

**PRESERVING MALE FERTILITY WITH SPERMATOGONIAL STEM CELLS**

by

**Hanna Valli**

B.S in Biology, University of Arkansas – Fort Smith

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School of Medicine in partial fulfillment  
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This dissertation was presented

by

Hanna Valli

It was defended on

September 30, 2014

and approved by

Stephen F. Badylak, Professor, Surgery

James L. Funderburgh, Associate Professor, Molecular Genetics and Developmental Biology

William H. Walker, Associate Professor, Molecular Genetics and Developmental Biology

Thesis Advisor: Kyle E. Orwig, Associate Professor, Molecular Genetics and Developmental

Biology

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Hanna Valli, PhD

University of Pittsburgh, 2014

Improved therapies for cancer and other conditions have resulted in a growing population of long-term survivors. Infertility is an unfortunate side effect of some cancer therapies that impacts the quality of life of survivors who are in their reproductive or pre-reproductive years. Some of these patients have the opportunity to preserve their fertility using standard technologies that include sperm, egg or embryo banking, followed by in vitro fertilization and/or embryo transfer. However, these options are not available to all patients, especially the prepubertal patients who are not yet producing mature gametes. For these patients, there are several stem cell technologies in the research pipeline that may give rise to new fertility options and allow infertile patients to have their own biological children. Spermatogonial stem cells are the foundation of spermatogenesis and may have application for preserving and restoring male fertility. However, majority of the knowledge about spermatogonial stem cells (SSCs) comes from rodents and not much is known about humans. In Chapter 2, I demonstrate that human spermatogonia have the phenotype of UTF1<sup>+</sup>, SALL4<sup>+</sup>, ENO2<sup>+</sup>, UCHL1<sup>+</sup>, ZBTB16<sup>+</sup>, ITGA6<sup>+</sup>, THY1<sup>dim</sup>, EPCAM<sup>dim</sup>, KIT<sup>-</sup> and that using the cell surface markers ITGA6, THY1, EPCAM it is possible to enrich human SSCs. In Chapter 3, I used this knowledge about the phenotype of human spermatogonia to show that the best method to cryopreserve intact human testicular pieces is controlled slow-freezing. In Chapter 4, I used the phenotype of human spermatogonia from Chapter 2, to show that it is possible to separate potentially therapeutic human spermatogonial stem cells from

malignant contamination. This is important because a majority of our prepubertal patients will have a testicular biopsy taken prior to initiation of chemotherapy so we want to make sure there would be no malignant contamination in the sample. Progress represented by this thesis research will facilitate translating SSC technologies toward the clinic for preservation and restoration of male fertility.

## TABLE OF CONTENTS

<b>PREFACE.....</b>	<b>XII</b>
<b>1.0 INTRODUCTION.....</b>	<b>1</b>
<b>1.1 SPERMATOGONIAL STEM CELLS AND SPERMATOGENESIS .....</b>	<b>3</b>
<b>1.2 MALE INFERTILITY AFTER CANCER .....</b>	<b>6</b>
<b>1.3 SPERMATOGONIAL STEM CELL TRANSPLANTATION.....</b>	<b>8</b>
<b>1.4 EXPERIMENTAL METHODS TO TRACK AND QUANTIFY HUMAN SPERMATOGONIAL STEM CELLS.....</b>	<b>11</b>
<b>2.0 CHARACTERIZATION OF HUMAN SPERMATOGONIAL STEM CELLS .</b>	<b>15</b>
<b>2.1 INTRODUCTION .....</b>	<b>15</b>
<b>2.2 MATERIALS AND METHODS .....</b>	<b>19</b>
<b>2.3 RESULTS .....</b>	<b>25</b>
<b>2.3.1 Acquisition of human testicular tissue .....</b>	<b>25</b>
<b>2.3.2 Immunohistochemical staining of human testicular sections .....</b>	<b>26</b>
<b>2.3.3 Correlation of spermatogonial markers with dark and pale descriptions of nuclear morphology and clone size .....</b>	<b>29</b>
<b>2.3.4 Immunohistochemical evaluation of cell surface markers in adult human testes</b>	<b>31</b>
<b>2.3.5 Expression of THY1 in adult human testicular cell suspensions.....</b>	<b>33</b>

2.3.6	Expression of ITGA6 in adult human testicular cell suspension.....	35
2.3.7	Expression of EPCAM in adult human testicular cell suspension .....	36
2.3.8	Enrichment of human spermatogonia using MACS .....	39
2.4	DISCUSSION.....	41
3.0	CRYOPRESERVATION OF HUMAN SPERMATOGONIAL STEM CELLS.	48
3.1	INTRODUCTION .....	48
3.2	MATERIALS AND METHODS .....	53
3.3	RESULTS.....	57
3.3.1	Acquisition of human testicular tissue .....	57
3.3.2	UTF1 staining of cryopreserved cells and tissues .....	58
3.3.3	Colonizing activity in cryopreserved cells and tissues.....	60
3.4	DISCUSSION.....	62
4.0	ELIMINATING MALIGNANT CONTAMINATION FROM THERAUPEUTIC HUMAN SPERMATOGONIAL STEM CELLS.....	66
4.1	INTRODUCTION .....	66
4.2	MATERIALS AND METHODS .....	69
4.3	RESULTS .....	75
4.3.1	Surface antigen expression on human testicular cells and MOLT-4 lymphoblastic leukemia cells.....	75
4.3.2	Expression of HLA-ABC and CD49e in adult human testicular cell suspension .....	77
4.3.3	Analysis of FACS sorted fraction for human spermatogonia.....	79

4.3.4	Tumor formation of FACS sorted cells following transplantation into the testes of nude mice. ....	82
4.4	DISCUSSION.....	88
5.0	SUMMARY AND CONCLUSIONS .....	94



## LIST OF TABLES

Table 1. Descriptive statistics for UTF1 positive cells per gram of tissue for human testicular cell and tissue cryopreservation.....	58
Table 2. Descriptive statistics for xenotransplant colonies per gram of tissue for human testicular cell and tissue piece cryopreservation.....	61
Table 3. Expression pattern of various cell surface antigens on MOLT-4 lymphoblastic leukemia cells and human testicular cell suspensions (expressed in percentage) .....	76
Table 4. Quantitative assessment of tumor formation in recipient mouse testes.....	84

## LIST OF FIGURES

Figure 1. Mammalian Spermatogenesis.....	2
Figure 2. Current model of rodent, nonhuman primate and human spermatogenesis. ....	5
Figure 3. Testicular cell transplantation.....	10
Figure 4. Human-to-nude mouse xenotransplantation assay. ....	12
Figure 5. Rabbit anti-primate antibody and ENO2 co-staining of recipient mouse testes xenotransplanted with human testis cells in cross-section.....	13
Figure 6. Expression of ZBTB16, UTF1, SALL4, UCHL1, ENO2 and KIT in human seminiferous epithelium.....	28
Figure 7. Summary of marker expression in adult human testis.....	29
Figure 8. UCHL1 expression in adult human testis.....	30
Figure 9. Whole-mount immunohistochemistry of seminiferous tubules from mouse and human testes.....	31
Figure 10. ITGA6 and EPCAM expression in adult human testis sections.....	32
Figure 11. FACS sorting and characterization of THY1 expression in adult human testes. ....	34
Figure 12. FACS sorting and characterization of ITGA6 expression in adult human testes. ....	36
Figure 13. FACS sorting and characterization of EPCAM expression in adult human testes.....	38
Figure 14. MACS sorting of human testicular cells for THY1, EPCAM and ITGA6. ....	41

Figure 15. Standard and experimental options for preserving male fertility. ....	50
Figure 16. ICC for UTF1 of cryopreserved human testicular cells and tissue pieces. ....	60
Figure 17. Colonization of cryopreserved human testicular cells and tissue pieces.....	62
Figure 18. SALL4–positive human spermatogonia do not express HLA-ABC or CD49e. ....	79
Figure 19. The EPCAM dim /CD49e negative/HLA-ABC negative fraction of MOLT-4–spiked human testis cell suspension is enriched for human spermatogonia.....	82
Figure 20. EPCAM <sup>-</sup> /CD49e <sup>+</sup> /HLA-ABC <sup>+</sup> cells form testicular tumors following transplantation into nude mice, but EPCAM <sup>dim</sup> /CD49e <sup>-</sup> /HLA-ABC <sup>-</sup> cells do not form tumors. ....	83
Figure 21. Testicular tumors observed after transplantation of the EPCAM <sup>-</sup> /CD49e <sup>+</sup> /HLA-ABC <sup>+</sup> fraction following FACS are of human origin.....	86
Figure 22. Separation of TF-1a–GFP cells and human spermatogonia from a contaminated human testis cell suspension using a multi-parameter FACS approach. ....	87

## **PREFACE**

The journey to get here has been challenging and not without ups and downs but I could not have done it without the help and support of a lot of people. First and foremost, I would like to thank Dr. Kyle Orwig, my advisor and mentor, who has provided a lot of guidance and support throughout this journey. He has taught me all the skills needed to be a great scientist. From him I have learned how to plan my experiments carefully, speak concisely and to the point, and how to write thoughtfully. He has also given me freedom to test my ideas and develop my research. In addition to being a great mentor, Kyle has also provided a collaborative lab environment. I have all of them to thank for helping me get to the point where I am now. I could not have done my work without Meena, who has performed all of the cell transplants for my experiments and helped with analyzing the data. Brian, who taught me the basics of working in the lab when I first joined the lab as a summer student and helped me do my first experiments. Bart, who was always willing to answer all my questions and help out whenever I needed help, but most importantly I learned from him how to always stay positive. Serena, who I co-first authored my first paper with. Kathrin, who has been great helping me keep up with all the literature, and Karen, who has been helping me process the human tissues. I would like to also thank other current or previous lab members who have helped me with my projects or just contributed to the lab environment - Yi Sheng, Chi Cheng, Julia, Min, Jessica, Matrika, Gosia and everyone else who have come through the lab over the years.

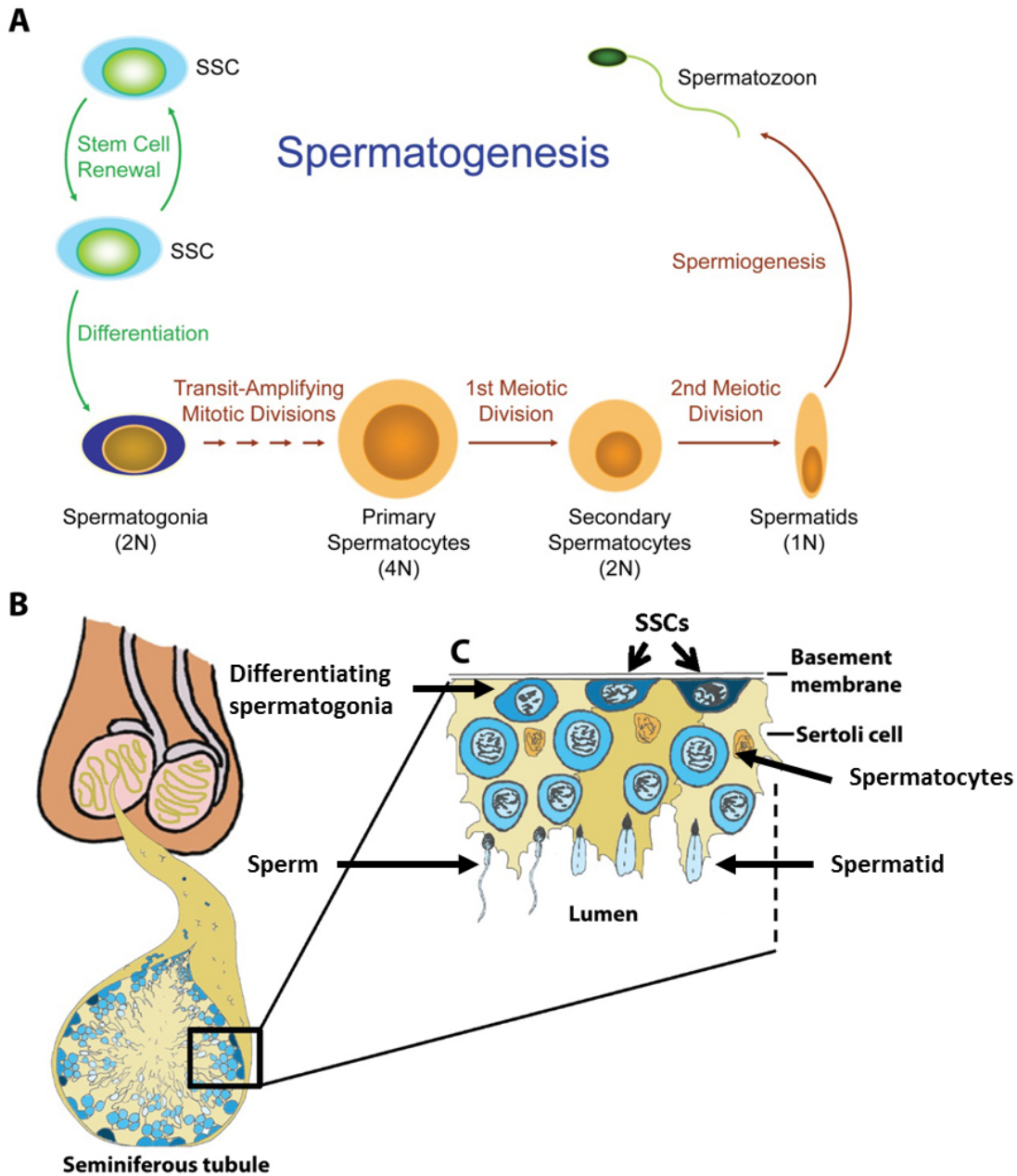
Outside the lab, I also owe a big thank you to a lot of important people. First, my mom who has fully supported me in everything I do, from deciding not to go to college and instead play volleyball for two years, to moving across the ocean without knowing what to expect (or know a single person). Also, my significant other Ken, who has supported me throughout the past few years and helped me get to the finish line.

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## 1.0 INTRODUCTION

Spermatogenesis is a highly organized process that produces millions of sperm each day in postpubertal mammals [1-3]. This productivity is dependent on the activity of spermatogonial stem cells (SSCs), which are the adult tissue stem cells in the testes that balance self-renewing divisions with differentiating divisions that maintain the stem cell pool and fuel spermatogenesis, respectively [4, 5]. When SSCs differentiate, they give rise to spermatogonia that undergo a species-specific number of transit amplifying mitotic divisions, followed by two meiotic divisions and spermiogenesis to produce terminally differentiated sperm (Figure 1A). SSCs reside in a specialized niche located on the basement membrane of the seminiferous tubules where they are in direct contact with Sertoli cells, which produce some of the paracrine factors required to regulate self-renewal and differentiation fate decisions (Figure 1B and C). Stem, progenitor and differentiating spermatogonia are all located on the basement membrane of the seminiferous tubules (Figure 1C). Differentiating spermatogonia give rise to spermatocytes that initiate meiosis and migrate off the basement membrane and produce spermatids and then sperm (Figure 1C).



**Figure 1. Mammalian Spermatogenesis.**

(A) The pool of diploid (2N) spermatogonial stem cells (SSCs) balance self-renewing and differentiating divisions to maintain the stem cell pool and also continuously produce sperm. Once committed to differentiate, SSCs give rise to undifferentiated and differentiating spermatogonia (2N), which undergo a species dependent number of transit-amplifying mitotic divisions that can dramatically increase the yield of sperm from a single stem cell. Differentiating spermatogonia give rise to primary spermatocytes (4N), which undergo two meiotic

divisions to produce haploid spermatids (1N). The meiotic divisions are followed by spermiogenesis to produce terminally differentiated sperm. **(B)** Spermatogenesis takes place inside the seminiferous tubules of the testis. **(C)** Cut out of the seminiferous epithelium. Spermatogonia (including SSCs) located on the basement membrane of seminiferous tubules give rise to primary spermatocytes, which initiate meiosis and migrate off the basement membrane. Two meiotic divisions and spermiogenesis give rise sequentially to secondary spermatocytes, spermatids and terminally differentiated sperm, which are released into the lumen of the seminiferous tubule. Reprinted from Valli et al., Fertil Steril. 2014 Jan;101(1):3-13, Copyright (2014), with permission from Elsevier Ltd.

## 1.1 SPERMATOGONIAL STEM CELLS AND SPERMATOGENESIS

The majority of the knowledge about SSCs comes from rodents, where the SSC pool is considered to reside in the population of isolated type  $A_{\text{single}}$  ( $A_s$ ) spermatogonia. In the rodent testis,  $A_s$  spermatogonia are rare, comprising 0.03% of all germ cells in the mouse testis [5]; they are evenly distributed along the basement membrane of seminiferous tubules, have a relatively large nuclear to cytoplasmic ratio and diffuse chromatin. When  $A_s$  spermatogonia divide, they produce  $A_{\text{pair}}$  ( $A_{\text{pr}}$ ) spermatogonia that either undergo complete cytokinesis to produce two new  $A_s$  (self-renew) or remain connected by an intercytoplasmic bridge to produce a chain of four  $A_{\text{aligned}}$  ( $A_{\text{al4}}$ ) spermatogonia (Figure 2B). Thus, at least a portion of  $A_{\text{pr}}$  must function as stem cells because they contribute to self-renewal of the  $A_s$  pool. Successive divisions of  $A_{\text{al4}}$  produce chains of 8, 16 and sometimes 32  $A_{\text{al}}$  spermatogonia. The synchronized development of spermatogonial clones is facilitated by protein and messenger RNA exchange via the intercytoplasmic bridges that connect the individual cells within a chain [6]. Types  $A_s$ ,  $A_{\text{pr}}$  and  $A_{\text{al}}$  spermatogonia are collectively termed  $A_{\text{undifferentiated}}$  ( $A_{\text{undiff}}$ ) spermatogonia (Figure 2A and B). In rodents, undifferentiated spermatogonia give rise to differentiating types A1, A2, A3, A4,



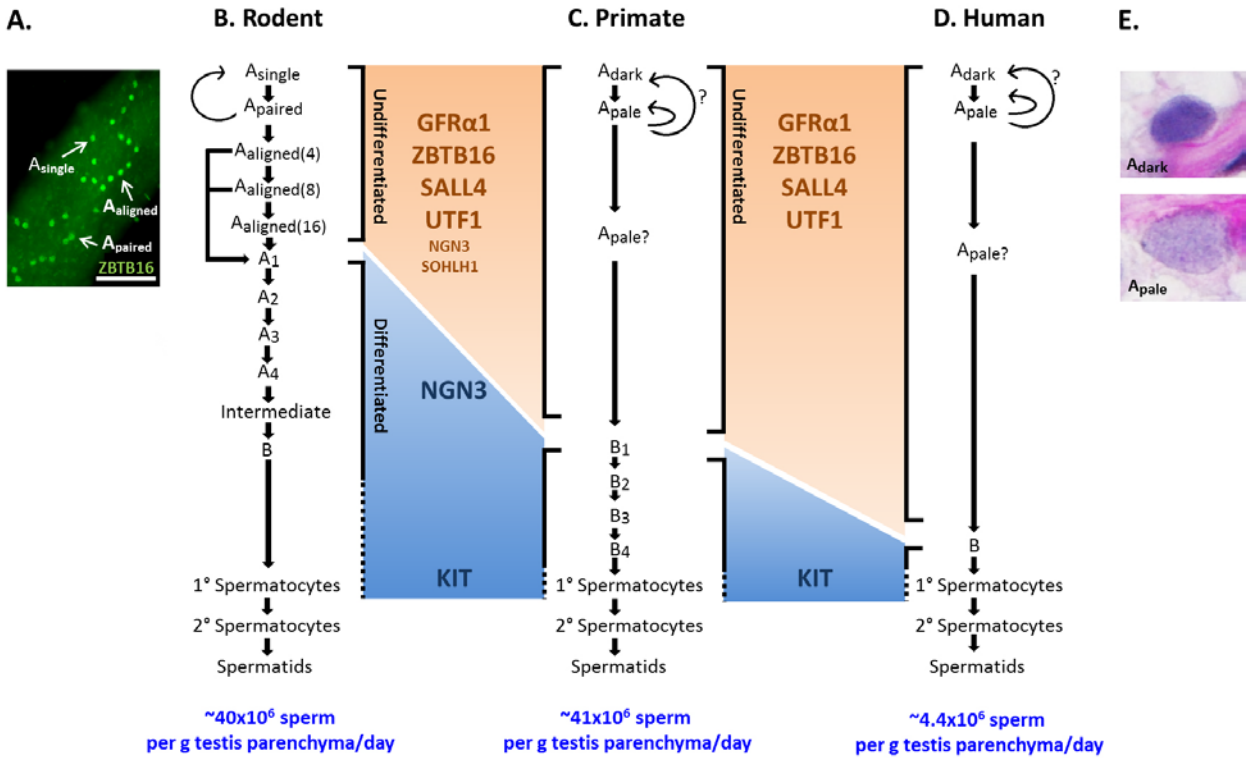
Intermediate and B spermatogonia, followed by primary spermatocytes, secondary spermatocytes, spermatids and sperm.

Depending on whether the transition from  $A_{al}$  to differentiating A1 spermatogonia occurs from  $A_{al}$  chains of 4, 8, 16 or 32 spermatogonia, a clone generated from a single SSC can theoretically give rise to between 1024 and 8192 sperm in rodents [7, 8]. The actual yield of sperm is 50-80% less than what would be predicted by the clonal amplification scheme described above and this is due to apoptosis that occurs primarily in the A2-A4 spermatogonia in rodents [9-12].

In rodents, no SSC specific marker has been identified but several markers have been described that are expressed by stem and/or progenitor cells (e.g.  $GFR\alpha 1$ ,  $POU3F1$ ,  $POU5F1$  (OCT4),  $ZBTB16$  (PLZF),  $NGN3$ ,  $NANOS2$ ,  $NANOS3$ ,  $SOHLH1$ ,  $SOHLH2$ ,  $FOXO1$ ,  $ITGA6$  ( $\alpha 6$ -integrin,  $CD49f$ ),  $LIN28$ ,  $ID4$ ,  $PAX7$ ,  $UTF1$ ,  $CDH1$ ,  $GPR125$ ,  $ITGB1$  ( $\beta 1$ -integrin,  $CD29$ ),  $EPCAM$  ( $CD326$ ),  $CD9$  and  $THY1$  ( $CD90$ ) [13-43]). When the spermatogonia go through differentiation, expression of stem and progenitor markers is reduced and expression of differentiation markers (e.g.,  $NGN3$  and  $KIT$ ) increases (Figure 2B). When coupled with whole mount immunofluorescence, clonal arrangement of spermatogonia can be determined (Figure 2A and B).

In contrast to rodents, undifferentiated (Type A) spermatogonia in primates are termed  $A_{dark}$  and  $A_{pale}$ , based on differences in nuclear architecture and staining with hematoxylin in histological sections (Figure 2E) [44-48].  $A_{dark}$  and  $A_{pale}$  are both found on the basement membrane of primate seminiferous tubules, but in contrast to rodents (Figure 2A and B) there are limited data describing their molecular characteristics or clonal arrangement [46, 48, 49] and conflicting views on whether one or both populations function as active stem cells in steady state

spermatogenesis [7, 48, 50-53]. This is an important knowledge deficit because spermatogonial stem cells may have application for treating male infertility.



**Figure 2. Current model of rodent, nonhuman primate and human spermatogenesis.**

(A) Whole-mount immunohistochemistry staining for ZBTB16 in adult mouse seminiferous tubules. ZBTB16<sup>+</sup> spermatogonia are identified as A<sub>single</sub>, A<sub>paired</sub> or A<sub>aligned</sub>. Scale bar = 100μm. (B) Rodent undifferentiated spermatogonia, including the SSC pool, is comprised of A<sub>single</sub> and some A<sub>paired</sub> spermatogonia and based on whole-mount staining analysis their phenotype is of GFRα1<sup>+</sup>, ZBTB16<sup>+</sup>, SALL4<sup>+</sup>, UTF1<sup>+</sup>, NGN3<sup>+/-</sup> and KIT<sup>-</sup>. Transit amplifying progenitors include some A<sub>paired</sub> spermatogonia and A<sub>aligned</sub> spermatogonia (chains of 4-16 cells), with a phenotype of GFRα1<sup>+</sup>, ZBTB16<sup>+</sup>, SALL4<sup>+</sup>, UTF1<sup>+</sup>, NGN3<sup>+/-</sup> and KIT<sup>+/-</sup>. The differentiating spermatogonia that are made up of A1-A4, Intermediate and B spermatogonia, have a phenotype of GFRα1<sup>-</sup>, ZBTB16<sup>-</sup>, SALL4<sup>-</sup>, UTF1<sup>-</sup>, NGN3<sup>+/-</sup> and KIT<sup>+</sup>. (C and D) In nonhuman primate and human testis, the undifferentiated spermatogonia are the Type-A spermatogonia that are designated A<sub>dark</sub> and A<sub>pale</sub> based on nuclear staining intensity with hematoxylin. The B spermatogonia are considered to be the differentiating

spermatogonia and in nonhuman primates they go through 4 divisions before producing primary spermatocytes, whereas in human there is only one division of B spermatogonia. (E) Sections of human testis stained using Periodic Acid-Schiff method and counterstained with hematoxylin to show nuclear morphology. Abbreviations: ZBTB16, zinc finger and BTB domain containing 16; GFR $\alpha$ 1, GDNF family receptor alpha-1; UTF1, undifferentiated embryonic cell transcription factor 1; SALL4, Sal-like 4; NGN3, neurogenin 3; SOHLH1, spermatogenesis and oogenesis specific helix-loop-helix 1. Reprinted with permission from Valli, H. et al., (in publication) Chapter 15: Spermatogonial Stem Cells and Spermatogenesis. In Plant TM and Zeleznik AJ, *Knobil and Neill's Physiology of Reproduction*.

## 1.2 MALE INFERTILITY AFTER CANCER

High dose chemotherapy, whole body radiation or radiation to the gonads can cause permanent infertility [54]. This is a significant human health concern because over 75,000 people under the age of 40 in the United States are diagnosed with cancer each year and most are cured [55]. Thus, cancer patients can look beyond their diagnosis and treatment to quality of life after cancer. Parenthood is important to cancer survivors and distress over infertility can have long-term psychological and relationship implications [56]. Therefore, the American Society for Clinical Oncology (ASCO) [57] and the American Society for Reproductive Medicine (ASRM) Ethics Committee [58] recommend that the reproductive risks of gonadotoxic therapies and options for preserving fertility be discussed with patients before initiating treatment. While adoption and third-party reproduction provide alternative family building options, the available data indicate that most cancer survivors prefer to have their own biological children [57].

Post-pubertal adolescent and adult males have the option to cryopreserve sperm prior to oncologic treatment. This is a simple and established method for preserving fertile potential and

allows men to father their own genetic children. Nearly 17,000 men between the ages of 15 and 44 are diagnosed with cancer each year in the United States and nearly 2385 survivors will receive a treatment that puts them at high risk of azoospermia [55, 59]. Unfortunately, only about 24% of men in this age range cryopreserved semen prior to their oncologic treatment [60]. Therefore, I calculate that each year in the United States, over 1800 adult cancer survivors will be infertile with azoospermia and have limited options to have their own biological children because they did not save a semen sample. In some cases, sperm can be recovered surgically from small focal areas of spermatogenesis in the testes using the testicular sperm extraction (TESE) method and used to fertilize oocytes by intracytoplasmic sperm injection (ICSI) [61].

There are no options to preserve the fertility of prepubertal boys, who are not yet making sperm. This is a significant problem because about 5131 boys under the age of 15 in the United States are expected to develop cancer each year and 83% are expected to survive [55]. A report from the Childhood Cancer Survivor Study indicates that the cytotoxic therapies for cancer reduce the number subsequently able to have children by 44% [59, 62]. Based on these statistics, I calculate that each year in the United States, 1874 young male cancer patients will become sterile due to their treatment. In addition to cancer survivors, over 500 patients under the age of 20 receive hematopoietic stem cell (HSC) transplants each year in the United States for non-malignant conditions (e.g., bone marrow failure, blood and immune deficiencies, autoimmune disorders) [63]. Myeloablative conditioning therapy prior to bone marrow transplantation is associated with a high risk of infertility [57, 62, 64, 65]. The ASCO report notes that “Impaired future fertility is difficult for children to understand, but potentially traumatic to them as adults” [57]. The available data indicate that most parents are interested in preserving fertility on behalf of their children who receive gonadotoxic therapies [66, 67].

