SPERMATOGONIAL STEM CELLS: STUDY OF RECOVERY KINETICS AND POTENTIAL ROLE IN RESTORATION OF MALE FERTILITY AFTER CYTOTOXIC TREATMENT

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Abstract

Gonadotoxic therapies including radiotherapy and chemotherapy used to treat cancers are extremely damaging to germinal epithelium inducing transient or permanent azoospermia. Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis. They represent the basis of a male fertility restoration strategy after treatment. This work presented herein addressed two critical aspects in this strategy; markers to identify and isolate human SSCs, and the kinetics of SSC recovery after chemotherapy.

In chapter 2, the expression of various rodent SSC markers by undifferentiated human spermatogonia, which include SSCs, was examined by immunohistochemistry, and CD9 expression was detected in the basal compartment of human seminiferous tubules. After immunological sorting, human CD9 positive male gem cells showed 3 to 4-folds enrichment when transplanted into immune-deficient nude mice testes, confirming that CD9 is expressed on human putative human SSCs and can be used to enrich for this population.

In chapter 3, I studied the contribution of SSC recovery to fertility recovery after chemotherapy in a mouse model, and provided functional evidence that the restoration kinetics of male fertility follows those of the SSC population after damage. I also addressed the question whether there is a critical SSC number to confer fertility and suggested a 30% of original SSC population as a threshold of the SSC population size required for the onset of male fertility restoration. Though the study did not identify a faithful physiological parameter related to male reproduction (sperm count, testis weight, testosterone level, Glial cell-derived neurotropic factor (GDNF) transcripts level in testes), which would allow for monitoring the degree of SSC recovery in a non-invasive manner, yet it paved the way for future efforts to predict the timing of male fertility restoration after chemotherapy.

In conclusion, this work identified a new marker for human putative SSCs and proved it to be effective to enrich for this cell population. In addition, it provided functional evidence that restoration of male fertility results from that of SSC and identified for the first time a threshold level of SSC population size permitting recovery.

Resumé

Les thérapies gonadotoxiques y compris la radiothérapie et de la chimiothérapie utilisées pour traiter les cancers sont extrêmement dommageable pour l' épithélium germinal, induisant l'azoospermie transitoire ou permanente. Les cellules souches germinales (CSG) sont à la base de la spermatogenèse. Elles représentent la base d'une stratégie de restauration de la fertilité masculine après traitement. Ce travail a porté sur deux aspects essentiels de cette stratégie; identifier des marqueurs pour isoler les CSG humains, et étudier la cinétique de la récupération des CSG après une chimiothérapie.

Dans le chapitre 2, l'expression de différents marqueurs des CSG de rongeurs a été examinée dans les CSG humaine par immunohistochimie, et l'expression de CD9 a été détectée dans le compartiment basal des tubes séminifères de l'homme. Après un triage cellulaire immunologique, les cellules CD9 positives ont montré un enrichissement de trois à quatre lorsqu'elles sont transplantées dans les testicules des souris nude immunodéficientes, confirmant que CD9 est exprimé sur les CSG mâles et peut être utilisé pour enrichir cette population.

Dans le chapitre 3, j'ai étudié la corrélation entre la cinétique de récupération des CSG et la restauration de la fertilité après une chimiothérapie chez un modèle de souris, et fourni une preuve fonctionnelle que la restauration de la fertilité masculine est parallèle à celui de la population CGS après l'insulte. J'ai également abordé la question de savoir si il y a un nombre critique de CSG pour récupérer la fertilité. Mes résultats suggèrent que 30% de la population d'origine CSG doit être seuil pour supporter la restauration de la fertilité masculine. Bien que l'étude n'a pas identifié un paramètre physiologique mesurable indicateur de la capacité reproductive des mâles pour l'apparition de la restauration de la fertilité masculine (nombre de spermatozoïdes, poids des testicules, niveau de testostérone, niveau de transcription du facteur neurotrope dérivé des cellules gliales (GDNF) dans les testicules), qui

permettrait de surveiller le degré de récupération de la CSG, mais il a ouvert la voie à de futurs efforts.

En conclusion, ce travail a identifié un nouveau marqueur des CSG humaines et il s'est avéré être efficace pour enrichir cette population cellulaire. En outre, il a fourni des preuves que la restauration fonctionnelle de la fertilité masculine est le résultat de la récupération des CSG, et a identifié pour la première fois un seuil de taille de la population CSG permettant la récupération de fertilité masculine.

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Format of the thesis

This thesis is comprised of four chapters. Chapter 1 is a general introduction that includes an overview of the male reproductive system and SSC transplantation and culture as well as a literature review on SSC markers and previous work on regeneration of spermatogenesis after chemotherapy. Chapters 2 and 3 are data chapters that are presented as the duplicated text of published papers in respect with the “Guidelines for Thesis Preparation”. Chapter 2 was published in *Biology of Reproduction* in 2012, and Chapter 3 in *Human reproduction* 2011. The copyright agreements permit the inclusion of these manuscripts in this thesis. Chapter 4 contains a discussion of all the results of this thesis and potential future studies. The appendices contain the ethics certificates for work on animal and human subjects and the copyright notices of *Biology of Reproduction* and the *Human reproduction***.**

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List of abbreviations

- AR ANDROGEN RECEPTOR

- Bcl6b B-CELL CLL/LYMPHOMA 6 MEMBER B
  BMP BONE MORPHOGENETIC PROTEIN
  BTB BLOOD-TESTIS BARRIER

- C-RET REARRANGED IN TRANSFORMATION
  CSF-1 COLONY STIMULATING FACTOR -1
  CySC CYST STEM CELLS

- Dpc POSTCOITUM

- ECM EXTRACELLULAR MATRIX
  Etv5 ETS VARIANT GENE 5

- FGF2 FIBROBLASTS GROWTH FACTOR 2
  FRS-2 FIBROBLAST GROWTH FACTOR RECEPTOR SUBSTRATE 2
  FSH FOLLICLE-STIMULATING HORMONE

- GDNF GLIAL CELL-DERIVED NEUROTROPHIC FACTOR
  GFRα-1 GLYCOSYLPHOSPHATIDYLINOSITOL- (GPI)-ANCHORED BINDING MOLECULE GDNF FAMILY RECEPTOR ALPHA-1
  GnRH GONADOTROPIN RELEASING HORMONE
  GSC GERMLINE STEM CELL
  GFP GREEN FLUORESCENT PROTEIN-LABELED

- JAK-STAT JANUS KINASE-SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION

- LH LUTEINIZING HORMONE
  Lhx1 LIME HOMEOBOX PROTEIN 1

- MAPK MITROGEN-ACTIVATED PROTEIN KINASE

- Oct4 OCTOMER-BINDING TRANSCRIPTION FACTOR 4

- PGC PRIMORDIAL GERM CELLS
  PI3K PHOSPHATIDYLINOSITOL 3-KINASE
  Plzf PROMYELOCYTIC LEUKEMIA ZINC FINGER

- RA RETINOIC ACID

- SF STEEL FACTOR
  SFK SRC FAMILY KINASES
  SSC SPERMATOGONIAL STEM CELLS
  SSEA-1 STAGE-SPECIFIC ANTIGEN 1

- Tnap TISSUE NON-SPECIFIC ALKALINE PHOSPHATASE

Contribution of authors

I performed all experiments described in this thesis. I performed spermatogonial stem cell transplantation described in Chapters 2 and 3 in collaboration with X. Zhang. I conducted all data analyses with M Nagano, and designed experimental scheme and wrote manuscripts in collaboration with M Nagano and Peter Chan.

Chapter 1

Introduction, Background, Rationale & Hypothesis

1. Introduction

Spermatogonial stem cells (SSCs) are stem cells of the male germ line. They are the foundation of spermatogenesis, the process by which mature male germ cells are produced. SSCs have the dual biological function to reproduce themselves to sustain the stem cell pool and to concurrently generate progenitors committed to differentiation to produce spermatozoa. They are present in the testicular seminiferous tubules at birth and perform this dual function throughout the adult life. Because some aspects of spermatogenesis include active cell proliferation process and DNA replication, it is sensitive to anticancer treatment (chemotherapy and radiotherapy) and can be lost permanently in cancer survivors rendering them infertile. This has become a major health and social concern worldwide with the marked improvement of cancer survival rates and the prevalence of cancer survivors among young adults estimated to be 1 in 250 persons (1). In contrast to the availability of treatment regimens for different cancers, sperm banking is currently the only option to preserve male patient fertility using assisted reproduction techniques; it is, however, not an option for prepubertal patients who do not produce mature sperm at the time of therapy. As SSCs are the foundation of spermatogenesis, if SSCs can resume their dual function in self-renewal to rebuild stem cell pool, together with differentiation and production of differentiated germ cells, then fertility can be restored. This is the principle of two strategies currently suggested*; pharmaceutical approach*, where manipulation of hormone levels is used to stimulate functional recovery of the somatic environment and the activity of surviving SSCs, *and non-pharmaceutical, SSC surgical transplantation approach,* where sufficient number of SSCs are collected prior to anticancer therapy to be autologously transferred to patient after therapy to restore spermatogenesis. The SSC surgical transplantation approach has the advantage over pharmaceutical approach in that SSCs are not exposed to anticancer therapy and thus are -if not affected by cancer- genetically and

physiologically intact. Yet this approach has two considerations that have to be addressed before applicability in the human fertility clinic; the potential risk of reintroducing malignant cells in the testis of cured patient and the efficiency of transplanted SSCs to restore fertility.

Decontaminating the SSC population from malignant cells relies on the availability of SSC markers that identify and isolate this population from cancer cells. In Chapter 2, I examined the expression of CD9, a known marker of rodent SSCs, in human germ cells and determined if CD9 can identify and enrich for putative human SSCs using immunomagnetic sorting against CD9 and SSCs transplantation assay.

The efficiency of SSC transplantation in restoring spermatogenesis and fertility is associated with the number of SSCs transplanted. In Chapter 3, I examined the kinetics of fertility restoration and SSCs recovery after chemotherapy in a mouse model using functional transplantation assay. I determined the relation between these two profiles as well as the size of SSC population that confers fertility upon transplantation to infertile mouse recipients. I also examined various physiological parameters related to male reproduction (testis weight, sperm count, testosterone level, histological analyses of spermatogenesis and levels of GDNF transcript in the testis) and attempted to identify a parameter that faithfully monitors the degree of recovery of SSC population.

2. Background

1. Anatomy of the male reproductive system

The mammalian male reproductive tract consists of the testis and the system of excurrent ducts formed of the epididymis, vas deferens, efferent ducts and urethra that store and transport spermatozoa to the exterior. In addition, the male tract includes seminal vesicles, prostate and bulbouretral glands which together form the accessory sex glands that empty into the excurrent ducts and the penis. In the following sections, I will describe briefly the anatomy and function of components relative to this thesis.

The mammalian testis:

In mammals, the testis is the male reproductive organ responsible for the production of testosterone and sperm (Fig.1). The name testis is thought to be derived from the Latin word “testis” which means “witness”(2). The origin of this nomenclature is believed to be from “the witness of virility”, as people used to swear upon their testicles in courts in the days of Roman and Babylonians. Morphologically, the testis is an oval-shaped organ that initially lies in the abdomen. The chronology of testis descent into the scrotum depends on the species; descent in rodents and dogs takes place postnatally whereas in human, pig, horse, cattle and sheep the descent is completed before birth (3). Its presence in the scrotum helps to maintain a testicular temperature 2-3 degrees lower than the core body temperature, a requisite that is essential for spermatogenesis. Structurally, the testis is covered by the tunica albugina, which is a dense fibrous layer of connective tissue, and is composed of multiple seminiferous tubules where spermatogenesis takes place. The proximal open ends of seminiferous tubule join together at a region called the rete, from where spermatozoa in seminiferous fluid are released into the efferent duct exiting the testis at the time of ejaculation.

Inside the testis, the intratesticular environment is divided in two discrete compartments: *extratubular interstitium or interstitial space,* and *seminiferous tubules*.

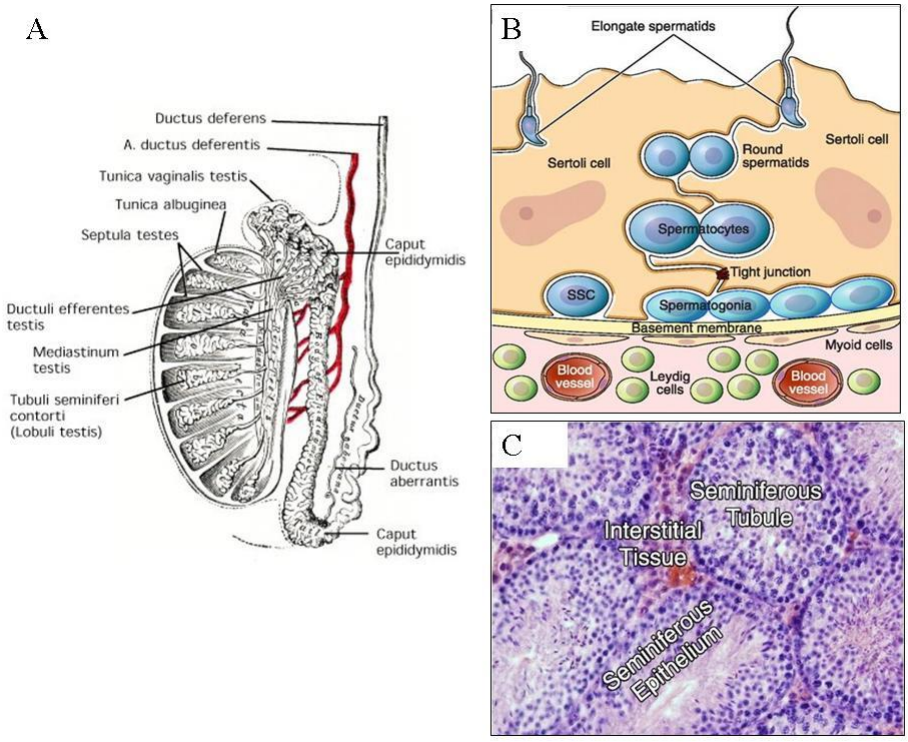

Figure 1: Anatomy of the testis

(A) Cross-section in the testis and epididymis, (B) Histological section in seminiferous tubule, (C) Illustration of the seminiferous epithelium and adjacent interstitial space. Adapted from Grays anatomy and Oatley et al.(4).

The extratubular compartment is usually referred to as the interstitial space. In this compartment lie large polyhedral cells often found in small groups known as Leydig cells. They are responsible for secretion of sex hormones, primarily testosterone, and other steroids such as estradiol. Testosterone is a steroid hormone involved in the expression of the secondary male phenotype including masculinization of the brain and sexual behavior, the differentiation of the male sex organs, and secondary sex characteristics. More importantly, testosterone is crucial for the

initiation and maintenance of spermatogenesis. The absence of testosterone results in the arrest of spermatogenesis and loss of fertility (5). Finally, the interstitial space of the testis contains numerous vascular and lymphatic vessels that provide endocrine factors and nutrients to the cells of the testis.

The seminiferous tubules

The testis consists of long, highly convoluted seminiferous tubules connected at both ends to the rete testis. This structure increases the surface area of the seminiferous epithelium and consequently the number of spermatozoa produced. Inside these seminiferous tubules, which make up to approximately 90% of the weight of the testis, spermatogenesis takes place. Surrounding each seminiferous tubule is a single layer of contractile smooth muscle cells known as peritubular myoid cells. These cells are involved in the transport of spermatozoa and tubular fluid out of the testis to the efferent duct through their contractility (6). In addition, they secrete extracellular matrix components (including laminin, type I and IV collagens, fibronectins and proteoglycans) which form the basement membrane. The basement membrane is a semi-permeable barrier that provides structural support to the cells within the seminiferous tubules. Germ cells on the basement membrane express specific receptors that recognize extracellular matrix proteins. Binding of these extracellular matrix receptors to their ligands has been shown to promote cell survival, proliferation, and differentiation in different cell types (7).

Two distinct types of cells characterize the seminiferous epithelium of the testis: the germ cells and the supporting Sertoli cells. Germ cells include spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa. In the center of each tubule is a fluid-filled lumen containing spermatozoa. In the following sections I will review both types of cells.

The Sertoli cell

They were named after Enrico Sertoli, an Italian physiologist who published a description of these cells in 1865 (8). They are large columnar cells extending from the basal lamina of the tubule wall to the adluminal space or lumen constituting the only somatic cells found in the testis seminiferous epithelium (Fig.1) (9, 10). With their large surface area, they interact with germ cells at all stages of differentiation. They are fully differentiated, non-dividing and thus non-renewing cells in adult testes. In addition, adjacent Sertoli cells are connected by tight junctions that form the blood-testis barrier (BTB), which divide the seminiferous epithelium into two functional compartments: the basal compartment and the adluminal compartment. The basal compartment contains spermatogonia and early spermatocytes accessible to the vascular system, while the adluminal compartment contains late spermatocytes, spermatids and spermatozoa (Fig.1). The role of BTB is to maintain an optimal environment within the seminiferous tubules by preventing the diffusion of potential toxic substances and the displacement of immune cells from the interstitial tissue (11, 12).

In addition to providing structural support over which germ cells proliferate and differentiate, Sertoli cells secrete extracellular factors that were shown to regulate spermatogenesis, for example inhibin, androgen binding protein, Steel factor (SF), and glial cell-derived neurotrophic factor (GDNF) (13-16). Through these growth factors, Sertoli cells control spermatogenesis and thus maintain quantitatively normal sperm production. In addition, as the number of Sertoli cells is fixed, and as each Sertoli cell supports a fixed number of germ cells, it is thus logical to think that the total number of Sertoli cells defines the sperm production capacity of an adult testis depending on the species (14).

Sertoli cell function in supporting normal spermatogenesis in turn is regulated vice the hypothalamo-pituitary axis by gonadotropin releasing

hormone (GnRH), a decapeptide produced by specialized neurons in the hypothalamus (17). Pulsatile GnRH production signals gonadotroph cells in the anterior pituitary to produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that then act on the testis to regulate spermatogenesis. LH binds to receptors on the surface of Leydig cells in the testis and stimulates the production of testosterone that diffuses into the seminiferous tubules. Within the seminiferous tubules only Sertoli cells have receptors for testosterone, androgen receptor (AR), and FSH (8). Sertoli cells transduce signals from testosterone and FSH into the production of factors that are required by germ cells as they mature into spermatozoa (15, 17). In the absence of testosterone or the AR on Sertoli cells, the formation of the BTB is compromised, germ cells are unable to progress beyond meiosis, germ cells that have progressed beyond meiosis detach from supporting Sertoli cells and die, and mature sperm cannot be released from Sertoli cells resulting in infertility (18).

2. Embryonic germ cell development

After fertilization and the development of the blastocyst, the cell of the inner mass gives the three germ layers; endoderm, mesoderm and ectoderm. Mouse germ cells are first recognized at the base of the allantois in the extraembryonic mesoderm at approximately 7 days post-coitum (dpc) as a cluster of cells exhibiting alkaline phosphatase activity and called primordial germ cells (PGCs) (19) (Fig. 2). These cells are not stem cells but rather a precursor of the germ line stem cells, the spermatogonial stem cells (SSCs). Note that PGCs exist transiently till they become irreversibly committed to germ cell lineage after contacting the cells of the developing gonad as discussed in the following paragraph.

