: Composition of IMDM-SFM used to culture human SSCs

Item	Stock Solution	Final Concentration
BSA	-	5mg/ml
Glucose	-	6mg/ml
Fetuin	-	1mg/ml
Pen/Strep		
Insulin	10mg/ml	25µg/ml
Transferrin	100mg/ml	100µg/ml
Pyruvic Acid	1.25g/ml	200µg/ml
Putrescine	60mM	60µM
Sodium Selenite	$3x10^{-4}M$	30nM
Lactic Acid	-	1ul/ml
L-Glutamine	200mM	2mM
BME	7µl/1000µl	$5x10^{-5}M$
MEM Vitamin Solution	100x	1x
Non-essential amino acids	100x	1x
Ascorbic Acid	100mM	$10^{-4}M$
d-Biotin*	1mg/ml	5µg/ml
β-estradiol	0.5mg/ml	30ng/ml
Progesterone	0.5mg/ml	60ng/ml
CD lipid concentration	-	10µl/ml
Knockout Serum Replacement	-	50µl/ml

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