The summed incidence of chemotherapy or radiation-induced male infertility that cannot be treated with existing reproductive therapies is approximately 4000 individuals each year in the United States. Therefore, responsible development of novel therapies to help these patients have biological children has a significant potential impact.

### **1.3 SPERMATOGONIAL STEM CELL TRANSPLANTATION**

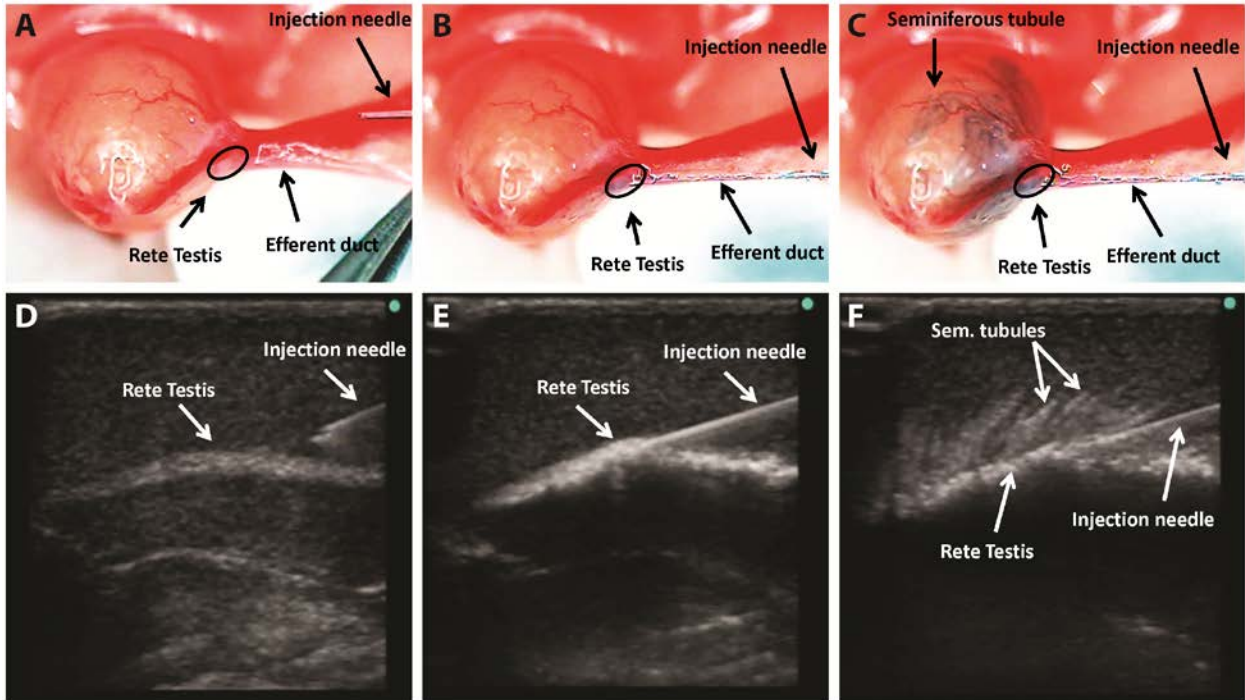
Ralph Brinster pioneered the technique for spermatogonial stem cell transplantation in mice in 1994, demonstrating that donor SSCs could engraft the seminiferous tubules of chemotherapy-treated recipient mice and produce spermatogenesis that was competent to produce viable progeny [68-73]. The SSC transplantation technique has become the experimental gold standard for quantifying stem cell activity and may have application for treating male infertility. Homologous species SSC transplantation has now been reported in mice, rats, pigs, goats, bulls, sheep, dogs and monkeys, including the production of donor-derived progeny in mice, rats, goats and sheep [70, 73-86]. SSCs from donors of all ages, newborn to adult, can regenerate spermatogenesis [74, 87] and SSCs can be cryopreserved and retain spermatogenic function upon thawing and transplantation [86, 88, 89]. We recently demonstrated that prepubertal and adult rhesus SSCs could be frozen, thawed and transplanted to regenerate spermatogenesis and produce fertilization competent sperm [83, 90]. Thus, prepubertal boys or adult men should be able to cryopreserve testicular tissue containing SSCs prior to treatment and have these cells reintroduced into their testes at a later date to regenerate spermatogenesis.

Radford and colleagues initially introduced the autologous SSC transplantation technique to the human clinic in 1999 [91]. In Manchester, the United Kingdom, testicular tissue from 12

male non-Hodgkin's lymphoma patients was cryopreserved as a cell suspension prior to the initiation of chemotherapy. At later dates, seven of the patients had the cells injected back into their testes [92, 93]. To our knowledge, there have been no follow up reports on the fertility status of those patients so the outcome of the experiment is unknown. Even if the men in that study fathered children, it would be difficult to demonstrate unequivocally (in the absence of a unique genetic marker) that those offspring resulted from sperm produced by transplanted stem cells rather than from surviving endogenous stem cells. There have been no other reports of SSC transplantation in humans since 1999. Nonetheless, this bold, pioneering study demonstrated that patients are willing to pursue experimental stem cell approaches to achieve fertility. To date, I estimate that testicular tissue or cells have been cryopreserved for more than 150 prepubertal and adult male patients worldwide [66, 67, 94-100].

For SSC transplantation in rodents, the testes are typically accessed via a mid-ventral abdominal incision. Testicular cells (including SSCs) are injected using a pulled glass capillary pipet inserted via the efferent ducts into the rete testis space, which can be visualized on the surface of the testis and is contiguous with all seminiferous tubules [101] (Figure 3A-C). Testis anatomy in larger animals, including nonhuman primates and humans is different than rodents, with the rete testis being centrally located in the testes. Stefan Schlatt and colleagues [102] demonstrated that ultrasound can be used to visualize the rete testis and guide an injection needle into the rete testis space. Ultrasound-guided rete testis injection has now been employed for SSC transplantation in several large animals species, including nonhuman primates [76-80, 84, 85, 90]. In contrast to the standard method in rodents, surgery is not required for ultrasound-guided rete testis injection. An injection needle is simply inserted under ultrasound guidance through the scrotal skin and testicular parenchyma into the rete testis space [90] (Figure 3D-F). Clinical

translation of the SSC transplantation technique appears eminent considering successes in several large animal models and that many patients have already cryopreserved testicular tissue or cells.



**Figure 3. Testicular cell transplantation.**

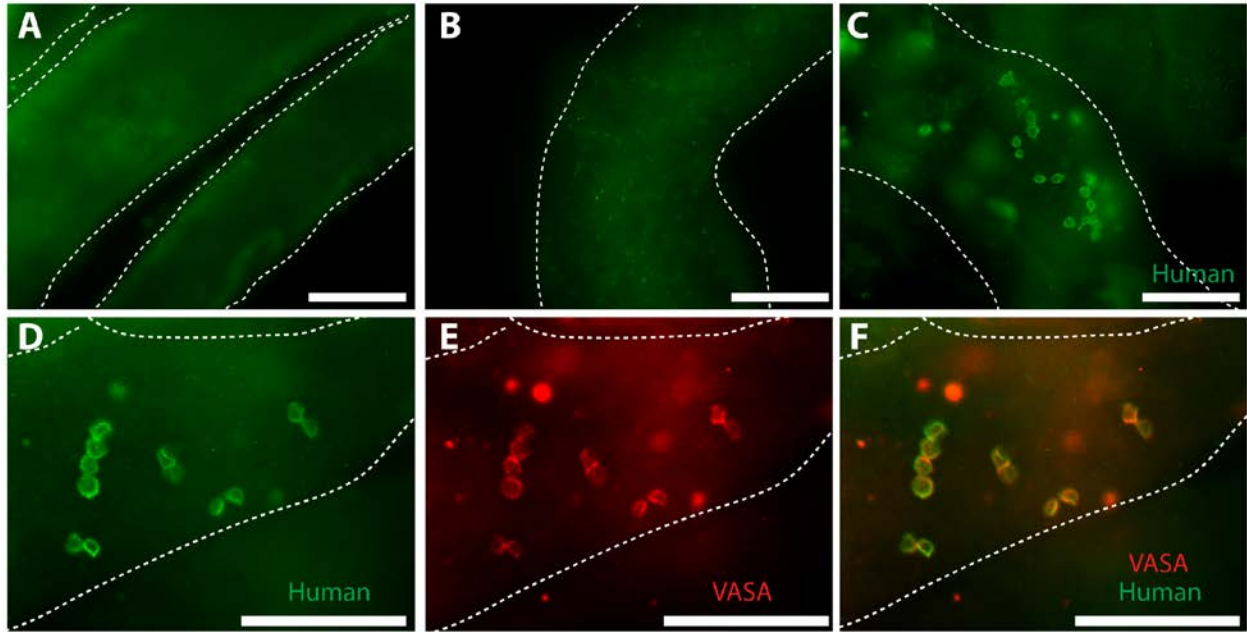
(A-C) In rodents, the testicular cells are injected via the efferent ducts into the rete testis space, which can be visualized on the surface of the testis and is contiguous with all seminiferous tubules. (C) Trypan blue is injected with the testicular cells to visualize the filling of the seminiferous tubules. (D-F) Testis anatomy in large animals is different than rodents, with the rete testis being more centrally localized and therefore more difficult to visualize and access. Therefore, ultrasound is used to guide injections. (D) Rete testis (echo-dense structure) is visible on ultrasound. The injection needle is inserted under ultrasound guidance through the scrotal skin into the rete testis space, which is continuous with the seminiferous tubules. (E) Positive pressure is applied to the needle so the cells are slowly injected into the rete testis and seminiferous tubules. (F) The filling of the seminiferous tubules is observed using microbubbles. Reprinted with permission from Valli, H. et al., (in publication) Chapter 15: Spermatogonial Stem Cells and Spermatogenesis. In Plant TM and Zeleznik AJ, *Knobil and Neill's Physiology of Reproduction*.

## 1.4 EXPERIMENTAL METHODS TO TRACK AND QUANTIFY HUMAN SPERMATOGONIAL STEM CELLS

Studies on human cells and/or tissues are a valuable stepping stone toward clinical translation. However, these studies are challenged by the limited experimental tools for quantifying human spermatogonia and testing their function. Here I propose that reliable markers of human spermatogonia are those with expression limited to germ cells located on the basement membrane of human seminiferous tubules. Proteins that meet these criteria, based on personal experience and review of the literature include PLZF, GFR $\alpha$ 1, GPR125, SALL4, LIN28, UCHL1, UTF1, FGFR3, EXOSC10, DSG2, CBL, SSEA4, CD9, OCT2 and SSX [103-113].

In rodents, SSC transplantation is the gold standard that allows investigators to quantify spermatogonial stem cells by observing their biological potential to produce and maintain spermatogenesis in infertile recipient animals. Homologous species transplantation to test the function of human spermatogonial stem cells is not possible. Our laboratory previously established and validated a primate-to-nude mouse xenotransplantation assay for monkey SSCs [53, 114]. To enable this assay, we generated a rabbit anti-primate testis cell polyclonal antibody that specifically recognizes antigens in primate testis cells. This antibody did not exhibit immunoreactivity with untransplanted mouse seminiferous tubules (Figure 4A), but it does recognize colonies of human spermatogonia in mouse seminiferous tubules 2 months after transplantation (Figure 4C and D). Monkey and human SSCs do not produce complete spermatogenesis in mouse seminiferous tubules (probably due to evolutionary distance between primates and mice). However, the colonization foci are considered to be SSC derived, because (a) they exhibited typical spermatogonial appearance, including arrangement as singles, pairs, and chains on the basement membrane of seminiferous tubules, and expressed the germ cell





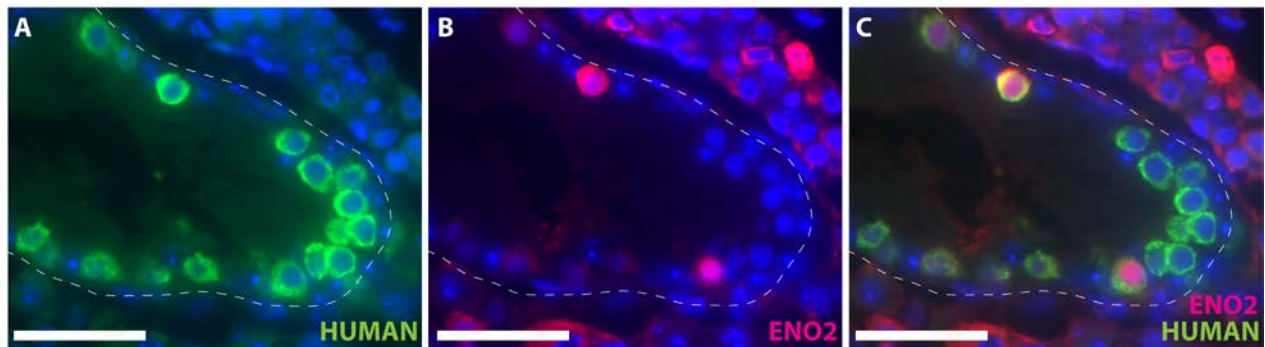
**Figure 4. Human-to-nude mouse xenotransplantation assay.**

A rabbit anti-primate testis cell polyclonal antibody was previously generated that specifically recognizes antigens on primate (human and nonhuman) testis cells. (A) The antibody does not exhibit immunoreactivity with untransplanted mouse seminiferous tubules. (B) An isotype control antibody (rabbit IgG) does not exhibit immunoreactivity with mouse seminiferous tubules transplanted with human testicular cells. (C and D) The primate testis cell antibody cross-reacts with human testis cells and can be used to identify colonies of human spermatogonia in mouse seminiferous tubules 2 months after transplantation. Cells in colonies have a typical spermatogonial appearance, with large nuclear-to-cytoplasmic ratios, and are arranged as singles, pairs, and chains located on the basement membrane of seminiferous tubules. (E and F) The colonizing cells recognized by the primate testis cell antibody also express the germ cell marker VASA. Mouse seminiferous tubules are demarcated by dashed white lines. Scale bar: 100  $\mu$ m. Reprinted with permission from Dovey SL and Valli H et al., *J Clin Invest.* 2013 Apr 1;123(4):1833-43, Copyright (2014).

marker, VASA (Figure 4E and F). (b) Clusters are not “just survivors of the transplant,” because the transplanted cells were a single cell suspension (confirmed visually on a hemocytometer) that was filtered through a 35- $\mu$ m strainer. The presence of chains of human germ cells clearly

indicates proliferation after engraftment. (c) These colonies are unlikely to arise from differentiating B spermatogonia, because a colonizing human B spermatogonia would produce a spermatocyte at its next division and migrate off the basement membrane. Therefore, spermatogonial colonies with 4 or more cells located on the basement membrane of seminiferous tubules must originate from human A<sub>dark</sub> or A<sub>pale</sub> spermatogonia, which are considered reserve and active SSCs, respectively [114-117].

Additionally, immunohistochemical assessment of human colonizing events in recipient mouse testes indicate that colonizing cells are located on the basement membrane of seminiferous tubules and contain enolase 2 (ENO2) positive undifferentiated human spermatogonia as well as ENO2 negative human cells that are presumably more differentiated germ cells (Figure 5).



**Figure 5. Rabbit anti-primate antibody and ENO2 co-staining of recipient mouse testes**

Immunofluorescence co-staining for the primate antibody (A and C) and ENO2 (B and C) in human to nude mouse xenotransplants testis. DAPI staining (blue) identifies all the nuclei. Scale bars = 50 μm. Reprinted from Valli et al., Fertil Steril. 2014 Aug;102(2):566-580, with permission from Elsevier Ltd.

At present, human to nude mouse xenotransplantation is the best functional assay to test the spermatogonial stem cell-like potential of a test cell population [94, 95, 104, 106, 112, 118-120]. This method does not recapitulate complete spermatogenesis from transplanted cells like

mouse to mouse SSC transplantation, probably due to evolutionary distance between humans and mice. However, human-to-nude mouse xenotransplantation does assay the ability of transplanted cells to migrate to the basement membrane of seminiferous tubules, proliferate to produce characteristic colonies of spermatogonia and persist long term [104, 106, 112, 118, 119].

## 2.0 CHARACTERIZATION OF HUMAN SPERMATOGONIAL STEM CELLS

### 2.1 INTRODUCTION

Spermatogenesis is a process that produces millions of sperm per day in postpubertal mammals [1-3]. At the foundation of spermatogenesis are spermatogonial stem cells (SSCs) that balance self-renewing divisions with differentiating divisions to maintain the stem cell pool and fuel spermatogenesis, respectively [4, 5, 44]. Despite their importance to male fertility, there is limited knowledge about the molecular characteristics of the human SSCs, which are typically described as  $A_{\text{dark}}$  and  $A_{\text{pale}}$  spermatogonia based on nuclear staining intensity with hematoxylin [44, 48, 121].

The majority of information about the molecular phenotype of spermatogonia has been generated using rodent models and although no SSC specific marker has been identified several markers that are expressed by stem and/or progenitor cells have been described (e.g.  $GFR\alpha 1$ ,  $POU3F1$ ,  $POU5F1$  (OCT4),  $ZBTB16$  (PLZF),  $NGN3$ ,  $NANOS2$ ,  $NANOS3$ ,  $SOHLH1$ ,  $SOHLH2$ ,  $FOXO1$ ,  $ITGA6$  ( $\alpha 6$ -integrin, CD49f),  $LIN28$ ,  $ID4$ ,  $PAX7$ ,  $UTF1$ ,  $CDH1$ ,  $GPR125$ ,  $ITGB1$  ( $\beta 1$ -integrin, CD29),  $EPCAM$  (CD326),  $CD9$  and  $THY1$  (CD90) [13-43]). Rodent SSCs are only definitively identified by their ability to produce spermatogenesis when transplanted into the testes of infertile recipient mice, an assay that was first described by Brinster and colleagues [122, 123]. In the transplant bioassay, each colony of spermatogenesis produced in

the recipient testis arises from a single SSC and therefore allows quantification of the starting population of stem cells [124-127]. The combination of the transplant technique with fluorescence activated cell sorting (FACS) has provided insights about additional phenotypic features that can be used to isolate and enrich mouse spermatogonia. Mouse spermatogonia have the phenotype: ITGA6<sup>+</sup>, ITGB1<sup>+</sup>, THY1<sup>+</sup>, CD9<sup>+</sup>, GFR $\alpha$ 1<sup>+</sup>, mitochondrial membrane potential<sup>high</sup>, Rhodamine 123 (Rho123)<sup>low</sup>, ITGAV ( $\alpha$ v-Integrin, CD51)<sup>-</sup>, KIT (cKIT, CD117)<sup>-</sup>, MHC-I, ALDH (aldehyde dehydrogenase) activity<sup>-</sup> and CD45<sup>-</sup> [20, 29, 31, 128-133]. There is a lack of consensus about whether SSC activity can also be recovered in the Hoechst side population fraction of mouse testes [19, 134-136].

In humans, undifferentiated stem and progenitor spermatogonia have been described by classical descriptions of nuclear morphology as A<sub>dark</sub> and A<sub>pale</sub> spermatogonia [48, 137]. Information about the molecular phenotype of human spermatogonia has begun to emerge in the last few years. Based on immunofluorescence and colorimetric staining of adult human testicular sections, human spermatogonia on the basement membrane of the seminiferous tubules express UTF1, SALL4, ZBTB16, GFR $\alpha$ 1, UCHL1, GPR125, LIN28, EXOSC10, FGFR3, DSG2, CBL, SSX2 and OCT2 [26, 108, 109, 138-146]. Less is known about cell surface markers that could be used to isolate and enrich human SSCs. A few studies have reported enrichment of putative human SSCs by sorting based on cell surface marker expression of GPR125, SSEA4, ITGA6 and CD9 [112, 143, 145, 147], but currently only two studies have confirmed their results by demonstrating SSC colonizing activity in the xenotransplant assay. Magnetic activated cell sorting (MACS) revealed enrichment of SSC colonizing activity in the SSEA4<sup>+</sup> and CD9<sup>+</sup> fractions of human testis cells [112, 145].

THY1, a glycoposphatidylinositol anchored cell surface protein, that belongs to the immunoglobulin-like superfamily of genes [148], has been shown to be expressed by neuronal cells, CD34 positive hematopoietic stem cells, fibroblasts and endothelial cells [149-155]. THY1 is involved in diverse processes, including cell migration, cell-cell/cell-matrix interactions [156] and T-cell activation [157]. In testis, THY1 has been shown through transplantation assay to be a conserved spermatogonial stem cell marker in mice [19], rats [29] and non-human primates [53]. However, the expression of THY1 in human spermatogonia has been contradictory. He *et al.* [143] showed that THY1 expression is limited to a few rare cells on the basement membrane of seminiferous tubules, whereas Izadyar *et al.* [112] showed staining in the germ cells located toward the lumen of the tubule and also in peritubular and interstitial cells. Both of these reports are based on immunofluorescence staining and no transplants were performed. Human to human transplants are not possible as a routine bioassay, but xenotransplants into the testes of infertile nude mice has emerged as a quantitative assay for human and nonhuman primate spermatogonia [26, 53, 94, 95, 112, 114, 118, 119, 145, 158, 159].

To help clarify this issue of whether THY1 is expressed by human SSCs, I fractionated human testis cell suspensions based on THY1 expression using FACS and MACS. The presence of undifferentiated stem or progenitor spermatogonia in the sorted fractions was evaluated by immunocytochemistry for SALL4 and human to nude mouse xenotransplantation. Similar experiments were performed for the cell surface markers ITGA6 (CD49f) and EPCAM (CD326), which are established markers of rodent spermatogonia [20, 29, 160].

ITGA6 is the integrin alpha chain 6. Integrins are cell surface proteins that are made up of an alpha chain and a beta chain and they provide a link between extracellular matrix proteins and the cytoskeleton [161]. ITGA6 has been shown to regulate glioblastoma stem cells [162] and

is expressed by mouse mammary stem cells [163] and is crucial for the survival of the MCF-7 cell line stem cells [164]. EPCAM (epithelial cell adhesion molecule) is a transmembrane glycoprotein that mediates homophilic cell-cell adhesion [165]. Modulation of *Epcam* activity is thought to affect cell migration, proliferation and invasion [165, 166] and overexpression of *Epcam* plays a role in cancer development [166-168].

Currently, no human data are available regarding whether spermatogonial markers used in FACS are also appropriate for MACS and vice versa. The choice of whether to use FACS or MACS depends on the desired output. FACS has limited throughput ( $\sim 30 \times 10^6$  cells per day); it is fairly time consuming and requires specialized equipment and a skilled operator, but it allows high resolution selection of sorting gates. MACS has a lower resolving power, but is generally a faster and is a higher throughput sorting strategy that can be performed on the laboratory bench and does not require specialized equipment. A single adult human testis that can be obtained for research through an organ donor program can contain over 1 billion cells, which is far beyond the typical sorting capacity of FACS. MACS can easily be scaled to accommodate this number of cells and maximize the use of this valuable human tissue resource for fundamental research. In addition, MACS is technically accessible and affordable, which will facilitate application for enriching SSCs in the clinical setting.

FACS fractions were analyzed by immunocytochemistry for the human spermatogonial marker SALL4 [118, 139] and human-to-nude mouse xenotransplantation. SALL4 is a member of *sal*-gene family of transcription factors that is highly conserved between species [169-175]. SALL4 is expressed by the cells in an early embryo and is important for maintaining pluripotency of ES cells [176, 177]. In addition SALL4 is a conserved marker of spermatogonia

[139, 178, 179] and has been implicated in the regulation of spermatogonial differentiation in mice [178]. MACS fractions were analyzed by human-to-nude mouse xenotransplantation.

Analyses of FACS fractions indicated that, all three cell-surface markers, EPCAM<sup>dim</sup>, ITGA6<sup>+</sup> and THY1<sup>dim</sup> can be used to effectively isolate and enrich human SSCs from a heterogeneous testis cell suspension. In contrast, only ITGA6 was suitable for sorting human SSCs by MACS, as THY1 and EPCAM provided no enrichment.

## 2.2 MATERIALS AND METHODS

### *Animals*

All experiments utilizing animals were approved by the Institutional Animal Care and Use Committees of the Magee-Womens Research Institute and the University of Pittsburgh and were performed in accordance with the National Institute of Health guidelines for the care and use of animals (assurance # A3654-01).

### *Preparation of Human Testicular Tissue*

Deidentified, normal adult human testicular tissue was obtained through the University of Pittsburgh Health Sciences Tissue Bank and Center for Organ Recovery and Education (CORE) under University of Pittsburgh IRB #0506140. Following the removal of tissue, it was transported to the laboratory on ice in Lactated Ringer's solution. Cells were recovered from human testicular tissue using a two-step enzymatic digestion described previously [53, 114, 118]. Briefly, testicular tissue was digested with collagenase type IV for 5 minutes at 37°C on the shaker (250 rpm), then shaken vigorously and incubated for another 3 minutes and if necessary 2



additional minutes at 37°C on the shaker. The tubules were then sedimented by centrifugation at 200xg for 5 minutes and washed with Hank's Balanced Salt Solution (HBSS, Gibco). The tubules were then digested with 0.25% trypsin/EDTA and DNase I. The suspension was triturated vigorously 3-5 times and incubated at 37°C for 5 minutes. The process was repeated in 5 minute increments for up to 15 minutes total. The digestion was stopped by adding 10% fetal bovine serum (FBS) and the cells were strained through 70µm strainer (Becton Dickson). The cells were pelleted by centrifugation at 600xg for 15 minutes. Cells were then suspended in minimal essential medium  $\alpha$  (MEM  $\alpha$ ) + 10% FBS at a concentration of  $40 \times 10^6$  cells/mL and aliquoted in cryovials. An equal volume of cryopreservation medium consisting of MEM $\alpha$  + 20% FBS + 20% dimethylsulphoxide (DMSO) was added drop-wise, making the final concentration  $20 \times 10^6$ /mL in MEM $\alpha$ /15% FBS/10% DMSO). The vials were frozen at a controlled rate using Nalgene freezing containers (Nalgene-Nunc International) or a CryoMed controlled-rate freezer (Thermo Scientific) and then stored in liquid nitrogen. For experiments, the cells were thawed rapidly at 37°C, washed and suspended in MEM $\alpha$  medium containing 10% FBS.

#### *Fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS)*

For FACS, the frozen and thawed human testis cell suspension was stained on ice in Dulbecco's phosphate-buffered saline (D-PBS) containing 10% FBS for 20 min with fluorescent-conjugated antibodies (THY1-APC, clone 5E10,  $0.5 \mu\text{g}/10^6$  cells and ITGA6 -PE clone GoH3,  $20 \mu\text{l}/10^6$  cells; Becton Dickinson; EPCAM-PE, clone 9C4,  $20 \mu\text{l}/10^6$  cells; BioLegend). Cells were then washed twice with D-PBS to remove unbound primary antibody, and filtered through a 35µm strainer (Becton Dickinson). Propidium iodide ( $0.5\mu\text{g}/\text{ml}$ , BD

Bioscience) was added to distinguish between live and dead cells. FACS analysis was done using FACSVantage SE (Beckton, Dickinson) and the positive staining was identified by comparison to appropriate isotype control in order to correct for non-specific binding. Sorting gates were established based on level of marker expression as well as exclusion of dead cells stained with propidium iodide and exclusion of cells exhibiting non-specific binding or autofluorescence. The MACS protocol was similar to that of FACS, except after fluorescent-conjugated antibody staining (THY1-PE, ITGA6 –PE; Becton Dickson; and EPCAM-PE; BioLegend) and washes, anti-PE Microbeads (2  $\mu$ l/10<sup>6</sup> cells; Miltenyi Biotec) were used to detect the fluorophore on the primary antibody. The cells were then sorted on a MACS column (Miltenyi Biotec) into positive (bound) and negative (flow through) fractions.