Cells of the proximal region of the epiblast at the junction of the extraembryonic ectoderm are believed to be the origin of PGCs (20). Around 5.5 days post-coitum (dpc), the specification of these cells occurs when extraembryonic ectoderm starts to express the bone morphogenetic protein

(BMPs) 4 and 8b (21). BMP signaling leads to the expression of a zinc-finger transcriptional repressor called Blimp1 that inhibits the somatic programing of the cell (22) (Fig. 2). Although BMP4 and BMP8b were shown to be expressed uniformly by the extraembryonic ectoderm, only the cells at the proximal end of the epiblast receive enough signals to inhibit the somatic program and specify as PGCs (22). At 6.25 dpc, BMP4 and BMP8b induce expression of the transmembrane protein Fragilis in the PGC population (21-24). Between 6.5 and 7 dpc, approximately 40 cells expressing high levels of Fragilis and forming a small cluster starts to express the first marker of developing PGCs; Dppa3 (Stella) in addition to stage-specific antigen 1 (SSEA1) and tissue non-specific alkaline phosphatase (Tnap). Cells that do not express high level of Fragillis, do not express Stella and start expressing somatic genes (22). Between 7.25 and 12.5 dpc, PGCs undergo rapid mitosis and the 40 cells proliferate into 25,000 cells as they migrate from the base of the allantois into the genital ridge (25).

Till 12.5 dpc, both male and female PGCs are identical and bipotential (26, 27). It was shown that XY germ cells can develop as oocytes if transplanted into female embryos and XX germ cells develop as prospermatogonia if cultured with male urogenital ridge (28). PGCs remain uncommitted until they come in contact with the sexually differentiated gonadal environment leading to PGCs commitment to respective germ cell fate at 12.5 dpc in males and 13.5 dpc in females.

In the females gonads, PGCs differentiate into oogonia and enter meiosis but arrest at the diplotene stage and remain as such until ovulation. Retinoic acid (RA) from the surrounding mesonephros stimulates the expression of gene stimulated by retinoic acid 8 (Stra8) in the developing female germ cells thus promoting their entry into meiosis at 13.5 dpc (29). Stra8 is thought to be an important regulating factor in meiotic initiation as targeted disruption of Stra8 prevents entry into meiosis in the presence of RA (29).

In male embryos, PGCs do not enter meiosis but proliferate for a short period of time and are referred to as gonocytes. Later, gonocytes arrest mitotically at the G0/G1 phase of cell cycle and remain arrested until birth when

they resume mitotic divisions and establish the male postnatal diploid germ cells population, SSCs (23). Embryonic testes do not express Stra8, because Sertoli cells express Cyp26b1, a P450 cytochrome that degrades RA (30). By preventing the expression of Stra8 in the male gonad, male PGCs escape entry into meiosis. Interestingly, it was shown that exposure of embryonic testes to high levels of RA in culture leads to the expression of Stra8 and initiation of meiosis in male germ cells (29).

In summary, female germ cells lose their self-renewal potential before birth, as the entry to meiosis results in the loss of stem cells in the postnatal female germ line, while male germ line has a population of self-renewing SSCs throughout life.

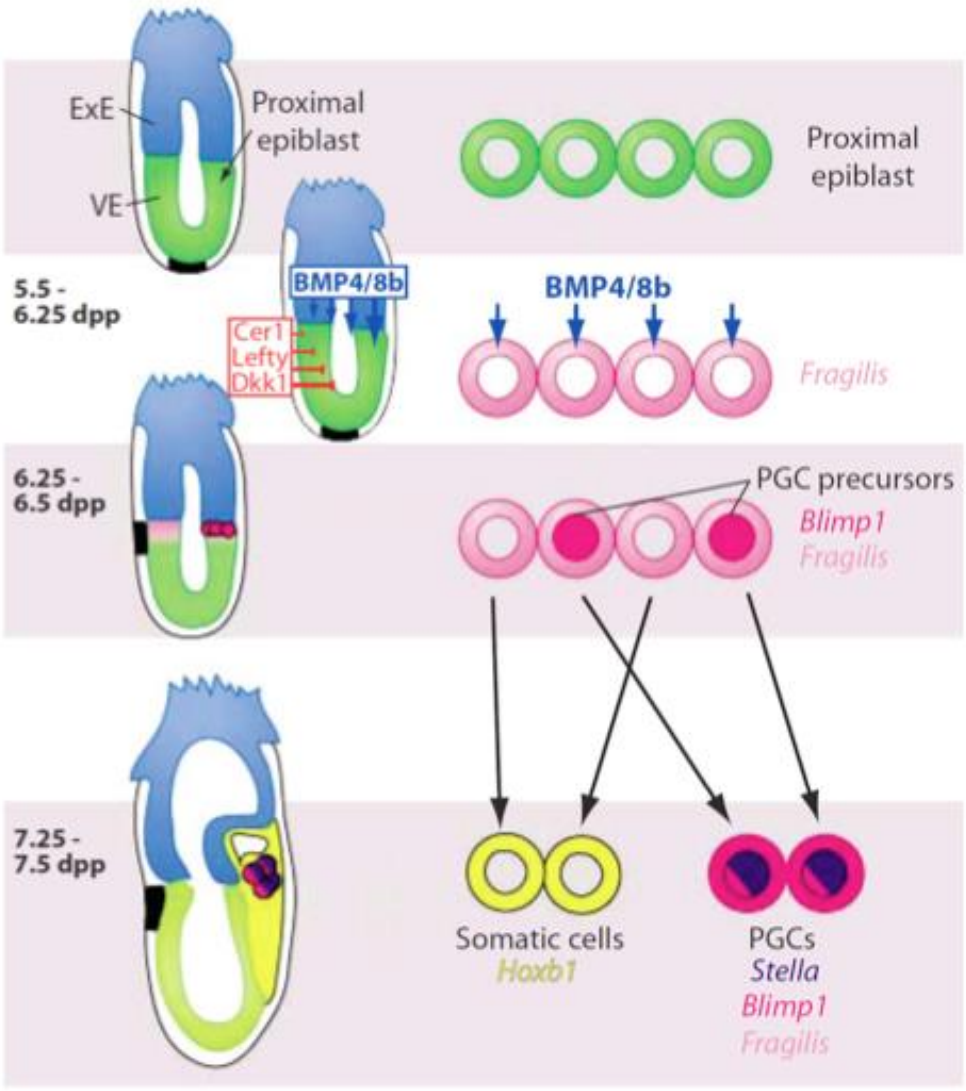

Figure 2: Development of early post-implantation embryo from E5.0 to E7.5, showing the formation of PGCs. Figure adapted from Hayashi (23).

3. Spermatogonial stem cells and Spermatogenesis

SSCs are the foundation of spermatogenesis, as a pool of these SSCs balancing self-renewal and differentiating divisions maintains the spermatogenic lineage. Spermatogonia have ovoid nucleus with a high nucleus to cytoplasm ratio and lie in a basal localization in the seminiferous tubules (31). Compared to human, rodents have more

spermatogonia subtypes. In the murine testis, the population of spermatogonia can be divided into A single (A_{single}), A paired (A_{paired}), A aligned ($A_{aligned}$), A1-4, Intermediate (In), and B spermatogonia (Fig.3). The development of these types is discussed in details in the next section. In contrast, in primates three types of spermatogonia are identified: A dark (A_{dark}), A pale (A_{pale}), and B spermatogonia (32). The A_{pale} spermatogonia proliferate regularly and are considered self-renewing progenitors (33, 34). The A_{dark} are mitotically quiescent and are considered as reserve stem cells that do not divide in stable condition, but start to proliferate after severe testicular damage (33).

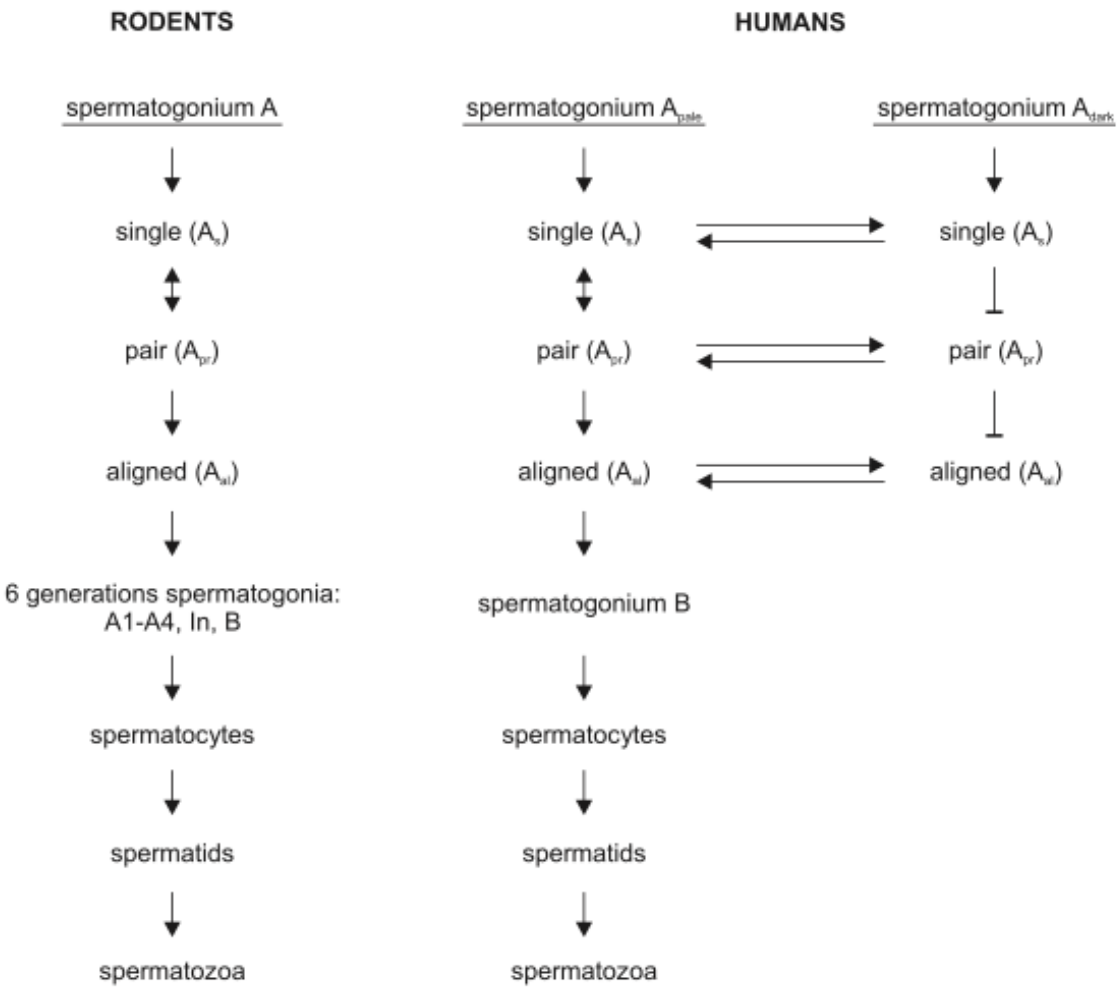

Figure 3: Spermatogonial stem cell fate in rodents and humans. Adapted from Kolasa (35).

Phases of Spermatogenesis:

Spermatogenesis begins at puberty and continues throughout life. During spermatogenic differentiation, germ cells migrate from the basal compartment towards the lumen of the seminiferous tubules. The process can be divided into three phases: mitotic, meiotic, and spermiogenic (Fig. 4).

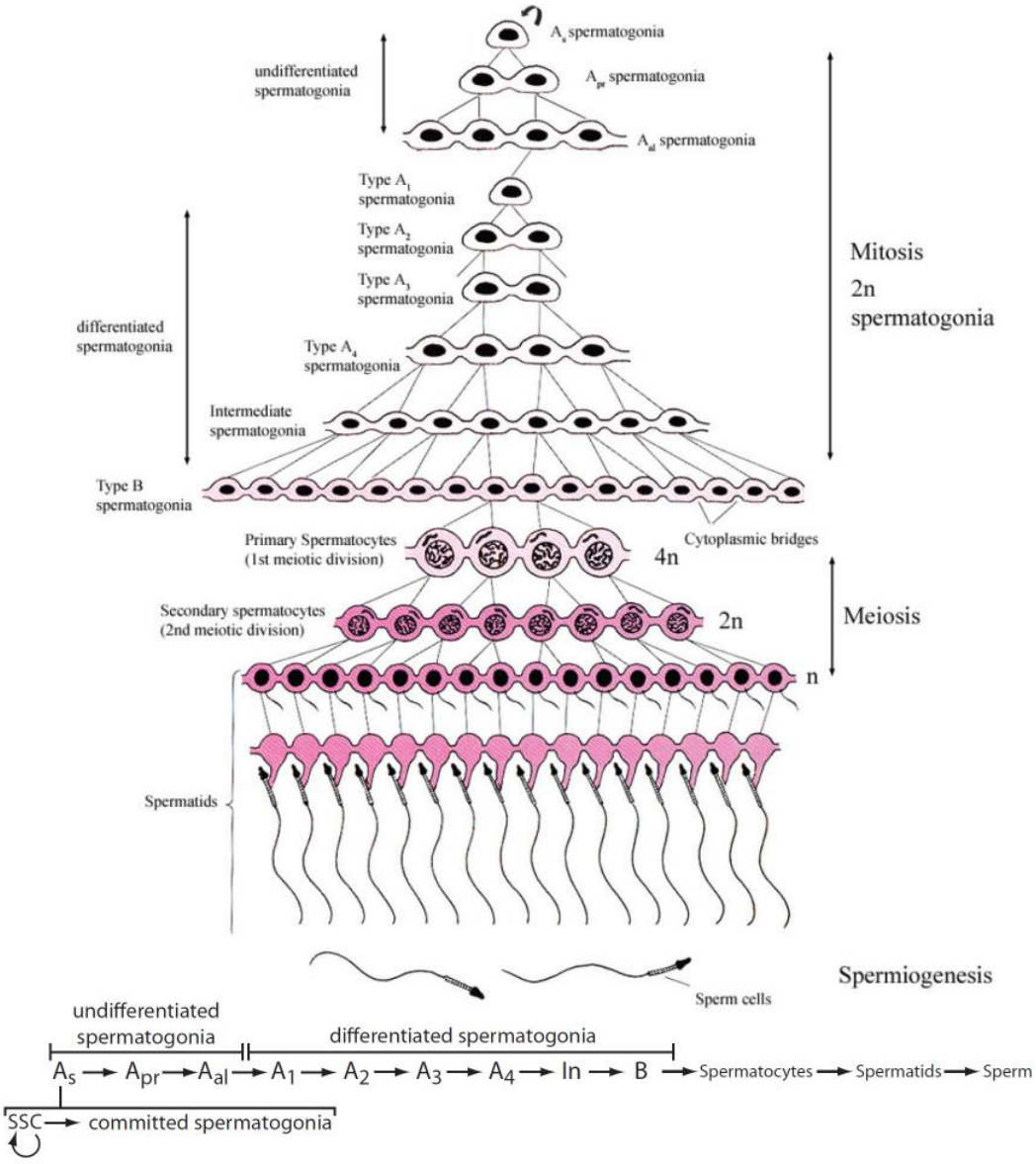

Figure 4: Phases of spermatogenesis in mouse. Adapted from Fawcett et al. (36).

a) Mitotic phase

Steps of germ cell differentiation are characterized based on their morphology and location within the seminiferous tubules. In the mouse, it is believed that SSCs are present within the most primitive undifferentiated spermatogonia; the A_{single} (A_s) spermatogonia located on the basement membrane. A_s spermatogonia cell division either generates two A_s spermatogonia or a pair of cells connected by an intercellular bridge, which occurs due to incomplete cytokinesis. These connected cells are referred to as A_{paired} (A_{pr}) spermatogonia. The formation of intercellular bridges after cell divisions is a unique feature of male germ cells and marks the start of differentiation process. It is believed that this incomplete cytokinesis allows for the formation of clones of spermatogonia that differentiate together at the same time. With the sequential divisions of A_{pr} spermatogonia, chains of 4 to 16 spermatogonia connected by intercellular bridges, termed $A_{aligned}$ (A_{al}) spermatogonia, are produced. When these clones of A_{al} spermatogonia differentiate together without cell division into A1 spermatogonia, they are then called “differentiated” spermatogonia. With subsequent divisions A1 gives rise to A2, A3, A4, intermediate, and type B spermatogonia. This nomenclature is according to the amount of heterochromatin in their nuclei; type A, intermediate and type B cells, have none, scarce and abundant heterochromatin, respectively (37). These mitotic divisions enlarge the germ cell population that will proceed into meiosis. Finally, type B spermatogonia undergo mitotic division to generate the primary spermatocyte population, which must cross the blood-testis barrier to the adluminal compartment and complete meiosis. The number of divisions of B spermatogonia differs between different primate species. For example, one division is described in man and four divisions (B1-4) in macaques.

b) Meiotic phase:

Throughout meiosis, partial cytokinesis resulting in intercellular bridges allows synchronous development and communication between

germ cells (38). Primary spermatocytes undergo two meiotic divisions to form the haploid spermatid. In the first meiotic division, the number of chromosomes is reduced to half and one tetraploid primary spermatocyte divides to form two diploid secondary spermatocytes. Germ cells spend most of its time during meiosis in the prophase stage of the first meiotic division characterized by chromosomal conformation. The meiotic prophase is further divided into five substages: leptotene, zygotene, pachytene, diplotene and diakinesis. Leptotene chromosomes are seen as fine threads attached to the nuclear envelope. Then homologous chromosomes align and synapse to form synaptonemal complex, which will mediate chromosome alignment, pairing and recombination (39). This is followed by the pachytene substage which lasts about two weeks in the rat and 16 days in human and is therefore considered as the longest substage in prophase (40). When homologous chromosomes complete synapse and undergo recombination, germ cells are called pachytene. This is followed by the diplotene substage where homologous chromosomes desynapse although they remain connected through chiasmata considered as sites of crossover. In the last stage, the diakinesis, condensation of chromosome occurs. Chromosomes align at the equatorial plate during the metaphase 1 stage, segregate at anaphase, and at telophase cytokinesis occurs. By the end of meiosis 1, germ cells are now called secondary spermatocytes and enter the second meiotic division, where sister chromatids segregate to form two round spermatids from each secondary spermatocyte.

c) Spermiogenesis

Spermiogenesis is the process by which postmeiotic haploid spermatids undergo cellular, molecular and morphological changes to give mature spermatozoa. It involves the condensation of genetic material in the nucleus of the spermatid head, development of the acrosome and, formation of the tail. It starts immediately after meiosis II with histones replaced by transition proteins, displaced in turn by protamines

condensing sperm DNA (41). In addition, spermatids develop an axoneme and a flagellum to acquire mobility. The energy required for this motility is generated by mitochondria arranged in helix form around the mid-piece (36). Over the anterior half of the spermatozoa head, a cap-like structure called acrosome develops from golgi apparatus and contains enzymes that allow sperm to break down the zona pellucida of the ovum, thus allowing the entry of sperm to the ovum (42). Finally, spermatids shed most of its cytoplasm into a residual body before the release of spermatozoa from the seminiferous tubules (43). Most sperm of different species have this same basic morphology and essential cellular components, but vary in size (generally ranging from 30 to 250 μm long) and number.

Spermatogenic cycle

Spermatogenesis occurs in a highly synchronized way with each round of spermatogenesis, from A1 to spermatozoa consistent in the same species: 35 days in mouse, 52 days in rat and 64 days in human (44). This cyclic initiation of spermatogenesis is known as spermatogenic cycle (Fig. 5). Moreover, each spermatogenesis cycle shows a specific intervals; 8.6 days in mouse and 12.9 days in rat (44). In this way spermatogenesis appears to process in waves, and a cross-section of a seminiferous tubule will show between four and five separate clones of cells at different steps along the differentiation process, with the clones that have entered more recently into the differentiation process located at the basement membrane and the clones that have nearly completed the differentiation process located towards the lumen. Staggering spermatogenesis into different stages along the seminiferous tubules maintains thereby the continuous release of sperm and continuous fertility compared to female cyclical fertility. When rat SSCs were transplanted into the seminiferous tubules of mouse testis, spermatogenesis took 52 days, i.e that of rat cycle, demonstrating that the duration of spermatogenic cycle is inherent

to germ cells of the species and not determined by the somatic environment (45).

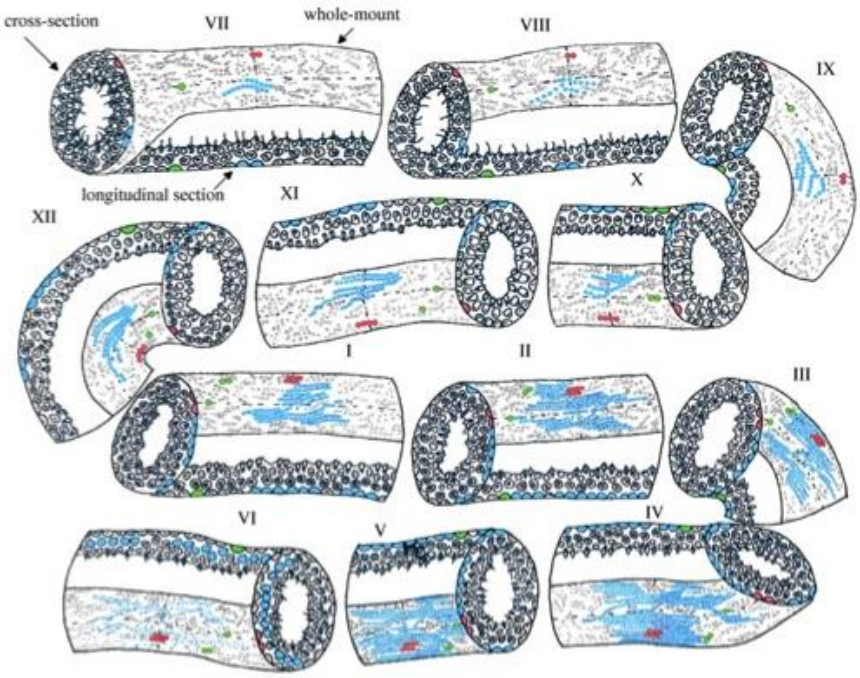

Figure 5: The mouse spermatogenic cycle showing twelve stages (I–XII). Adapted from Phillips et al. **(46).**

4. SSC niche and factors regulating SSCs fate

To determine how the local microenvironment can support stem cells and influence their behavior it is necessary to determine where stem cells reside. In 1978, Schofield proposed that stem cells reside within fixed compartments or “niches” defined as anatomical structure including cellular and acellular components, that integrates local and systemic factors to regulate stem cell proliferation, differentiation, survival and localization (47). Five common components appear to be part of stem cell niche of different tissues. This includes: i) stem cells; ii) stromal support cells interacting directly with stem cells as well as with each other through cell-surface receptors, gap junctions and soluble factors; iii) ECM proteins providing structure, organization and mechanical signals to the niche; iv) blood vessels carrying systemic signals; v) and neural inputs

communicating distant physiological cues to the stem cells microenvironment (Fig. 6).

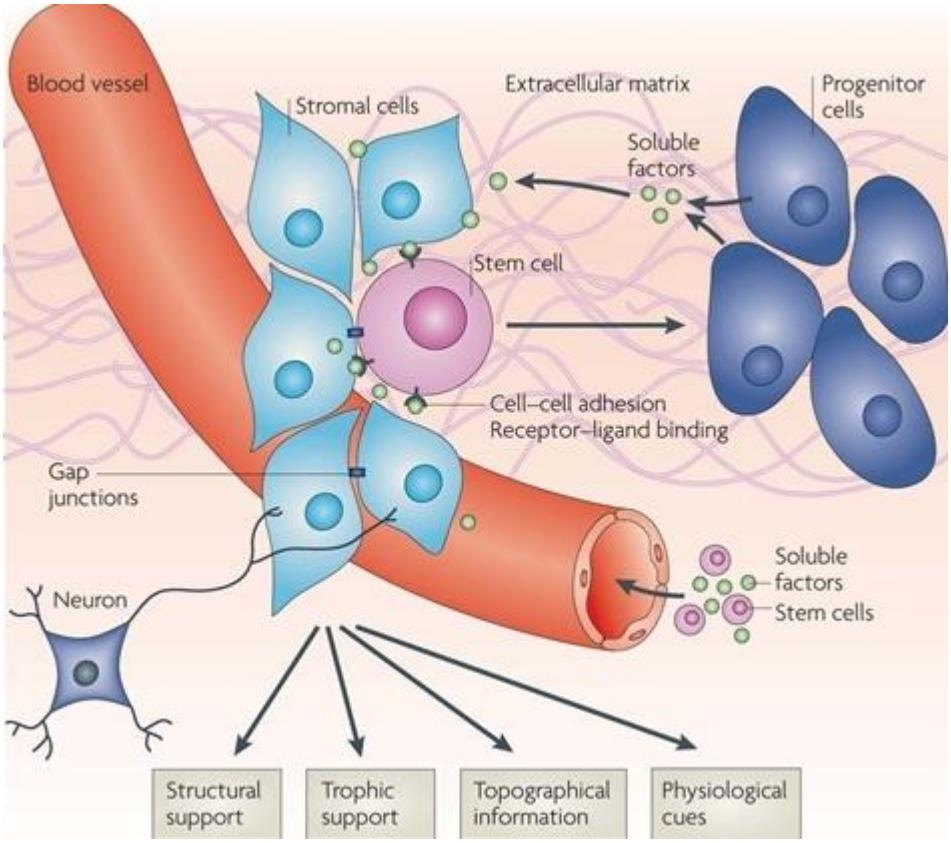

Figure 6 : Components and functions of stem cell niches. Adapted from Jones **(47).**

SSCs are also thought to reside in a “niche” microenvironment in the mammalian testis with these five common components. They reside in the basal compartment of the seminiferous epithelia with Sertoli cells and on the ECM proteins of the basement membrane offering support. Moreover, Leydig cells, myoid cells and differentiated germ cells are all thought to contribute. These different cells are thought to communicate with one another influencing SSC behavior. To better explain this, Drosophila testes offer a good example on how niche interactions allow for the self-renewal and maintenance of the germ stem cell population.

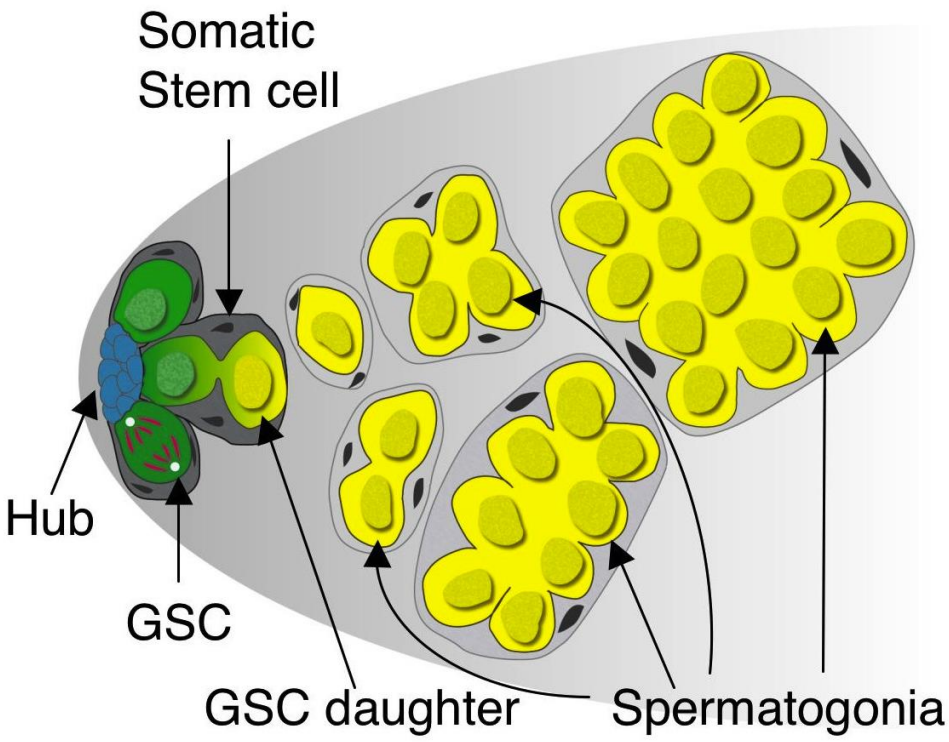

Figure 7: Drosophila male GSC niche (adapted from thenode.biologists.com)

Drosophila testis is a polarized tube with one end opening into the ejaculatory duct and the other closed end hosting the germline stem cell in a niche. In this niche, hub cells reside with germline stem cell (GSC) attached to them (Fig. 7). In turn, each GSC is surrounded by two somatic stem cells. Yamashita et al. showed that cell-cell attachment is critical to maintain GSC stemness, as cell division and loss of attachment to the niche leads to differentiation (48). With every asymmetric division of GSC, one daughter cell remains attached to the hub and becomes a GSC, while the other loses its attachment and starts differentiation to become a goniablast. Somatic stem cells known as cyst stem cells (CySC) also divide in a similar pattern with one somatic cell remaining in the hub while

the other cell known as cytocyte associates with goniablast throughout spermatogenesis. That is to say, GSCs undergo asymmetric divisions where differentiating cells are forced out of the stem cell niche. The attachment of GSC to the hub cells involves DE-cadherin homophilic binding (48, 49). Expression of DE-cadherin at the GSC-hub junction is important to arrange the GSC-hub interface in a perpendicular form with one GSC daughter cell remaining attached to the hub while the second one away from the hub and starting differentiation. When DE-cadherin expression is altered spindle misorientation occurs leading to both cells remaining attached to the hub and differentiation is thus arrested (48).

In addition to cell adhesion, the hub cells also control GSC fate through secreting soluble factors. One of these factors is the BMP homolog Unpaired (Upd) that was found to activate the Janus kinase-signal transducer and activator of transcription (JAK-STAT) cascade (50, 51), which promotes Drosophila GSC self-renewal. The loss of JAK-STAT signaling in mutant male flies leads to the loss of GSCs to differentiation while overexpression of Upd results in abnormal accumulation of GSCs (50, 51). These data together demonstrate that Drosophila GSC fate decision relies in part on direct cell interactions and in other on extrinsic secreted factors.

As with the Drosophila GSCs, the regulation of mammalian SSCs is believed to be mediated via cell-cell interaction and secreted extracellular factors. Unlike the simple unidirectional tube in Drosophila, mammalian testis is formed of multiple seminiferous tubules each of which contact 2 to 8 adjacent seminiferous tubules. This arrangement makes that each seminiferous tubule has peritubular regions that contact either the interstitial space or an adjacent seminiferous tubule (52). Yoshida et al. proposed that the mammalian germline niche is established in relation to the vasculature pattern (Fig. 8). Using green fluorescent protein-labeled (GFP) undifferentiated spermatogonia, they showed that undifferentiated spermatogonia preferentially localize in regions contacting the interstitium

in particular blood vessels. Furthermore, using real time imaging of seminiferous tubules, they showed that differentiation is associated with a movement of differentiating spermatogonia chains away from the undifferentiated spermatogonia compartment (53). These findings suggest that the distribution of undifferentiated spermatogonia might not be random and that this arrangement in close to the testicular vascular network is providing SSCs subpopulation included in the undifferentiated spermatogonia with extrinsic factors from the blood stream regulating their fate.

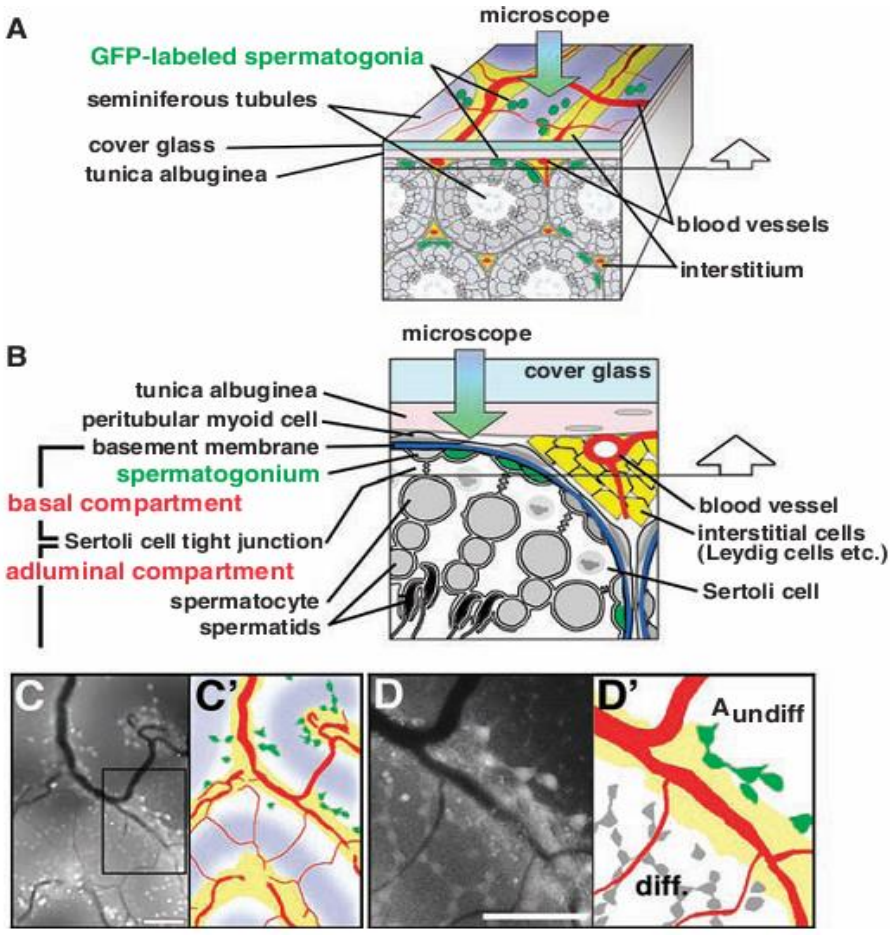

Figure 8: Anatomy of the testis (A,B) and localization of GFP-labeled undifferentiated spermatogonia (C,D), C' & D' showing spermatogonia in green (from Yoshida et al,2007 (53)).

The niche environment is also contributed to by several cell types; mainly Sertoli cells that provide structural support and soluble factors believed to regulate SSC fate (14). Other cell types include Leydig cells and myoid cells which secrete colony stimulating factor -1 (CSF-1) that was shown to promote expansion of SSCs in vitro (54). In the following section, I discuss molecular mechanisms regulating SSC fate decision.

Molecular mechanisms regulating SSC fate decision

1. Extrinsic growth factors

As previously discussed, Sertoli cells reside at the basement membrane in close association with the SSC population and constitute an important component of the SSC “niche” environment. They secrete several factors thought to be involved in the regulation of SSC fate decision. The best characterized of these factors is the glial cell line-derived neurotropic factor (GDNF), a distinct member of the transforming growth factor β superfamily. GDNF is involved in directing ureteric bud branching during kidney morphogenesis (55) and in promoting the survival and proliferation of several neural cell types, including dopaminergic neurons, cranial parasympathetic ganglia, enteric neuron and motor neuron (56). GDNF mRNA was detected in high levels in the embryonic testis (57). It was first detected at 14 dpc and the expression level increased thereafter till it peaked at postnatal day 7, followed by a gradual decrease reaching low levels in adult testis (57-59). To produce the mature GDNF molecule, the mRNA transcript for GDNF is first translated into pre-pro-GDNF. During mRNA translation, intracellular processing cleaves the pre-domain consisting of signal sequence from this peptide, giving rise to two pro-GDNF isoforms: α-pro-GDNF stored in the Golgi complex and, β-pro-GDNF stored in secretogranin II secretory vesicles (56). Upon secretion, furin endoproteinase and membrane bound pro-protein convertase 4, 5 or 7 (PACE4, PC5A, PC5B or PC7) cleaves the pro-domain from these proteins, giving rise to the mature GDNF peptide

(60). As GDNF was detected in both pro-GDNF and mature GDNF forms inside the cells, this suggests that the proteolytic cleavage process can take place outside the cell, most likely in the extracellular matrix, as well as intracellular (60).

GDNF exerts its action by binding a receptor complex expressed on undifferentiated spermatogonia (61) consisting of the transmembrane tyrosine kinase molecule, “Rearranged in Transformation” (c-RET), and the Glycosylphosphatidylinositol- (GPI)-anchored binding molecule GDNF family receptor alpha-1 (GFRα-1). A GDNF dimer first binds to two molecules of GFRα-1, which in turn recruit two molecules of c-Ret (Fig. 9). In the Ret intracellular domain, the transphosphorylation of specific tyrosine residues serves as binding sites for intracellular signaling proteins: phospholipase Cγ, Src family kinases (SFK), fibroblast growth factor receptor substrate 2 (FRS2), phosphatidylinositol 3-kinase (PI3K) and Mitrogen-activated protein kinase (MAPK), which in turn activate downstream signaling cascades. With the activation of PI3K & SFK intercellular mechanisms, Akt signaling is activated and in turn, promotes cellular proliferation and survival (62, 63).