### *Immunocytochemistry*

Cells from FACS and MACS were spotted on Superfrost slides and fixed with methanol. The cells were then rehydrated with D-PBS and blocked with a buffer containing 3% bovine serum albumin and 5% normal goat serum in order to eliminate nonspecific binding. Rabbit anti-SALL4 (1:500; ab29112, Abcam) antibody was added to the cells and incubated for 90 min at room temperature. Isotype matched normal IgG was used as negative control. Primary antibody was detected using goat anti-rabbit AlexaFluor-488 conjugated secondary antibody (1:200, Invitrogen). The slides were mounted with VectaShield (Vector Laboratories) mounting medium containing DAPI for detection of all nuclei and the staining was observed with a Nikon Eclipse E600 Fluorescence microscope and images captured with MetaView Digital Imaging software.

### *Immunofluorescence*

Human testicular tissue fragments were fixed with 4% paraformaldehyde (PFA) overnight, paraffin-embedded and sectioned (5  $\mu$ m). The tissue slides were de-paraffinized, rehydrated, incubated for 30 minutes in sodium citrate buffer (10 mM sodium citrate, pH 6.0, 0.05% Tween-20) for antigen retrieval. The tissue was then blocked with a buffer containing 3% bovine serum albumin and 5% normal serum from the host species of the secondary antibody. Subsequently, sections were stained for 90 minutes at room temperature with the following primary antibodies in antibody diluent: mouse anti-UTF1 (1:50, MAB4337, Millipore) goat anti-ZBTB16 (1:50; AF2944, R&D Systems), rabbit anti- KIT; goat anti-KIT (1:400; A4502, DakoCytomation; 1:50; AF332, R&D Systems), rabbit anti-SALL4 (1:500; ab29112, Abcam; 1:40; ab181087, Abcam), mouse anti-ENO2 (1:500, LS-B2890, LSBio), rabbit anti-UCHL1 (1:1000, 7863-0507, Biogenesis), rabbit anti-EPCAM (1:200; ab71919, Abcam), rabbit anti-ITGA6 (1:100; ab75737, Abcam). Isotype matched normal IgG was used as negative control. Primary antibodies were detected using AlexaFluor-488 or AlexaFluor-568 conjugated secondary antibodies (1:200, Invitrogen). The slides were mounted with VectaShield mounting medium containing DAPI (Vector Laboratories) for detection of nuclei. Sections were observed with a Nikon Eclipse E600 fluorescence microscope and images captured with MetaView Digital Imaging software. For the quantification of marker overlap, single-positive cells for each marker and double-positive cells were counted in cross-sections of seminiferous tubules. Total stained cell numbers were divided by the number of tubular cross-sections (at least 100 per sample x 3 replicate samples).

### *Colorimetric immunohistochemistry*

Human testicular tissue fragments were fixed with 4% PFA overnight, paraffin-embedded and sectioned (5  $\mu$ m). The tissue slides were de-paraffinized, rehydrated, incubated for 30 minutes in sodium citrate buffer (10 mM sodium citrate, pH 6.0, 0.05% Tween-20) for antigen retrieval. The tissue was then incubated in peroxidase block for 10 minutes and washed in PBS and blocked with a buffer containing 3% bovine serum albumin and 5% normal goat serum. Subsequently, sections were stained for 90 minutes at room temperature with rabbit anti-UCHL1 (1:1000, 7863-0507, Biogenesis). Isotype matched normal IgG was used as negative control. Primary antibody was detected using goat anti-rabbit HRP conjugated secondary antibody (1:200, sc-2054, Santa Cruz Biotechnology) for 30 minutes. Metal enhanced DAB substrate kit was used to detect staining (Thermo Scientific). The tissue was then counterstained with Periodic acid-Schiff and hematoxylin (Sigma-Aldrich) to enable identification of A<sub>dark</sub> and A<sub>pale</sub> spermatogonia.

### *Whole mount immunohistochemistry*

Human testicular tissue was teased apart using Collagenase type IV (1mg/mL) and DNase I (1mg/mL) in D-PBS. The tissue was then fixed overnight with 4% PFA. The tubules were permeabilized using PBS and 0.1% Triton-X and blocked with a blotto milk solution in D-PBS (D-PBS +0.02 mg/mL blotto dry milk powder + 5% Triton-X) and stained with a rabbit anti-UCHL1 (1:500, 7863-0507, Biogenesis) and goat anti-KIT (1:50; AF332, R&D Systems) primary antibodies overnight at 4°C. The primary antibodies were detected with donkey anti-rabbit IgG AlexaFluor568 and donkey anti-goat IgG AlexaFluor488 (1:200, Invitrogen). Finally,

the seminiferous tubules were mounted with VectaShield mounting media containing DAPI (Vector Laboratories) with raised cover slips and imaged with fluorescent microscopy.

*Xenotransplantation and whole mount immunofluorescent quantification of human SSC colonizing activity in mouse seminiferous tubules*

The human-to-nude mouse xenotransplantation was performed as a biological assay to investigate colonizing activity of putative human SSCs. Following FACS and MACS , unsorted and sorted testicular cell fractions were transplanted into the testes of busulfan-treated (40 mg/kg; Sigma, at 5–6 weeks of age), immune-deficient nude mice (NCr nu/nu; Taconic, Germantown, NY), as previously described [53, 114, 118, 159]. Briefly, xenotransplantation was performed 5 weeks after busulfan treatment by injecting cell suspensions containing 10% trypan blue (Invitrogen) into the seminiferous tubules of recipient mouse testes via the efferent ducts. Approximately 7  $\mu$ l of cell suspension was injected per testis. For quantitative analysis of colonization by human donor spermatogonia, the testes were recovered 8 weeks following transplantation, the tunica was removed, and the intact seminiferous tubules were dispersed gently with Collagenase IV (1mg/mL) and DNase I (1mg/mL) in D-PBS. The tubules were fixed for 4 hours in 4% PFA and the whole mount immunofluorescence was carried out by dehydrating samples in a graded series of methanol dilutions before incubating in MeOH:DMSO:H<sub>2</sub>O<sub>2</sub> (4:1:1) solution for three hours. The tubules were then rehydrated, blocked with a blotto milk solution in D-PBS (D-PBS + 0.02 mg/mL blotto dry milk powder + 5% Triton-X) and stained with a rabbit anti-primate testis cell primary antibody [114] at a 1:800 dilution overnight at 4°C. The primary antibody was detected with goat anti-rabbit IgG AlexaFluor488 (1:200, Invitrogen). Finally, the seminiferous tubules were mounted with VectaShield mounting

media containing DAPI (Vector Laboratories) with raised cover slips and imaged with fluorescent microscopy. Spermatogonial colonies were counted based on the following criteria: at least 4 cells exhibiting spermatogonial morphology (ovoid shape with high nuclear to cytoplasmic ratio) and located on the basement membrane in a continuous area of recipient seminiferous tubule ( $\leq 100 \mu\text{m}$  between cells).

### *Statistical Analysis*

I analyzed the data using linear mixed effect models, and performed Tukey's tests, as described in [180], to compare differences among the percent of SALL4<sup>+</sup> cells in unsorted versus sorted cell fractions in the immunocytochemistry experiments and colonizing activity in the human-to-nude mouse xenotransplant bioassay.

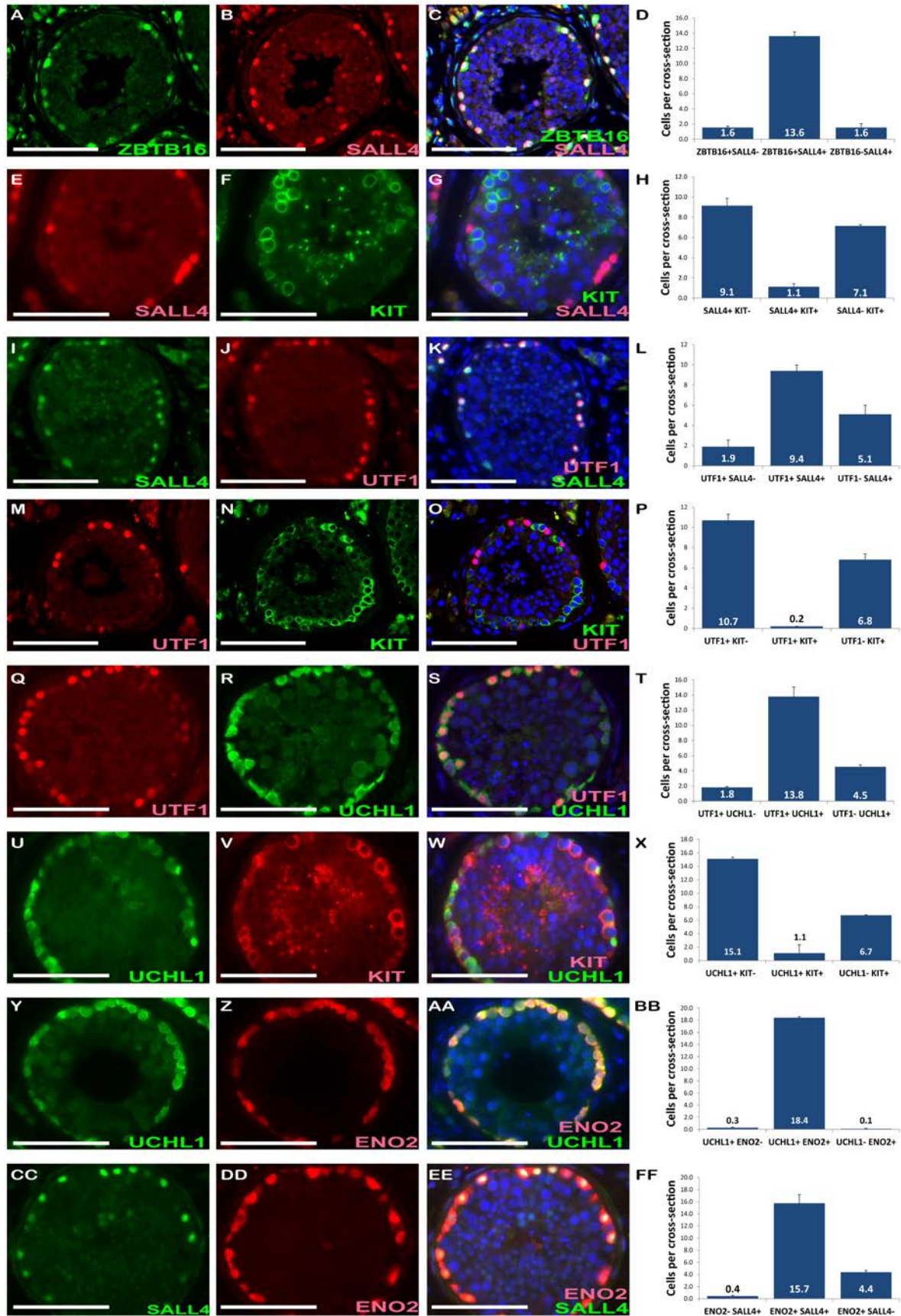
## **2.3 RESULTS**

### **2.3.1 Acquisition of human testicular tissue**

Testicular tissues used in this study were obtained from a total of 12 post-pubertal organ donors (Age 14-50). Testes weighed 11.3 to 26.0 grams and produced a theoretical yield (after correcting for tissue removed for pathology and immunofluorescence studies) of  $1.4 \times 10^9 \pm 0.14 \times 10^9$  cells per donor. All human testis cell suspensions used in this study were cryopreserved as described above and thawed at a later date for experimentation. Human testicular cells used in this study were frozen for periods of time ranging from 1 month to 15 months.

### 2.3.2 Immunohistochemical staining of human testicular sections

Immunohistochemical co-staining analysis was done to investigate the co-expression of known mouse and/or non-human primate spermatogonia markers in adult human testis. ZBTB16 and SALL4, which mark most stem and progenitor spermatogonia in rodents [179], were expressed in cells located on the basement membrane, consistent with location of undifferentiated spermatogonia. Roughly 89% of ZBTB16 positive cells were also positive for SALL4 (Figure 6 A-D), but also a small population of ZBTB16 positive cells (11%) did not express SALL4. Similarly, a sub-population of SALL4 positive cells also did not express ZBTB16 (11%) (Figure 6 D). Co-staining with SALL4 and KIT, an established marker of differentiating spermatogonia, revealed almost no overlap between these two markers (Figure 6E-H). These results suggest that SALL4 is not expressed by differentiating spermatogonia in human testis. UTF1 expression was also restricted to cells on the seminiferous tubule basement membrane (Figure 6 I-L). Co-staining with UTF1 and SALL4 indicated that 65% of the SALL4 positive cells express UTF1, whereas 35% of expressed SALL4 only. Seventeen percent of UTF1 positive cells express UTF1 only (Figure 6L). To confirm that UTF1 is not expressed by differentiating spermatogonia, I co-stained UTF1 with a differentiation marker KIT (Figure 6M-P) and found that there is no overlap between these two markers. Based on these results, I believe that UTF1 is a more restricted marker of stem and progenitor spermatogonia than SALL4. This interpretation is consistent with results of van Bragt and colleagues [28] who concluded that UTF1 is restricted to  $A_{\text{single}}$ ,  $A_{\text{paired}}$  and  $A_{\text{aligned4}}$  spermatogonia in rats. Similar to SALL4, UCHL1 expression is less restricted than UTF1 (Figure 6Q-T) with 75% of UCHL1 positive cells co-expressing UTF1 and 25% expressing UCHL1 only. UTF1 positive cells were UCHL1 positive 87% of the time and UTF1 only positive 13% (Figure 6T). Co-staining with KIT, confirms that UCHL1 is not expressed

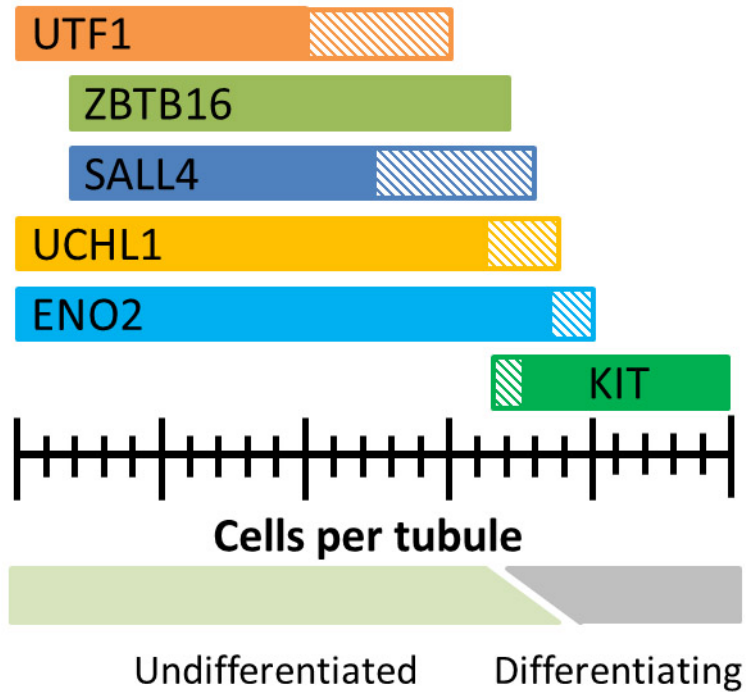




**Figure 6. Expression of ZBTB16, UTF1, SALL4, UCHL1, ENO2 and KIT in human seminiferous epithelium.**

Immunofluorescence co-staining for SALL4 and ZBTB16 (A-D), SALL4 and KIT (E-H), UTF1 and SALL4 (I-L), UTF1 and KIT (M-P), UTF1 and UCHL1 (Q-T), UCHL1 and KIT (U-X), UCHL1 and ENO2 (Y-BB) and SALL4 and ENO2 (CC-FF) in adult human testis. DAPI staining (blue) identifies all the nuclei. The bar graphs show quantification and relative proportion of each co-staining. The quantification is shown as the mean number of positive cells per cross-section of a seminiferous tubule. At least 100 seminiferous tubules were counted from 3 different organ donors. Bar graphs in D, H, L, P, T, X and BB indicate the mean number of marker positive cells per cross-section. Error bars represent SEM. Scale bars = 100  $\mu$ m. Reprinted from Valli et al., *Fertil Steril.* 2014 Aug;102(2):566-580, with permission from Elsevier Ltd.

by differentiating cells, demonstrated by limited overlap with KIT (Figure 6U-X). I also analyzed the expression pattern of a novel marker, ENO2, which exhibited nearly complete overlap of expression with UCHL1 (Figure 6Y-BB). By transitive logic, ENO2 is a marker of undifferentiated spermatogonia in humans because it exhibits nearly complete overlap with UCHL1, which has very little overlap with KIT. The overlap between ENO2 and SALL4 is less complete, with 78% of the ENO2 positive cells expressing SALL4 and 12% expressing ENO2 only (Figure 6CC-FF). These results indicate that ENO2 expression is slightly broader than SALL4 expression in human undifferentiated spermatogonia. Figure 7 summarizes our interpretation of these results in terms of the order and breadth of marker expression by human spermatogonia.



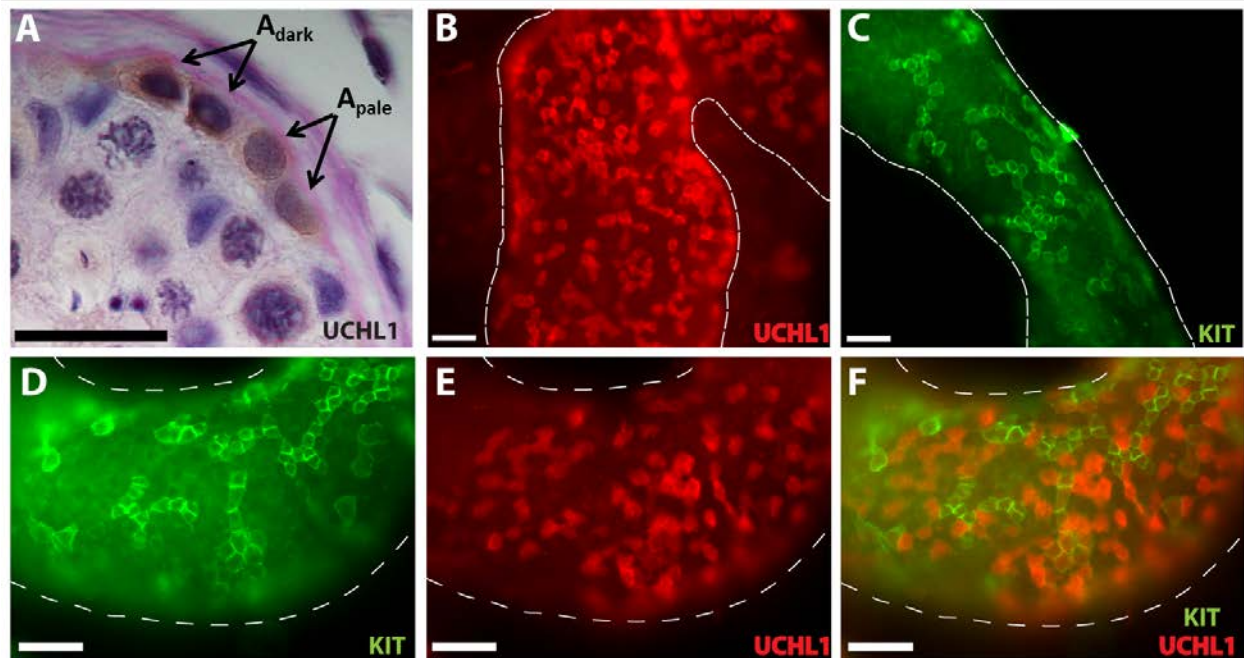
**Figure 7. Summary of marker expression in adult human testis.**

Colored bars indicate the overlap of markers based on data from Figure 6. Shaded area indicates range in data. UTF1 seems to be the most restricted marker of human spermatogonia, followed by ZBTB16 and SALL4. There is also almost no overlap between these markers and differentiation marker KIT. UCHL1 and ENO2 are more widely expressed in cells on the basement membrane of the seminiferous tubule and have slightly more overlap with KIT. Reprinted from Valli et al., *Fertil Steril.* 2014 Aug;102(2):566-580, with permission from Elsevier Ltd.

### **2.3.3 Correlation of spermatogonial markers with dark and pale descriptions of nuclear morphology and clone size**

To correlate molecular markers of human spermatogonia described in this study with classical descriptions of nuclear staining intensity ( $A_{\text{dark}}$  and  $A_{\text{pale}}$ ), I performed colorimetric immunohistochemistry for UCHL1 followed by Periodic Acid-Schiff and hematoxylin

counterstaining. The results in Figure 8A confirm that UCHL1 is expressed by human  $A_{\text{dark}}$  and  $A_{\text{pale}}$  spermatogonia.

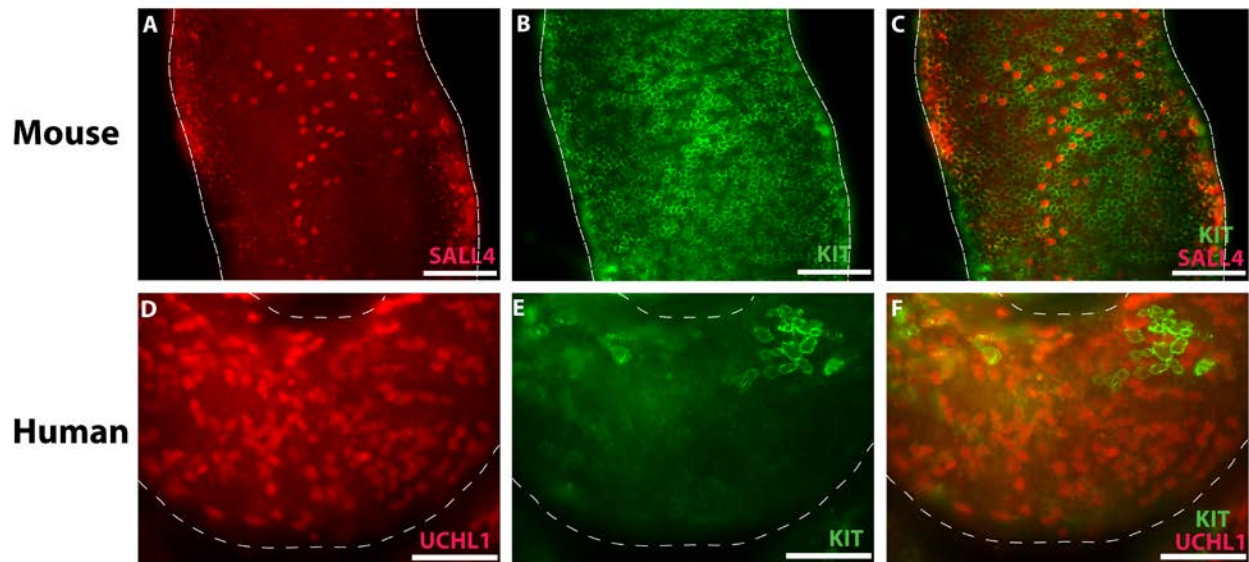


**Figure 8. UCHL1 expression in adult human testis.**

(A) UCHL1 staining in Periodic Acid-Schiff & Hematoxylin stained adult human testis section. UCHL1 is expressed by  $A_{\text{dark}}$  and  $A_{\text{pale}}$  spermatogonia. (B and D) UCHL1 and (C and E) KIT staining in whole mount staining of adult human testis. (F) UCHL1 clones are smaller (mostly 1-4 cells), whereas KIT clones tend to be bigger (more than 8). Scale bar = 50  $\mu\text{m}$ . Reprinted from Valli et al., *Fertil Steril*. 2014 Aug;102(2):566-580, with permission from Elsevier Ltd.

To correlate UCHL1 expression with clone size, I performed immunofluorescent spermatogonia, which are considered the reserve and active stem cells of the human testis, respectively [51, 181]. To correlate UCHL1 expression with clone size, I performed immunofluorescent analysis of UCHL1 expression in whole mount preparations of human seminiferous tubules. UCHL1 was expressed by cells located on the basement membrane of the

seminiferous tubules and arranged as single cells and clones of 2, 4 and sometimes 8 interconnected cells. In contrast, KIT expressing cells were typically arranged in clones of 4, 8 and sometimes 16 interconnected cells (Figure 8B-F). The density of undifferentiated spermatogonia on the basement membrane of human seminiferous tubules appears greater than in rodents (Figure 9A and D), whereas KIT<sup>+</sup> differentiating spermatogonia are considerably less dense in human tubules than in mouse (Figure 9B and E).



**Figure 9. Whole-mount immunohistochemistry of seminiferous tubules from mouse and human testes.**

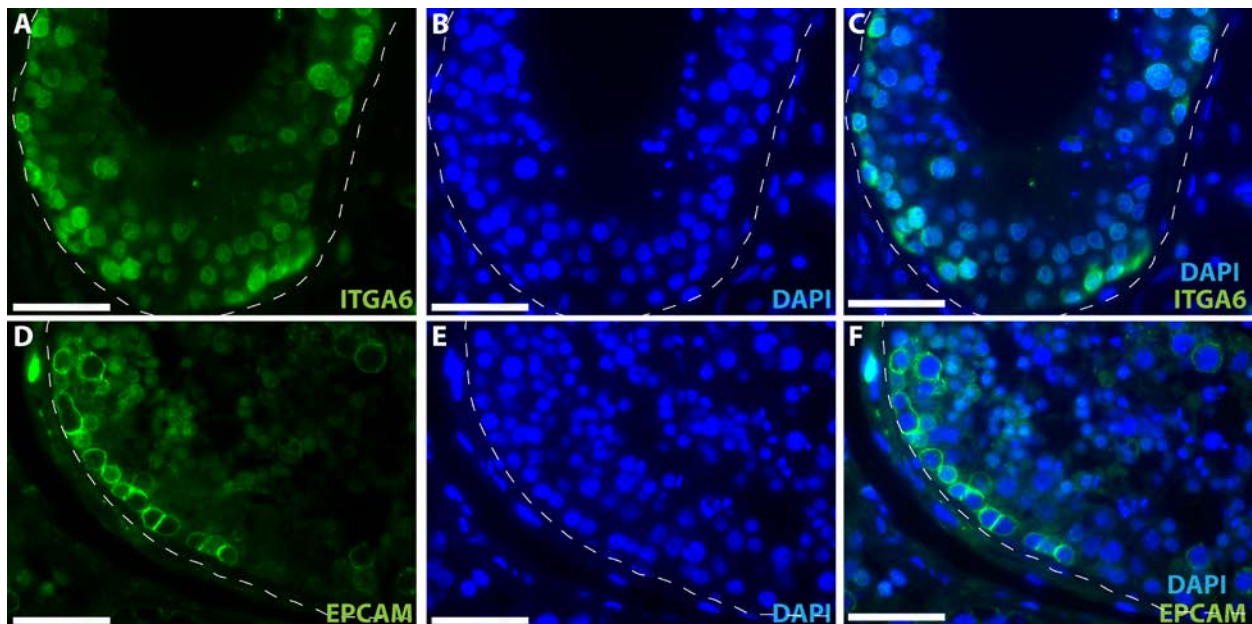
Whole-mount immunofluorescence analysis of the undifferentiated spermatogonia marker, SALL4 (A), differentiating spermatogonia marker, KIT (B) and co-staining of SALL4 and KIT (C) in mouse. Whole-mount immunofluorescence analysis of undifferentiated spermatogonia marker UCHL1 (D), differentiating spermatogonia marker KIT (E) and co-staining of UCHL1 and KIT (F) human. Scale bar = 100 $\mu$ m.

### 2.3.4 Immunohistochemical evaluation of cell surface markers in adult human testes

THY1, ITGA6 and EPCAM are cell surface markers that have each been used to isolate and enrich spermatogonial stem cells in other species [19, 20, 29, 53]. Previous studies indicated that

these cell surface markers are conserved in human testes [112, 118, 143, 147] and I hypothesized that each could be used to isolate and enrich human SSCs by FACS and/or MACS. I was not able to confirm the expression of THY1 in adult human testes by immunohistochemistry in this study. However, others have reported that this marker is expressed in human testes [112, 143, 147].

Immunohistochemical analysis of ITGA6 expression in normal adult human testis sections indicated that this antigen is expressed by many germ cells, including cells located on the basement membrane of seminiferous tubules (Figure 10A-C) and that EPCAM is expressed primarily by cells on the basement membrane of the seminiferous tubules, as well as a few cells located more towards the lumen (Figure 10D-F).