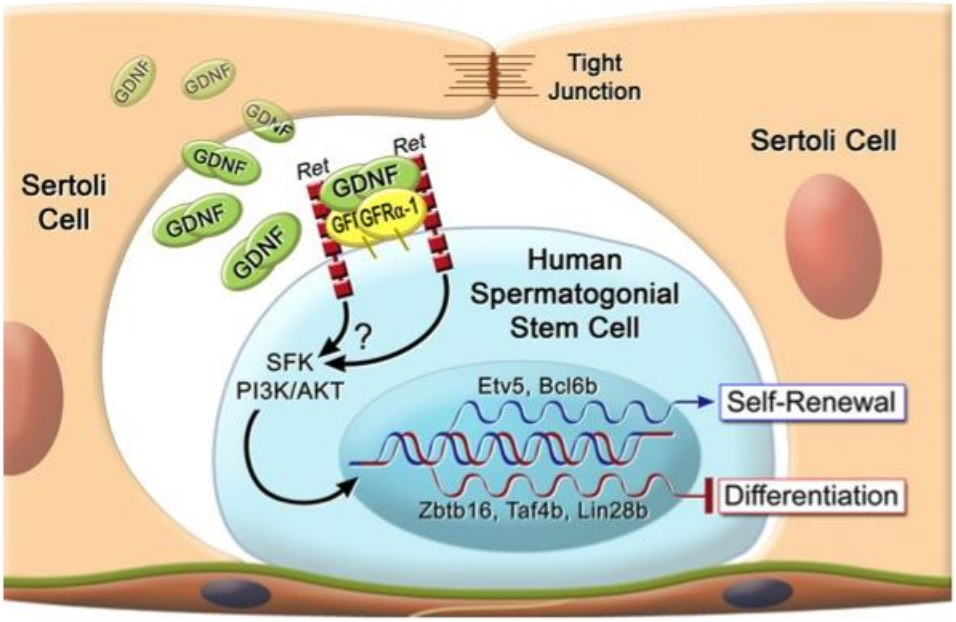

Figure 9: Signaling pathways involved in spermatogonial stem cells fate regulation by glial cell line-derived neurotrophic factor (GDNF). Adapted From Wu et al (64).

The role of GDNF in the testis was examined by Meng et al. who investigated GDNF loss of function and overexpression in mutant mice (65). Loss of function mutation GDNF -/- is lethal within 24 hours after birth due to defects in renal and neuronal development (66, 67). Nevertheless, these mice present with morphologically normal testes (65), a phenotype similar to Ret and GFRA1 null mutations (68, 69). Meng et al thus analyzed heterozygous mutant GDNF+/-mice, which are viable and fertile. GDNF+/- males showed disrupted spermatogenesis in a number of tubules with the progressive depletion of spermatogonia eventually leading to a sertoli cell only phenotype in older mice (65). When Naughton et al

grafted GDNF-/-, Ret-/- and GFRA1-/- neonatal testes subcutaneously into the back of castrated adult mice to examine the effect of disruption of GDNF-mediated Ret signaling (70). They reported significant reduction of germ cells at postnatal day 7 with failure of spermatogonia differentiation, and by 8 weeks, the loss of all germ cells in mutant testes. These observations show that GDNF is critical for SSCs maintenance in vivo as the reduction or loss of GDNF expression depletes undifferentiated spermatogonia.

Next Meng et al. explored the role of GDNF in proliferation of undifferentiated spermatogonia by examining GDNF overexpression. They reported that although the mutant mice showed normal spermatogenesis at birth, 3 weeks later they presented with the accumulation of excessive numbers of undifferentiated spermatogonia. These germ cells aggregations were mitotically active and expressed Ret and GFRα1 but not c-kit, a marker for differentiating spermatogonia, indicating that these cells are undifferentiated spermatogonia (65). This work showed that GDNF promotes survival of undifferentiated spermatogonia and enhances self-renewal by blocking SSCs differentiation.

In Meng et al. work GDNF overexpression was in both germ cells and testicular somatic cells. Yomogida et al examined whether GNDF affects SSC as an extrinsic factor by overexpressing GDNF exclusively in Sertoli cells (71). They transfected Sertoli cells with a GDNF-expression vector with an EGFP reporter gene using an in vivo electroporation method. Results showed that similar to Meng et al study, GDNF overexpression resulted in the formation of aggregations of undifferentiated spermatogonia. When they transplanted cells from these aggregations of undifferentiated spermatogonia into the testes of infertile recipients, they found a 20-fold increase in number of colonies compared to control (71). These results demonstrate that GDNF works as an extrinsic factor promoting SSC proliferation in vivo.

In vitro studies supported the GDNF role in regulation of SSC fate. Kubota et al. showed that when pup testes cells enriched for SSCs by selection for CD90 (Thy-1), a known surface marker of mouse SSCs (72, 73), were grown on mitotically inactivated STO (SIM mouse embryo-derived thioguanine and ouabain resistant) feeder cells in the presence of GDNF, they formed aggregations or “clusters” of undifferentiated germ cells reminiscent of those observed in testes of mice with GDNF overexpression (74). They confirmed that cells in these aggregations expressed various SSC markers, such as Promyelocytic leukemia zinc finger (Plzf), Octomer-binding transcription factor 4 (Oct4), integrin-α6 and c-Ret. In this culture system, SSCs expanded >5000-folds over 70 days, indicating self-renewal of SSCs in vitro. Other studies showed that GDNF supplementation was essential for long-term self-renewal of SSCs in mouse (75), rat (76, 77), and hamster (78), showing the functional conservation of GDNF in SSC self-renewal in rodent species.

Other factors beside GDNF were also suggested to be extrinsic niche factors. For example, Fibroblasts growth factor 2 (FGF2) secreted by Sertoli cells allowed for long-term SSC maintenance and expansion in vitro (79). The colony stimulating factor-1 (CSF1) expressed by Leydig cells and myoid cells (54), and the Wnt5a expressed by Sertoli cells (80) were shown to promote SSC expansion in vitro. Yet, more work is needed to explore niche-derived factors that control SSCs fate.

2. SSC intrinsic regulators

Extrinsic stimuli from niche microenvironment mediate their effects on SSCs via activation of various intrinsic regulators. Because GDNF is regarded as an essential extrinsic growth factor regulating SSCs fate, intrinsic regulatory factors are classified based on being regulated by GDNF or not.

a) GDNF regulated intrinsic factors:

Several GDNF-regulated transcription factors were identified to play a role in SSCs regulation, such as the transcriptional repressor B-cell CLL/Lymphoma 6 member B (Bcl6b) and, the transcription factors Ets variant gene 5 (Etv5) and lime homeobox protein 1 (Lhx1) (81, 82). Localization of each of these factors in testis sections were reported in spermatogonia (81-83). However, this localization was not restricted to spermatogonia as Bcl6b and Lhx1 were also expressed in spermatids and Etv5 in Sertoli cells. This distribution suggests that these factors might have diverse roles in spermatogenesis.

To study the role of these factors in SSCs function, the expression of each of these transcription factors was transiently reduced independently in SSC cultures using siRNA targeting. Number of SSCs in these cultures were determined using functional transplantation assay where SSCs within the transplanted cells are identified by the production of donor derived colonies of spermatogenesis within the infertile recipient mice testes as discussed later. With the reduction of each of these factors, significant reduction in SSCs numbers was observed (82, 83). This shows that each of these factors is important to maintain SSCs in vitro.

In vivo, the inactivation of Bcl6b causes a subfertile phenotype in male mice. The testes of Bcl6b-/- are smaller compared to those of wild type mice with ≈24% of seminiferous tubules showing degenerating spermatogenesis and Sertoli cell-only phenotypes (82). In contrast, the targeted disruption of Etv5 cause male infertility by 10 weeks of age (81). In these mice, spermatogonia are lost during the early waves of spermatogenesis with the progressive development of Sertoli cell-only phenotype due to impairment of SSC self-renewal. Lhx1-/- mice die at 10 days of embryonic development due to severe defects and thus studying Lhx1 deletion was not shown till present (82). These observations suggest that these factors have important role in SSC maintenance that yet have to be fully defined.

b) Non-GDNF regulated transcription factors

Intrinsic factors, for example the transcription repressor Plzf , the transcription factors Oct4, and the TBP associated factor 4b (Tab4b), were implicated to regulate SSC function (84-86). These factors were shown in cultured mouse SSCs not to be regulated by GDNF (82, 83, 87). I will discuss PLZF as an example of these factors.

In male mice germline, Plzf expression is restricted to undifferentiated spermatogonia from A_s to A_{al} spermatogonia stages (84, 88). Disruption of Plzf expression results in impaired spermatogenesis with the progressive absence of spermatogonia along the basal layer of the seminiferous tubules and the abolishment of developing germ cells leading to infertility (84, 88). Testes of these mice were reduced in size although their morphogenesis and germline development during embryogenesis were normal (84). When SSCs from Plzf-/- mice were transplanted into wild-type recipient infertile mice, they failed to re-establish spermatogenesis (84). Moreover, when wild-type SSCs were transplanted into Plzf-/- mice, donor derived spermatogenic colony formed (84, 88). This indicates that loss of Plzf causes an intrinsic defect in SSC function.

5. Functional assessment of spermatogonial stem cells:

Cell transplantation as a stem cell functional assay

Stem cell activity is characterized with long term self-renewal and differentiation. As these properties are functional properties, it is not possible to identify these cells by descriptive parameters such as markers, morphology or cell cycle activity, and they have to be identified retrospectively by using long-term self-renewal and differentiation as functional parameters. In the history of stem cell research, efforts have focused on developing functional stem cell assays. In 1961, Till and McCulloh reported the first functional assay for stem cells where they prepared single cell suspensions from the femora of normal mice and

injected them intravenously into lethally irradiated recipient mice (89). Eleven days later, the spleen of recipient mice showed nodules of differentiated cells including erythropoietic, granulocytic and megakaryocytic cells arranged into a colony-like structure, and donor-derived hematopoiesis was reconstituted in the recipient donor. Furthermore, when single cell suspensions were prepared from individual colonies in the spleen and injected into another set of lethally irradiated recipients, new colonies formed in the spleen of the recipients. Only a population of stem cells can confer this restoration of hematopoiesis in these donors. These results showed that the bone marrow cells initially injected contained a stem cell population capable of self-renewal and differentiation. Later on, the same authors with others studied the clonal origin of each colony and showed that each colony was derived from a single cell. They used sub-lethal irradiation on donor mice to induced unique chromosomal marks in their bone marrow before injecting bone marrow cells into recipient mice. The results showed that an individual colony formed in the spleen was composed of cells expressing the same chromosomal mark. Together these results showed that bone marrow contain a population of stem cells capable of self-renewal and differentiation and that the activity of these cells can be detected by counting the number of colonies formed after injection. This work laid the foundation for the development of a definition of stem cells as well as providing a method to quantify their activity (89).

Spermatogonial stem cell transplantation

In my work, SSC transplantation represented an important tool to validate my hypotheses; therefore I will discuss it in details in the following sections.

The direct study of the properties of the SSCs was not possible until a functional transplantation assay was developed. In 1994, R. Brinster et al. developed spermatogonial transplantation using the mouse as a model

species. SSC transplantation has since become the gold standard bioassay for experimental assessment of SSC activity (46). This procedure involved injecting a single cell suspension from testes of a transgenic reporter mouse expressing the *lacZ* transgene in post-meiotic germ cells into the seminiferous tubules of wild-type infertile mice. Transplantation resulted in regeneration of complete spermatogenesis derived from donor cells; recipients became fertile and sired offspring carrying donor genotype (90, 91). Donor-derived spermatogenesis was established in the form of segments or “colonies” along the recipient seminiferous tubules (90, 91). This work showed that donor cells could produce differentiated cells after transplantation. Moreover, colonies were observed 4 months after transplantation in the recipient mice, and knowing that one round of spermatogenesis in mouse takes 35 days, SSCs must have had to self renew to support spermatogenesis during these 4 months. Both long term self-renewal and differentiation of SSCs are thus demonstrated by colony formation in recipient testis (Fig.10).

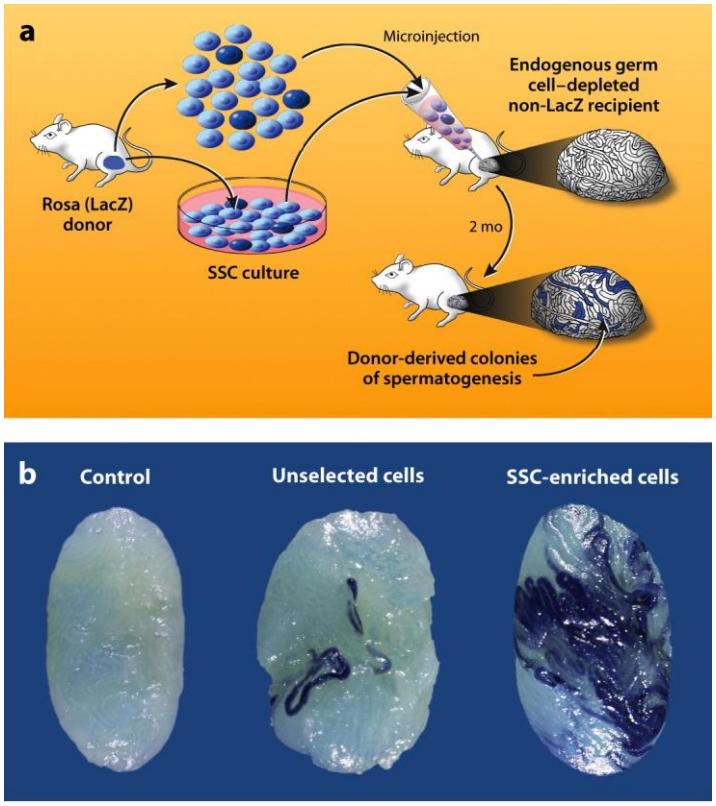

Figure 10: Spermatogonial stem cell transplantation in mice using transgenic mice. (A) injection of fresh or cultured testicular cells into infertile recipient (B) abundant colony formation in right testis injected with cell suspension enriched for SSCs compared to unselected testis cells in middle testis. Adapted from Oatley (79).

Colony formation

In 1999, Nagano et al. studied kinetics of colony formation after transplantation (92). In this work, colony formation was seen to develop in three continuous phases. The initial phase takes place during the first week following transplantation. Donor cells are visualized on the basement membrane and start to proliferate forming chains of cells. In the second phase, one week to one month after transplantation, donor cells in distinct testis regions form a monolayer spreading laterally and circumferentially along the basement membrane in what can be counted as colony. Finally, in the third phase from one month and on, each colony grows in size and

differentiated germ cells extend from basement membrane to the lumen of seminiferous tubule while the lateral edge of the colony expand in length by repeating phase one and two. An important finding was that spermatozoa could be identified in colonies 2 months after transplantation and that the number of colonies did not change after 1 month of transplantation to 4 months. Therefore, in my work, I determined the number of SSCs by analyzing recipient testis 2 months after transplantation.

Quantification of SSCs after transplantation

Several transplantation studies have shown that each colony of donor-derived spermatogonia arises from a single SSC (93, 94). Thus the number of colonies observed in recipient testes directly correlates with the number of functional SSCs that have successfully colonized the recipient testis and regenerated spermatogenesis. As a consequence, functional SSCs can be quantified by simply counting the number of colonies.

Species specificity

The SSC transplantation technique has been used successfully in rodents and non-rodent species. In mouse, transplantation of SSCs into infertile mouse recipients results in robust regeneration of spermatogenesis with the production of donor derived offspring through natural mating of recipients (90). Notice that as SSCs reside in the basal compartment of the seminiferous tubules, which is not protected by the blood-testis barrier, immune-compatible donors and recipients must be used or recipients must be animals with naturally or artificially depressed immune systems. In rat similar results were obtained when donor rat SSCs were transplanted into the testes of immunodeficienct mice; complete regeneration of rat spermatogenesis was observed in mouse testes (45) with fully functional spermatozoa (95).

Transplantation of SSCs in non-rodent species is more complicated due to the difficulty to generate recipients devoid of endogenous spermatogenesis and technical difficulties in the delivery of SSCs to the seminiferous tubules. Nevertheless, several studies reported complete donor-derived spermatogenesis after transplantation of SSCs in goats (96), dogs (97) and pigs (98, 99). Other reports have shown successful SSCs colonization in sheep (100, 101) and cattle (102).

In primates, studies reported that non-human primates SSCs successfully colonize testes of mice (103) or non-human primate (104) but with no differentiation. A more recent study, which was published during the writing of this thesis, reported the successful regeneration of spermatogenesis in busulfan-treated rhesus macaques with the production of functional sperm, which are capable of fertilizing oocytes and the production of preimplantation embryo (105).

In the case of human SSCs, they were able to colonize, proliferate and survive at least up to 6 months in mouse recipient testes after transplantation. However, no differentiation beyond spermatogonia or meiotic germ cells of human origin were generated (106). Despite the absence of complete spermatogenesis, the long-term survival of human spermatogonia in the mouse testis can provide a method for evaluating the stem cell potential in a human germ cell population. In studies of hematopoietic stem cells (HSCs), it was shown that long-term survival in host environment is a characteristic of stem cells (107). This also applies for SSCs. For example, SSCs from infertile adult mutant Steel-Dickie (SI_d) mice, which lack differentiating spermatogonia, survive in the testis from neonatal to adult ages and regenerate complete spermatogenesis when transferred to appropriate recipient host (108). Therefore, in chapter 2 I used human germ cell transplantation to mice testes to evaluate the stem cell potential in the target population as discussed later.

Homing efficiency

The ability of SSCs to home to the “SSC niche” is considered as an essential prerequisite for transplanted SSCs to colonize and undergo spermatogenesis in the recipient testis. After transplantation, only a fraction of donor SSCs forms colonies in the recipient testes. The percentage of these cells is referred to as “homing efficiency” of an SSC population. A remarkable critic to transplantation assay is that it does not allow a direct quantification of absolute numbers of SSCs. This is due to the fact that when adult mouse testis cells are transplanted into the testes of adult recipient mice, the homing efficiency is only 10%. Hence, only one in 10 SSCs transplanted can successfully migrate, survive, and reestablish spermatogenesis in the seminiferous epithelium of adult recipient mice (109). When Kanatsu-Shinohara et al. transplanted cells from germ cell- or Sertoli cell-specific knockout of β1-integrin, a known receptor for laminin proteins of seminiferous tubules basement membrane, they reported the death of all transplanted cells suggesting a major defect in attachment to the niche (110). Moreover, it was reported that approximately 9-fold more colonies of donor-derived spermatogenesis can be formed after transplantation into the testes of immature mice than those of adult mouse (111). It was proposed that this higher colonization efficiency occurs because the blood-testis barrier is not established in the seminiferous epithelium of immature mice (111). If this situation is applicable in human SSCs, the use of SSC transplantation (discussed later) could be inefficient in clinical settings when transplantation is done into adult testes and requires improvement to the technique itself. Therefore, SSC homing appears to involve variable factors affecting attachment, migration through the BTB, survival and regeneration. Yet transplantation assay allows for the only unequivocal examination of SSC function.

Frequency of SSCs

A common characteristic observed in all stem cell systems is that stem cells are a rare population in a cell lineage. Using spermatogonial transplantation, it was estimated that adult mouse SSCs represent 0.01% of total cells in the seminiferous tubule or 3000 SSCs/testis (109). This frequency is similar to that observed with HSC estimated to be 0.007% of nucleated bone marrow cells in mouse (112). Using the transplantation assay, it was shown that the total number of functional SSCs in a testis increases up to 40-fold from the day of birth to adulthood in mice (111) while in rats the increase is 70-fold (113, 114). Thus the number of SSCs differs with age.

6. Spermatogonial stem cell culture

The extreme rarity of SSCs in the adult testis had long hampered the study of their biology. Importantly, it was shown that SSCs could survive in culture and regenerate spermatogenesis after transplantation (115).

Over the past years, many protocols were developed for culturing mouse and rat SSCs that allow for rodent SSCs to be maintained for virtually indefinitely with a significant amplification in numbers without significant genetic or epigenetic properties changes [doubling time: mouse, 5.6 days (74), rat, 3–4 days (77) or 11 days (76)]. Importantly, studies have shown that the elimination of most testicular somatic cells prior to plating is a prerequisite for the success of culture (74). This can be done through immunological cell separation discussed later or differential plating (74, 116).

Long-term culture and propagation of human SSCs remains challenging due to the difficulties in maintaining SSCs in culture conditions that support their self-renewal, expansion, and molecular and cellular characteristics. Using testicular tissue biopsies from boys aged 1.1-9.9 years with cryptorchidism, Larsen et al cultured testicular fragments and

were able to maintain a significantly reducing number of spermatogonia for 3 weeks (117). Recently, several studies reported successful long-term culture and propagation of human spermatogonia in vitro (116, 118,119). The human testicular tissue in these studies was initially cryopreserved and thawed prior to cell isolation. In the first published study, Sadri-Ardekani et al used testicular tissue from six adult men who underwent orchiectomy for prostate cancer treatment for cell isolation and culture (116). After initial depletion of somatic cells by differential plating on plastic overnight, human spermatogonia were maintained on uncoated plastic in StemPro-34 medium containing growth factors in the absence of additional feeder cells for up to 15 weeks with up to 53-fold amplification in colonization activity as determined by xenotransplantation. Subsequent subculture of these cells using laminin-coated dishes allowed for up to 28 weeks maintenance and up to 18,450-fold amplification of xenotransplanted cells. Later, He et al depleted somatic cells from testicular tissue of five organ donors by differential plating on plastic for 3 hours (118). Next, they used immunomagnetic cell sorting using antibody against the G-protein coupled receptor 125 (GPR125) to enrich for human spermatogonia and maintained GPR125-positive spermatogonia on gelatin-coated plates in supplemented StemPro-34 medium for 2 weeks with a fivefold increase in cell numbers. More recently, Kokkonaki et al showed that SSEA-4 is a more efficient marker to enrich for human spermatogonia as SSEA-4 positive cell fraction express the highest level of SSC genes compared to other fractions isolated by other markers, and reported that SSEA-4 positive cells can be maintained for over 16 weeks (119).

Culturing of SSCs has many important implications on human reproduction, animal management, in addition to basic stem cell research. It was suggested that amplifying SSCs prior to transplantation into the testis of individuals treated with chemotherapy and/or radiation would make SSC-based fertility restoration more efficient. This should

particularly be the case for prepubertal boys because the amount of testis tissue biopsy is expected to be highly limited. The same approach could be utilized for the amplification of SSCs obtained from valuable livestock or endangered species. Finally, culturing SSCs would be the foundation for the experiments to develop a system for in vitro spermatogenesis allowing for the production of spermatozoa without SSC transplantation.

7. SSCs identification

While SSCs are functionally defined and detected, attempts have also been made to identify them based on morphology and intracellular or cell-surface molecules or “markers”.

1. Morphological identification:

As previously discussed, spermatogonia can be identified morphologically based on their location and nuclear morphology in addition to their unique characteristic of cell division; their incomplete cytokinesis and cell chain formation. The most primitive spermatogonia exist as isolated single cells which upon division either complete cytokinesis to give two isolated spermatogonia or give rise to a two-cell chain of spermatogonia. This chain formation is the first morphological differentiation that can be visually observed among spermatogonia and thus isolated single cells were commonly called stem cells. By definition, SSCs are detected retrospectively by their regenerative capacity, therefore, this morphological characteristic as a prospective marker cannot detect how a cell will behave at subsequent cell divisions. That is to say, stem cells could be isolated spermatogonia, but isolated spermatogonia might not be stem cells, since morphological parameters cannot predict the future course of cell actions.

2. Intracellular and extracellular markers

SSCs were shown to express several intracellular and extracellular molecules (table. 1). Yet many of these markers are also expressed in

differentiating spermatogonia and till date no unique marker that distinguishes A_{single} including SSCs is known (Fig. 11). Moreover, recent studies had shown that cells expressing neurogenin 3 generally commit to differentiation, but some of them retain stem cell properties (i.e., regenerative capacity), suggesting that rodent SSCs exhibit phenotypic heterogeneity (120-122). Another report showed that the expression of GFRA1 is heterogeneous in A_{single} spermatogonia in mice and among human A_{dark} and A_{pale} (123). Therefore, combining expression profiles of multiple markers is thought to be critical to provide phenotypic information and an isolation tool for SSCs.

Table 1: Cell-surface markers of primates and rodents spermatogonia/ SSCs.

Marker	Human	Rodent	Reference
ITGA6	+	+	(116, 124)
ITGB1	-	+	(124, 125)
CD9	?	+	(126)
GFRA1	+	+	(116, 127)
GPR125	+	+	(75, 128)
CD90	+	+	(72, 116)
c-kit	_	_	(72, 129, 130)
MHC-I	_	_	(72, 123, 131-133)
ITGAV	?	_	(129)
E-cadherin	?	+	(134)

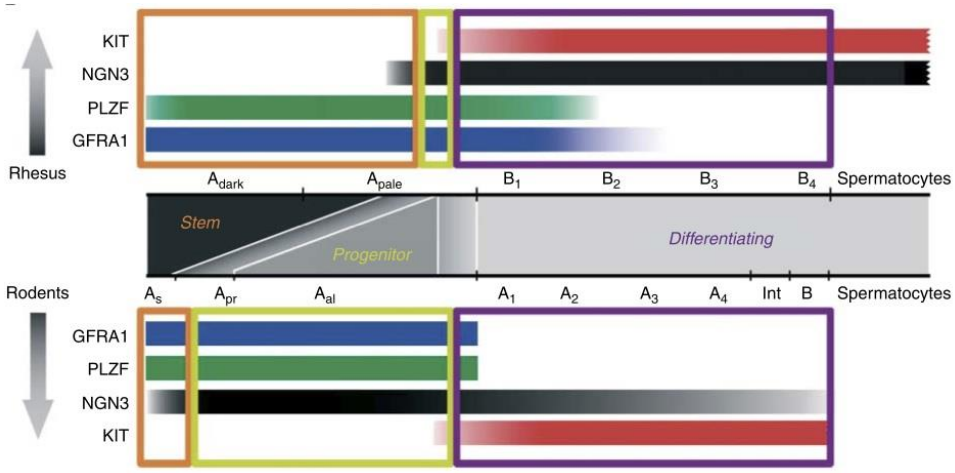

Figure 11: Expression of intracellular and cell-surface markers in primates and rodents spermatogonia. Colored boxes indicate functional descriptors ‘stem’ (orange), ‘progenitor’ (yellow), and ‘differentiating’ (violet). Adapted from Hermann et al. (135).

Similar to cell-surface markers, intracellular markers like Plzf and NGN3 are not exclusively expressed by SSCs but also expressed by differentiated spermatogonia. On the other hand, sorting SSCs based on intracellular markers is a labor-intensive task with challenges and disadvantages (136, 137). Perhaps, the greatest disadvantage is that immunological detection of an intracellular molecule is not suitable to obtain live cells, since its detection by an antibody requires permeablization of cell membrane; thus, regenerative activity of marker-positive cells cannot be verified. Live cell isolation based on an intracellular molecule is possible if cells are engineered to express a reporter gene, such as green fluroscent protein (GFP), driven by a promoter of the intracellular molecule. Obviously, however, this is not an option in clinical setting.

Nevertheless, identification of these markers gives the opportunity to further explore the characterization of SSCs. In human, MAGEA4 was shown to be a marker of spermatogonia (138). MAGEA4 is one of the

melanoma-associated antigen (MAGE) gene family of cancer-testis antigens that are expressed in certain neoplasm and the testis, but not in other healthy tissues. Using a mouse monoclonal antibody (MAb) against MAGE A4, Takahashi et al. showed that MAGEA4 proteins are expressed in the nucleus and cytoplasm of an entire population of human spermatogonia and to a lesser extent in early primary spermatocytes, specifically preleptotene spermatocytes. This was further confirmed by several studies that showed a similar expression pattern on A_{dark}, A_{pale} and B spermatogonia in adult and fetal human testicular tissues (119, 139, 140). Thus, MAGEA4 expression in sorted cells assists human SSC research by confirming that the sorted cells are at least spermatogonia and not germ cells at an advanced stage or somatic cells.

Although 40.7 ± 0.9 million years separate human and mice/rats (141), a comparison of the markers for spermatogonia in rodents and in primates shows that human and rodents share many, but not all, markers (Table 1). Thus, evolutionary conservation of SSC markers between mice and humans was suspected. Although all antigens listed in Table 1 have been confirmed to be expressed on mouse SSCs (thus, SSC markers), very little is known about the markers of human SSCs. Up to now, expression of CD49f, CD133, GFRA1, GPR125, MAGEA4, PLZF, SSEA4 and CD90 in human spermatogonia has been reported (Table 1) (84, 116, 119, 128, 130). Importantly, however, only SSEA4+ cells were functionally examined by transplantation assay to repopulate recipient mice testes after transplantation. Thus, the identity of these molecules as a human SSC marker has remained elusive, and more studies are needed to identify reliable markers for human SSCs. My study of CD9 expression in human spermatogonia (Chapter 2 in this thesis) represents an effort towards this end.

8. CD9

CD9 is a member of the tetraspanin protein family and has been identified to be a cell-surface marker of mouse and rat SSCs (126). The tetraspanin protein family contains 33 distinct members. The characteristic feature of this family is that each of them has four transmembrane domains, short N- and C-terminal cytoplasmic domains, a small intracellular loop and two extracellular loops (Fig. 12) (144). The larger extracellular loop allows tetraspanins to interact with themselves and with other proteins including membrane-bound growth factors, immunoglobulin (Ig) proteins, signaling enzymes and integrins (142). These protein-protein interactions allow tetraspanin to modulate the functions of associated proteins and thereby regulating many physiological and pathological processes such as cell adhesion, fertilization, motility, tumour invasion and transendothelial migration (143-145).

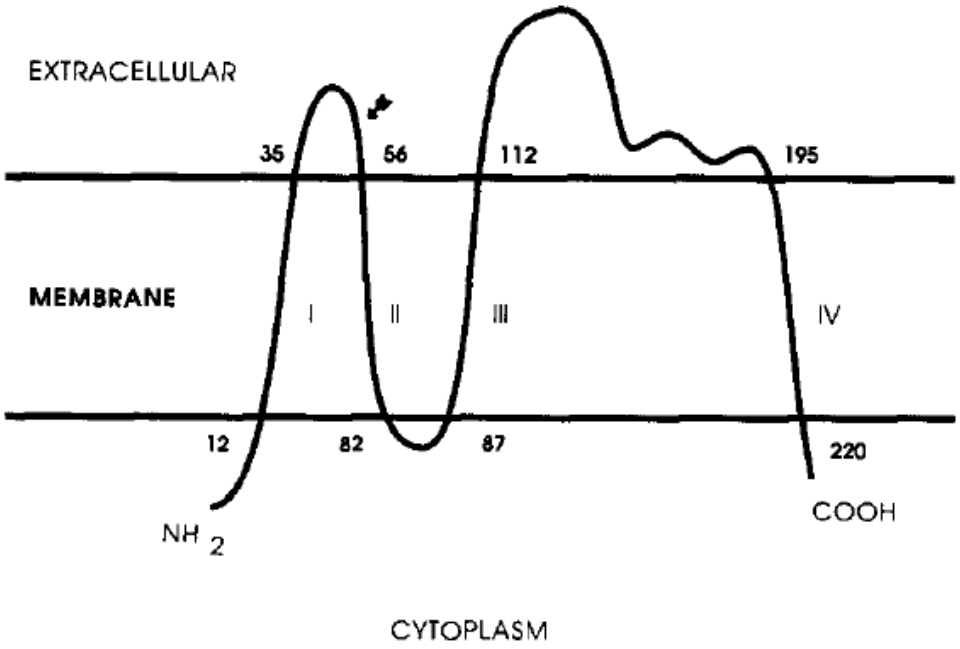

Figure 12: Schematic representation of CD9 molecule. Adapted from Boucheix et al (144).

In addition to mouse and rat SSCs, CD9 is expressed in multiple cell types, including hematopoietic cells, endothelial cells, epithelial cells, smooth muscle cells, pre-B cells (146). CD9 was also found to be expressed by multiple cancer cells namely; ovarian, colon, lung, gastric, breast, endometrium, leukemia and melanoma (147). While CD9 $^{-/-}$ mice showed no obvious abnormalities and appeared healthy with CD9 $^{-/-}$ males showing normal fertility, fertility of CD9 $^{-/-}$ females was severely impaired with completely inhibited sperm-egg fusion albeit normal sperm-egg binding (148-149). CD9 was also shown to regulate myoblast (150) and monocyte fusion (151), and HIV-induced syncytia formation (152). CD9 has tumor- suppressor-like functions in many tumor cell types, and can inhibit cell invasion and metastasis (153). In addition, CD9 was shown to contribute to cell signaling (154), and in regulation of cell adhesion (155), migration (156), apoptosis (157), membrane protein shedding (158), and diphtheria toxin binding (159). CD9 also interacts with other proteins, including other tetraspanins, a subset of integrins, other adhesion molecules, membrane proteases, choline receptors and G proteins (148).

CD9 was detected in the basal compartment of the seminiferous epithelium in human testes (160), but its expression on human SSCs was not reported. Based on the hypothesis that some SSC markers are evolutionarily conserved in rodents and humans, I examined if CD9 allows for functional identification of human SSCs in Chapter II.

9. Immunological cell sorting

Using antibodies against cell surface molecules, two methods have been employed widely to analyze and harvest live cells; immunomagnetic cell separation (or commonly called magnet-activated cell sorting; MACS) and fluorescent-activated cell sorting (FACS).

The antibody used these techniques is conjugated to either magnetic bead (MACS) or excitable fluorescent molecules, or fluorochromes, (FACS) and reacted with a single cell suspension of testis cells. The cell–

antibody mixture is then applied to a magnetic cell sorter or to a flow cytometer. Cells bound by the antibody are thus separated from those not bound based on the presence or absence of the bead or fluorescent dye. MACS is considered a quick and simple technique but only allows for a crude cell isolation and thus may be more suitable to remove a bulk stem cell-negative population. FACS on the other hand requires more sophisticated experience and expensive equipment. It also requires more cells than MACS for analyses. However, FACS is more advantageous over MACS because it allows for multi-parameter cell separation based on multiple cell surface markers using multiple lasers and detectors. It is also more powerful and versatile as many parameters that cannot be used in MACS are integrated in FACS analyses, such as cell size and complexity, cell cycle phase, viability and apoptosis.

10. Cancers

In the past years, the incidence of cancers commonly diagnosed in the adolescent and young adult population, such as Hodgkin and non-Hodgkin lymphoma, acute lymphocytic leukemia and testicular germ cells tumors (TGCT), showed remarkable increase (161). For instance, the incidence of TGCT, which represent the most frequently diagnosed malignant solid tumors in men aged between 15 and 35 years (162), has doubled during the last 40 years with an annual increase of 3-6% reported in Caucasian population (162). In 2009, the American Cancer Society had estimated that about 8400 new cases of TGCT are diagnosed every year in the USA (1). Yet, the prognosis of many of these cancers had improved dramatically during the same 40 years with the introduction of efficient combination chemotherapy as standard care, which raised the five-years disease-free survival rate over 90% (163-165).

However, germinal epithelial damage resulting in oligo- or azoospermia with the use of certain chemotherapeutic agents and radiotherapy has been well documented. Even before treatment,

impairment of spermatogenesis has been demonstrated in cancer patients (166-169). This impact on fertility is a major concern for testicular cancer survivors because of the young age of these patients, the achieved high cure rate and the long life expectancy following the treatment.

Cytotoxic treatment

Radiotherapy is done using one of two sources: electromagnetic radiation (X-ray) and corpuscular radiation (electrons) produced by a linear accelerator or rays generated by the decay of the cobalt 60 radioisotope. Both kinds of radiation can cause damage to the testes directly or diffusely during abdomino-pelvic radiation. Agents with anti-cancer properties can be grouped into: 1) Alkylating agents, such as cyclophosphamide, 2) anti-metabolites such as methotrexate and 5-flurouracil, 3) antimitotics such as vinblastine and vincristine, 4) enzymes, such as asparaginase. Studies investigate the effect of specific drugs and their doses on testicular damage aiming to achieve balance between the highest cure results and smallest side effects (165).

Chemotherapy and fertility

Ninety % of Hodgkin disease patients treated with MVPP and MOPP (mustine, vinblastine/vincristine, procarbazine and prednisolone) suffer from azoospermia up to 10 years after treatment. An alternative hybrid regime consisting of ABVD (doxorubicin hydrocholoride [Adriamycin], bleomycin, vinblastine, and dacarbazine) alternating with MOPP has shown to be less gonadotoxic with recovery of spermatogenesis in 40% of patients 27 months after treatment (170). After treatment with bleomycin, etoposide and cisplatin (BEP) used in testicular cancer , the majority of patients shortly become azoospermic (171). This condition is usually transient with 50 to 80% of patients showing recovery of spermatogenesis two to five years after treatment. However, with high dose-treatment in which cumulative doses of cisplatin exceed 400 mg/m^2, irreversible

impairment of spermatogenesis is usually observed (172-173). This suggests that fertility impairment is drug specific and dose related.

Somatic cells of the testis survive most chemotherapies although in some species they might suffer functional damage (174). This damage is not immediate and is believed to be secondary to the loss of germ cells, which decreases inhibin secretion by the Sertoli cells, thereby increasing the secretion of follicular stimulating hormone (FSH) by the pituitary (175). While testosterone secretion is not affected, germinal aplasia reduces the size of the testis and the blood flow, with less testosterone being distributed to the circulation, triggering the increase of luteinizing hormone (LH) secretion by the pituitary in response.

Chemotherapy and SSCs

Assessment of SSCs after chemotherapy has been done in animal models by i) morphologically quantifying numbers of spermatogonia (A_s Spermatogonia in rodents and A_{pale} in primates) or ii) quantifying numbers of spermatogenic colonies that spontaneously arise from surviving stem cells or primitive spermatogonia, or iii) recently, functionally using transplantation assay.

In mice, it is known that the recovery kinetics of spermatogenesis are similar after radiotherapy and chemotherapy (176). After a single dose of 1 and 2 Gy irradiation, the number of spermatogonia decreases and 45 days later, sperm count went down to 53 and 34 % of normal level respectively (177). A_s spermatogonia start to divide within a week after single dose of 1 Gy irradiation to increase their numbers with about a 2-day cell cycle time to replenish a stem cell pool. Although the first divisions are almost always self-renewing, A_{pr} and A_{al} differentiating cells develop within 6 days after irradiation (178, 179). Recovery continues, and by 5 weeks sperm production begins (180). With higher doses of 6 Gy, A_s spermatogonia are lost to 10% of control at 8 weeks and recovery does not start until about 8 weeks (181). Using transplantation assay, Kanatsu–Shinohara et al.

examined the recovery of the number of functional stem cells after treatment with busulfan, an alkylating agent known to preferentially kill SSCs. Using a low dose of 15 mg/kg, the number of SSCs was reduced to 4% of control at 3 days after treatment and showed a 4-fold increase between 3 and 15 days (182).