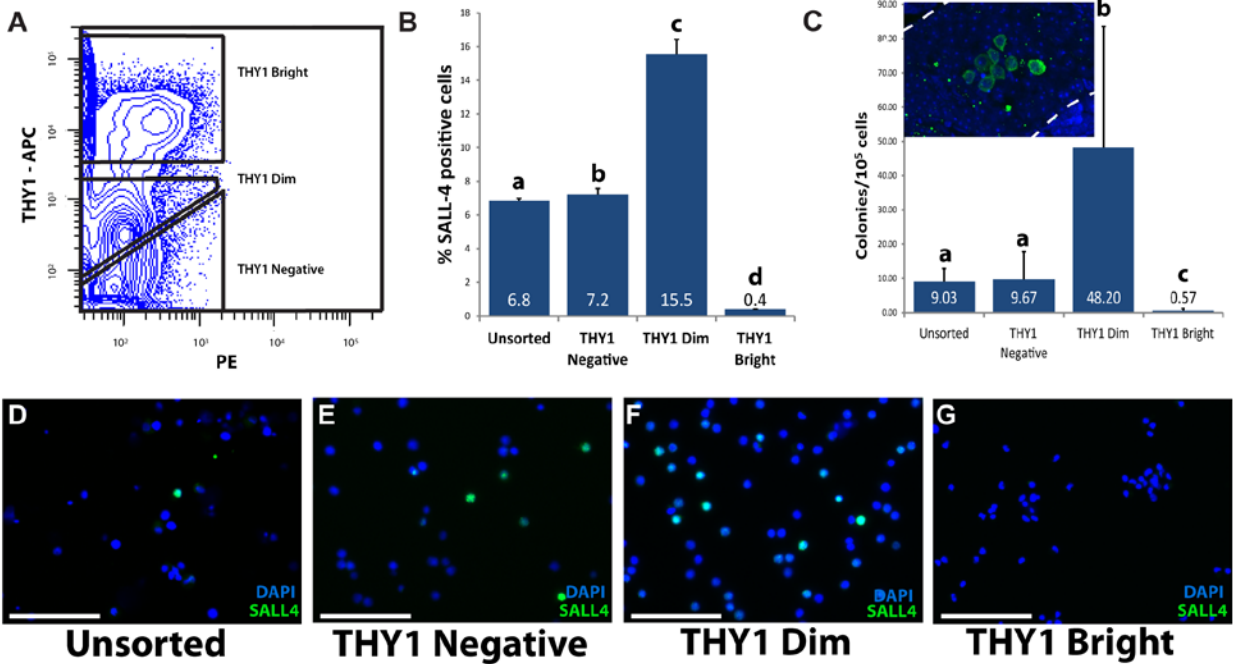


**Figure 10. ITGA6 and EPCAM expression in adult human testis sections.**

Immunofluorescence staining for ITGA6 (**A and C**) and EPCAM (**D and F**) in adult human testis. DAPI staining (blue) (**B and E**) identifies all the nuclei. Scale bars = 50  $\mu$ m. Reprinted from Valli et al., *Fertil Steril*. 2014 Aug;102(2):566-580, with permission from Elsevier Ltd.

### 2.3.5 Expression of THY1 in adult human testicular cell suspensions

THY1 is a marker of mouse, rat and non-human primate SSCs [19, 29, 53] as well as a marker for mouse and human hematopoietic stem cells [182-184]. Therefore, I hypothesized that THY1 is a marker for human SSCs and analyzed the expression on adult human testicular cells using FACS and MACS. Staining of adult human testis cell suspensions with THY1 identified three populations of cells, designated THY1 bright, THY1 dim and THY1 negative, based on their level of fluorescence, plotted against a negative PE axis, which helps to identify and eliminate autofluorescence (Figure 11A). The THY1 bright, dim and negative fractions represented  $12.2 \pm 4.2\%$ ,  $19.0 \pm 4.0\%$  and  $46.5 \pm 7.0\%$  of the live cells, respectively. Immunofluorescence staining revealed that  $6.8 \pm 0.1\%$  of unsorted human testicular cells express human spermatogonia marker SALL4, compared to  $7.2 \pm 0.3\%$  in the THY1 negative fraction ( $p < 0.01$ ),  $15.5 \pm 0.9\%$  in the THY1 dim fraction ( $p < 0.01$ ) and only  $0.4 \pm 0\%$  in the THY1 bright fraction ( $p < 0.01$ ) (Figure 11B). To confirm the immunocytochemistry results and to functionally correlate THY1 expression in adult human testis to SSC colonizing activity, the human-to-nude mouse xenotransplantation assay was performed. The transplant results confirm that SSC colonizing activity was depleted from THY1 bright fraction ( $0.57 \pm 0.6$  colonies/ $10^5$  cells;  $p < 0.01$  compared to the unsorted controls). The majority of SSC colonizing activity was recovered in the THY1 dim fraction ( $48.2 \pm 36.3$  colonies/ $10^5$  cells;  $p < 0.01$  compared to the unsorted controls), compared to  $9.03 \pm 3.8$  and  $9.67 \pm 8.1$  colonies/ $10^5$  cells in unsorted and THY1 negative fractions, respectively (Figure 11C). Based on these results, there is roughly a 5-fold enrichment of SSC colonizing activity in the THY1 dim fraction of human testis cells.

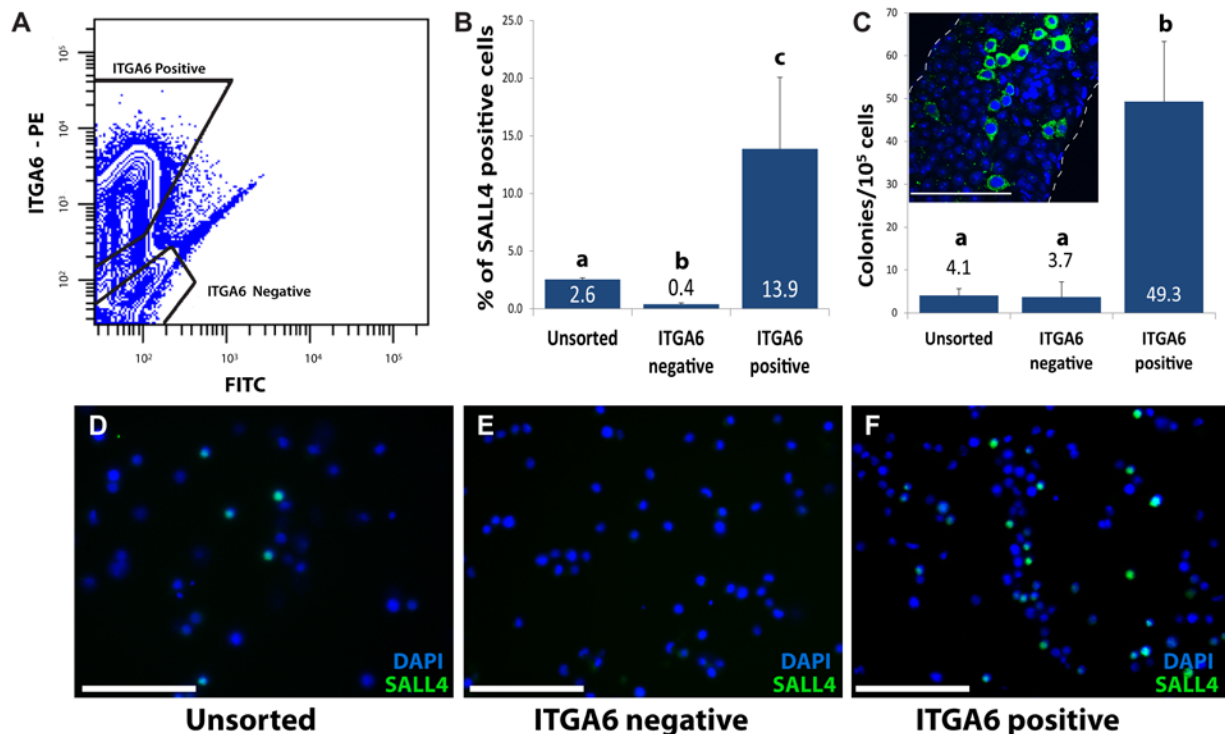


**Figure 11. FACS sorting and characterization of THY1 expression in adult human testes.**

(A) FACS was used to characterize and sort human testicular cells based on the level of THY1 expression. Based upon THY1-APC staining intensity and negative PE autofluorescence, three populations were identified – THY1<sup>bright</sup>, THY1<sup>dim</sup> and THY1<sup>neg</sup>. Negative gates were defined by analysis of human testis cells stained using APC-conjugated isotype control antibodies. (B) After the sort, all sorted fractions, as well as the unsorted cells, were fixed and immunocytochemistry for SALL4 was performed. SALL4 positive cells were enriched in the THY1<sup>dim</sup> fraction compared to the unsorted cells. (C) To confirm the ICC results, human-to-nude mouse xenotransplants were also performed. Two months after transplant, colonies of human spermatogonia were identified in mouse recipient testes. (C inset) Examples of colonies of human spermatogonia in whole mount preparations of recipient mouse seminiferous tubules stained with the rabbit anti-primate antibody. Colonies in each recipient testis were counted and normalized to 10<sup>5</sup> viable cells transplanted per testis. (D-G) Representative images of SALL4 staining from each sorted fraction and unsorted cells. At least 10 views were counted from each fraction based on DAPI staining and SALL4 staining. Different letter indicate P < 0.01, same letters indicate P > 0.05. Bar graphs in B and C are presented as mean ± SEM. Scale bar = 100 μm. Reprinted from Valli et al., Fertil Steril. 2014 Aug;102(2):566-580, with permission from Elsevier Ltd.

### 2.3.6 Expression of ITGA6 in adult human testicular cell suspension

To determine whether ITGA6 is expressed on human spermatogonia and could be used as a positive selection marker to enrich human SSCs, adult human testicular cell suspensions were stained with a PE-conjugated antibody against ITGA6 and sorted by FACS. Two distinct populations of cells were gated; ITGA6 negative and ITGA6 positive (Figure 12A), which represented  $27.6 \pm 7.6\%$  and  $11.6 \pm 3.0\%$  of the live cells, respectively. Immunocytochemistry of the ITGA6 sorted fractions and unsorted cells revealed that  $13.8 \pm 6.2\%$  of cells in the ITGA6 positive fraction were SALL4 positive (Figure 12B and F), compared to  $2.6 \pm 0.2\%$  in the unsorted cell population ( $p < 0.01$ ) (Figure 12B and D). SALL4 positive cells were depleted from the ITGA6 negative fraction ( $0.38 \pm 0.1\%$ ;  $p < 0.01$  compared to the unsorted controls; Figure 12B and E). To confirm the immunocytochemistry results, colonizing activity in ITGA6 sorted and unsorted cells was assessed by xenotransplantation into nude mouse testes.





**Figure 12. FACS sorting and characterization of ITGA6 expression in adult human testes.**

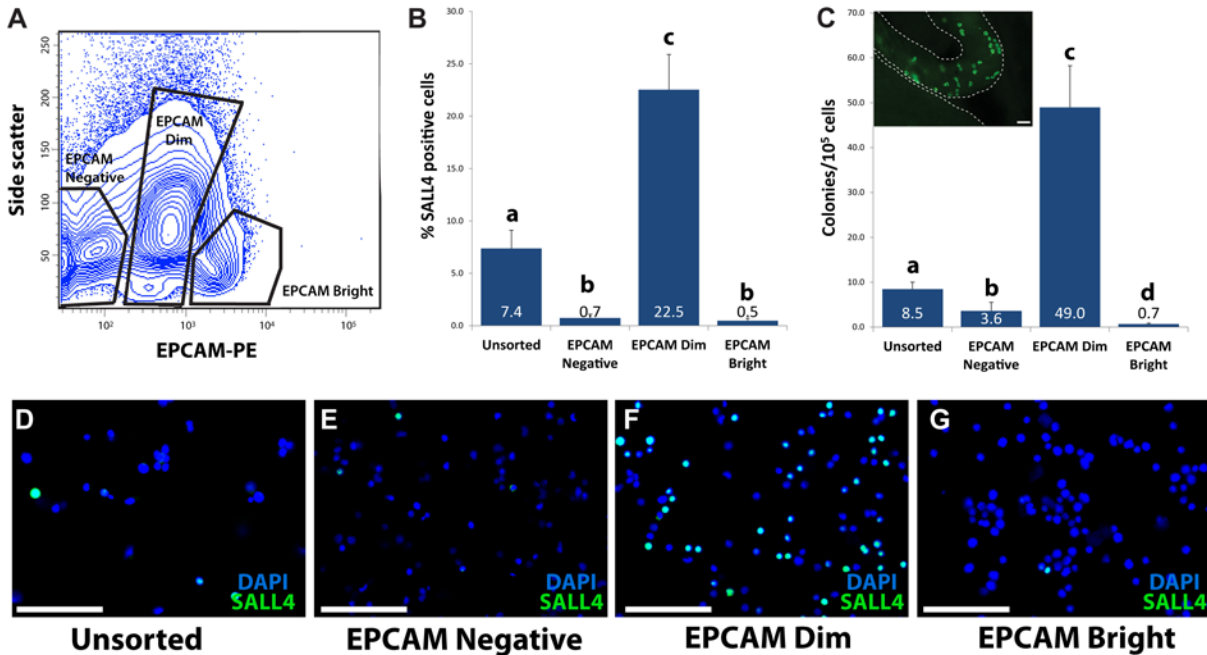
(A) FACS sorting for ITGA6 in human testis resulted in 2 different populations based upon ITGA6 -PE staining intensity and negative FITC autofluorescence – ITGA6 positive and ITGA6 negative. Negative gates were defined by analysis of human testis cells stained using PE-conjugated isotype control antibodies. (B) After the sort, all sorted fractions, as well as the unsorted cells, were fixed and immunocytochemistry for SALL4 was performed. SALL4 positive cells were enriched in the ITGA6 positive fraction compared to the unsorted cells. (C) To confirm the ICC results, human to nude mouse xenotransplants were also performed. Two months after transplant, colonies of human spermatogonia were identified in mouse recipient testes. (C inset) Example of a colony of human spermatogonia in whole mount preparations of recipient mouse seminiferous tubules stained with the rabbit anti-primate antibody. Colonies in each recipient testis were counted and normalized to  $10^5$  viable cells transplanted per testis. (D-F) Representative images of SALL4 staining from each sorted fraction and unsorted cells. At least 10 views were counted from each fraction based on DAPI staining and SALL4 staining. Different letters indicate  $P < 0.01$ , same letters indicate  $P > 0.05$ . Bar graphs in B and C are presented as mean  $\pm$  SEM. Scale bar = 100  $\mu$ m. Reprinted from Valli et al., Fertil Steril. 2014 Aug;102(2):566-580, with permission from Elsevier Ltd.

On average, cells in the ITGA6 positive fraction produced significantly more colonies in recipient mouse testis ( $49.3 \pm 14.0$  colonies/ $10^5$  cells transplanted) than the unsorted controls ( $4.1 \pm 1.5$  colonies/ $10^5$  cells,  $p < 0.01$ ) or ITGA6 negative cells ( $3.7 \pm 3.5$  colonies/ $10^5$  cells) (Figure 12C). Thus, SSC colonizing activity resides predominantly in the ITGA6 positive fraction of human testis cells and is enriched approximately 12-fold compared to the unsorted population.

### **2.3.7 Expression of EPCAM in adult human testicular cell suspension**

To determine whether EPCAM is expressed on human spermatogonia and could be used as a positive spermatogonial selection marker, human testicular cell suspensions were stained with a PE-conjugated antibody against EPCAM and sorted using FACS. As demonstrated in Figure 13

populations of cells were identified following staining with EPCAM, based on their level of fluorescence and on side scatter of incident light, which provides a measure of intracellular complexity: EPCAM negative, EPCAM dim, and EPCAM bright. EPCAM is known to be expressed on SSCs in rats [29, 185]. Following sorting, each fraction of cells was fixed and stained with an antibody directed against SALL4 to quantify undifferentiated human spermatogonia. The majority of SALL4 positive spermatogonia were recovered in the EPCAM dim fraction (Figure 13B and F). Compared with  $7.4 \pm 1.8\%$  of cells expressing SALL4 in the unsorted testicular cell population,  $22.5 \pm 3.3\%$  of cells in the EPCAM dim fraction expressed SALL4 ( $P < 0.0001$ ). The EPCAM negative and EPCAM bright fractions were virtually depleted of SALL4-expressing cells ( $P < 0.0001$  compared with unsorted). The human-to-nude mouse xenotransplantation assay was used to quantify SSC activity in unsorted, EPCAM negative, EPCAM dim, and EPCAM bright fractions. Unsorted human testicular cells produced  $8.5 \pm 1.5$  colonies of spermatogonia per  $10^5$  viable transplanted cells (Figure 13C). The EPCAM dim fraction produced  $49 \pm 9.2$  colonies of spermatogonia per  $10^5$  viable transplanted cells, representing an approximate 6-fold enrichment compared with the unsorted population ( $P < 0.0001$ ). Mirroring the SALL4 data, colony numbers were significantly reduced in the EPCAM negative and EPCAM bright fractions ( $P < 0.01$  compared with unsorted controls). Thus, I conclude, based on SALL4 immunocytochemistry (Figure 13B and D-G) and the xenotransplantation results (Figure 13C), that SSC activity resides in the EPCAM dim fraction of human testis cells.

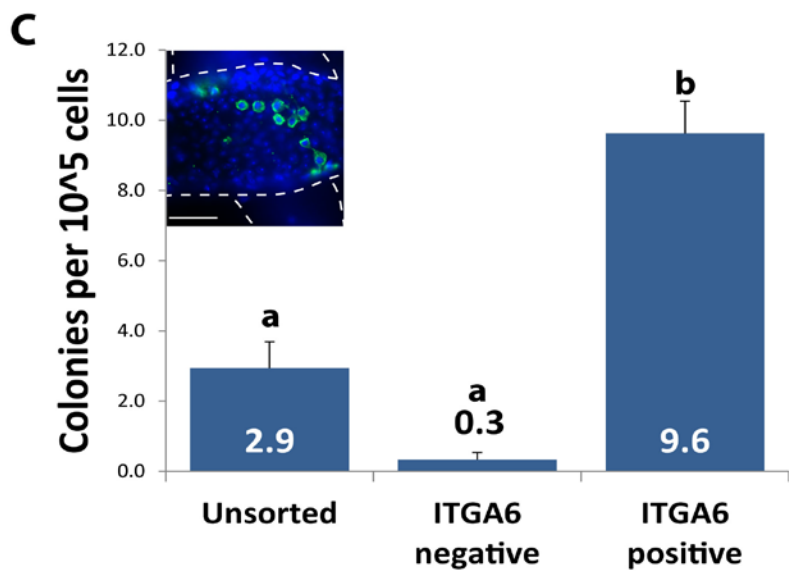
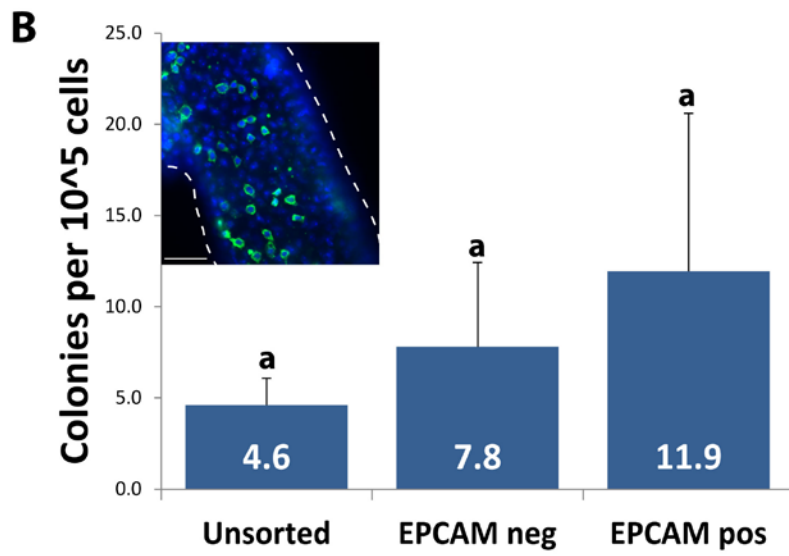
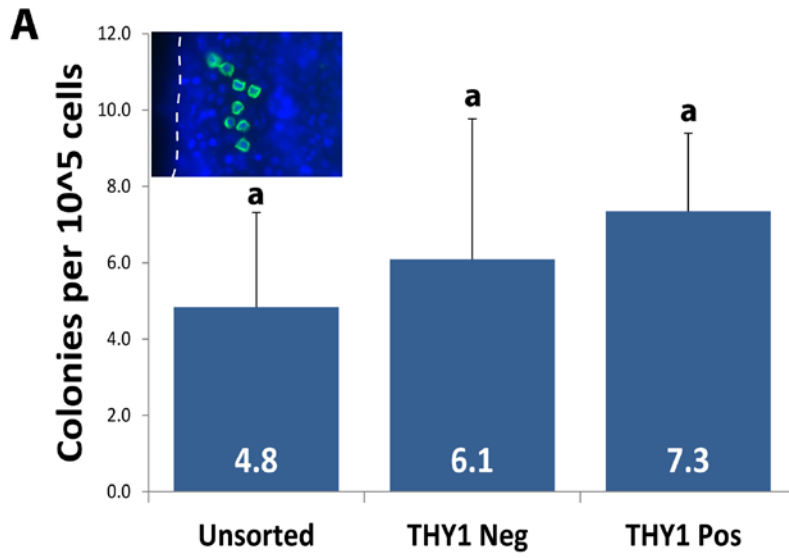


**Figure 13. FACS sorting and characterization of EPCAM expression in adult human testes.**

(A) FACS was used to characterize and sort human testicular cells based on the level of EPCAM expression. Based upon EPCAM-PE staining intensity and negative PE autofluorescence, three populations were identified –  $THY1^{bright}$ ,  $THY1^{dim}$  and  $THY1^{neg}$ . Negative gates were defined by analysis of human testis cells stained using APC-conjugated isotype control antibodies. (B) After the sort, all sorted fractions, as well as the unsorted cells, were fixed and immunocytochemistry for SALL4 was performed. SALL4 positive cells were enriched in the  $THY1^{dim}$  fraction compared to the unsorted cells. (C) To confirm the ICC results, human-to-nude mouse xenotransplants were also performed. Two months after transplant, colonies of human spermatogonia were identified in mouse recipient testes. (C inset) Examples of colonies of human spermatogonia in whole mount preparations of recipient mouse seminiferous tubules stained with the rabbit anti-primate antibody. Colonies in each recipient testis were counted and normalized to  $10^5$  viable cells transplanted per testis. (D-G) Representative images of SALL4 staining from each sorted fraction and unsorted cells. At least 10 views were counted from each fraction based on DAPI staining and SALL4 staining. Different letters indicate  $P < 0.01$ , same letters indicate  $P > 0.05$ . Bar graphs in B and C are presented as mean  $\pm$  SEM. Scale bar = 100  $\mu$ m. Reprinted with permission from Dovey SL and Valli H et al., J Clin Invest. 2013 Apr 1;123(4):1833-43, Copyright (2014).

### **2.3.8 Enrichment of human spermatogonia using MACS**

Analysis of FACS indicated that ITGA6, THY1 and EPCAM can be used to effectively isolate and enrich human SSCs from a heterogeneous testis cell suspension. However, the FACS sorting approach has limited throughput ( $\sim 30 \times 10^6$  cells per day). Therefore, I evaluated a higher throughput sorting approach (MACS) to maximize the use of human testicular cells and compare the results to FACS. I evaluated the fractionation of human testis cells by THY1 MACS where there is no option to distinguish between bright and dim expression of THY1. The cells were sorted into THY1 positive (bound) and negative (flow through) fractions using MACS and then transplanted into nude mouse testes to analyze SSC colonizing activity relative to unsorted human testis cells. Unsorted cells produced  $4.8 \pm 2.5$  colonies/ $10^5$  cells, compared to  $6.1 \pm 2.0$  and  $7.3 \pm 3.7$  colonies/ $10^5$  cells in THY1 negative and THY1 positive fractions, respectively ( $P > 0.05$ , compared to unsorted and each other), indicating that MACS did not effectively fractionate SSC colonizing activity based on THY1 expression (Figure 14A). Similar to the THY1 FACS results in this study, the SSC colonizing activity is enriched in the EPCAM dim fraction of human testis cells. Therefore, it is not surprising that MACS did not effectively fractionate SSC colonizing activity from human testis cells based on EPCAM expression (Figure 14B).



**Figure 14. MACS sorting of human testicular cells for THY1, EPCAM and ITGA6.**

Human testicular cells were MACS sorted into 2 fractions – negative (flow through) and positive (bound). Both positive and negative fractions from MACS, as well as unsorted cells, were transplanted into nude mouse testis. **(Inset A, B and C)** Two months after transplant, colonies of human spermatogonia were identified in whole mount preparations of recipient mouse seminiferous tubules using the rabbit anti-primate antibody. Colonies in each recipient testis were counted and normalized to  $10^5$  viable cells transplanted per testis. **(A and B)** For THY1 and EPCAM, no significant difference was found between the unsorted cells and the sorted fractions ( $P > 0.05$ ). **(C)** ITGA6 positive fraction was enriched roughly 3 fold compared to unsorted cells ( $P < 0.05$ ). Bar graphs are presented as mean  $\pm$  SEM. Scale bar = 100  $\mu$ m. Reprinted from Valli et al., Fertil Steril. 2014 Aug;102(2):566-580, with permission from Elsevier Ltd.

In contrast, MACS was effective for isolation and enrichment of human SSC colonizing activity based on ITGA6 expression (Figure 14C). SSC colonizing activity in the ITGA6 positive MACS fraction was enriched over 3-fold ( $9.6 \pm 0.9$  colonies/ $10^5$  cells) compared to the unsorted fraction ( $2.9 \pm 0.8$  colonies/ $10^5$  cells ;  $P < 0.05$ ; Figure 14C). SSC colonizing activity was nearly depleted in the ITGA6 negative fraction, which produced only  $0.3 \pm 0.2$  colonies/ $10^5$  cells, indicating that almost all SSCs were recovered in the ITGA6 positive fraction.

## 2.4 DISCUSSION

In rodents, SSCs are defined by their ability to establish and maintain spermatogenesis when transplanted into infertile mouse testis [101, 122, 123, 186]. Although there is no specific molecular marker of rodent SSCs (except possibly ID4 and PAX7 [22, 43]), stem and progenitor spermatogonia can be described collectively by expression of some or all of the following markers GFR $\alpha$ 1, POU3F1, POU5F1, ZBTB16, NGN3, NANOS2, NANOS3, SOHLH1,

SOHLH2, FOXO1, ITGA6, LIN28, ID4, PAX7, UTF1, CDH1, GPR125, ITGB1, EPCAM, CD9 and THY1 [13-43, 160, 187], and by their clonal arrangement on the basement membrane of seminiferous tubules ( $A_{\text{single}}$ ,  $A_{\text{paired}}$ ,  $A_{\text{aligned}}$ ; [188]). In humans, stem spermatogonia are described primarily as  $A_{\text{dark}}$  and  $A_{\text{pale}}$  based on the intensity of nuclear staining with hematoxylin [44, 48, 121]. There is limited information about how dark and pale descriptions of nuclear morphology correlate with transplantation potential, molecular markers or clone size.

Here I show that spermatogonia on the basement membrane of human seminiferous tubules have the phenotype of  $SALL4^+$ ,  $ZBTB16^+$ ,  $UTF^+$ ,  $UCHL1^+$  and  $ENO2^+$  (Figure 6). The expression of  $SALL4$ ,  $ZBTB16$ ,  $UTF1$  and  $UCHL1$  in human testes has been reported previously [26, 109, 138, 139, 143, 146].  $ENO2$  is a gene that was identified by Oatley and co-workers because it is upregulated in  $ID4$ -GFP positive spermatogonia [189]. This is the first study to demonstrate that  $ENO2$  is expressed by human spermatogonia and co-expressed with established markers of human stem and progenitor spermatogonia (i.e.,  $UCHL1$  and  $SALL4$ ) [139, 143]. This is also the first study to quantify the expression of these markers at the cellular level and describe their expression relative to other stem and progenitor markers by co-staining. I believe this systematic molecular profiling will identify subpopulations of cells (e.g., putative stem, progenitor and differentiating cells) that will become the subject of future investigations.