In rats, numbers of A_s spermatogonia start to decline after treatment with 6 Gy irradiation (183, 184), and continue till reaching 10% of control at 8 weeks after treatment (181). Interestingly, although complete regeneration of the number of A_s spermatogonia after 6 Gy irradiation occurs between 8 and 16 weeks (181), recovery of sperm counts after similar treatment varies according to the strain. Sprague-Dawley outbred rats for instance showed vigorous recovery after irradiation and chemotherapy compared to the inbred rat LBNF1(185-187). Yet, Sprague-Dawley rats showed a lower percentage of seminiferous tubules with differentiating cells than did mice when given the same dose of radiation (188, 189). This is explained by the observation that although A_s spermatogonia are completely recovered, differentiation is blocked and differentiating progeny undergo apoptosis (190). Zhang et al. transplanted spermatogonia from rats irradiated at 6 Gy into recipient irradiated nude mice (174). Although spermatogenic differentiation did not occur in situ in rat testes, it did occur in mouse testes, producing rat spermatozoa in mouse testes. Moreover when they transplanted spermatogonia from untreated prepubertal rats into irradiated rats testes, donor spermatogonia colonized the basement membrane of the seminiferous tubules but failed to differentiate. Collectively, these data denote that failure to undergo differentiation was due to damage to the rat somatic environment, an observation not seen with mice (174).

In primate, the rate of depletion of spermatogonia is slow compared to non-primates when the same dose of irradiation was given. The difference may lay in the difference between spermatogonial cells types; while primates has two types of spermatogonia, actively cycling A_{pale} and

the reserve resting A_{dark}, non-primates do not. During the first 11 days after radiation dose between 0.5 and 4 Gy to macaques testes, A_{pale} spermatogonia decreased to about 13% of pretreatment level while A_{dark} did not show significant reduction (191). At day 14 A_{dark} showed significant decrease and A_{pale} increased denoting an activation of A_{dark} proliferation to A_{pale} spermatogonia (191). With a dose of 0.5 and 2 Gy, A_{pale} spermatogonia reached 10 and 5% respectively of pretreatment numbers on day 44, then on day 200 reached 90 and 70% respectively of pretreatment numbers (191, 192). With a dose of 4 Gy, progressive recovery of sperm count lasting for over 1 year suggests a gradual recovery of the SSC population and regeneration of spermatogenesis from surviving SSCs (193). With a dose of 7 Gy, seminiferous tubules cross-sections showed no or few spermatogonia yet without progressive recovery (194). Hermann et al. using busulfan showed complete depletion of all germ cells in all seminiferous tubules of macaques with prolonged reduction in sperm counts in a dose-dependent manner; 47% of seminiferous tubules were devoid of spermatogenesis 60 weeks after treatment with 8 mg/kg body weight of busulfan while all tubules were empty 63 weeks after treatment with 12 mg/kg of busulfan (195). Collectively, these results indicate that in non-human primate, regeneration of spermatogenesis and recovery of fertility are dose dependent after cytotoxic treatment.

In human, a single dose of 6 Gy can produce permanent azoospermia (196). Testicular biopsies from azoospermic men who received cytotoxic treatment including alkylating agents and cisplatin revealed complete absence of germ cells several years after the treatment suggesting the complete killing of stem cells. Table 2 summarizes the impact of cytotoxic treatment on sperm production in humans.

Table 2: Cytotoxic effect on sperm production in humans (adapted from (197))

Treatment Impacting Sperm Production			
Radiation		Chemotherapy	
Testicular Dose (cGy)	Effect on Spermatogenesis	Agent	Dose Effecting Spermatogenesis
<10	No Effect	Cyclophosphamide	7 g/m^2
10–30	Temporary reduction in counts	Ifosfamide	42–60 g/m^2
30–50	Temporary azoospermia: 100% recovery 48 mos	BCNU and CCNU	1 gm/m^2 and 500 mg/m^2
50–100	100% temporary reduction 3–17 mos after treatment. Recovery begins 8–26 months post treatment.	Chlorambucil	1.4 g/m^2
100–200	100% azoospermia 2–9 mos. Recovery begins 11–20 months post treatment	Melphalan	140 mg/m^2
200–300	100% azoospermia 1–2 months. May be permanent. If recovery, may take years.	Busulfan	600 mg/m^2
1,200 TBI	Permanent azoospermia	Procarbazine	4 g/m^2
2,400 Gonadal	Permanent azoospermia	Cisplatin	500 mg/m^2

Very little is known about the regeneration of SSCs after cytotoxic treatment in humans, because no SSC markers are defined and no functional assay has been applied. Hence, most of the available data come from sperm count.

It was shown that type A_{pale} spermatogonia start to regenerate their numbers and reach their pre-irradiation levels 2 years after patients are treated with a single dose of 1 Gy (198). Moreover, Meistrich and Van Beek observed that in patients receiving 1 Gy irradiation, the ratio of spermatocytes to spermatogonia is 10-fold lower than control levels, suggesting that spermatogonial differentiation is not blocked but rather occurs at reduced efficiency. In addition, Kreuser et al. observed only spermatogonia in tubule cross-sections from azoospermic patients treated with chemotherapy, while Chan et al. observed that testicular sperm was present in 9 patients out of 20 undergoing testicular sperm extraction for azoospermia treated with various chemptherapeutic agents (199, 200). Clinical studies have reported the cases in which spontaneous recovery of sperm production in azoospermic patients was not observed up to 15 years after treatment with cytotoxic treatment (201-203). These observations show that in man, after cytotoxic treatment, spermatogonia

are recovering but sperm do not follow at the same rate. This dissociation between the recovery of spermatogonia and the recovery of sperm might be related to temporary somatic environment damage after cytotoxic treatment similar to that seen in rat (174), although no data are available to support this assumption. Another explanation is that the number of spermatogonia must first reach a critical number before differentiation takes place.

11. SSC-based strategy to restore male fertility

Currently, semen cryopreservation prior to initiation of cancer therapy remains the cornerstone for fertility preservation in majority of cancer patients. This technique, however, involves significant drawbacks. First, only adolescents who have reached “spermarche” and produce mature sperm in their testes can have spermatozoa in the semen for cryopreservation, and the technique cannot be used to preserve fertility among patients treated for cancer prior to adolescence (204). Second, semen collection by masturbation may be viewed by some patients as inappropriate for cultural or religious reasons which might make collection of semen unfeasible (205). Third, semen parameters even before anti-cancer treatment are impaired (198, 206). Compared to fertile controls, patients with testicular and systemic malignancies show lower sperm concentration, motility and cryosurvival rates and a higher DNA fragmentation index (206). After cancer treatment, further deterioration of semen quality is seen (207). Sperm chromosomal aneuploidy rate increases after therapy and sperm chromatin integrity declines years after therapy (208). Patients with reduced sperm quality usually require assisted reproduction techniques (ART) to achieve pregnancy. Note that with the use of ART, the chance of passing defective genetic material might be higher with the loss of natural selection mechanisms with these techniques. Sperm banking and ART in fact do not restore male fertility rather than bypassing reconstitution of a man’s physical ability to father a

genetic child. To overcome the drawbacks of sperm banking as a method of fertility preservation, other approaches are therefore investigated.

Due to the dual function of SSCs in self-renewal and progenitor generation, they are an important target cell population for male fertility restoration after cytotoxic treatment. The manipulation of SSCs has been well established in experimental animals, although not yet realized in humans. Two potential strategies have been proposed for male fertility recovery for boys and men of any age; pharmacological manipulation and SSCs-based approach.

Pharmaceutical manipulation

This approach involves the use of hormonal suppression to preserve or restore spermatogenesis in cancer patients undergoing cytotoxic treatment. Originally it was suggested by Glode et al. who claimed that pretreatment with gonadotropin-releasing hormone (GnRH) protected spermatogenesis in mice from damage after chemotherapy by reducing the rate of spermatogenesis (209). This is based on the observation that non-cycling cells are generally more resistant to killing by anticancer agents compared to cycling cells, and therefore by interruption of pituitary-gonadal axis, spermatogenesis is slowed and testis becomes more resistant to the effect of chemotherapy. Later on, it was shown that the suppression of gonadotropins and testosterone only blocks the completion of spermatogenesis with no effects on SSCs, which appear not to be affected by hormonal suppression (210).

After cytotoxic treatment, surviving SSCs proliferate to repopulate the testis and differentiate to recover sperm production. In contrast to mice (211) and monkeys (212), surviving SSCs in rats fail to differentiate and their progeny undergo apoptosis. Upon examination of their hormonal status after chemotherapy, rats showed decreased inhibin secetion by Sertoli cells, elevated levels of FSH and LH, and 2- to 4-fold increase in intratesticular testosterone concentration while serum testosterone level

remained unchanged (190, 213, 214). Testosterone was shown to be primarily responsible for the inhibition of spermatogonial differentiation while FSH played a minor role (215). When GnRH agonists (Leuprorelin) (216) or antagonists (Cetrorelix) (214) were administrated in rats to reduce the high levels of intratesticular testosterone caused by cytotoxic treatment, germ cells were released from differentiation block (190, 217-219). Note that when hormonal suppression was introduced to rats only after cytotoxic treatment, the numbers of differentiated germ cells still increased dramatically compared to control which did not receive hormonal suppression; however, spermatogenesis proceeded only to round spermatids with no production of sperm after GnRH treatment (217). Only when the suppression effect was over spermatids gave mature sperm reflecting the role of testosterone in spermatogenesis. In addition to the use of GnRH agonists and antagonists alone, other studies used GnRh antagonist (Nal-Glu) with antiandrogens (Flutamide) (220), systemic physiological doses of testosterone (221), progestins (medroxyprogesterone acetae) and estrogens (estradiol E2) (222) to suppress gonadotropin and intratesticular testosterone in rats and reported effective recovery of spermatogenesis.

However, in men and primates, hormonal therapy has not been successful in preserving fertility or facilitating recovery of spermatogenesis (223-225). Out of seven clinical trials performed, six indicated no protection. This might be due to the fact that intratesticular testosterone is normally more than 10-folds higher in humans compared to rats (609 ng/ml vs 50 ng/ml) (226). Results of the 7 studies are shown in table 3. Only one study by Masala et al. reported that all patients receiving cyclophosphamide for nephrotic disorders restored normal sperm counts 6 months after therapy when they were injected with 100 mg intramuscular testosterone injection every 15 days starting 30 days before cyclophosphamide and continuing during treatment, a response not seen in the control group receiving cyclophosphamide alone (227).

Table 3: Results of hormonal suppression treatments given before and during cytotoxic therapy in men (adapted from (228)).

Study	Disease	Cytotoxic therapy	Hormone treatment	Recovery[a]	
				Hormone treated	Controls
Johnson *et al.* (1985)	Hodgkin's	MOPP 3–6 cycles	GnRH agonist	1 of 5	No controls
Waxman *et al.* (1987)	Hodgkin's	MVPP, ChlVPP	GnRH agonist +testosterone	0 of 20	0 of 10
Redman & Bajorunas (1987)	Hodgkin's	MOPP ~4 cycles	Testosterone	≈70% of 23[b]	≈70% of 22[b]
Fossa *et al.* (1988)	Testis Ca	PVB, ADR/CY, Radiation	Medroxyprogesterone	0 of 4 (2 of 12)[c]	2 of 3 (7 of 13)[c]
Kreuser *et al.* (1990)	Testis Ca	PVB	GnRH agonist	6 of 6	8 of 8
Brennemann *et al.* (1994)	Testis Ca (Seminoma)	Radiation	GnRH agonist+antiandrogen	12 of 12	8 of 8
Masala *et al.* (1997)	Nephritis	Cyclophosphamide	Testosterone	5 of 5	1 of 5

Although hormonal suppressive treatment was not successful in protecting and stimulating spermatogenesis recovery in man, efforts are ongoing to develop better combinations. Several advantages are foreseen with this approach. First, SSCs are not removed from their natural "niche" and not exposed to toxic effect of cryoprotectants (229). Second, the approach does not involve complicated protocols or need experienced laboratories to sort cells or cryopreserve tissues. Third, it can be offered at remote clinics and during or after cytotoxic therapy, which is important in cases where malignancy needs urgent intervention. Fourth, no risk of reintroducing malignant cells after treatment is implicated in this approach. On the other hand, hormonal suppressive treatment exposes SSCs to the hazardous effect of cytotoxic therapy, depends on surviving SSCs which might be lost in some cases especially with high does of cytotoxic therapy and exposes patient to known side effects of hormonal therapy.

SSCs-based surgical approach

The SSCs-based surgical approach uses sperm-producing stem cells by surgically manipulating SSCs. This surgical strategy is further divided into two approaches; implantation of testes biopsy and transplantation of SSCs. In the implantation approach, testes biopsies are grafted into testes or ectopically under the skin, and in both locations spermatogenesis is

restored. In the transplantation approach, SSCs are injected into the seminiferous tubules to regenerate spermatogenesis.

Testis tissue implantation

In this technique a testicular tissue is harvested from the patient before cytotoxic therapy and transplanted to an ectopic site such as under a patient's skin or into testes after successful cancer therapy (auto-grafting) or into animals (xenografting). It maintains cell-to-cell contacts between germ cells and therefore preserves the stem cell niche necessary for their survival and subsequent maturation. Proper storage of gonadal tissue and optimal amount of collected material are the factors to guarantee success of future fertility preservation.

The long term preservation of testicular tissue by cryopreservation was shown to not affect the results of implantation later (230, 231). Recent studies implanting cryopreserved testicular tissue pieces of mice, hamsters, rabbits, pigs, and goats in the testis or under the skin of immunodeficient mice established spermatogenesis in these implants (230-233). Moreover microinsemination using spermatozoa from these implants gave offspring of donor mice and rabbits. Recently, Sato et al. demonstrated successful spermatogenesis in the xenograft of human infant testicular tissue grafted into nude mice until pachytene spermatocyte stage (234).

Recipients in these studies were adult males castrated prior to or during the implantation surgery. Castrating recipient mice resulted in improved graft survival (233). One of the most significant parameters for the success of implantation was the developmental stage of the donor testis. Tissue from adult donors show poor survival and a marked tendency to degenerate, while the use of tissue from prepubertal males gives better survival and outcome than the use of pubertal or adult donors (231, 235). This was suggested to be due to the lack of proliferation of Sertoli cells in adult donor tissue or the increased sensitivity to ischemia

(231, 235-238). Suppression of spermatogenesis in adult donor mouse testes by GnRH antagonists or experimentally induced cryptorchidism before grafting improved graft survival and spermatogenesis restoration after implantation suggesting that the poor graft survival of adult donor might be due to the increased metabolic activity and therefore the liability to hypoxic damage (239).

It is speculated that testicular tissue implantation for fertility preservation of cancer survivors, especially prepubertal boys, can probably be applied to humans. In this novel strategy, testis biopsies are obtained prior to anticancer treatment, dissected in fragments suitable for implantation and cryopreserved. When needed, fragments are thawed and implanted into the patient or an immune-deficient recipient where they mature and sperm can be recovered and subsequently used for microinjection of oocytes

Although this strategy has the advantage that SSCs remain in their microenvironment (niche), purging cancer cells, which may have invaded into testicular biopsy before harvesting it, is not possible and therefore tumor cells might be reintroduced to the cancer survivor in the autografting scheme. Xenografting can overcome this risk. However, the possibility of host pathogens having an effect on the developing testis tissue and its resulting sperm must be considered in this case (240). Therefore, use of this technique for fertility preservation in humans needs more studies to address ethical and safety issues before testis xenografting can be considered a feasible and safe option for fertility preservation for cancer survivors.

Spermatogonial transplantation:

In a potential clinical application of spermatogonial transplantation for male fertility restoration, SSCs are first harvested from a patient testis before cancer therapy and cryopreserved. After the patient is cured and when he is ready to father his offspring, preserved SSCs are transplanted

back into the seminiferous tubules of his testes to regenerate complete spermatogenesis and restore fertility (Fig.13).

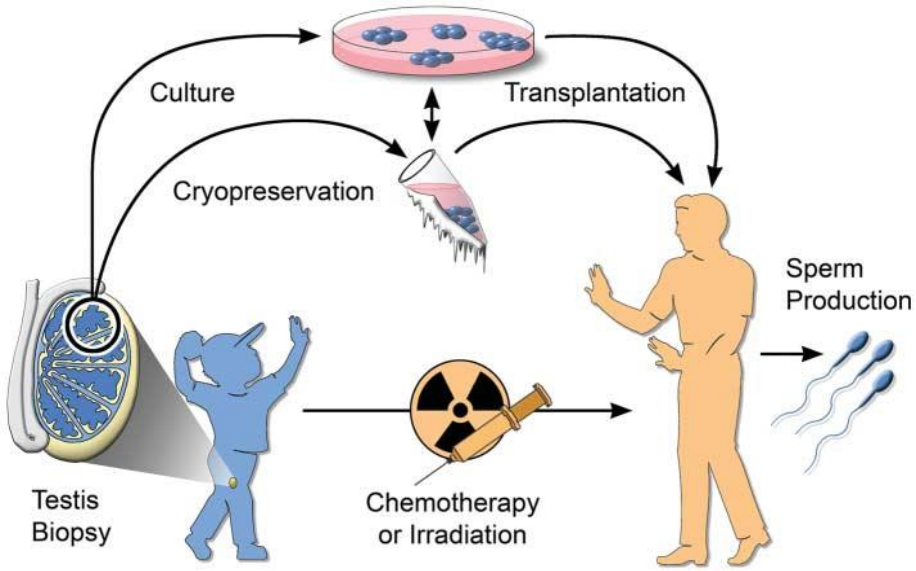

Figure 13: Proposed application of SSCs transplantation to preserve male fertility. Adapted from Oatley **(241).**

Besides restoring natural fertility and producing unlimited number of sperm, the most significant advantage of this approach is that it allows for SSC selection accompanying purging of tumorigenic cells. This is critical in clinical application to avoid reintroducing cancer cells to the patient. A study on rats showed that testis cell preparation contaminating only 20 leukemic cells can lead to the recurrence of leukemia after spermatogonial transplantation (242). To purge cancer cells from testicular cell preparation, immunological cell sorting using antibodies raised against markers expressed on SSCs and on different cancer lineages can be used in a combination of positive and negative selection (243).

To apply this surgical strategy to human, three major steps are essential. The first step, harvesting testis biopsies, is currently a routine procedure. Protocols to prepare single cell suspensions from these human

testis biopsies are currently available using two-step enzymatic digestion with a cocktail of multiple enzymes for digesting human testis biopsies. These single cell preparations can be cryopreserved without the apparent loss of SSC activity (106). Studies had tried to optimize the cryopreservation efficiency and the viability of thawed cells by adding different cryoprotectants achieving up to 59 % viability (244). The final step involves transplantation of thawed germ cells. In a non-human primate model, different groups had successfully injected germ cells into the rete testis space of monkeys (245), goats and dogs (246, 247), using ultrasound to guide the injection needle.