The majority of cells that express  $SALL4$ ,  $ZBTB16$ ,  $UTF1$ ,  $UCHL1$  and  $ENO2$ , do not express the differentiation marker  $KIT$ , as demonstrated by direct co-staining (i.e.,  $UCHL1/KIT$ ,  $SALL4/KIT$  and  $UTF1/KIT$ ) or transitive logic ( $UCHL1/ENO2$ ; Figure 6). These results suggest that  $SALL4$ ,  $ZBTB16$ ,  $UTF1$ ,  $UCHL1$  and  $ENO2$  mark human undifferentiated spermatogonia and immunohistochemical analysis confirms that  $UCHL1$  is expressed by  $A_{\text{dark}}$  and  $A_{\text{pale}}$  spermatogonia, the putative SSCs in human testes (Figure 8). Examination of these markers in

whole mount preparations of seminiferous tubules provides novel insights about human spermatogenic lineage development. Our results indicate that UCHL1 tended to be expressed by smaller clones (1-4 cells) while KIT is expressed in larger clones (usually 8 or more cells). Collectively, these results indicate that several markers of rodent stem and progenitor spermatogonia are conserved in humans and that spermatogonial differentiation in humans is correlated with increased clone size and initiation of KIT expression, similar to rodents [21, 179].

Spermatogenesis is an extremely productive system that produces millions of sperm per gram of testicular tissue each day in rodents and humans [1-3]. However, our results suggest that the dynamics of spermatogenic lineage development in humans may be different than rodents. In rodents, rare undifferentiated spermatogonia are heavily outnumbered by transit-amplifying differentiated spermatogonia [9]. In contrast, I found that number of undifferentiated spermatogonia in human testes was greater than the number of KIT<sup>+</sup> differentiated spermatogonia (Figure 6, 8 and 9). Thus, it appears that the highly productive spermatogenic system in rodents depends on a small pool of stem and progenitor spermatogonia and a large pool of transit-amplifying cells while the human spermatogenic lineage is characterized by a relatively larger pool of undifferentiated stem and progenitor cells and a smaller pool of transit amplifying cells.

FACS is suitable for characterizing relatively small cell populations ( $\leq 30 \times 10^6$ ) and can be used to achieve significant enrichment of spermatogonial stem cells [19, 29, 53, 118, 133, 160, 190-193]. When coupled with molecular marker screening (using markers that are restricted to stem and progenitor spermatogonia) and the stem cell transplant assay to validate sorted fractions, FACS can be a powerful tool for dissecting the molecular phenotype of SSCs. In the



current study, I used SALL4 immunocytochemistry (ICC) to screen sorted cell populations. I considered SALL4 an excellent marker for screening human stem and progenitor spermatogonia because it is conserved in mice [139, 178, 179], rats (Gassei and Orwig, unpublished), monkeys [139] and humans [139], including expression by human A<sub>dark</sub> and A<sub>pale</sub> spermatogonia [139]. SALL4 ICC provided a rapid assessment of sorted fractions and was an excellent predictor of the results from human-to-nude mouse SSC xenotransplantation, which has an inherent two month delay to analysis. Based on the data presented here, I believe that UTF1, ZBTB16, UCHL1 and ENO2 would also be good markers to rapidly screen for human stem and progenitor spermatogonia.

SSC transplantation is the experimental “gold standard” for assaying spermatogonial stem cells [194, 195]. SSC transplantation in humans may someday be feasible in the clinical setting [196], but cannot be used as a routine bioassay. However, Nagano and coworkers demonstrated that human SSCs can engraft the testes of infertile, immune compromised mice [197]. Human SSCs do not produce complete spermatogenesis in mouse seminiferous tubules, but they do execute several functions that are consistent with the activity of SSCs: 1) they migrate to the basement membrane of seminiferous tubules without being phagocytosed by mouse Sertoli cells; 2) they proliferate to produce characteristic chains and networks of spermatogonia and 3) they persist for several months. Human-to-nude mouse xenotransplantation is becoming a routine bioassay for human SSCs [26, 94, 95, 112, 118, 145, 197].

Studies employing FACS followed by transplantation of sorted fractions have established that ITGA6, THY1 and EPCAM are markers of SSCs in rodents [19, 20, 29]. Similar methodology with FACS or MACS sorting followed by human-to-mouse xenotransplantation

has been used to demonstrate that CD9 and SSEA4 are markers of human SSCs [112, 145]. Human testis cells have also been fractionated by MACS based on expression of GPR125, THY1 and ITGA6 [143, 147, 198], but stem cell activity in sorted fractions was not tested by transplantation.

Flow cytometry analyses in the current study identified two distinct THY1 positive populations in the human testis that I designated dim and bright. SALL4 staining as well as xenotransplant results suggested that the majority of the SSCs were in the THY1 dim fraction and SSC colonizing activity in that fraction was enriched approximately 5-fold compared to unsorted human testis cells (Fig. 11C). Almost no SSCs are found in the THY1 bright fraction. I obtained similar results for EPCAM, where the SSC colonizing activity was recovered in the EPCAM dim fraction of human testis cells and depleted in the EPCAM bright and EPCAM negative fractions (Figure 13). Interestingly, neither of these markers could be used to effectively fractionate and enrich SSC colonizing activity from the human testis using MACS. SSC colonizing activity was recovered in both the bound and flow through fractions and colonizing activity in each fraction was similar to unsorted controls (Figure 14A and B). Perhaps this result can be attributed to the low expression level of these two antigens in human SSCs. Considering our MACS results, it is noteworthy that THY1 MACS is routinely used to sort SSCs from mouse testes [192, 199-203]. These results may indicate that there are species-specific differences in the level of THY1 expression. Alternatively, these results may indicate technical differences between direct labeling with bead-conjugated THY1 primary antibodies (mouse) and indirect labeling using bead conjugated secondary antibodies (current study). The bead conjugated anti-mouse THY1 antibodies did not cross-react with the human THY1 antigen (data not shown). Flow cytometric analysis of ITGA6 in human testis cells revealed only two distinct populations,

positive and negative, and the majority of SSC colonizing activity was recovered in the ITGA6positive fraction, which was enriched 12-fold compared to unsorted controls (Figure 12C). In contrast to THY1 and EPCAM, cells with SSC colonizing activity could be effectively isolated and enriched from heterogeneous human testis cell suspensions using ITGA6 MACS. However, the level of enrichment achieved by ITGA6 MACS (3.3-fold) was less than ITGA6 FACS (12-fold). Sorting resolution by FACS is typically greater than MACS because FACS allows for gating of cell populations based on simultaneous evaluation of several parameters, including viability (PI), cell size (forward scatter of incident light), cell complexity (side scatter of incident light) and specific immunoreactivity (autofluorescent<sup>-</sup>, nonspecific binding<sup>-</sup>).

I identified several proteins with expression limited primarily to undifferentiated spermatogonia (KIT<sup>-</sup> cells) located on the basement membrane of seminiferous tubules in human testes. These markers may provide insights into the molecular mechanisms that regulate the function of human SSCs and can be used to screen human cell populations or tissues for putative SSCs. In addition they can be used to validate newly discovered markers of human stem and progenitor spermatogonia using co-staining approaches similar to those employed in the current study to validate the expression of ENO2 in human undifferentiated spermatogonia. In this study I demonstrated that human SSCs have the cell surface phenotype THY1 dim, EPCAM dim, ITGA6 positive. SSEA4 and CD9 are also cell surface markers of human SSCs that have been validated by human-to-mouse xenotransplantation [112, 145]. These markers can now be used alone or in combination to achieve significant enrichment of human SSCs for downstream studies. MACS can also be used for isolation and enrichment of SSCs prior to initiation of SSC cultures, as previously described for mice [192, 204]. ITGA6 (current study), CD9 [145] and SSEA4 [112] are also amenable to immunomagnetic sorting, which has virtually unlimited cell

sorting capacity and will facilitate isolation of SSCs from human testes that can contain over one billion cells.

### **3.0 CRYOPRESERVATION OF HUMAN SPERMATOGONIAL STEM CELLS**

#### **3.1 INTRODUCTION**

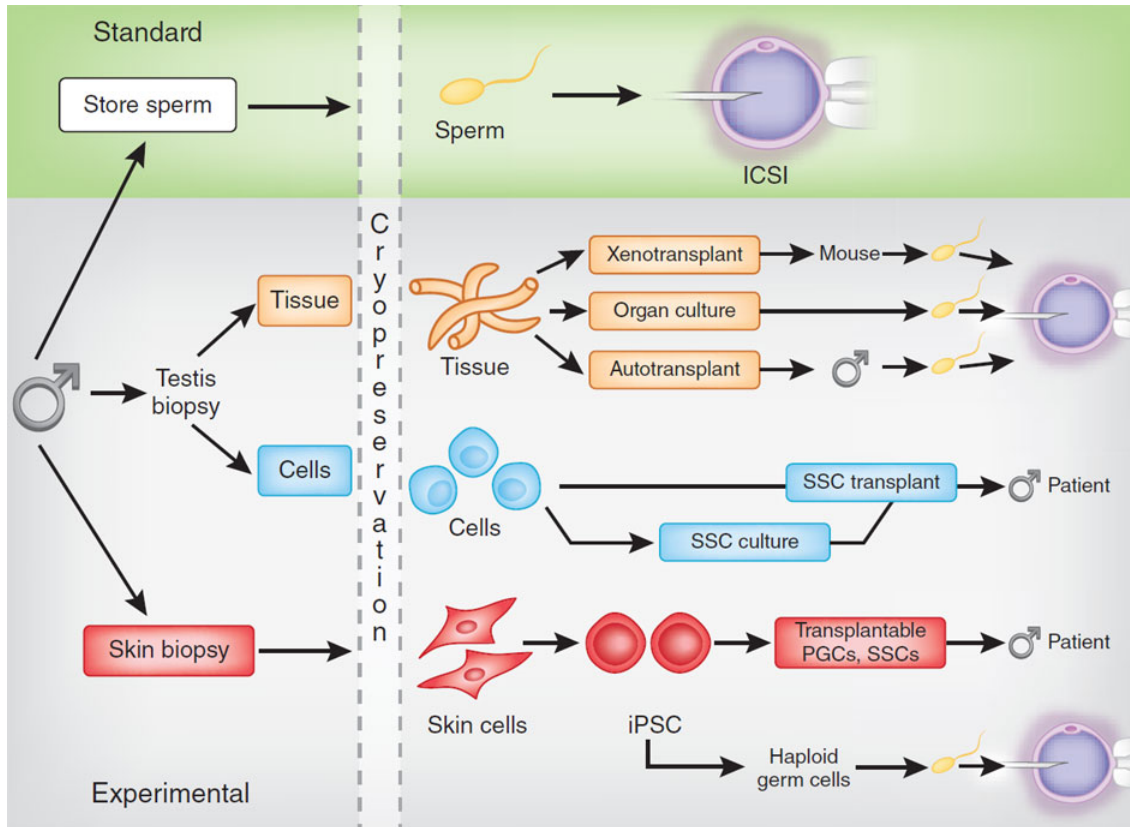
Improved therapies for cancer and other conditions have resulted in growing population of long-term survivors. Unfortunately, some cancer treatments, like whole body radiation or alkylating chemotherapy, can render the patient infertile [205]. For grown men and pubertal boys, the established fertility preservation protocol involves cryopreserving a semen sample (Figure 15, top). However, that is not an option for prepubertal boys who do not make sperm yet. For these patients, there are several stem cell based technologies in the research pipeline that in the future may offer novel techniques to preserve and restore their fertility. Even though these techniques are not yet available, prepubertal patients could in the future benefit from testicular tissue cryopreservation now. The technique(s) might be available by the time the patients are ready to have a family. In that case, the prepubertal patient and his parents are counseled on the reproductive risks of the cancer therapy and if the parents decide to cryopreserve testicular tissue, a testicular biopsy is taken from the patient prior to the initiation of cancer treatment. The testicular biopsy is then cryopreserved in liquid nitrogen for possible future use [196, 206].

The techniques in the research pipeline that may be available for these patients in the future include testicular tissue grafting (Figure 15, bottom, yellow boxes), organ culture (Figure 15, bottom, yellow boxes), induced pluripotent stem cell (iPSC) derived germ cells (Figure 15,

bottom, red boxes) and SSC transplantation (Figure 15, bottom, blue boxes). Testicular tissue grafts from newborn mice, pigs and goats produced complete spermatogenesis when grafted under the skin of nude mice [207] and sperm obtained from the grafts were used to produce offspring in mice [208]. Prepubertal monkey testis tissue also produced complete spermatogenesis with fertilization-competent sperm after xenografting into nude mice [209]. Xenografting human testicular tissue has been less successful. No studies report production of full spermatogenesis; the most advanced stage of germ cell development reported is a spermatocyte [210-212]. Sato and colleagues demonstrated that organ culture of mouse testicular tissue pieces produces sperm that can be used to fertilize an oocyte and generate live offspring [213, 214]. Spermatogonial stem cell transplantation technique has been reported to regenerate spermatogenesis in mice, rats, goats, pigs, bulls, dogs and monkeys; donor derived progeny were produced in mice, rats, goats and sheep [70, 73-85, 90].

For patients who did not cryopreserve sperm or spermatogonial stem cells or testicular tissue before cancer treatment, generation of transplantable germ cells or haploid gametes from patient-derived induced pluripotent stem cells (iPSCs) has been investigated (Figure 15, red boxes). Mouse ESCs and iPSCs can give rise to primordial germ cells, that when transplanted into an infertile mouse testes, restored spermatogenesis [215]. The sperm from the recipient mice were capable of fertilizing oocytes and produced live offspring. Generation of germ cells from nonhuman primate ESCs [216, 217] and human ESCs and/or iPSCs [218-221] has been reported, including evidence of haploid cells in some cases.

To date, published reports document that over 150 prepubertal and adult males have cryopreserved their testicular tissue or cells [91, 93-95, 100, 222-224] worldwide. Therefore, it is incumbent on the medical and research community to responsibly develop technologies



**Figure 15. Standard and experimental options for preserving male fertility.**

Top, sperm obtained by ejaculation or surgical retrieval from the testes or epididymis are competent to fertilize oocytes using assisted reproductive techniques including intrauterine insemination (IUI), in vitro fertilization (IVF) or IVF with intracytoplasmic sperm injection (ICSI) that are standard in most fertility clinics. These options are not available to prepubertal boys who are not producing sperm or to adult azoospermic men. Bottom, testis tissue obtained via biopsy from prepubertal boys contains SSCs that can produce sperm in the context of the intact tissue by xenotransplant, organ culture or autologous transplantation back into the individual (orange boxes). Sperm retrieved from cultured or transplanted tissue can be used for ICSI. Cells in suspension obtained from biopsied testicular tissue can be transplanted back into the endogenous seminiferous tubules of the patient (blue boxes). SSCs in the suspension can regenerate spermatogenesis and, in some cases, fertility. For infertile individuals who did not preserve germ cells before gonadotoxic therapy, induced pluripotent stem cells (iPSCs) may be produced from his somatic cells (e.g., skin or blood) to differentiate into transplantable germ cells (PGCs or SSCs) or haploid germ cells that can be used for ICSI (red boxes). Reprinted by permission from Macmillan Publishers Ltd: Clark AT, Phillips BT, Orwig KE, Nat Med. 2011 Dec 6;17(12):1564-5.

that will allow patients to use their tissue for reproductive purposes in the future. Our laboratory demonstrated previously that testicular tissue could be obtained from rhesus macaques by biopsy prior to the initiation of gonadotoxic therapy [86]. The testicular tissue (containing SSCs) was then digested with enzymes to produce a cell suspension that was cryopreserved. At a later date, cells were thawed and transplanted by ultrasound-guided rete testis injections into the testes of chemotherapy treated animals. The frozen and thawed cells engrafted recipient testes, regenerated spermatogenesis and produced functional sperm. Thus, clinical translation of the SSC transplantation technique appears to be on the horizon.

To maximize the use of cryopreserved SSCs for future use, I investigated the colonization activity and UTF1 expression of cryopreserved intact testicular tissue pieces compared to cryopreserved cell suspension. Intact tissue pieces have the advantage that they can be used for tissue based or cell based approaches; whereas a cell suspension can only be used for cell culture or SSC transplantation. In case organ culture or testicular tissue grafts are a viable option to restore male fertility in the future, an optimal cryopreservation technique needs to be established.

Slow-freezing is the preferred method for cryopreserving intact testicular tissue pieces in mice [225-227] and it has been validated with achieved live births using spermatozoa from the tissue grafts [225]. Some laboratories are also starting to cryopreserve intact human testicular tissue by slow-freezing [212, 224, 228, 229] as well as vitrification [230-232]. In both cases, differentiation of spermatogonia up to pachytene spermatocyte stage was observed in prepubertal testicular tissue after tissue grafting into nude mice [211, 212, 232].

Slow-freezing and vitrification are both cryopreservation techniques that are designed to minimize damage by ice crystal formation within the cells. Slow-freezing involves cooling the cells at a low rate and as the temperature decreases, ice crystals form in the extra-cellular



solution [233, 234]. The concentration of cryoprotectant increases around the cells and that draws out the water from inside the cell, minimizing the formation of intracellular ice crystals in the cell cytoplasm. Slow cooling rates are necessary to allow enough time for the water to efflux from the cells. Vitrification is a method of cryopreservation that uses higher concentrations of cryopreservation agent and faster cooling rates [235]. With this technique the transformation process from a liquid to a solid glass-like state happens rapidly without crystallization.

It has been reported that both slow-freezing and vitrification do equally well at cryopreserving intact testicular tissue pieces [231, 232, 236-238] and maintain at least some functionality as demonstrated by tissue grafting experiments [212, 232]. Additionally, Pacchiarotti and colleagues compared slow-freezing of sexual reassignment patient testicular tissue to a cell suspension and showed that there is a trend that cryopreserving testicular tissue has a better viability and recovery of SSEA4 (undifferentiated spermatogonia marker) positive and VASA (germ cell marker) positive cells than cryopreserved cell suspension. The results in this study were not statistically significant [239] and spermatogonial stem cell activity was not assessed.

In this study, I compared cryopreservation efficiency of slow-frozen and thawed cell suspension to small (3-5 mm<sup>3</sup>) and large (6-10 mm<sup>3</sup>) intact tissue pieces with slow-freezing (SF) and vitrification. I hypothesized that the recovery of stem and progenitor spermatogonia is better from frozen and thawed cell suspension than from frozen and thawed intact testicular tissue pieces. In a cell suspension the distribution of cryopreservation media is uniform and all the cells are cryopreserved at a similar rate, compared to an intact piece of tissue, where I can only control the freezing rate and cryopreservation media concentration on the outside of the tissues [240].

The experiments were analyzed by ICC staining for a marker of human stem and progenitor spermatogonia, UTF1, (see Figure 6) and also human-to-nude mouse xenotransplantation (see Section 1.4). ICC for UTF1 revealed that cryopreserved intact tissue has more UTF1 positive cells per gram of tissue than cryopreserved cell suspension. Human to nude mouse xenotransplantation demonstrates that cryopreservation of intact pieces does at least as well as cryopreserving a cell suspension and slow-freezing small and large pieces of intact tissue is significantly better than cryopreserving a cell suspension.

### 3.2 MATERIALS AND METHODS

#### *Animals*

All experiments utilizing animals were approved by the Institutional Animal Care and Use Committees of the Magee-Womens Research Institute and the University of Pittsburgh and were performed in accordance with the National Institute of Health guidelines for the care and use of animals (assurance # A3654-01).

#### *Preparation of Human Testicular Tissue*

Normal adult human testicular tissue was obtained through the University of Pittsburgh Health Sciences Tissue Bank and Center for Organ Recovery and Education (CORE) under University of Pittsburgh IRB #0506140. Following the removal of tissue, it was transported to the laboratory on ice in Lactated Ringer's solution. The tissue was either cut in to small pieces (3-5mm<sup>3</sup>) or large pieces (6-10mm<sup>3</sup>), or made into a cell suspension with a two-step enzymatic digestion described above (see section 2.2).

### *Controlled slow freezing and vitrification*

Testicular tissue was cryopreserved either as cell suspension or intact pieces of tissue. Cell suspension was cryopreserved using controlled slow freezing and intact pieces of tissue were cryopreserved with either controlled slow freezing (SF) or vitrification. For cell suspension, the vials were cooled at 1°C per minute in a -80°C freezer using Nalgene freezing containers (Nalgene-Nunc International) and then stored in liquid nitrogen. For slow freezing of intact pieces of tissue, 4-5 pieces of small tissue or 1-2 pieces of large tissue was put into 2.0ml cryovials containing 1.5ml of cryoprotectant medium consisting of 5%DMSO, 5% Serum Supplement Substitute (SSS) (Irvine Scientific) in Quinn's Advantage Blastocyst (QAB) (SAGE) medium (as described in [224], except clinical grade media was used). The tissue was then equilibrated on ice for 30 minutes and then cooled using a programmable freezer as described before [224, 230]. The cooling rate was 1°Cmin<sup>-1</sup> with holding at 0°C for 5 min, followed by cooling at 0.5°Cmin<sup>-1</sup> until -8°C. At this temperature, the tissue was manually seeded and held for 10 min. The program continued to cool to -40°C at a rate of 0.5°Cmin<sup>-1</sup>, held for 10min and continued to -70°C at 7°Cmin<sup>-1</sup>, the cryovials were then plunged into liquid nitrogen. The tissue was thawed in 37°C water bath and washed in clinical grade PBS (Irvine Scientific).

For vitrification of tissue pieces, the tissue was submerged into equilibration solution consisting of 7.5% DMSO, 7.5% ethylene glycol and 20% Dextran Serum Supplement (DSS, Irvine Scientific) in QAB medium for 10 minutes. The tissue was then transferred into vitrification solution consisting of 15% DMSO, 15% ethylene glycol, 20% DSS and 0.5M sucrose in QAB medium for 5 minutes (according to Vitrification Kit from Irvine Scientific).

The pieces were then placed in cryovials and stored in liquid nitrogen. Samples were thawed in pre-warmed (37°C) thawing solution consisting of 20% DSS and 1M sucrose in QAB medium for 5 minutes (Vitrification Kit; Irvine Scientific). They were then transferred into dilution solution consisting of 20% DSS and 0.5M sucrose in QAB medium for 5 minutes and finally washed in 20% DSS in QAB medium for 10 minutes, followed by two 5 minute washes.

#### *Human to nude mouse xenotransplantation*

Human to nude mouse xenotransplantations were done to analyze colonizing activity of putative human SSCs. All cryopreserved intact testicular tissue pieces were made into cell suspensions after thawing using a two-step enzymatic digestion with clinical grade enzymes, as described above in Preparation of Human Testicular Tissue (see section 2.2). Cell suspensions were transplanted into the testes of busulfan-treated (40 mg/kg; Sigma, at 5–6 weeks of age), immune-deficient nude mice (NCr nu/nu; Taconic, Germantown, NY) as described above (see section 2.2).

#### *Whole mount immunofluorescent quantification of human SSC colonizing activity in mouse seminiferous tubules*

Human to nude mouse xenotransplantation was analyzed by whole mount immunofluorescence. The testes were recovered 8 weeks following transplantation, the tunica was removed, and the intact seminiferous tubules were dispersed gently with Collagenase IV (1mg/mL) and DNase I (1mg/mL) in D-PBS. The tubules were fixed for 4 hours in 4% PFA and the whole mount immunofluorescence was carried out as described in section 2.2. Spermatogonial colonies were counted based on the following criteria: at least 4 cells exhibiting

spermatogonial morphology (ovoid shape with high nuclear to cytoplasmic ratio) and located on the basement membrane in a continuous area of recipient seminiferous tubule ( $\leq 100 \mu\text{m}$  between cells).

### *Immunocytochemistry*

Frozen and thawed cells were spotted on Superfrost slides and fixed with methanol. The cells were then rehydrated with D-PBS and blocked with a buffer containing 3% bovine serum albumin and 5% normal goat serum in order to reduce nonspecific binding. Rabbit anti- UTF1 (1:500; MAB4337, Millipore) antibody was added to the cells and incubated for 90 min at room temperature. Isotype matched normal IgG was used as negative control. Primary antibody was detected using goat anti-rabbit AlexaFluor-488 conjugated secondary antibody (1:200, Invitrogen). The slides were mounted with VectaShield (Vector Laboratories) mounting medium containing DAPI for detection of all nuclei and the staining was observed with a Nikon Eclipse E600 Fluorescence microscope and images captured with MetaView Digital Imaging software.

### *Statistical analysis*

I present descriptive statistics (mean, standard deviation, min, median, and max) of the number of colonies per gram of tissue and UTF1 positive cells per gram of tissue for each of the five groups (frozen thawed cell suspension, vitrified large tissue pieces, vitrified small tissue pieces, slow-freezing large tissue pieces and slow-freezing small tissue pieces). The number of colonies per gram of tissue and the number of UTF1 positive cells per gram of tissue were compared between groups using the Dwass, Steel, Critchlow-Fligner (DSCF) multiple comparison analysis, which is based on pairwise two-sample Wilcoxon rank sum comparisons. I

chose this technique instead of parametric analysis of variance (ANOVA) because the skewed distribution of colonies per gram of tissue violates the assumptions required for proper application of ANOVA, necessitating the use of a non-parametric test. The DSCF analysis compares the median dependent variable in each possible combination of the five groups (frozen thawed cell suspension, vitrified large tissue pieces, vitrified small tissue pieces, slow-freezing large tissue pieces and slow-freezing small tissue pieces). The DSCF test is an extension of the standard Wilcoxon rank-sum test, but adjusts for pairwise comparisons of multiple groups. Within this test, a statistically significant result for any particular comparison (i.e. slow-freezing small tissue pieces vs. frozen thawed cell suspension) indicates that the center of the distribution of the dependent variable in one group differs significantly from the center of the distribution in the other group (i.e. that the median number of colonies per gram of tissue in slow-freezing small tissue pieces is significantly greater than the median number of colonies per gram of tissue in frozen thawed cell suspension).

### **3.3 RESULTS**

#### **3.3.1 Acquisition of human testicular tissue**

All the tissue used in the experiments came from 5 organ donors between the ages 15-49. The testis weight ranged from 8.5 to 23.9 g (after pathology) and yielded  $44.7 \times 10^6 \pm 5.8 \times 10^6$  cells per gram of tissue. The tissues were cryopreserved between 1 and 1.5 month before performing the experiments.