This progress demonstrates that spermatogonial transplantation is feasible towards restoring fertility in cancer survivors. Regeneration of spermatogenesis arising from transplanted SSCs may allow recovery of spontaneous fertility or may at least facilitate subsequent procedures using assisted-reproductive technologies. This approach is also amenable to the amplification of SSCs in vitro before transplantation. Recent progress in human SSC culture protocols previously discussed could now allow for harvesting a small testicular biopsy from a patient and producing enough therapeutic SSCs in culture for future transplantation. In summary, SSCs transplantation is a feasible approach that could be applied in fertility clinics after optimizing the necessary techniques.

3. Rationale and Hypothesis

Spermatogonial stem cells (SSCs) are the stem cells of the male germ line and are the foundation of spermatogenesis. They are present in the testis since birth and throughout male life. Many anti-cancer therapies result in male infertility and with the increasing survival rate of cancer patients, this becomes a significant concern. SSCs are expected to provide powerful tool to preserve and restore fertility. A proposed SSC-based strategy includes harvesting and isolating SSCs from patient testis biopsy before anti-cancer therapy, expansion in vitro and

cryopreservation. After a patient is cured, preserved SSCs are transplanted back into the seminiferous tubules of his testes to regenerate complete spermatogenesis with the restoration of fertility. This strategy was successful in fertility restoration in mice, which forms the foundation of the proposed strategy in humans. Several issues remain to be addressed. First, the potential transmission of tumorigenic cells contaminated in testis biopsy is a major concern. It is thus crucial to be able to isolate SSCs from contaminating cancer cells. This is successfully applied in human hematopoietic stem cell (HSC) transplantation, which benefit from a long list of cell surface markers. In chapter II, I hypothesized that CD9, a known cell surface marker of rodent SSCs, is expressed on human male germ cells showing colonizing/repopulating activity and can be used to enrich for human SSCs. I tested this hypothesis using a functional xenotransplantation assay, combined with immunomagentic cell sorting of human testis biopsies.

Second, It was suggested that spermatogenesis recovery depends on the surviving SSCs, yet an important question remains to be addressed: do the SSC recovery kinetics influence the process of fertility restoration? In the study presented in Chapter III, I hypostatized that the recovery of SSCs following cytotoxic damage induced with anti-cancer therapy reflects the restoration of male fertility. To test this hypothesis, I used the mouse as a model and studied the kinetics of SSC recovery with transplantation assay after treatment with three doses of busulfan, a known alkylating agent that preferentially kills SSCs.

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Chapter 2

Manuscript 1

CD9 is expressed on human male germ cells that have a long-term repopulation potential after transplantation into mouse testes.

Running Title: CD9 is a marker of human spermatogonial progenitors.

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Abstract

Human spermatogonial stem cells (SSCs) play critical roles in life-long maintenance of male fertility and regeneration of spermatogenesis. These cells are expected to provide an important resource for male fertility preservation and restoration. A basic strategy has been proposed to involve harvesting testis biopsy specimens from a cancer patient prior to cancer therapies, and transplanting back to the patient at a later time; then, SSCs included in the specimens regenerate spermatogenesis. To clinically apply this strategy, isolating live human SSCs is important. In this study, we investigated if CD9, a known rodent SSC marker, is expressed on human male germ cells that can repopulate recipient mouse testes upon transplantation. Testicular tissues were obtained from men with obstructive azoospermia. Using immunohistochemistry, we found that CD9 was expressed in human male germ cells in the basal compartment of the seminiferous epithelium. Following immunomagnetic cell sorting, CD9-positive cells were enriched for germ cells expressing MAGEA4, which is expressed by spermatogonia and some early spermatocytes, compared to unsorted cells. We then transplanted CD9-positive cells into nude mouse testes and detected a ~3 to 4-fold enrichment of human germ cells that repopulated mouse testes for at least four months after transplantation, compared to unsorted cells. We also observed that some cell turnover occurred in human germ cell colonies in recipient testes. These results demonstrate that CD9 identifies human male germ cells with capability of long-term survival and cell turnover in the xenogeneic testis environment.

Introduction

The activity of spermatogonial stem cells (SSCs) is the foundation of spermatogenesis. These cells can self-renew for a lifetime and give rise to progeny that are committed to differentiation, thereby supporting continuous sperm production during steady-state spermatogenesis. Owing to this unique potential, SSCs also play a critical role in regeneration of spermatogenesis following testicular injuries. It has been demonstrated in the mouse that treatment with an alkylating agent, busulfan, induces male infertility. Upon transplantation into testes of these infertile mice, SSCs migrate to the basal compartment of the seminiferous epithelium and colonize recipient testes, leading to regeneration of spermatogenesis and restoration of fertility (1, 2). This experimental scheme has been proposed to be applicable in clinical settings to safeguard male fertility for patients who undergo cytotoxic cancer therapies (3). SSCs can be harvested prior to anti-cancer therapies, and following in vitro amplification, autologously transplanted back to a patient's testes to restore male fertility. Since mouse male germ cells in fetal tests are known to possess the capacity to colonize and regenerate spermatogenesis upon transplantation into postnatal testes (4), the SSC transplantation approach can be expected to be beneficial for men of any age.

A caveat of this approach is the potential transmission of tumorigenic cells infiltrating testis tissues. To circumvent this risk, immunological separation of germ cells from cancer cells has been investigated. An animal study showed that leukemic cells can be largely purged from testis cells by immunological cell separation using two cell-surface antigens, a major immunohistocompatibility complex class I (MHC-I) molecule and CD45 pan-hematopoietic antigen, as markers (5). Transplantation of the resulting cells in the marker-negative fraction prevented cancer transmission while allowing for generation of functional sperm. However, Geens et al. showed that positive selection of germ cells only or negative selection of leukemic cells alone was insufficient to decontaminate

testicular cell preparations (6). Another study concluded that germ cell selection in combination with leukemic cell depletion prevented leukemia transmission in association with transplantation of the sorted cells (7). With emerging evidence that leukemic stem cells may evolve and change their immunophenotypes (8, 9), identification of multiple human SSC markers for cell enrichment is critical.

Accumulating evidence suggests that SSC markers are conserved considerably from mice to non-human primates (10); thus, rodent SSC markers are expected to be applicable for human SSCs. However, a recent study suggests that some differences appear to exist between human SSCs and SSCs of other species (11), even though a degree of conservation has been observed across species (10-13). Such a finding highlights the importance that the applicability of rodent SSC markers to human SSCs needs to be verified on a molecule-by-molecule and species-by-species basis.

To identify SSC markers in various animal species, transplantation into nude mice has been used as a functional assay (10-13). This transplantation assay is an unequivocal functional assay of SSCs when complete spermatogenesis is regenerated, as seen in the mouse-to-mouse transplantation (14). For human SSCs, a xenotransplantation approach using nude mice as recipients has been used as a functional assay. However, human spermatogenesis can not be completed in this xenogeneic environment (15), and thus, this technique has a weakness to detect the ability of SSCs to support differentiation. Nonetheless, it allows for identifying human germ cells that can migrate to the basal compartment of mouse seminiferous epithelium where daughter cells proliferate and survive for a long time (12, 13, 15). These functions represent the key characteristics of SSCs and are essential when human SSCs are to be used for male fertility restoration through transplantation. Thus, xenotransplantation is a logical functional detection method available for human spermatogonial stem/progenitor cells (10,13).

SSEA4 is thus far the only marker identified for human stem/progenitor spermatogonia through the functional transplantation assay (11). Here, we tested a hypothesis that CD9 is expressed on human male germ cells with colonizing/ repopulating activity. CD9 is a known cell-surface marker of rodent SSCs (16) and belongs to the tetraspanin superfamily. Tetraspanins interact with other proteins, such as integrins, immunoglobulins, proteoglycans, complement-regulatory proteins, and growth factor receptors. CD9 is involved in cell adhesion and contributes to functional regulation of integrins (17). It can also regulate cell migration and proliferation as well as egg-sperm fusion (18, 19). While various types of differentiated somatic cells express CD9 (17, 20-23), some stem cells, such as embryonic stem cells (24), hematopoietic stem cells (25), and neural stem cells (26) also express this molecule. In human testes, CD9 expression was detected in the basal compartment of the seminiferous epithelium using immunohistochemistry (27), but its expression on human SSCs has not been documented.

In this study, using immunological cell sorting and xenotransplantation, we evaluated repopulation potential of CD9-positive ($CD9^+$) human male germ cells. Our results indicated that upon transplantation into mouse testes, $CD9^+$ cells generated more colonies of human germ cells that were maintained for at least 4 months, compared to unsorted human testis cells. We also obtained results that are suggestive of cell-turnover taking place in these colonies. The data thus support that CD9 is expressed not only on mouse SSCs but also on human male germ cells with repopulation potential and may help the development of clinical approach to the male fertility restoration through transplantation.

Material and Methods

Patients

Human testis tissues were obtained from 18 adult males with obstructive azoospermia, aged on average 44.6 ± 1.3 years (39 – 50

years). Tissues were obtained through aspiration using a minimal invasive technique using a 16-18 gauge intravenous catheter inserted percutaneously under local anesthesia, as previously described (28). Among the samples from 18 patients, those from 9 patients were used for immunostaining with or without cell sorting, while those from 6 patients were used for cell sorting followed by xenotransplantation. The remaining three samples were used in preliminary transplantation experiments. This study was approved by the institutional ethics review board, and informed consent was obtained from all subjects.

Immunohistochemistry

Human testicular biopsies were fixed with Bouin's solution for 6 hours. Paraffin-embedded samples were sectioned at 5 μm thickness. For immunostaining for CD9 or GFRA1, samples were treated with blocking solution (phosphate-buffered saline (PBS) with 5% donkey serum) overnight at 4°C and reacted with either polyclonal goat anti-human CD9 (sc 7639, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or polyclonal goat anti-human GFRA1 (sc 6157, Santa Cruz Biotechnology) both at a dilution of 1:50 overnight at 4°C. Following repeated washes with PBS, positive cells were visualized with FITC-conjugated donkey anti-goat IgG (Jackson Immunoresearch, West Grove, PA, USA). Images of immunostaining were captured under an epifluorescent microscopy. To co-stain for MAGEA4 expression, sections stained for CD9 or GFRA1 were extensively washed with PBS, then treated with 0.3% H_2O_2 (Sigma, St. Louis, MO, USA) and incubated in blocking solution (PBS with 5% normal goat serum) overnight at 4°C. The following day, sections were incubated with monoclonal mouse anti-human anti-MAGE antibodies (clone 57B, kindly provided by Dr. G.C Spagnoli) (29-34) at 1:100 overnight at 4°C. Samples were then reacted with peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch laboratories) at 1:500 for 1 hour at room temperature. Positive cells were visualized using 3,3'-

diaminobenzidine (DAB substrate kit, SK-4100, Vector Laboratories, Burlingame, CA, USA). Images of MAGEA4 staining were merged with those of CD9 or GFRA1 staining to assess the colocalization of positive signals.

To co-stain for CD9 and ITGA6 (integrin-α_6 or CD49f) expression, serial sections of 5 μm were used. Samples were treated for CD9 staining as described above. For ITGA6 staining in an adjacent section, samples were treated with blocking solution (PBS with 5% normal donkey serum) overnight at 4°C and reacted with polyclonal goat anti-human ITGA6 (sc 6596, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a dilution of 1:50 overnight at 4°C. Following repeated washes with PBS, positive cells were visualized with FITC-conjugated donkey anti-goat IgG (Jackson Immunoresearch, West Grove, PA, USA). Images of immunostaining were captured under an epifluorescent microscopy. Images of ITGA6 staining were merged with those of CD9 staining to assess the colocalization of positive signals.

Two sections from each patient were scored for CD9/MAGEA4 or GFRA1/MAGEA4 colocalization with a total of 198 and 173 cross-sections scored respectively. For ITGA6/CD9 colocalization, 4 sections from 2 patients for each antigen with 81 cross sections were scored.

Cell preparation

Single suspension of human testis cells were prepared by digesting biopsy specimens with mixture of collagenase I, collagenase IV, hyaluronidase, and DNase I (all from Sigma, St. Louis, MO, USA) at 1 mg/ml each in 5 ml Hanks' balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA, USA) at 33°C for 15 minutes with periodic shaking. After the initial digestion, human testis cells and tubules were centrifuged at 500 x *g* for 5 min and further digested at 33°C for 5 minutes using 0.5 mg/ml trypsin and 1 mg/ml DNase I in HBSS. The mean biopsy weight was 67.1 ± 8.3 mg per patient, while the mean cell recovery was $8.6 \pm 0.4 \times 10^4$

cells/mg testicular specimen; thus, an average of 5.8 x 10^6 cells were recovered per patient. Cell viability was 95.5 ± 1.7%.

Immunomagnetic cell sorting

Cell sorting was performed using samples from each individual subject; thus, cells were not pooled. A single cell suspension of donor testis cells was divided into control (unsorted) and experimental groups in each experiment. For the experimental group, immunomagnetic cell sorting was done according to the previously described protocol with some modifications (35). Briefly, cells were suspended in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 1% fetal bovine serum (FBS) at a concentration of 6 x 10^6 cells/ml and reacted with mouse anti-human leukocyte antigen (HLA)-ABC antibodies (555551, BD Biosciences Pharmingen, Franklin Lakes, NJ, USA) (5 µg/ml) on ice for 30 min. This antibody recognizes a human form of a monomorphic epitope of major histocompatibility class I antigens widely distributed on nucleated cells. Following washing, the cells were incubated with secondary sheep anti-mouse antibodies conjugated with a magnetic bead (catalogue number 11031, Invitrogen Dynal AS, Oslo, Norway) on ice for 30 min with gentle agitation. The negative fraction was recovered and incubated with polyclonal goat anti-human CD9 antibodies (5 µg/6 x 10^6 cells/ml) on ice for 30 min, followed by biotin-conjugated donkey anti-goat IgG (Jackson Immunoresearch laboratories) on ice for 30 min with gentle agitation. To capture the antibody-bound cells, the M280 streptavidin magnetic beads (catalogue number 112-05D, Invitrogen Dynal) were used at 20 µl/ml cell suspension on ice for 30 min with gentle agitation. Cells were then applied to a magnetic cell sorter (Invitrogen Dynal) for 3 min, and antibody-bound cells as well as unbound cells were recovered and cryopreserved at a cell concentration of 5 x 10^6 cells/ml. Cells were kept at -80°C overnight and stored in liquid nitrogen until transplantation.

In cytospin experiments, a single cell suspension from a testicular

biopsy was subjected to immunomagnetic cell sorting with anti-human CD9 or anti-human GFRA1 (both at 5 µg/ml) without HLA sorting. For GFRA1 sorting, the primary antibody was as described above, followed by biotin-conjugated donkey anti-goat IgG (Jackson Immunoresearch laboratories) on ice for 30 min with gentle agitation. Antibody-bound cells were recognized by magnetic beads, M280 streptavidin, as above. Cells were then applied to a magnetic cell sorter (Invitrogen Dynal) for 3 min. Sorted and unsorted cells were centrifuged at 200 x *g* for 15 min on glass slides at a cell concentration of 5 x 10^4 cells/ml in 1 ml per specimen chamber. Cells were air-dried and stained for MAGEA4 as in immunohistochemistry.

Transplantation

Ncr nude mice (nu/nu, Taconic, Germantown, NY) served as recipients for human testis cell transplantation. To destroy endogenous spermatogenesis, the recipient mice were treated at 8 weeks of age with busulfan (40 mg/kg) (Sigma) at least 6 weeks before donor cell transplantation. Frozen human testis cells were thawed and resuspended in DMEM with 0.1 mg/ml DNase and 0.04% trypan blue. Cell viability after thawing was 53.3 ± 1.9% across the cell fractions transplanted (HLA^-CD9^+ 53.6%, HLA^-CD9^- 53.0%, HLA^+ 53.6%, and unsorted 52.9%). Since no significant differences were detected among all the cell fractions, we transplanted all cells (i.e., viable and non-viable cells) into recipient mouse testes. The concentration of donor human cell suspension was 25 x 10^6 cells/ml across the groups. Approximately 7 µl of donor cell suspension were introduced into the seminiferous tubules through the rete testis of a recipient mouse (2). For HLA^-CD9^+, HLA^-CD9^-, HLA^+, and unsorted fractions, 15, 7, 7, and 11 testes were injected and analyzed respectively. All animal handling and care were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

Analysis of recipient testes

Whole-mount immunohistochemistry using anti-human mouse monoclonal MAGEA4 antibody (clone 57B, kind gift from Dr. G.C. Spagnoli) was performed 2 and 4 months after transplantation; staining results were analyzed throughout an entire testis. Recipient mouse testes were treated with a mixture of collagenase IV and DNase I (Sigma, St. Louis, MO, USA) at 1 mg/ml each in 5 ml Hanks' balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA, USA) to disperse the seminiferous tubules and subsequently fixed in 4% paraformaldehyde at 4°C for 2 hours. Following extensive washing, testis tubules were treated with 0.3% H_2O_2 and incubated in blocking solution (PBS with 5% normal goat serum) at 4°C overnight. The samples were then processed with the MAGEA4 antibody at 1:100 in PBS with 5% BSA at 4°C overnight and reacted with peroxidase-conjugated goat anti-mouse IgG at 1:250 at 4°C overnight. MAGEA4$^+$ human germ cells were visualized using DAB as described above. Stained tubules were fixed in 10% neutral-buffered formalin and kept in PBS at 4°C.

Statistical analyses

Statistical analyses were done using *t*-test or, for multiple comparisons, ANOVA followed by Tukey post hoc test. Significance was determined when $p < 0.05$. All data are expressed as the mean ± SEM.

Results

Testicular biopsies were derived from adult patients with obstructive azoospermia. Using immunohistochemistry in paraffin sections of human testes, we initially tested antibodies against various antigens known to be expressed by mouse SSCs for their reactivity to human spermatogonia. Among them, anti-CD9 and anti-GFRA1 antibodies gave clear positive signals (Fig. 1A). The cells stained with either antibody were localized in the basal compartment of human seminiferous epithelium, a typical

staining pattern of spermatogonia. We then double-stained $CD9^+$ or $GFRA1^+$ cells with MAGEA4 antibodies. MAGEA4 is an oncofetal protein expressed in a variety of malignant neoplasms and, among normal cells, only in spermatogonia and, to a lesser extent, early spermatocytes (29-34). Indeed, we observed positive MAGEA4 signals on germ cells in the basal compartment (Fig. 1A). Expression of MAGEA4 was clearly observed in $CD9^+$ or $GFRA1^+$ cells on the basal membrane, confirming that these two antigens are expressed in human spermatogonia, and possibly, early spermatocytes.