### 3.3.2 UTF1 staining of cryopreserved cells and tissues

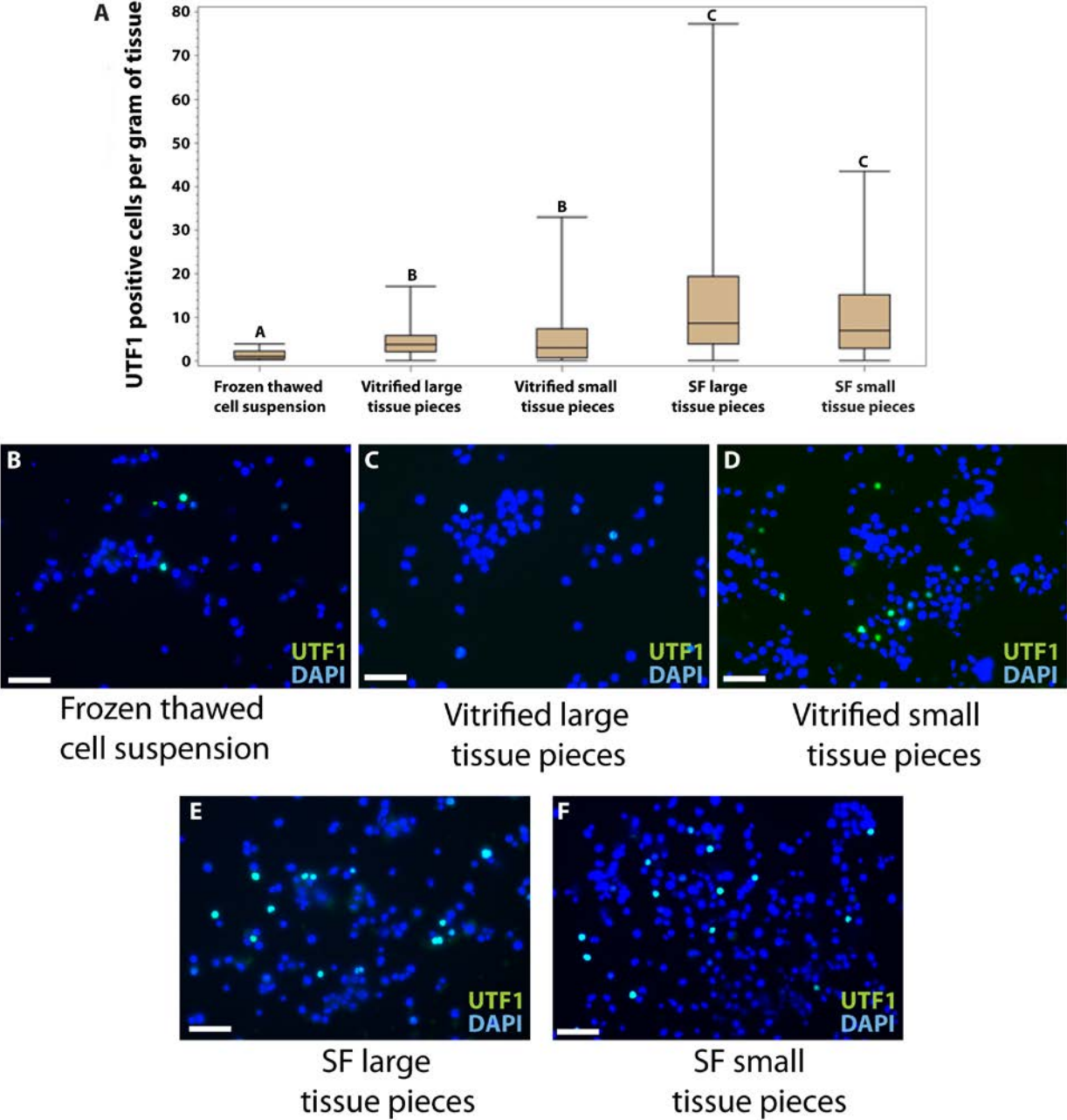
Human testicular tissue was cryopreserved with slow-freezing (SF) as a cell suspension and as small and large intact pieces of tissue. Testicular tissue pieces (small and large), were also cryopreserved by vitrification (Table 1). At a later date, the cells and tissues were thawed and all the intact pieces of tissues were made into cell suspensions. The cell suspensions from each group were stained for UTF1 (spermatogonia marker, see Figure 6) by immunocytochemistry.

**Table 1. Descriptive statistics for UTF1 positive cells per gram of tissue for human testicular cell and tissue cryopreservation.**

	<b>Mean</b>	<b>SD</b>	<b>Min</b>	<b>Median</b>	<b>Max</b>
<b>Frozen thawed cell suspension</b>	1.47	1.11	0.11	1.1	3.9
<b>Vitrified large tissue pieces</b>	4.4	3.6	0	3.7	17.1
<b>Vitrified small tissue pieces</b>	5.4	7.2	0	2.9	32.9
<b>Slow-freezing large tissue pieces</b>	14.9	16.9	0	8.6	77.2
<b>Slow-freezing small tissue pieces</b>	10.4	9.9	0.003	6.9	43.4

The results were normalized to UTF1 positive cells per gram of tissue frozen. The median UTF1 positive cells per gram of tissue was highest in the SF large tissue piece samples (median=8.6, range [0-77]) and SF small tissue piece samples (median=6.9, range [0.003-43.4]) (Table 1 and Figure 16). These were significantly greater than the median UTF1 positive cells per gram of tissue in the frozen thawed cell suspension group (median=1.1, range [0.11-3.9],  $P < 0.0001$ ), vitrified large tissue pieces group (median=3.7, range [0-17.1],  $P < 0.01$ ), and the vitrified small

tissue pieces group (median=2.9, range [0-32.9],  $P < 0.01$ ). Recovery of the UTF1 positive cells in the SF large tissue pieces group was not significantly different than the SF small tissue piece group ( $P = 0.7712$ ).





**Figure 16. ICC for UTF1 of cryopreserved human testicular cells and tissue pieces.**

Human testicular cells and tissue pieces were cryopreserved and thawed. All the tissue pieces were made into a cell suspension after thawing and all groups were stained with UTF1. **(A)** Slow-freeze (SF) large and SF small tissue pieces have significantly higher UTF1 positive cells per gram of tissue than other groups. Different letter indicate  $P < 0.05$ , same letters indicate  $P > 0.05$ . Black line in the brown box indicates median, the bottom of the brown box is quartile 1(Q1), the top is quartile 3 (Q3). The top error bar indicates max and bottom error bar indicates minimum. **(B-F)** Representative images of UTF1 staining from each sorted fraction and unsorted cells. At least 10 views were counted from each fraction based on DAPI staining and UTF1 staining.  $N=5$ . Scale bar = 50  $\mu\text{m}$ .

Similar to the SF small and large tissue pieces, recovery of UTF1 positive cells was significantly greater from vitrified small and large tissue pieces than from the frozen thawed cell suspension ( $P = 0.0119$  and  $P < 0.0001$ , respectively) (Table 1 and Figure 16).

### **3.3.3 Colonizing activity in cryopreserved cells and tissues**

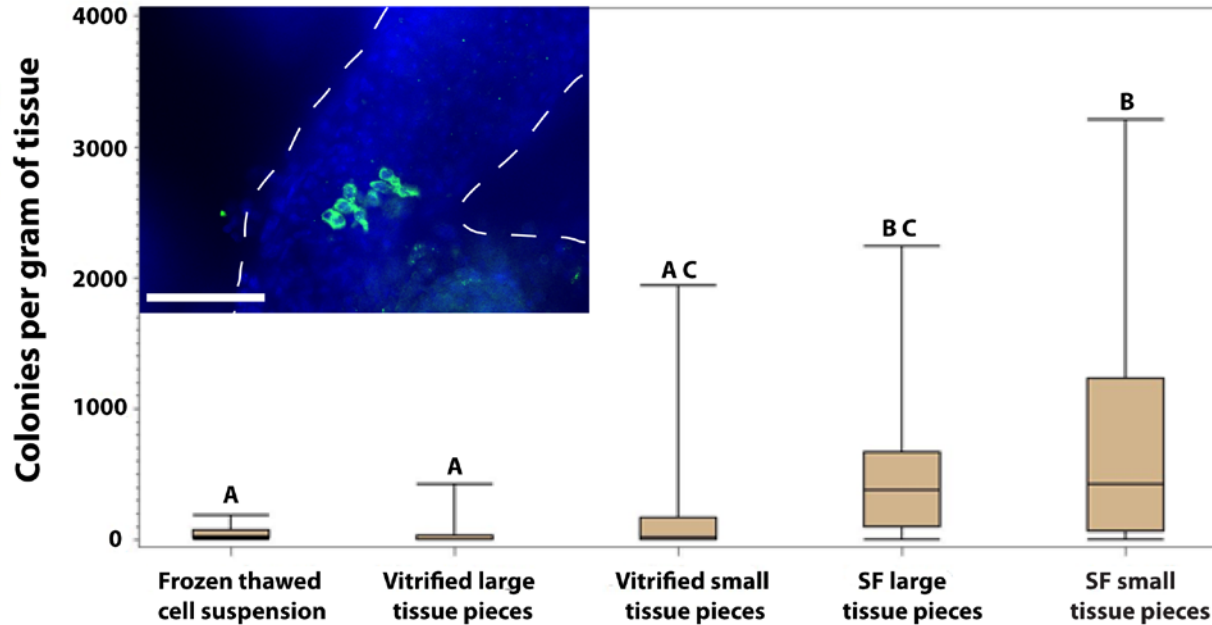
The results from UTF1 staining of cryopreserved cells and tissues were confirmed by human-to-nude mouse xenotransplantation experiments. The cryopreserved cell suspension and intact tissue pieces were thawed and the intact tissue pieces were made into a cell suspension before xenotransplantation into nude mice. The median number of colonies per gram of tissue frozen was highest in the SF small tissue piece samples (median=426.3, range [0-3209]) (Table 2 and Figure 17). This was significantly greater than the median number of colonies per gram of tissue in the frozen thawed cell suspension group (median=28.0, range [0-185],  $P = 0.0003$ ), vitrified large tissue piece group (median=0, range [0-420],  $P < 0.0001$ ), and the vitrified small tissue piece group (median=17.9, range [0-1941],  $P = 0.0472$ ). Colonies per gram of tissue in the SF

small tissue piece group was not significantly different was the SF large tissue piece group (median=375.9, range [0-2243], P = 0.9960).

**Table 2. Descriptive statistics for xenotransplant colonies per gram of tissue for human testicular cell and tissue piece cryopreservation**

	<b>Mean</b>	<b>SD</b>	<b>Min</b>	<b>Median</b>	<b>Max</b>
<b>Frozen thawed cells</b>	49.6	52.8	0	28.0	185.2
<b>Vitrified large tissue pieces</b>	48.0	108.8	0	0	420.6
<b>Vitrified small tissue pieces</b>	370.3	661.5	0	17.9	1941.1
<b>Slow-freezing large tissue pieces</b>	487.3	501.9	0	375.9	2243.9
<b>Slow-freezing small tissue pieces</b>	760.2	922.1	0	426.3	3209.1

SF large tissue piece group was also significantly greater than frozen thawed cell suspension (P < 0.0001), vitrified large tissue piece group (P < 0.0001), and borderline significant compared to vitrified small tissue piece group (P = 0.0528). Colonizing activity in the frozen thawed cell suspension, vitrified large tissue pieces, and vitrified small tissue pieces groups were not significantly different (Table 2 and Figure 17).



**Figure 17. Colonization of cryopreserved human testicular cells and tissue pieces.**

Human testicular cells and tissue pieces were cryopreserved and thawed. All the tissue pieces were made into a cell suspension after thawing and all groups were transplanted into nude mouse testis. **(Inset)** Two months after transplant, colonies of human spermatogonia were identified in whole mount preparations of recipient mouse seminiferous tubules using the rabbit anti-primate antibody. Colonies in each recipient testis were counted and normalized to colonies per gram of frozen tissue. Different letter indicate  $P < 0.05$ , same letters indicate  $P > 0.05$ . Black line in the brown box indicates median, the bottom of the brown box is quartile 1(Q1), the top is quartile 3 (Q3). The top error bar indicates max and bottom error bar indicates minimum.  $N = 5$ . Scale bar = 100  $\mu\text{m}$ .

### 3.4 DISCUSSION

Spermatogonial stem cells are the adult tissue stem cells of the testes that may have the potential to treat some cases of male infertility. For example, the SSC freezing and transplantation methods could be used to preserve and restore fertility of prepubertal boys who

are undergoing chemotherapy or radiation for cancer or other conditions that may render them infertile. These boys do not make sperm yet, so they do not have the option to cryopreserve a semen sample. Currently, there are several stem cell based techniques in the research pipeline that may in the future offer opportunities for these prepubertal patient to have their own genetically related children (Figure 15, bottom). These techniques are still experimental, but several centers around the world are cryopreserving testicular tissues from prepubertal boys because it is anticipated that stem cell therapies will be available in the future [91, 93-95, 100, 222-224]. Methods currently in the research pipeline include SSC transplantation [70, 73-85, 90], testicular tissue grafting [207-212, 241-247], testicular organ culture [213, 214] and induced pluripotent stem cell (iPSC) technologies [215-221]. Because it is uncertain which of the fertility preservation/restoration technique(s) will be translated to the clinics in the future, it is important to preserve tissues in a way that will maximize access to downstream applications. Many studies have demonstrated that testicular cell suspensions can be frozen, thawed and transplanted to regenerate spermatogenesis [70, 72, 73, 86, 122, 248-252]. However, freezing testicular tissues as cell suspension eliminates the possibility of using testicular tissue grafting or organ culture to produce sperm. Some laboratories are cryopreserving patient tissues as intact tissue pieces and have shown that this method cryopreserves the SSCs for testicular tissue grafting in mice [212, 232]. Therefore, to maximize the potential use of the cryopreserved prepubertal patient tissues in the future, I wanted to test whether cryopreserving intact pieces of tissue will compromise the SSC activity for SSC transplantation technique.

Here, I evaluated the recovery of stem and progenitor spermatogonia from human testicular tissue frozen as a cell suspension versus pieces of intact tissue. The intact tissue pieces were cryopreserved either by slow-freezing or vitrification. Slow-freezing has been used to

successfully cryopreserve rodent testicular tissue [225-227] and human testicular tissue for xenografting purposes [230-232]. Vitrification is a technique that is used in oocyte and embryo cryopreservation [253, 254]. Vitrification and slow-freezing are both techniques that minimize ice crystal formation and therefore prevent cell damage [233, 234]. Vitrification uses higher cryopreservative concentration and faster cooling rates to eliminate ice crystal formation in the intracellular and extracellular space [234, 235]. Slow-freezing uses a slow cooling rate to allow water to exit the cell to minimize intracellular ice crystal formation [234]. Vitrification has the advantage that it does not require expensive freezing machines and can be performed fast in almost any clinical laboratory.

I hypothesized that the recovery of stem and progenitor spermatogonia is more efficient from frozen and thawed cell suspension than from frozen and thawed intact pieces of tissue. I analyzed the cryopreservation conditions with ICC for UTF1 (spermatogonia maker, Figure 6) for a quick readout assay and also confirmed the results by human-to-nude mouse xenotransplantation assay. I found that all freezing conditions for intact pieces of tissues were at least as effective, if not more effective than slow-freezing a cell suspension (Figures 16 and 17). To our surprise, the recovery of stem and progenitor spermatogonia from slow-frozen small or large pieces of intact tissues was significantly better than slow-freezing a cell suspension. Recovery of UTF1 positive cells from vitrified small and large pieces of testicular tissue pieces was also significantly better than frozen thawed cell suspension, but not as good as from slow-frozen tissue pieces. Colonization activity in xenotransplants assay between vitrified tissue pieces and frozen thawed cell suspension was not statistically significant, the reason for that could be high variability within each sample. The variability is due to variation between different human samples. Cryopreserving a cell suspension requires more processing steps before (cell

digestion, washing, centrifugation) and after freezing (washes to remove cryoprotectant) than cryopreserving intact tissue pieces. The additional processing steps may reduce the recovery of stem and progenitor spermatogonia.

Vitrification of intact testicular tissue has been shown to be at least as effective as slow-freezing in some cases [231, 232, 236-238] but that was not the case here. One of the reasons could be that the functional endpoint in some of these studies was grafting of the cryopreserved tissue into nude mice. In tissue grafting, the SSCs remain in their niche, compared to SSC transplantation, where the tissue is made into a cell suspension. This difference could explain why one method works better for SSC transplantation than the other. It could also be that there are small differences in the technique between laboratories. In any case, it is advantageous to cryopreserve prepubertal patient testicular biopsies as intact pieces of tissues so that the potential to use it for different techniques is maximized.

These studies were conducted using human tissues and the same clinical grade reagents that one used to process patient testicular tissues in our fertility preservation center. Therefore the results can be immediately implemented in protocols for processing patient tissues.

## **4.0 ELIMINATING MALIGNANT CONTAMINATION FROM THERAPEUTIC HUMAN SPERMATOGONIAL STEM CELLS**

### **4.1 INTRODUCTION**

Over 12,000 children are diagnosed with cancer every year in the US, and it has been estimated that a male infant has a 1 in 300 chance of being diagnosed with a malignancy by the age of 20 [255]. Fortunately, success rates in treating childhood cancer have increased dramatically over the past few decades, and now over 80% of children survive following treatment [256, 257].

Given this growing cohort of adult survivors of childhood cancers, emphasis is now being placed on quality of life issues following successful treatment. Many therapies to treat cancer are gonadotoxic and can lead to infertility, and fertility potential has an important impact on quality of life according to cancer survivors [57, 258-260]. In fact, the American Society of Clinical Oncology [57] and American Society of Reproductive Medicine [58] now recommend that the reproductive risks of cancer therapies and fertility preservation options should be routinely discussed with patients before beginning treatment.

In men, freezing semen samples is an efficient and well-established technique to preserve fertility for those facing gonadotoxic treatments, such as chemotherapy or radiation. Unfortunately, this is not an option for boys who have not yet entered puberty and do not have sperm. However, these boys do have spermatogonial stem cells (SSCs) in their testes that are

poised to produce spermatogenesis at the start of puberty [7, 95, 224]. SSCs maintain spermatogenesis throughout postpubertal life, and they are defined by their ability to undergo both self-renewing cell divisions and differentiation, leading to the production of haploid sperm. Brinster and colleagues provided the initial demonstration that testicular cells from a fertile mouse could be transplanted into the seminiferous tubules of an infertile recipient, in which they produced complete spermatogenesis and sometimes restored fertility [68-73]. Regeneration of spermatogenesis following SSC transplantation has now been established in several animal models, including rodents, goats, sheep, pigs, dogs, and monkeys [70, 72, 73, 86, 122, 248-252].

The potential of using SSCs to preserve and restore fertility in patients receiving gonadotoxic therapies has been extensively discussed [94, 222, 261-268]. In theory, testicular cells obtained via biopsy prior to cancer treatment could be cryopreserved and then retransplanted following clinical remission. Several centers around the world, including our own Fertility Preservation Program in Pittsburgh (<http://www.mwrf.org/220>), are now performing testicular biopsies on boys prior to the initiation of cancer therapy in hopes that this tissue can be used in the future to restore fertility [95, 222, 224, 267]. However, to make SSC transplantation a realistic clinical option for the prepubertal patient cohort, two major hurdles must be overcome. First, we need to learn the characteristics of human SSCs to facilitate their isolation and enrichment. Second, techniques to remove malignant contamination from the testis cell suspension are needed to eliminate the risk of reintroducing cancer back into survivors.

Unfortunately, there is a real potential for malignant contamination in testicular tissue obtained from patients prior to cancer treatment, especially for those with hematologic cancers. One study demonstrated that 20% of boys with acute lymphocytic leukemia possessed malignant cells in a testicular biopsy taken prior to the initiation of chemotherapy [269]. Furthermore, it has



been demonstrated in a rat model of leukemia that transplantation of testicular cells from leukemic donors consistently transmitted leukemia to healthy recipients [270]. Fujita and colleagues were the first to demonstrate that FACS could be used to successfully remove malignant cells from a testicular sample prior to SSC transplantation [262]. They used antibodies directed against cell surface antigens CD45 and MHC class I (HLA-ABC) to remove the malignant cells from testis cell suspension. Sorted and unsorted cell suspensions were then transplanted into the seminiferous tubules of infertile recipient mice. In this landmark study, recipient mice that received transplants with unsorted cells consistently developed leukemia, whereas those transplanted with sorted cells did not. Additionally, viable offspring were generated from the infertile recipients following transplantation of the sorted germ cells [262]. Fujita and colleagues followed up this initial report by demonstrating that 7 out of 8 human leukemic cell lines also expressed the cell surface antigens CD45 and MHC class I, and thus these leukemic markers could theoretically be used to separate leukemic cells from testicular cells in humans as well, but this was not assessed experimentally in that study and transplants were not performed [263]. Hermann and coworkers demonstrated the feasibility of removing contaminating leukemic cells from nonhuman primate testis cell suspensions by FACS sorting with THY1 (spermatogonial marker) and CD45 (leukemia marker) but also did not perform transplants to assess malignant potential [159].

However, not all studies using immune-based sorting technologies to separate malignant cells from testicular cell suspensions have been as successful [185, 264]. Using a leukemic rat model, Hou and colleagues concluded that a single marker sort is generally not adequate to remove malignant contamination [185]. Moreover, studies using human tissue to assess decontamination methods have been very limited to date, likely due to difficulties in obtaining

such material for research. However, human studies are needed to demonstrate feasibility and safety before SSC transplantation can be translated to the clinic.

In this chapter, I characterized the cell surface phenotype of human spermatogonia in testicular tissue obtained from organ donors as well as the MOLT-4 leukemic cell line derived from a patient with acute T cell lymphoblastic leukemia. I used this information to devise sorting strategies to isolate and enrich human SSCs and to remove malignant contamination from human testicular cell suspensions that had been “spiked” with MOLT-4 leukemia cells. A human-to-nude mouse xenotransplantation biological assay was used to assess SSC activity and malignant contamination in fractions obtained from FACS of MOLT-4-contaminated human testis cell suspensions.

## 4.2 MATERIALS AND METHODS

### *Animals*

All experiments using animals were approved by the Institutional Animal Care and Use Committees of the Magee-Womens Research Institute and the University of Pittsburgh and performed in accordance with the NIH guidelines for the care and use of animals (assurance no. A3654-01).

### *Procurement and processing of human testicular tissue.*

Deidentified human testicular tissue was obtained through the Center for Organ Recovery and Education and the University of Pittsburgh Health Sciences Tissue Bank under University of Pittsburgh IRB no. 0506140. Tissue was obtained from postpubertal male organ donors and

transported on ice in Lactated Ringer's solution following procurement. The time of tissue processing ranged from 7 to 21 hours following organ procurement. A single cell suspension of human testicular parenchyma was prepared with two step enzymatic digestion as described in Chapter 2.

#### *MOLT-4 cell line culture*

The MOLT-4 cell line, derived from a 19-year-old man with acute T cell lymphoblastic leukemia in relapse [271], was obtained from American Type Culture Collection (ATCC). Cultures were established in RPMI-1640 media (GIBCO, Invitrogen) with 10% FBS and supplemented with antibiotic-antimycotic solution containing penicillin, streptomycin, and amphotericin (Antibiotic-Antimycotic, GIBCO Cell Culture, Invitrogen). Fresh media was added every 2 to 3 days, and cells were subcultured at or before they reached a density of  $2 \times 10^6$  cells/ml, as per manufacturer recommendations.

#### *Flow cytometry*

Flow cytometry was used to characterize the expression of a panel of cell surface antigens on MOLT-4 and human testicular cells. To assess antigen expression,  $0.5 \times 10^6$  cells were stained with fluorophore-conjugated primary antibodies for 20 minutes on ice. Cells were also stained with isotype control antibodies to correct for nonspecific antibody binding. Preliminary titration experiments were carried out with each antibody to determine the optimal antibody concentration for both MOLT-4 cells and human testicular cells. Following staining, cells were washed 3 times with cold Dulbecco's PBS (D-PBS; GIBCO, Invitrogen) containing 10% FBS. A FACSDiva (Becton Dickinson) machine was used to perform flow cytometry, and the percentage of cells expressing the antigen of interest was determined by quantifying the

percentage of cells with higher fluorescence intensity than the isotype control. Each experiment was replicated 3–5 times.

#### *Fluorescence-activated cell sorting*

Based on flow cytometry results, markers that were expressed on >95% of MOLT-4 cells were considered markers of MOLT-4 leukemia cells. In contrast, markers expressed by <1% of MOLT-4 cells and 5% or more of human testis cells were considered potential SSC markers. CD49e ( $\alpha 5$  integrin) and HLA-ABC were expressed by >95% of MOLT-4 cells and therefore met the criteria for potential MOLT-4 markers. EPCAM was expressed by <1% of MOLT-4 cells and 5% or more of human testis cells and met the criteria for a potential SSC marker. These markers were selected for further analysis by FACS and immunocytochemical analysis of human testis cell fractions.

Human testis cell suspensions were stained with fluorescent-conjugated antibodies (anti-human CD49e clone NKI-SAM-1, BioLegend; anti-human HLA-ABC clone G46-2.6, BD Biosciences; anti-human EPCAM clone 9C4, BioLegend) and sorted using FACSVantage SE fluorescence-activated cell sorter (Becton Dickinson) as described in Chapter 2.

#### *Immunocytochemistry*

Cell fractions were collected in Opti-MEM (GIBCO, Invitrogen) supplemented with 10% FBS, spotted onto slides (Superfrost Plus; Fisher Scientific), and fixed with methanol. The slides were stained as described in Chapter 2 with rabbit anti-SALL4 (1:500, Abcam). To quantify the percentage of cells expressing SALL4 in each sorted fraction, at least 10 random images of each fraction were recorded and the number of SALL4 positive cells as well as the total number of cells was quantified. An unsorted sample of testicular cells was also stained to determine the

percentage of unsorted testicular cells that express SALL4. These experiments were replicated 3 times for each representative antibody (HLA-ABC, CD49e, and EPCAM) using testicular tissue from different male donors.

*Xenotransplantation and whole mount immunofluorescent quantification of colonization activity of undifferentiated spermatogonia*

Following FACS, unsorted and sorted testicular cell fractions were transplanted into the testes of busulfan-treated, immune-deficient nude mice (NCr *nu/nu*; Taconic) as previously described in Chapter 2. For experiments involving FACS of contaminated testicular cell suspensions, MOLT-4 cells were mixed with human testicular cells such that MOLT-4 cells made up approximately 10% of the final cell suspension prior to sorting. The concentration of cells transplanted into seminiferous tubules from each fraction varied based on the total number of cells collected following sorting. An average of 996,845 cells were transplanted per recipient mouse testis from the unsorted spiked cell suspension, 63,780 cells were transplanted from the EPCAM dim/CD49e negative/HLA-ABC negative (spermatogonial) fraction, and 5,000 cells were transplanted from the EPCAM negative/CD49e positive/HLA-ABC positive (MOLT-4) fraction. A prior sensitivity analysis demonstrated that as few as 10 MOLT-4 cells were capable of inducing tumor formation when transplanted into the testes of immunodeficient mice treated with busulfan, and injection of 1,000 MOLT-4 cells reliably induced tumor formation in the majority of mice (83%) [159]. This experiment was designed primarily to assess SSC activity in each fraction. Malignant contamination of each fraction was also evaluated by injection into the testicular interstitial space, which is an excellent environment for tumor formation (see *Human-to-nude mouse tumor bioassay* below).

### *Human-to-nude mouse tumor bioassay*

In addition to the intratubular transplant bioassay for human spermatogonia, xenotransplants into the interstitial space (between seminiferous tubules) of nude mouse testes were performed to assess tumorigenic potential of unsorted and sorted cell fractions. I found that the interstitial space was more conducive to tumor formation than the intratubular space and was therefore a more sensitive bioassay for malignant contamination. Approximately 10  $\mu$ l of cell suspension was transplanted into the interstitial space at cell concentrations of  $0.5 \times 10^6$  cells/ml to  $5 \times 10^6$  cells/ml (50,000 cells per recipient mouse testis in the unsorted spiked arm, 5,000 cells per testis in all other experimental arms) by initially cannulating the efferent duct and then advancing the needle through the rete testis into the interstitial space. As indicated above, as few as 10 MOLT-4 cells are sufficient to produce tumors following transplantation into the testes of nude mice [159]. Therefore, the human-to-nude mouse tumor assay has the sensitivity to detect a 0.2% contamination with cancer cells (10 cells in a transplanted fraction of 5,000 cells). Following interstitial transplantation, the mice were monitored and palpated regularly to assess for tumor formation and sacrificed for analysis when palpable tumors were present or by 4 months following transplantation. The testes were removed and examined grossly for tumor formation.

### *TF-1a lymphoblastic leukemia cell line: marking with GFP, phenotyping and sorting*

To determine if the multi-parameter FACS approach would be successful across different human malignancies, another human leukemic cell line, TF-1a was used in a second spiked sorting experiment. TF-1a, a lymphoblastic cell line derived from a 35 year old Japanese male with erythroblastic leukemia was obtained from ATCC [272].

Previous sensitivity analyses had demonstrated that TF-1a cells do not form solid tumors as consistently as MOLT-4 cells when transplanted into the testes of immune-deficient nude mice. Thus, TF-1a cells were transduced with a lentivirus containing GFP driven by the ubiquitin-C promoter (generously provided by Dr. Carlos Lois, University of Massachusetts [273]) to enable tracking of malignant cells through the multi-parameter FACS experiments. The cell culture was then expanded and cloned by limiting dilution. Cells derived from a single GFP-expressing clone, TF-1a (C2), were used for all experiments in this study. Cultures were established in RPMI-1640 media (GIBCO, Invitrogen) with 10% FBS and supplemented with Antibiotic-Antimycotic solution containing penicillin, streptomycin, and amphotericin (GIBCO Invitrogen Cell Culture). Fresh media was added every 2-3 days and cells were passaged at or before they reached a density of  $2 \times 10^6$  cells/mL as per manufacturer recommendations.

Initial flow cytometry experiments using the TF-1a-GFP clone demonstrated that over >95% of cells expressed the markers CD45 and CD49e, but not HLA-ABC (as I had observed for the MOLT-4 leukemic cells). Additionally, EPCAM was expressed on <1% of the TF-1a cells. Thus, our multi-parameter sorting approach with TF-1a utilized CD45-PE and CD49e-PE as markers for TF-1a leukemic cells, and EPCAM-APC as a marker of spermatogonia. Spiked sorting experiments were carried out as described above by adding TF-1a-GFP cells to a suspension of human testicular cells and performing multi-parameter FACS.

#### *Immunohistochemistry of testicular tumors with NuMA*

To demonstrate that the testicular tumors observed after transplantation of MOLT-4 cells and the EPCAM<sup>+</sup>/CD49e<sup>+</sup>/HLA-ABC<sup>+</sup> fraction resulted from the MOLT-4 cells injected and are of human origin, immunohistochemistry was performed with a human-specific polyclonal

antibody directed against the nuclear mitosis apparatus protein (NuMA), a protein involved in the formation and maintenance of the mitotic spindle. To accomplish this, the tumors were fixed with 4% paraformaldehyde overnight, paraffin-embedded and sectioned (5  $\mu$ m). The tissues were then stained as in Chapter 2.2 using anti-NuMA antibody (1:100, Abcam, Cambridge, MA).

### *Statistics*

Analysis of variance on nested linear mixed-effect models was used to compare differences among the percentage of SALL4 positive cells in unsorted versus sorted cell fractions in the immunohistochemistry experiments and colonizing activity in the human-to-nude mouse xenotransplant bioassay. *P* values of less than 0.05 were considered significant.

## **4.3 RESULTS**

### **4.3.1 Surface antigen expression on human testicular cells and MOLT-4 lymphoblastic leukemia cells.**

To characterize cell surface antigens on human testicular cells and MOLT-4 acute lymphoblastic leukemia cells [271], respectively, flow cytometry was performed for a panel of 24 markers, 15 of which exhibited positive immunoreactivity with human testis cells and/or MOLT-4 leukemic cells (Table 3). Our aim with this set of experiments was to characterize the cell surface phenotypes of human spermatogonia and human MOLT-4 leukemia cells to identify antigens that could be used to distinguish these 2 cell populations. For MOLT-4 markers, I selected



**Table 3. Expression pattern of various cell surface antigens on MOLT-4 lymphoblastic leukemia cells and human testicular cell suspensions (expressed in percentage)**

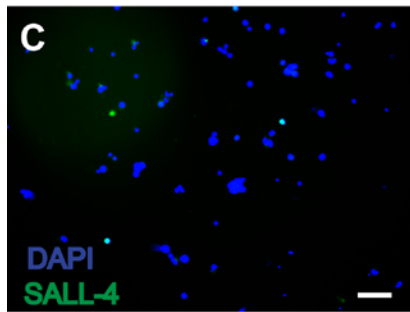
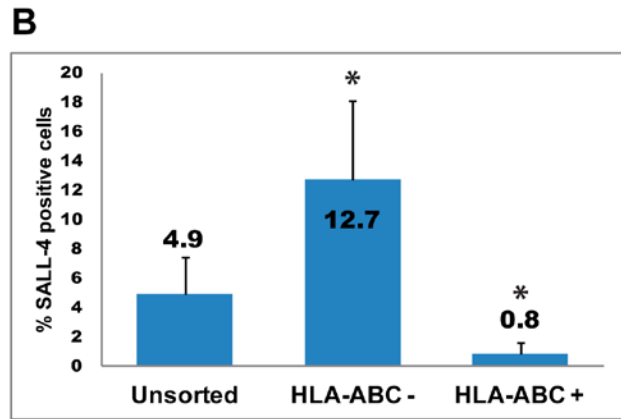
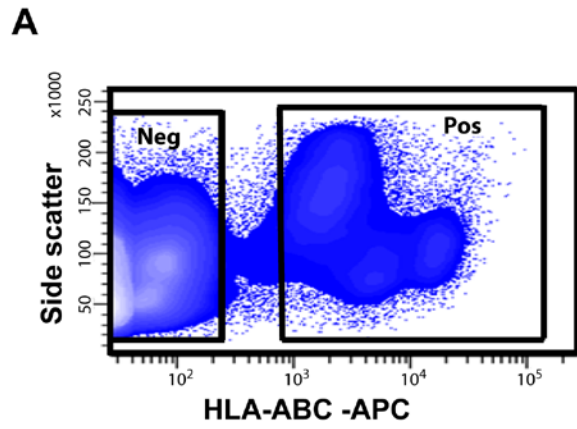
<b>Cell surface antigen</b>	<b>MOLT-4</b>	<b>Human testis</b>
CD4	75.9 ± 13.5	0.14 ± 0.02
CD9	68.9 ± 6.0	16.1 ± 0.4
CD24	0.17 ± 0.07	2.46 ± 0.3
ITGB1 (CD29)	98.3 ± 0.5	24.4 ± 3.7
CD31 (PECAM-1)	51.8 ± 2.3	1.1 ± 0.3
CD34	4.5 ± 1.7	15.96 ± 1.1
CD45	97.9 ± 0.9	3.56 ± 0.6
ITGA5 (CD49e)	97.6 ± 0.9	23.2 ± 4.2
ITGA6 (CD49f)	12.5 ± 1.7	53.2 ± 8.7
CD54	56.1 ± 7.7	26.1 ± 3.5
CD71	91.6 ± 1.0	0.46 ± 0.2
THY1 (CD90)	2.2 ± 0.5	21.1 ± 1.3
CD147	98.3 ± 0.5	53.1 ± 14.6
EPCAM (CD326)	0.1 ± 0.0	16.4 ± 3.9
HLA-ABC	96.9 ± 1.2	9.42 ± 0.7

antigens that were expressed on greater than 95% of MOLT-4 cells for further study. CD29, CD45, CD49e, CD147, and HLA-ABC met these criteria (Table 3), and HLA-ABC, CD49e, and CD147 were selected for use in subsequent experiments. To identify potential spermatogonial markers that were distinct from MOLT-4 cells, our goal was to identify antigens that were

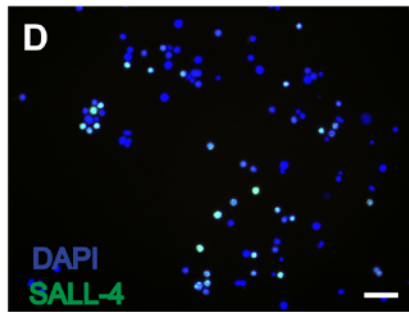
expressed by less than 1% of MOLT-4 cells and by 5% or more of human testis cells. ITGA6 (CD49f), THY1 (CD90), and EPCAM (CD326) were of particular interest because these markers are expressed by human spermatogonia (see Chapter 2). ITGB1 (CD29) has been demonstrated to be expressed on spermatogonia in other animal models [20, 29, 185, 263]. ITGA6, ITGB1, and THY1 were rejected for further consideration, because they were expressed by >1% of MOLT-4 cells. EPCAM satisfied the criteria (expressed on 0.1% of MOLT-4 cells and 16.4% of human testis cells; see Table 3) and was selected for further study.

#### **4.3.2 Expression of HLA-ABC and CD49e in adult human testicular cell suspension**

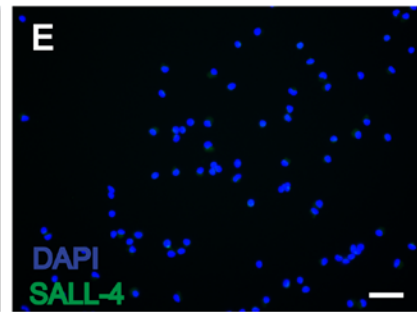
FACS experiments were performed to determine whether putative MOLT-4 markers CD49e, HLA-ABC, and CD147 (expressed by >95% of MOLT-4 cells) were also expressed by SALL4 positive spermatogonia in human testis cell suspensions. The goal of these experiments was to identify markers that can distinguish MOLT-4 leukemia cells from human spermatogonia. FACS analysis of human testis cells for HLA-ABC (Figure 18A), followed by immunocytochemistry of positive and negative fractions for SALL4 (Figure 18B–E), revealed that the majority of SALL4 positive human spermatogonia were recovered in the HLA-ABC–negative fraction ( $P < 0.0001$ ). Similarly, the majority of SALL4 positive spermatogonia were recovered in the CD49e-negative fraction of human testis cells ( $P < 0.0001$ ; Figure 18F–J). SALL4 positive spermatogonia were found in both the CD147-positive and -negative fractions (data not shown), and, thus, the CD147 marker was not deemed useful for separating MOLT-4 cells from spermatogonia.



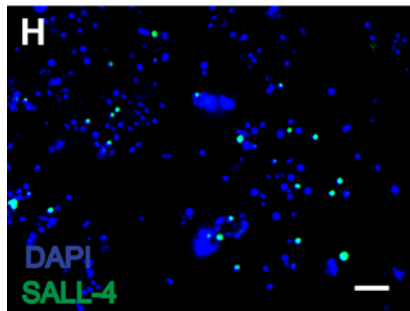
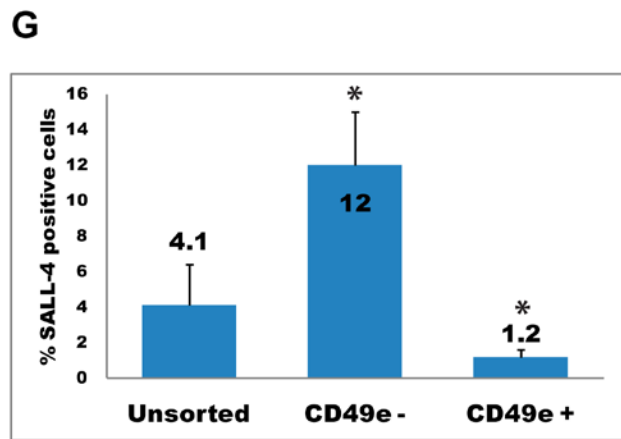
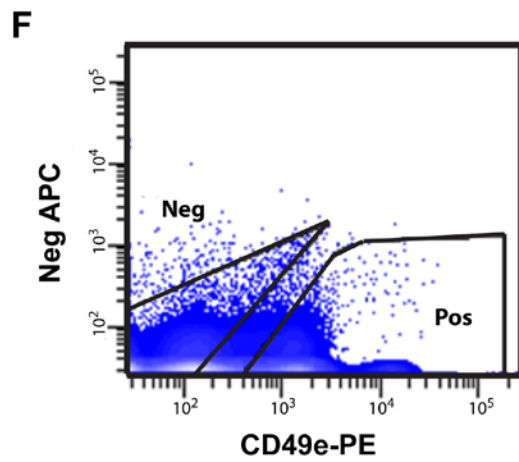
Unsorted



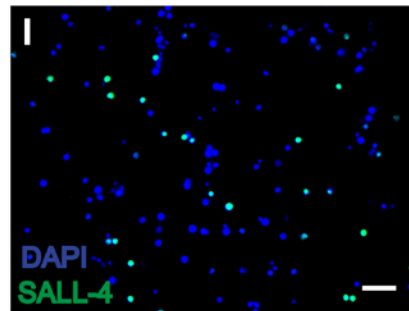
HLA-ABC -



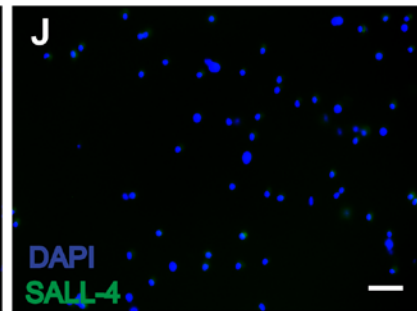
HLA-ABC +



Unsorted



CD49e -



CD49e +

**Figure 18. SALL4-positive human spermatogonia do not express HLA-ABC or CD49e.**

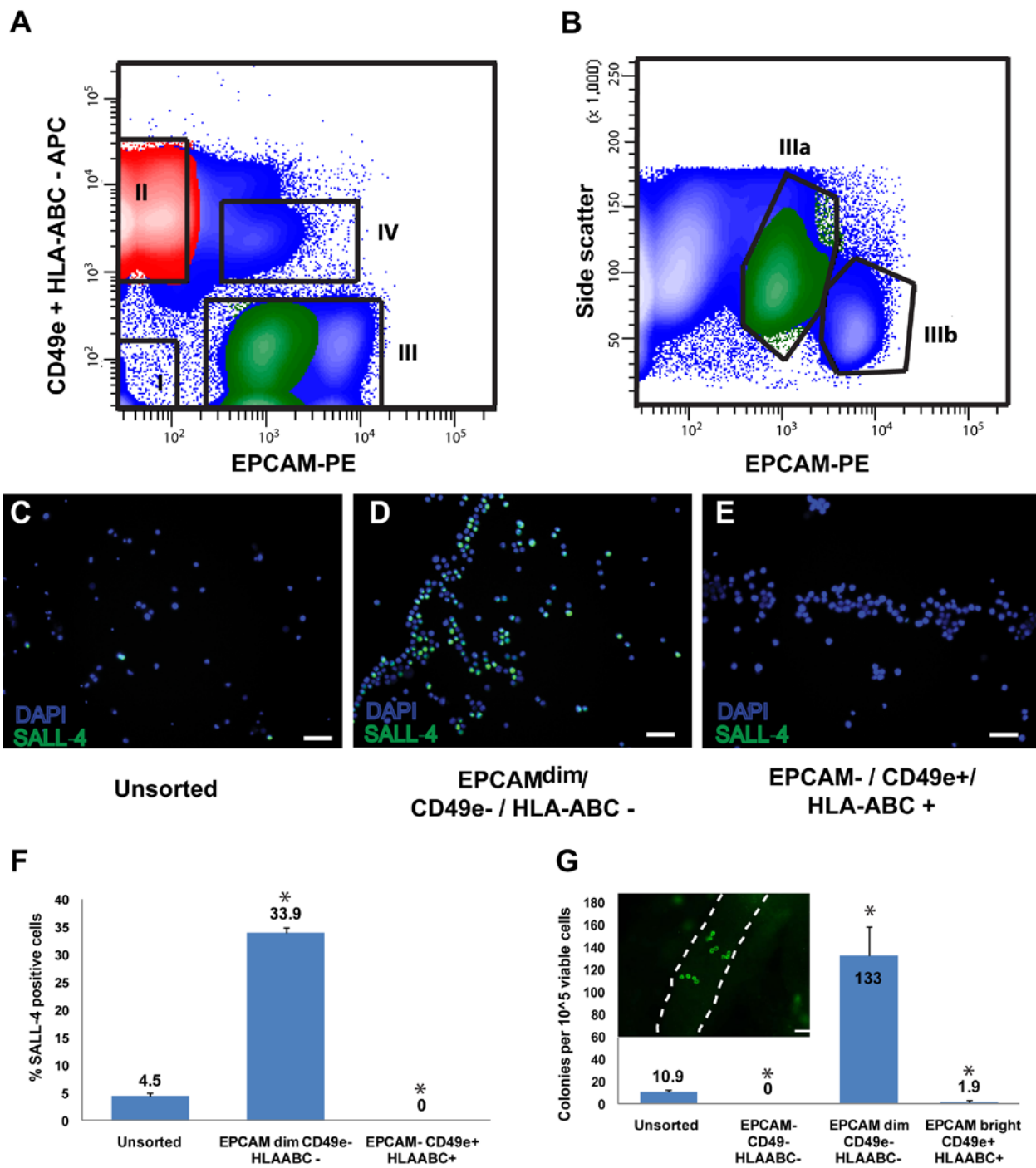
(A) To determine whether human spermatogonia express HLA-ABC, human testicular cell suspensions were stained with APC-conjugated HLA-ABC antibodies and sorted into positive and negative fractions by FACS. Negative gates were defined by analysis of human testis cells using APC-conjugated isotype control antibodies. (B–E) Following FACS, each fraction of cells was fixed and immunocytochemistry was performed to assess SALL4 expression; then, fractions were counterstained with DAPI to quantify total cells. (B) The percentage of cells in each unsorted and sorted fraction that displayed SALL4 staining (SALL4 positive green cells/DAPI-stained total cells). (F–J) A similar experiment was conducted using APC-conjugated CD49e antibodies. Scale bar: 50  $\mu\text{m}$  (C–E and H–J). Bars in B and G indicate the mean percentage of SALL4-positive cells (SALL4-positive cells/total cells) in each fraction. Error bars in B and G represent SEM from 3 replicate sorting experiments. \* $P < 0.001$ , compared with unsorted cells. Reprinted with permission from Dovey SL and Valli H et al., *J Clin Invest.* 2013 Apr 1;123(4):1833-43, Copyright (2014).

### **4.3.3 Analysis of FACS sorted fraction for human spermatogonia**

Human testicular cell suspensions were contaminated with 10% MOLT-4 cells to simulate a clinical situation in which a patient preserves a testicular biopsy that contains SSCs and might be used in the future to restore fertility (i.e., by SSC transplantation). To safely use the preserved tissue for autologous transplantation, the malignant cells must be identified and completely removed. The objectives of this set of experiments were to determine (a) whether spermatogonia could be successfully sorted from a contaminated testicular cell population, (b) whether spermatogonia could be enriched, and (c) whether contaminating malignant cells could successfully be separated from spermatogonia. To achieve these goals, I sorted the contaminated human testis cell suspension into fractions based on relative expression of EPCAM (spermatogonial marker) as well as CD49e and HLA-ABC (MOLT-4 markers). As shown in Figure 19 populations of cells were gated:  $\text{EPCAM}^-/\text{CD49e}^-/\text{HLA-ABC}^-$  (fraction I);  $\text{EPCAM}^-$

/CD49e<sup>+</sup>/HLA-ABC<sup>+</sup> (fraction II); EPCAM<sup>+</sup>/CD49e<sup>-</sup>/HLA-ABC<sup>-</sup> (fraction III); and EPCAM<sup>+</sup>/CD49e<sup>+</sup>/HLA-ABC<sup>+</sup> (fraction IV). The EPCAM<sup>+</sup>/CD49e<sup>-</sup>/HLA-ABC<sup>-</sup> fraction (III) was further fractionated based on level of EPCAM expression and side scatter of incident light (Figure 19B, fractions IIIa and IIIb) into EPCAM<sup>dim</sup>/SSc<sup>high</sup> (Figure 19A, fraction III, green) and EPCAM<sup>bright</sup>/SSc<sup>low</sup> (Figure 19A, fraction III, blue). Based on data in Figures 13 and 18 and Table 3, I hypothesized that human spermatogonia would be recovered in fraction IIIa (Figure 19A and B, green) and that MOLT-4 cells would be recovered in fraction II (Figure 19A, red).

As expected, immunocytochemical staining of sorted fractions revealed significant enrichment of SALL4 positive cells in the EPCAM<sup>dim</sup>/SSc<sup>hi</sup>/CD49e<sup>-</sup>/HLA-ABC<sup>-</sup> fraction (IIIa) compared with unsorted testicular cells ( $33.9 \pm 1.0\%$  vs.  $4.5 \pm 0.6\%$  SALL4 positive cells in the unsorted population,  $P = 0.0005$ ; Figure 19C–F). This fraction will be described as EPCAM<sup>dim</sup>/CD49e<sup>-</sup>/HLA-ABC<sup>-</sup> from this point forward and in Figures 19 and 20. No SALL4 positive cells were found in the EPCAM<sup>-</sup>/CD49e<sup>+</sup>/HLA-ABC<sup>+</sup> fraction (II) (Figure 19E and F). Furthermore, the xenotransplantation analysis of spermatogonial colonies in the seminiferous tubules of nude mice confirmed that colonization activity was enriched in the EPCAM<sup>dim</sup>/CD49e<sup>-</sup>/HLA-ABC<sup>-</sup> fraction compared with unsorted (unspiked) testicular cells ( $133 \pm 25.2$  colonies per  $10^5$  viable transplanted cells vs.  $10.9 \pm 2.1$  colonies per  $10^5$  viable transplanted cells in the unsorted control,  $P < 0.0001$ ; Figure 19G). This represents approximately 12-fold enrichment of spermatogonial colonizing activity in the human-to-nude mouse xenotransplant assay.

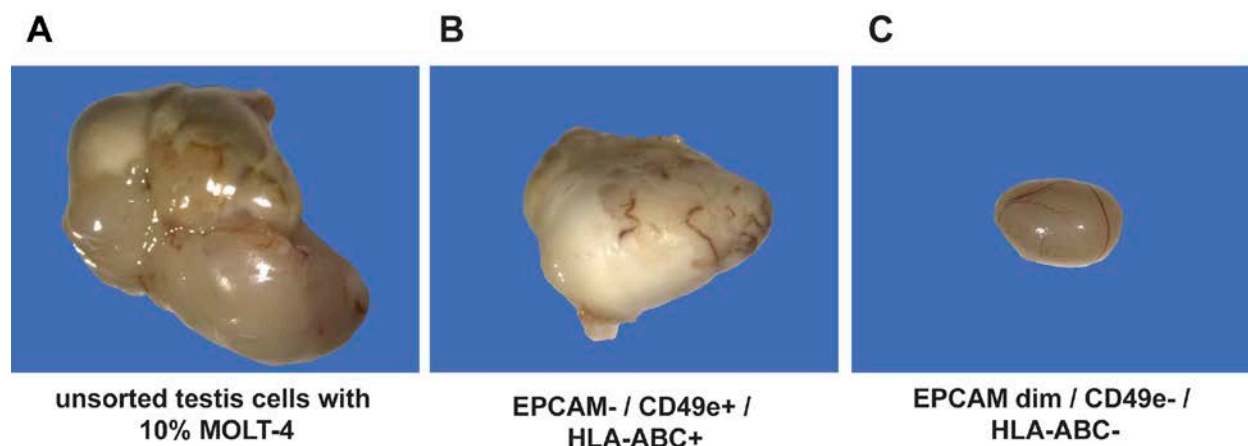


**Figure 19. The EPCAM dim/CD49e negative/HLA-ABC negative fraction of MOLT-4-spiked human testis cell suspension is enriched for human spermatogonia.**

(A) Human testicular cell suspensions were spiked with 10% MOLT-4 cells and then FACS sorted using EPCAM-PE, HLA-ABC-APC and CD49e-APC antibodies. (B) Fraction III in A was further analyzed with side scatter, as described in Figure 11, to identify the SSC fraction, EPCAM dim/side scatter high (green, Fraction IIIa). Only cells that (A) primarily fell within fraction III and (B) secondarily fell within fraction IIIa were collected. (C–F) Immunocytochemistry was performed to assess relative SALL4 expression in unsorted and sorted fractions. We focused specifically on fractions II and IIIa (green), because this is where we expected to find MOLT-4 leukemia cells and human spermatogonia, respectively, based on data in Figures 13 and 18. Scale bar: 50  $\mu$ m (C–E). Bars in F indicate the mean percentage of SALL4-positive cells (SALL4-positive cells/total cells) in each fraction. Error bars in F represent SEM from 6 replicate sorting experiments. (G) The human-to-nude mouse xenotransplantation assay was used to assess spermatogonial colonizing activity in unsorted (unspiked) and sorted (spiked) testis cell fractions (I, IIIa, and IV), as described in Figure ?. Bars indicate the mean number of colonies per  $10^6$  viable cells in each fraction. Error bars represent SEM from 6 replicate sorting experiments. \* $P < 0.001$ , compared with unsorted cells. A typical colony of human spermatogonia in recipient mouse seminiferous tubules is shown in the inset. Scale bar: 50  $\mu$ m. Reprinted with permission from Dovey SL and Valli H et al., J Clin Invest. 2013 Apr 1;123(4):1833-43, Copyright (2014).

#### **4.3.4 Tumor formation of FACS sorted cells following transplantation into the testes of nude mice.**

To determine whether MOLT-4 cells had been successfully removed from the sorted population of spermatogonia, tumor formation was assessed following transplantation of the sorted fractions into the testes of nude mice. In a prior sensitivity analysis, it was demonstrated that transplantation of as few as 10 MOLT-4 cells into the testes of nude mice could induce tumor formation [159].



**Figure 20. EPCAM<sup>-</sup>/CD49e<sup>+</sup>/HLA-ABC<sup>+</sup> cells form testicular tumors following transplantation into nude mice, but EPCAM<sup>dim</sup>/CD49e<sup>-</sup>/HLA-ABC<sup>-</sup> cells do not form tumors.**

(A and B) Unsorted spiked testicular cells and cells from fraction II (see Figure 19A) produced tumors in recipient mouse testes. (C) Cells from fraction IIIa (see Figure 19A and B) that contained human spermatogonia colonizing the seminiferous tubule of nude mice (see Figure 19G) did not produce tumors. Reprinted with permission from Dovey SL and Valli H et al., *J Clin Invest.* 2013 Apr 1;123(4):1833-43, Copyright (2014).

Following sorting of the spiked testicular cell population, the EPCAM<sup>dim</sup>/CD49e<sup>-</sup>/HLA-ABC<sup>-</sup> (putative SSCs, fraction IIIa) and EPCAM<sup>-</sup>/CD49e<sup>+</sup>/HLA-ABC<sup>+</sup>-sorted (putative MOLT-4 cells, fraction II) fractions (Figure 19A and B) were transplanted into the seminiferous tubules of nude mice. Uncontaminated testicular cells, a pure population of MOLT-4 cells, and unsorted spiked cells were transplanted in the same manner to serve as negative and positive controls, respectively. When a pure population of MOLT-4 cells was transplanted into the seminiferous tubules, tumor formation was observed 18% of the time (Table 4). The unsorted spiked population of cells produced tumors in 41% of testes transplanted (Figure 20A and Table 4). The EPCAM<sup>-</sup>/CD49e<sup>+</sup>/HLA-ABC<sup>+</sup> fraction produced tumors in 23% of transplanted testes (Figure 20B and Table 4), whereas tumors were never observed in the EPCAM<sup>dim</sup>/CD49e<sup>-</sup>/HLA-ABC<sup>-</sup> fraction (Figure 20C and Table 4).



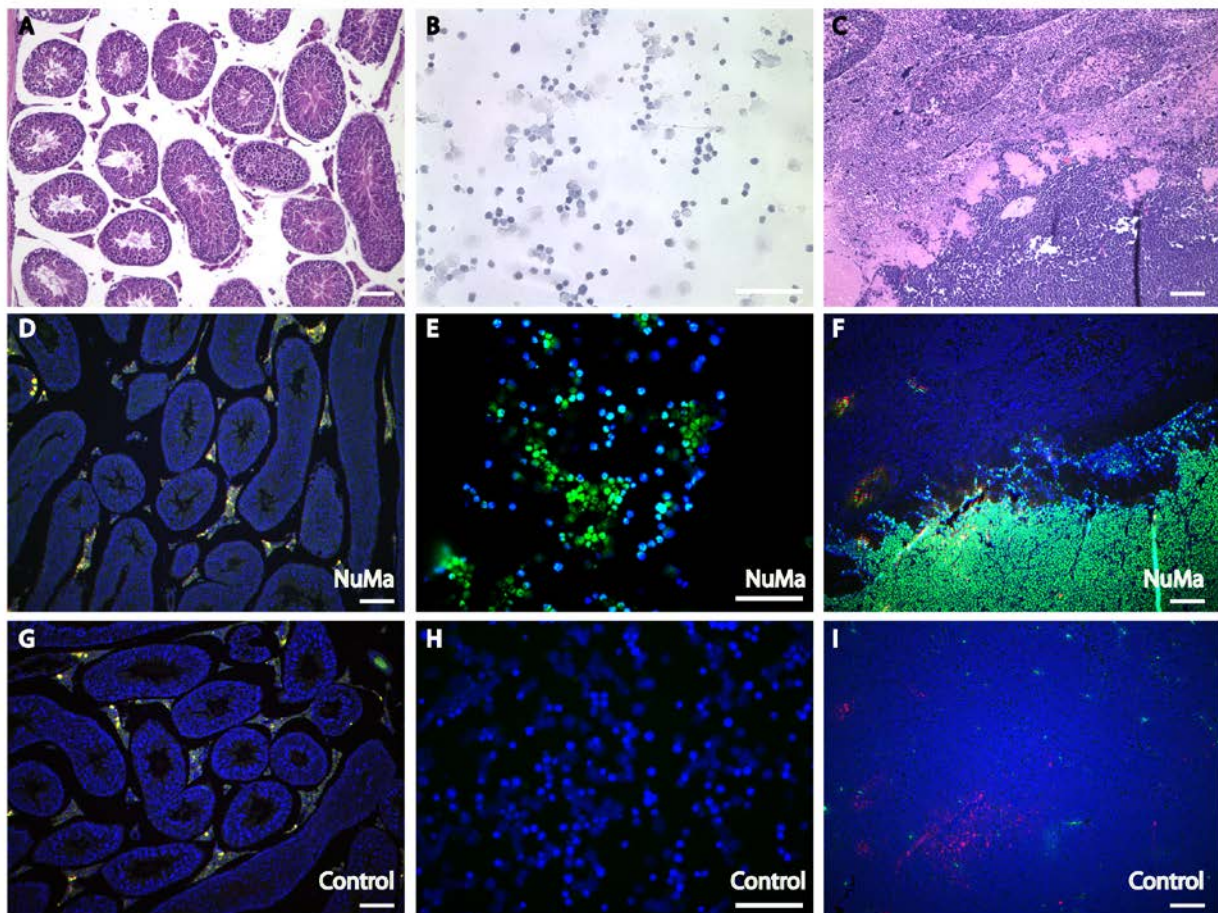
**Table 4. Quantitative assessment of tumor formation in recipient mouse testes**

	Testis number (n)		Tumor formation [n(%)]	
	Intratubular	Interstitial	Intratubular	Interstitial
<b>Before sort*</b>				
Testis cells	29	n/a	0 (0%)	n/a
MOLT-4 cells	28	25	5 (18%)	18 (72%)
Testis cells + 10% MOLT-4 cells	32	26	13 (41%)	16 (62%)
<b>After sort</b>				
EPCAM <sup>dim</sup> /CD49e <sup>-</sup> /HLA-ABC <sup>-</sup>	25	30	0 (0%)	0 (0%)
EPCAM <sup>-</sup> /CD49e <sup>+</sup> /HLA-ABC <sup>+</sup>	22	29	5 (23%)	16 (55%)

\*Unsorted (before sort) and sorted (after sort) cell fractions were transplanted into seminiferous tubules or interstitial space of recipient mouse testes. n/a – not applicable.

For additional confirmation that MOLT-4 contamination had been successfully removed from the EPCAM<sup>dim</sup>/CD49e<sup>-</sup>/HLA-ABC<sup>-</sup> fraction, interstitial testicular transplants were performed. Earlier work with MOLT-4 testicular transplantation suggested that tumor formation may be more efficient when cells are introduced into the interstitial space, thus increasing the sensitivity of the tumor bioassay. The same cell fractions were transplanted into the interstitial space of the testes in nude mice. Approximately 5,000 MOLT-4 cells were transplanted per testis in the control arms of this cancer cell–spiking experiment (i.e., 5,000 MOLT-4 cells or 50,000 unsorted testis cells spiked with 10% MOLT-4 cells). Unlike in the intratubular transplantation experiments above, tumor analysis was not performed until 16 weeks following transplantation, or sooner if palpable tumors were present, to maximize the sensitivity of the tumor bioassay. With the interstitial transplants, 72% of testes transplanted with pure MOLT-4 cells developed tumors, as did 62% of testes transplanted with an unsorted spiked suspension of cells (Table 4). Following sorting, tumor formation was observed in 55% of testes transplanted with the EPCAM<sup>-</sup>/CD49e<sup>+</sup>/HLA-ABC<sup>+</sup> (putative MOLT-4) fraction, whereas there was no tumor formation in any of the testes transplanted with the EPCAM<sup>dim</sup>/CD49e<sup>-</sup>/HLA-ABC<sup>-</sup> (putative

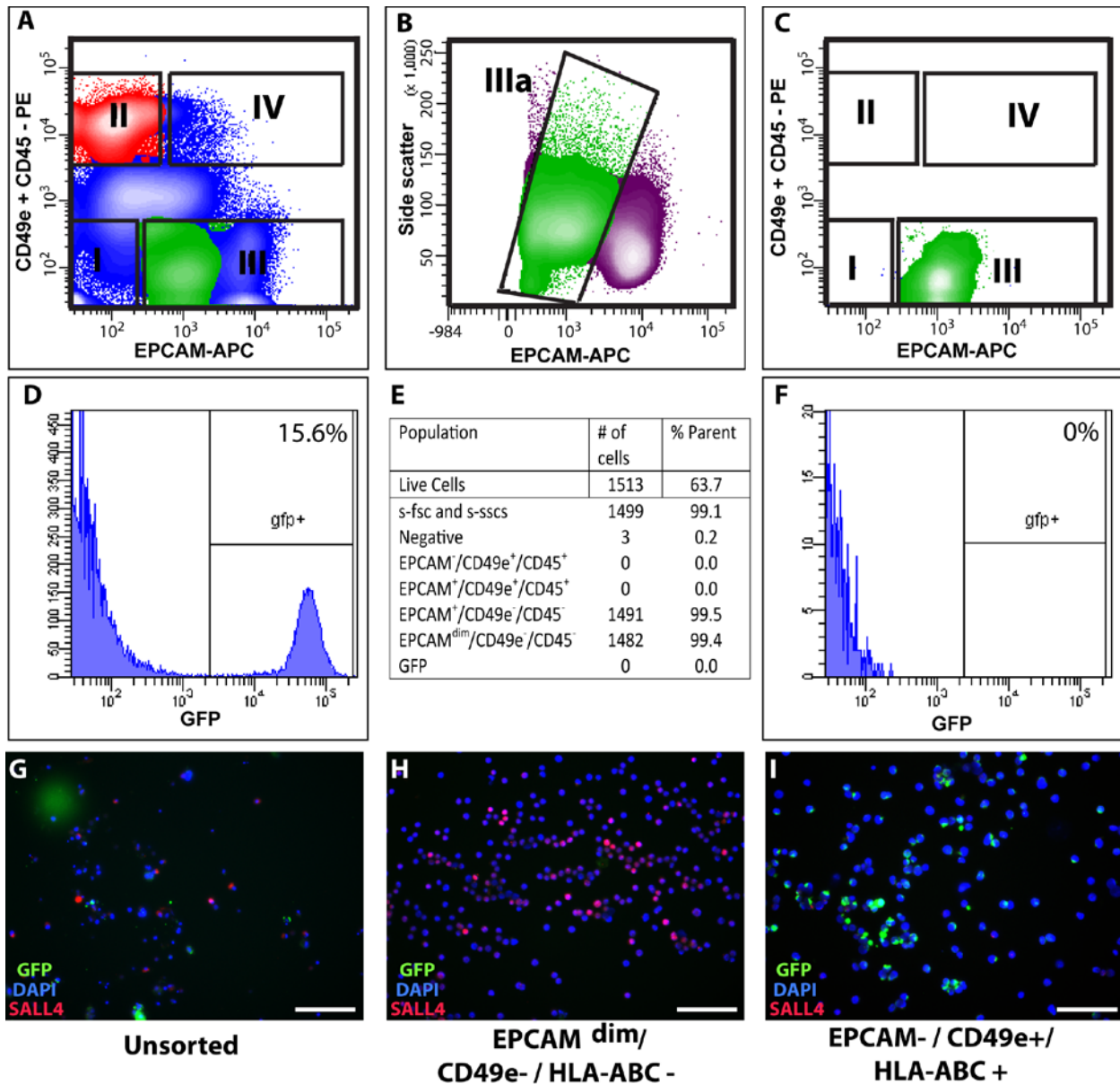
SSC) fraction. Pathological analyses of the samples (by C.A. Castro) indicated that they are consistent with lymphocytic tumoral growth, with characteristic malignant invasion through the tunica albuginea and into abdominal organs. Furthermore, immunohistochemical analyses of testicular tumors with a human-specific antibody directed against the nuclear mitosis apparatus protein (NuMA) demonstrated that the tumors are of human origin (Figure 21). Thus, a multiparameter sort strategy effectively segregated undifferentiated spermatogonia from MOLT-4 leukemia cells. FACS reanalysis of the  $EPCAM^{dim}/CD49e^{-}/HLA-ABC^{-}$  fraction demonstrated a purity range of 98.8%–99.8%.



**Figure 21. Testicular tumors observed after transplantation of the EPCAM<sup>-</sup>/CD49e<sup>+</sup>/HLA-ABC<sup>+</sup> fraction following FACS are of human origin.**

(A, D, G) Cross-section of a nude mouse testis showing normal morphology. (A) Stained with H&E, (D) staining with the human-specific nuclear mitotic apparatus protein (NuMA) and (G) is an IgG isotype control. (B, E and H) MOLT-4 leukemic cell suspension. (B) MOLT-4 cells stained with H&E, (E) NuMA antibody, and (H) with an IgG isotype control. NuMA is expressed by a variety of human malignancies, including MOLT-4 leukemic cells, as demonstrated in (E) but not expressed by mouse testicular cells (B). (C, F and I) Testis from nude mouse demonstrating gross tumor formation following transplantation of EPCAM<sup>-</sup>/CD49e<sup>+</sup>/HLA-ABC<sup>+</sup> cells. Disruption of the normal morphology of the seminiferous tubules by the MOLT-4 leukemic cells can be visualized in (C) (bottom right), and these cells stain positively for NuMA (F). (I) MOLT-4-derived tumor stained with an IgG isotype control. Scale bar = 100µm. Reprinted with permission from Dovey SL and Valli H et al., J Clin Invest. 2013 Apr 1;123(4):1833-43, Copyright (2014).

To demonstrate that the multiparameter sorting strategy could be generalized to other cancer cell lines, I contaminated human testis cell suspensions with TF-1a human leukemia cells [272]. TF-1a cells did not efficiently make solid tumors following xenotransplantation to nude mouse testes, so I labeled them with ubiquitin-C-GFP to enable tracking and assess contamination of sorted fractions (Figure 22). TF-1a cells did not express HLA-ABC to the same degree as the MOLT-4 cell line, so an alternate epitope, CD45, was used instead. A multiparameter FACS procedure was performed using EPCAM-APC (spermatogonial marker), CD49e-PE (TF-1a marker), and CD45-PE (TF-1a marker) on a human testicular cell suspension spiked with TF-1a cells, as described for the MOLT-4 line above (Figure 22). A purity check indicated that the putative spermatogonial fraction (IIIa) was 99.4% pure (Figure 22C and E). This fraction contained SALL4 positive spermatogonia (Figure 22H) but was devoid of GFP<sup>+</sup> TF-1a cells (Figure 22F and H).



**Figure 22. Separation of TF-1a-GFP cells and human spermatogonia from a contaminated human testis cell suspension using a multi-parameter FACS approach.**

(**A and D**) Human testicular cell suspensions were “spiked” with TF-1a-GFP cells (15.6%) and then FACS was performed using EPCAM-APC, CD49e-PE and CD45-PE. (**B**) Fraction III in (**A**) was further analyzed with side scatter, as previously described, to identify the spermatogonial fraction, EPCAM dim/side scatter high (green, fraction IIIA). (**C and E**) Purity check indicated that the EPCAM dim/side scatter high (green, fraction IIIA) fraction was 99.4% pure and contained no GFP positive cells (**E and F**), representing the TF-1a leukemic

cells. **(G-I)** Unsorted and sorted cell fractions were evaluated by immunocytochemistry for SALL4 (human spermatogonia) and GFP (TF-1a-GFP). We focused on fractions II (red) and IIIA (green) because these were expected to contain TF-1a leukemic cells and human spermatogonia, respectively. The EPCAM<sup>dim</sup>/CD49<sup>e-</sup>/CD45<sup>-</sup> fraction (IIIA) contained SALL4 positive spermatogonia, but not GFP positive TF-1a cells **(H)**. The EPCAM/CD49<sup>e+</sup>/CD49<sup>+</sup> fraction (II) contained GFP positive TF-1a cells, but not SALL4 positive spermatogonia **(I)**. Scale bars = 100  $\mu$ m. Reprinted with permission from Dovey SL and Valli H et al., J Clin Invest. 2013 Apr 1;123(4):1833-43, Copyright (2014).

#### 4.4 DISCUSSION

SSCs may have application for preserving and restoring spermatogenesis in men who are rendered infertile due to chemotherapy or radiation treatment for cancer or other conditions. A boy or man could theoretically cryopreserve testicular tissue or cells (containing SSCs) before the gonadotoxic therapy and have these cells reintroduced into his testis after he is cured of his primary disease. For a cancer survivor, this approach has the inherent and unacceptable risk of reintroducing malignant cells. I created this scenario in this study by contaminating human testis cell suspensions with MOLT-4 leukemia cells. I then used a multiparameter sorting approach to prove that it is feasible to isolate and enrich SSCs from a heterogeneous human testis cell suspension and also remove malignant contamination.

EPCAM, our selected spermatogonial marker, is a calcium-independent adhesion molecule that is expressed by murine embryonic stem cells, primordial germ cells of both sexes, and spermatogonia in adult mice [274]. Furthermore, Ryu and colleagues demonstrated that EPCAM could be used as a cell surface marker to isolate and enrich transplantable SSCs in the rat [29]. The majority of SALL4 positive spermatogonia were recovered in the EPCAM<sup>dim</sup> fraction of human testis cells, and xenotransplant colonizing activity in this fraction

was enriched nearly 6 fold compared with that in unsorted controls (Figure 13). It is important to confirm experimentally that rodent spermatogonial markers are conserved in humans. CD29 ( $\beta$ 1-integrin), for example, is a marker of rodent SSCs that does not appear to be conserved in humans [181] (Valli and Orwig, data not shown). Others have reported that SSEA4 [112] and GPR125 [143] are cell surface markers of human spermatogonia. I did not observe immunoreactivity for either marker with human testis cell suspensions in this study. These disparate results might be attributed to differences in cell processing (i.e., trypsin concentration) that affect cell surface antigens or the use of different antibodies.

I further refined our sorting strategy by adding 2 MOLT-4 leukemia cell markers (CD49e and HLA-ABC) to the staining cocktail that was then used to analyze and fractionate MOLT-4-contaminated human testis cell suspensions. The putative spermatogonial fraction ( $\text{EPCAM}^{\text{dim}}/\text{CD49e}^-/\text{HLA-ABC}^-$ ) was enriched 12-fold for colonizing activity in the human-to-nude mouse xenotransplant assay. This fraction never produced a tumor following transplantation into seminiferous tubules or into the testicular interstitial space. In contrast, the putative MOLT-4 leukemia cell fraction was depleted of SALL4 positive spermatogonia and produced tumors in seminiferous tubules as well as in the testicular interstitial space. Similar results were obtained by Hou and colleagues, who used EPCAM in combination with leukemia markers to remove malignant contamination in a rat model of Roser's T cell leukemia [185] and concluded that a multiparameter sorting strategy that included both spermatogonial and leukemia markers was required to eliminate malignant contamination and leukemia transmission.

I then replicated this finding using a different human leukemic cell line, TF-1a, to demonstrate that the multiparameter FACS strategy to remove malignant cells from therapeutic spermatogonia can be applied across different malignancies (Figure 22). It is important to note,

however, that I needed to use different cell surface antigens when sorting the TF-1a cells from spermatogonia, as their cell surface phenotype was somewhat different than that of MOLT-4 cells. Through a series of similar experiments, it may be possible to identify a broad panel of markers that can be used in a generalized approach to remove a variety of malignant cell types from human testis cell suspensions.

Two prior studies have attempted to separate spermatogonia from cancer cells in a human model. In 2006, Fujita and colleagues demonstrated via flow cytometry that several human leukemic cell lines uniformly expressed cell surface antigens MHC class I and CD45 [263]. They then performed FACS on human testicular cells and demonstrated that the MHC class I<sup>+</sup>/CD45<sup>-</sup> fraction contained germ cells (assessed qualitatively by RT-PCR for germ cell markers), suggesting that these cell surface antigens could be used to sort leukemic cells away from germ cells. However, the authors of that study did not report sorting and transplantation of contaminated human testis cell suspensions, as they had previously reported for mice [262]. Geens and coworkers did contaminate human testis cell suspensions with B cell acute lymphoblastic leukemic cells but were not able to remove the malignant contamination using FACS-based selection for HLA-ABC [264].

Our study adds significantly to the current literature by demonstrating that a multiparameter sorting strategy can enrich spermatogonia and eliminate cancer contamination from a human testis cell suspension. These conclusions are supported by quantitative in vitro and in vivo assessments, including transplant of selected fractions into the seminiferous tubules of recipient mice. This human-to-nude mouse xenotransplant assay is most relevant to the cancer survivor paradigm in which the ultimate objective will be to transplant a patient's cells back into the seminiferous tubules of his testes to reinitiate spermatogenesis. However, these bioassays

require a large number of cells and time. Ultimately, it will be necessary to identify specific, sensitive markers of SSCs and cancer cells so that assessment of stem cell activity and malignant contamination can be conducted quickly and with a relatively smaller portion of the patient's tissue. Molecular readouts, such as PCR, are rapid and likely have the best sensitivity to detect occult tumor cells, and, indeed, evaluation of minimal residual disease (MRD) with PCR is now being investigated as a more precise means to screen tissue for transplantation [275]. Alarmingly, assessment of MRD in ovarian tissue destined for autotransplantation in patients with leukemia identified malignant contamination in the majority of samples, even after a negative histology and immunohistochemistry examination [275, 276].

One current limitation to performing MRD screening routinely prior to transplantation is the need to identify a PCR target unique to the cancer of interest. However, identifying a distinctive PCR target for MRD screening is just half of the equation. What is the clinical significance of very-low-level contamination detected only by PCR for a given malignancy? How likely is this to result in clinical relapse if the tissue is transplanted? Courbiere and colleagues discussed this issue eloquently in an editorial describing a patient with chronic myeloid leukemia who underwent ovarian tissue harvesting in which autotransplantation of the tissue was debated after histology evaluation was negative but PCR demonstrated a small number of BCR-ABL transcripts in the cortical tissue (0.001%) [277]. Considering that the survival and engraftment of tumor cells will depend on the type of cancer and number infused, it was felt clinically that the likelihood of inducing relapse was low if transplantation was performed, but the absolute risk is virtually impossible to quantify.

The findings in our study parallel this clinical dilemma, in that the FACS reanalysis purity check demonstrated that the EPCAM<sup>dim</sup>/CD49e<sup>-</sup>/HLA-ABC<sup>-</sup> fraction was 98.8%–99.8%



pure. Furthermore, this fraction did not produce tumors in 55 transplanted testes (intratubular and interstitial). Do these results indicate that approximately 99% purity should be considered safe for autologous transplantation? In the bone marrow transplant field, “purging” malignant cells from HSC samples prior to autologous transplant has been studied extensively for over 2 decades, as autologous bone marrow transplant is considered standard treatment for patients with various malignancies [278]. Overall, there is limited convincing evidence that transfusing a small number of cancer cells in HSC grafts causes relapse or that purging HSC grafts decreases rates of relapse, and results from phase II and III clinical trials have been mixed [278, 279]. Clearly, HSC transplantation and spermatogonial and/or ovarian transplantation are not clinically equivalent, considering that HSC transplantation is required to treat or cure life-threatening conditions, whereas fertility preservation procedures are elective. Nonetheless, HSC graft purging studies do highlight the point that in vitro measures of decontamination efficiency, such as PCR, may not always be appropriate surrogates of clinical outcome. Short of performing a clinical trial, biological readouts, such as xenotransplantation, may be the most relevant end points to assess the adequacy of decontamination. Indeed, as our ability to detect MRD through molecular methods improves, it is likely that clinicians will face this challenging scenario on a more frequent basis. Thus, it will be important to not only improve MRD screening techniques, but also to correlate MRD screening results with xenotransplantation studies, so that the clinical risk of inducing a relapse following transplantation of tissue with trace MRD can be estimated.

Progress in culturing human SSCs has been reported by several laboratories in the past few years [26, 94, 95, 143, 280] and may provide an alternative approach for removing malignant contamination. In theory, it may be possible to amplify human SSCs clonally from individual cells or from small enriched fractions of testis cells and thereby alleviate malignant

contamination. This will require continued progress to establish robust culture conditions in which human SSCs survive and can be expanded over several passages to produce a sufficient number of stem cells for therapeutic application.

I have demonstrated that it is feasible to enrich SSCs and remove malignant contamination from a heterogeneous human testis cell suspension. As the panels of spermatogonial and cancer markers expand, it will be important to test sorting strategies on primary human cancers, which are likely to be more heterogeneous than the MOLT-4 and TF-1a leukemia lines used in this study. In addition, it will be important to develop methods to rapidly screen cell populations for malignant contamination and establish criteria for assessing safety prior to transplant. Continued work in this field is important, because clinics are already cryopreserving testicular tissue and ovarian tissue for patients with cancer in anticipation that this tissue can be used in the future to restore fertility. Autologous transplantation of tissue or cells is among the techniques being considered for both sexes, so risk of reintroducing cancer is of paramount concern.

## 5.0 SUMMARY AND CONCLUSIONS

Spermatogonial stem cells are adult tissue stem cells that balance self-renewal and differentiation to support spermatogenesis throughout a male's life. These cells may also one day be used in clinics to treat some cases of male infertility. The SSCs can only be definitively identified by their biological potential to produce and maintain spermatogenesis after transplantation. This assay was first described by Brinster and colleagues [122, 123] and is widely used to analyze SSC activity in any given rodent testis cell population. Obviously, human-to-human transplants as a routine bioassay are not possible and there is a lack of experimental tools to analyze SSCs in human testis tissues or cell suspensions.

For grown men and pubertal boys, the effective and well established method to preserve their fertility while undergoing cancer treatment or bone marrow transplant is to cryopreserve a semen sample (Figure 15, top). Unfortunately, many post-pubertal patients (especially adolescence boys) do not preserve a semen sample and this is not an option for prepubertal boys who do not yet make sperm. However, these boys do have spermatogonial stem cells in their testis that will initiate spermatogenesis at puberty. There are several experimental stem cell based options in the research pipeline that may be available for the patients in the future (Figure 15). Several centers around the world are already cryopreserving testicular biopsies from prepubertal patients in hopes that when these patients are ready to have kids, the techniques to restore their fertility are available in clinics [91, 93-95, 100, 222-224].

Feasibility and safety studies using human tissues are important to ensure responsible translation of stem cell reproductive technologies to the clinic. Techniques currently under investigation involve using either a cell suspension or intact pieces of tissues and it is uncertain which technologies will progress to clinical application. Therefore, it is important to optimize tissue processing and cryopreservation to maximize patient access to downstream applications. Additionally, the biopsies taken from prepubertal patient may have malignant contamination since they are obtained prior to the initiation of chemotherapy. Therefore, methods are needed to eliminate the risk of reintroducing cancers when using these cells.

To begin addressing these issues, I had to develop experimental tools to analyze and quantify human SSCs. A modification of the mouse SSC transplant method has been used in humans and is becoming the gold standard for quantifying human SSC-like activity [94, 95, 104, 106, 112, 118-120]. In our lab, we have generated a rabbit anti-primate antibody that recognizes primate cells (including human) in mouse testis [53, 114, 118, 120, 159] (Figures 4 and 5). The human-to-nude mouse xenotransplantation assay has 2 month delay from transplant to analysis. Therefore, I also developed a quick read out assay that involves staining for human spermatogonia markers by immunocytochemistry.

SALL4, PLZF, UTF1, ENO2 and UCHL1 were identified as markers of undifferentiated human spermatogonia. All of these markers are expressed by cells on the basement membrane of seminiferous tubules but do not co-express the differentiation marker KIT (Figure 6). Therefore, all of the markers can be used in immunocytochemistry to identify human stem and progenitor spermatogonia.

Next, I used ICC and human-to-nude mouse xenotransplantation to identify cell surface markers that can be used to isolate and enrich human spermatogonia. I demonstrated that cell

surface markers THY1, EPCAM and ITGA6 can all be used to enrich human SSCs with FACS sorting (Figures 11-13). Out of the three markers, only ITGA6 was amenable to MACS sorting (Figure 14), which is a higher throughput method than FACS.

It is not known which fertility restoration technique will be translated to the clinics in the future, therefore, optimization of testicular tissue cryopreservation methods is extremely important. The preferred cryopreservation technique should maximize the access to downstream technologies to restore fertility for the patients. Great progress has been made in SSC transplantation technique (Figure 15, blue boxes). Homologous species SSC transplantation has now been reported in mice, rats, pigs, goats, bulls, sheep, dogs and monkeys, including the production of donor-derived progeny in mice, rats, goats and sheep [70, 73-85, 90]. In contrast to SSC transplantation, which involves disaggregation of SSCs from their cognate niches, testicular tissue grafting and testicular tissue organ culture maintain the integrity of the stem cell/niche unit. Testicular tissues obtained from newborn mice, pigs and goats could produce complete spermatogenesis when grafted under the skin of nude mice [207]. In mice, the resulting sperm were used to fertilize eggs by ICSI and gave rise to live offspring [208]. Xenografting with prepubertal rhesus macaque also successfully produced complete spermatogenesis with fertilization competent sperm [281]. Survival and spermatogenesis from adult testicular tissue grafts have been less successful than immature grafts [242]. Human tissue grafting into nude mice has been less successful as no one has reported the production of haploid gametes or sperm [210, 211, 282-285]. The most advanced stage of germ cell development reported from human testicular tissue grafts to date has been pachytene spermatocytes [211, 212, 232]. The results of the monkey studies suggest that autologous transplantation may be an option if suitable cryopreservation conditions are developed. Similar to SSC transplantation, autologous grafting

will be problematic in cases where malignant contamination of the testicular tissue is suspected. Xenografting of human testicular tissue into animals could circumvent this problem, but is associated with additional concerns about xenobiotics and has been unsuccessful to date.

Sato and colleagues [213, 214] reported production of sperm and live offspring from an organ culture method (Figure 15, yellow boxes). If these results in mice can be translated to humans, testicular organ culture would circumvent the need to put tissues or cells back into the patient and may be a safe option for patients with malignancies that contaminate the testes.

I validated and compared methods for cryopreserving human testicular cells or tissues and subsequent recovery of stem and progenitor spermatogonia in order to optimize processing of patient tissues. I found that slow-freezing small ( $3\text{-}5\text{mm}^3$ ) or large ( $6\text{-}10\text{mm}^3$ ) tissue pieces is the optimal method to preserve SSC colonizing activity (Figure 17). In our hands, recovery of human spermatogonia after tissue vitrification was not as effective as slow-freezing. Nonetheless, this method was equal to or slightly better than freezing a cell suspension and therefore could be used if no slow-freezing machine is available. Freezing intact tissues retains the options for either tissue based or cell based therapies in the future [196].

The biopsies obtained from the prepubertal patients are taken prior to their cancer treatment and therefore have a chance of malignant contamination. It has been shown that 20% of boys with acute lymphocytic leukemia have cancer cells in a testicular biopsy taken prior to the initiation of chemotherapy [269]. That is an important concern because prior to translating the SSC transplantation technique to the clinics, we have to be sure that we do not reinitiate cancer in these survivors. Here, I provided proof in principle that by combining positive selection with human spermatogonia marker EPCAM with negative selection for MOLT-4 leukemia cell like markers HLA-ABC and CD49e in FACS, it is possible to remove the malignant

contamination from the potentially therapeutic SSCs. In this case, I used a fairly homogenous cancer cell line and therefore, these experiments have to be replicated using primary human cancers, which are more heterogeneous than a cell line. This is necessary to make sure no malignant contamination remains in the patient samples. Development of cell culture or organ culture methods to expand transplantable stem cells or produce sperm could also circumvent the concerns about transplanting malignant cells or tissues (Figure 15, bottom, blue and yellow boxes).

Stem cell technologies for treating male infertility have the potential to impact the clinic in the near future and therefore it is important to establish criteria to monitor progress and analyze the outcomes. Although it is not popular in the current era that prioritizes the highest impact, innovative and novel science; descriptive studies of human germ lineage development are essential to guide experimental design and enable accurate interpretation of results of human stem cell studies. This knowledge is critical, as I believe it is reasonable to expect that SSCs or other stem cells will be used to preserve and restore male fertility in the coming decades.

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