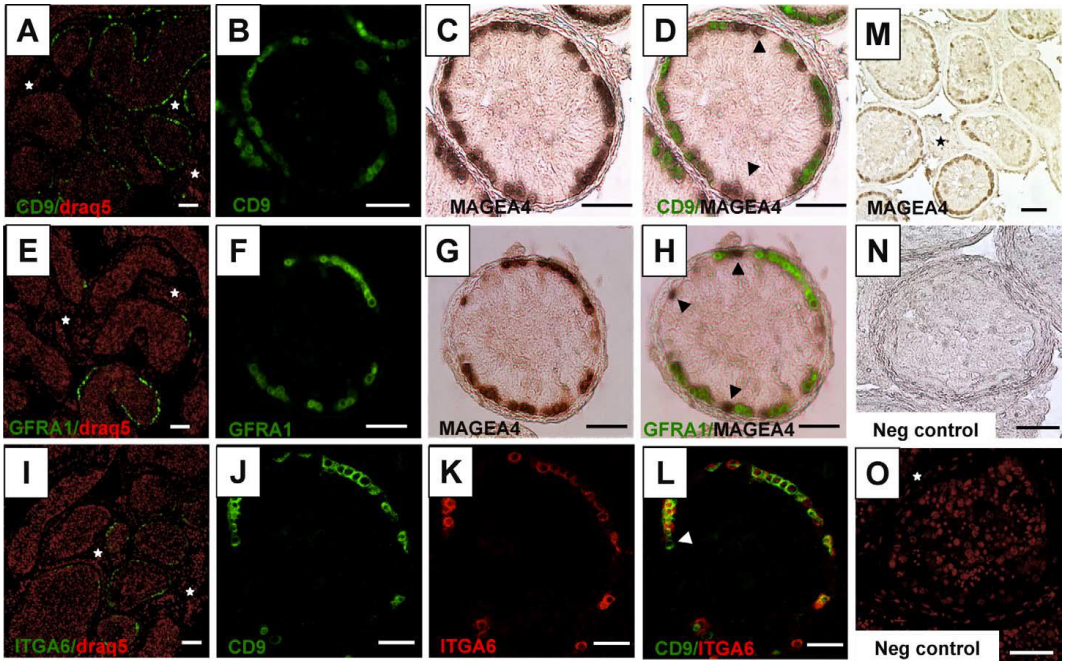


Figure 1: Expression of CD9, GFRA1, MAGEA4 and ITGA6 in human seminiferous epithelium. Immunostaining of human testicular sections with antibodies against CD9, GFRA1, ITGA6 and MAGEA4 showing positive cells resting on the basement membrane of the seminiferous tubules. Pictures are merged to show $CD9^+MAGEA4^+$cells, $GFRA1^+MAGEA4^+$ cells and $CD9^+ITGA6^+$. Note that some cells are $CD9^-MAGEA4^+$, $GFRA1^-MAGEA4^+$ or $CD9^-ITGA6^+$ (arrow heads). Negative control of IHC (stained with hematoxylin) and IF (stained with draq5) are

also shown. Stars mark negative staining in the interstitium. Scale bar = 50 μm.

$CD9^+MAGEA4^+$ cells accounted for nearly all (97%) of CD9 single-positive cells while representing 84% of MAGEA4 single-positive cells (Table 1). Likewise, $GFRA1^+MAGEA4^+$ cells were 98% of $GFRA1^+$ cells and 77% of $MAGEA4^+$ cells.

ITGA6 was previously shown to be expressed by human spermatogonia (11, 36) and non-human primate spermatogonial stem/progenitor cells (37). Thus, we examined if this molecule is expressed by $CD9^+$ cells. $CD9^+ITGA6^+$ cells accounted for 88% of CD9 single-positive cells while representing 90% of ITGA6 single-positive cells (Table 1). Thus, CD9 and GFRA1 are expressed by human spermatogonia, consistent with the results of previous studies (38, 39).

Table 1 : Proportion of double-positive cells in each single-positive cell population per seminiferous tubule section.

	MAGEA4+ cells	**CD9+ cells**
% of CD9+MAGEA4+	**83.8 ± 2** [a]	**96.9 ± 1**

	MAGEA4+ cells	**GFRA+ cells**
% of GFRA+MAGEA4+	**76.9 ± 2.1**	**97.9 ± 0.3**

	ITGA6+	**CD9+ cells**
% of CD9+ITGA6+	**88.4 ± 0.5**	**90 ± 3.1**

a: The value indicates that CD9/MAGEA4 double-positive cells represent 83.8% of MAGEA4 single-positive cells.

Based on these results, we next examined if CD9 and GFRA1 can be used to enrich human testis cells for MAGEA4$^+$ germ cells using immunomagnetic cell sorting. The sorting efficiency was analyzed by MAGEA4 staining following cytospin of selected cells, in comparison to unsorted cells. The results showed that the CD9$^+$ and GFRA1$^+$ cell fractions contained 4- and 4.5-fold more MAGEA4$^+$ cells, respectively (Fig. 2).

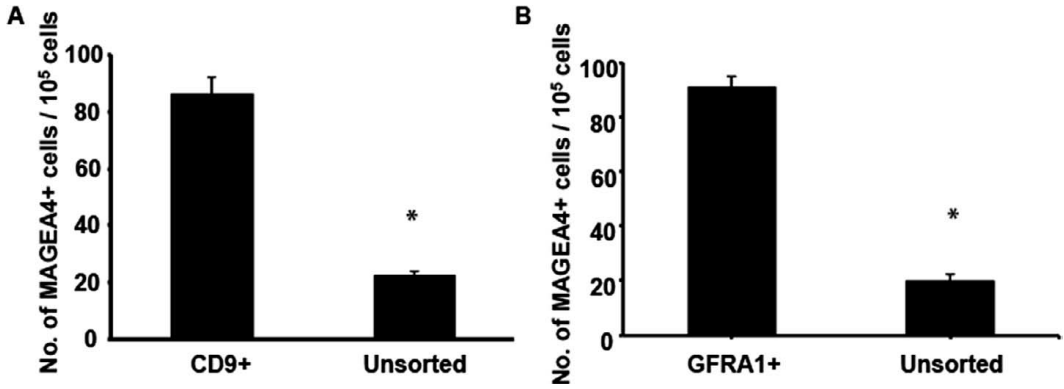


Figure 2: Detection of MAGEA4$^+$ cells in the CD9$^+$ or GFRA1$^+$ fractions. Numbers of MAGEA4$^+$ cells in the sorted and unsorted fractions using CD9 (**A**) or GFRA1 (**B**) as a marker. Sorted cells were stained for MAGEA4 expression following cytospin. The CD9$^+$ and GFRA1$^+$ cell fractions contain 4- and 4.5-fold more MAGEA4$^+$ cells, respectively ($p < 0.05$).

Although both cell-surface antigens allowed a similar enrichment degree of MAGEA4$^+$ human germ cells, a marked difference was noted in the efficiency of cell recovery. While ~12% of total cells were recovered in the CD9$^+$ cell population, only ~3% were collected in the GFRA1$^+$ cell population (Table 1). The low cell recovery using GFRA1 antibodies was anticipated to cause practical problems for further cell manipulation, particularly when a cell source was limited to testicular biopsies. Therefore, we focused on CD9 as a potential marker for human SSCs in further experiments.

We next asked whether $CD9^+$ human germ cells possess a capacity to colonize and repopulate nude mouse testes upon transplantation. A single cell suspension of human testis cells was first sorted immunomagnetically using an antibody against HLA, a MHC-I molecule. MHC-I has been known to be not expressed in human spermatogonia at the protein level (39-42). Thus, negative selection for HLA should be beneficial for SSC enrichment, particularly when SSCs are to be used for male fertility restoration in cancer patients. HLA^- cells were further sorted into the $CD9^+$ and $CD9^-$ cell populations. We recovered 66% of total human testis cells in the HLA^+ fraction, ~10% in the HLA^-CD9^+ fraction, and ~20% in the HLA^- $CD9^-$ fraction (Table 2).

Tables 2 : Cell recovery in percentage after immunomagnetic sorting of human testis cells

	Mean % ±SEM	**n**
$CD9^+$ only	**12.2 ± 0.4**	**8**
GFRα1 only	**3.2 ± 0.2**	**6**
HLA^-CD9^+	**9.6 ± 0.3**	**9**
HLA^-CD9^-	**19.4 ± 0.9**	**9**
HLA^+	**66.1 ± 0.8**	**9**

These three cell fractions as well as unsorted cells were transplanted into recipient testes of nude mice. Two or four months following transplantation, human germ cells that colonized and repopulated the xenogeneic testis environment were detected by whole-mount MAGEA4-staining of recipient testes (Fig. 3A-D, and supplemental Fig. S1). We confirmed that the MAGEA4 antibody did not cross-react with testis cells of intact mice, denoting that the antibody specifically identifies human germ cells in recipient mouse testes (Fig. 3E and F). To assess the

enrichment degree of human male germ cells with repopulating potential, we counted the number of cell groups, or colonies, and compared colony numbers across the cell fractions. We defined a colony as a group of 4 or more cells separated by less than one cell diameter.

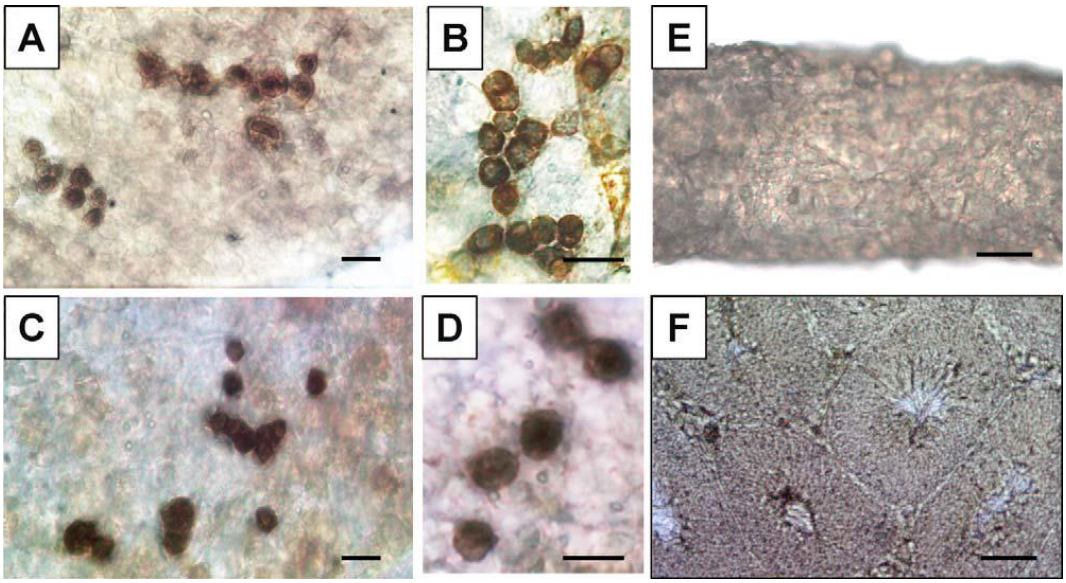
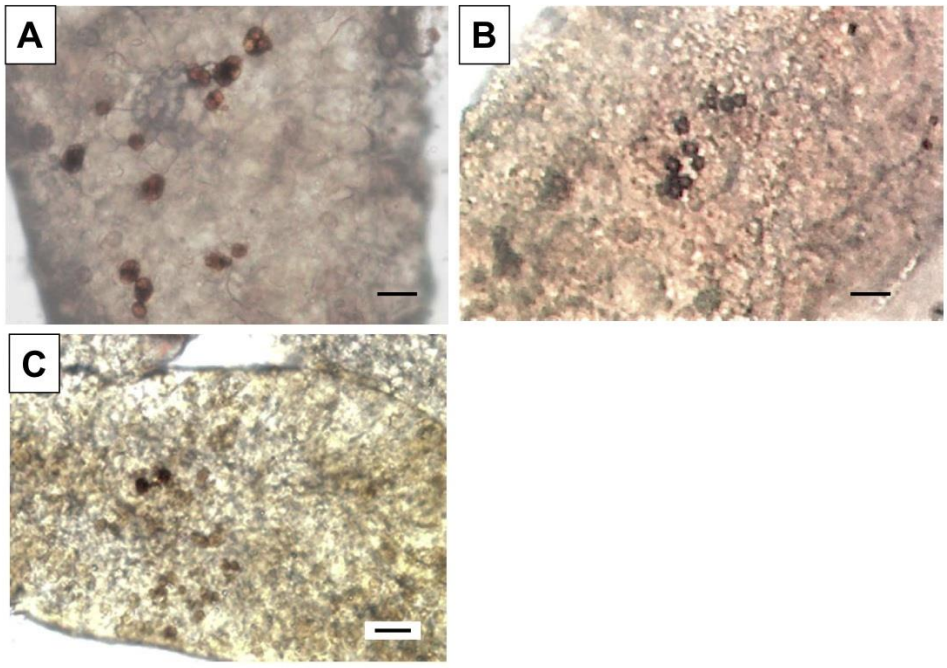


Figure 3: Whole-mount MAGEA4 staining of recipient mouse testes after transplantation. Detection of human germ cells in mouse testes using whole-mount MAGEA4 staining 2 months (**A,B**) and 4 months (**C,D**) after transplantation. An intact mouse seminiferous tubule (**E**) and tubule sections (**F**) stained with the MAGEA4 antibody show no positive signals, proving that the antibody does not cross-react with mouse testis cells. Scale bar = 50 µm.



Supplemental Figure 1: Whole-mount MAGEA4 staining of recipient mouse testes after transplantation. Images of MAGEA4-stained human germ cells in recipient testes are shown at a lower magnification. Scale bar = 50 μm.

Two months after transplantation, the number of colonies in the $HLA^{-}CD9^{+}$ cell population was approximately 4-fold greater compared to unsorted cells and 11.7-fold compared to $HLA^{-}CD9^{-}$ cells (Fig. 4). In the four month experiments, reflecting long term repopulation, the colony number declined in all groups in comparison to two month experiments. Nonetheless, the $HLA^{-}CD9^{+}$ population generated 3.3-fold greater numbers of colonies compared to the unsorted population and 37-fold greater compared to $HLA^{-}CD9^{-}$ cells (Fig. 4). The significantly increased colony numbers in $HLA^{-}CD9^{+}$ cells relative to unsorted cells at both post-transplantation times indicate that CD9 is expressed on human male germ cells that have the potential of long-term repopulation in the xenogeneic

testis environment, and that CD9-based cell sorting leads to enrichment of these cells.

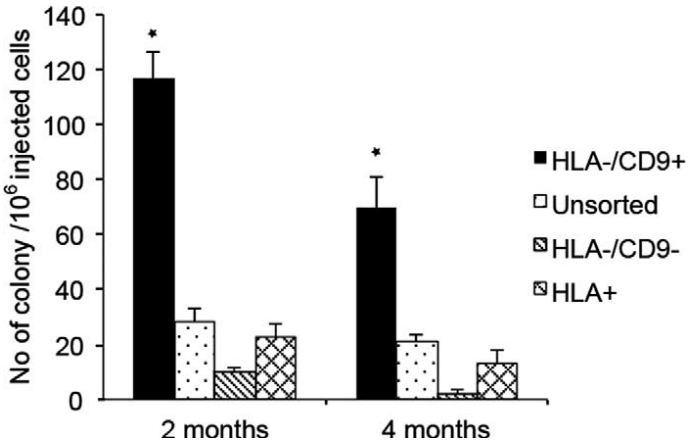


Figure 4: Number of colonies in recipient testes 2 and 4 months after transplantation. Colony numbers found with the $HLA^{-}CD9^{+}$ fraction are significantly greater than those found with any other fraction, indicating that CD9 is expressed on transplantable human germ cells ($p < 0.05$). Analyses for the $HLA^{-}CD9^{+}$, $HLA^{-}CD9^{-}$, HLA^{+}, and unsorted fractions were done using 15, 7, 7 and 11 recipient testes, respectively. Colonies are defined as a group of 4 or more cells separated by less than one cell diameter.

Finally, we compared the distribution of colony sizes (i.e., colonies containing different numbers of cells) between two and four months in the $HLA^{-}CD9^{+}$ and unsorted cell fractions. As shown in Fig. 5, the number of colonies containing 4 cells significantly declined from two to four months in both cell fractions, while that of colonies carrying 5 or more cells stayed constant or increased during the same time period.

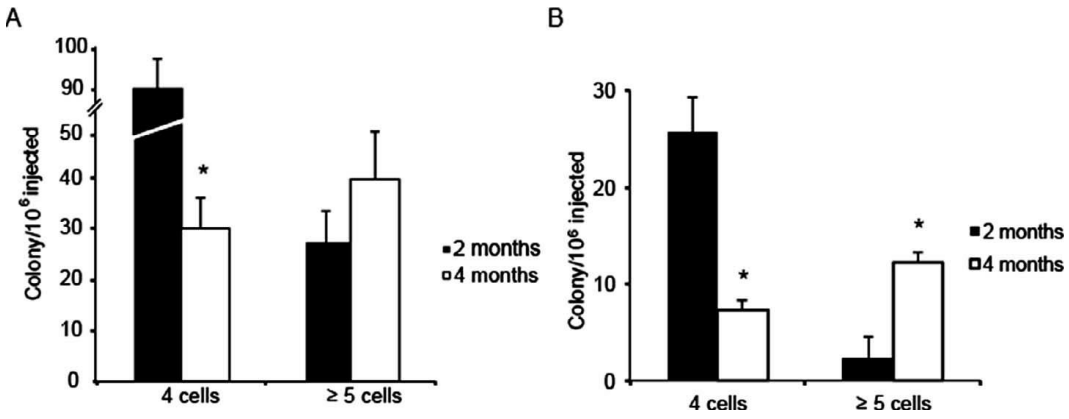


Figure 5: Colony size at two and four months after transplantation. Distribution of colony sizes between two and four months in the $HLA^{-}CD9^{+}$ cells (**A**) and unsorted cells (**B**). Colonies with 5 or more cells remain constant or increase in number from two to four months after transplantation, reflecting the long-term self-renewal capability ($p < 0.05$).

These results suggest that similar to mouse SSC transplantation (43), human germ cells in colonies did not remain dormant after transplantation and that sustainable turnover of these cells (e.g., proliferation/death) occurred from 2 months to 4 months after transplantation.

Discussion

In this study, we have found that human $CD9^{+}$ male germ cells possess the ability to migrate through and colonize the recipient seminiferous epithelium for at least 4 months upon transplantation. Hence, our results show for the first time that CD9 can be used as a cell-surface marker to enrich human testis cells for germ cells that repopulate recipient testes for a long time.

To detect human germ cells after transplantation, we used MAGEA4 as a human germ cell marker, which is expressed by human spermatogonia and, to a lesser extent, early spermatocytes. We confirmed that its antibody did not cross-react with mouse cells (Fig. 3E and F).

These characteristics of the marker are important because non-germ cells are known to colonize testes after transplantation (13, 44, 45). In xenotransplantation experiments, it is insufficient to visualize donor cells in recipient testes by simply using a species-specific marker alone, such as human-specific microsatellites, and therefore, additional parameters are required to confirm the cell type (12,13,45). The use of the MAGEA4 antibody in this study allowed for species and cell-type specific identification of colonizing donor cells as human germ cells. Since the consequence of germ cell transplantation is measured only by detection of differentiating germ cells, whole-mount detection for $MAGEA4^{+}$ cells allowed us to generate faithful readouts of human germ cell transplantation.

The markers for non-human primate SSCs have been analyzed with xenotransplantation (10). Using this approach combined with fluorescent-activated cell sorting (FACS), Hermann et al. have shown that Rhesus monkey spermatogonia expressing THY1 (CD90) exhibit enhanced activity to colonize mouse testes (13,45), suggesting that THY1 is conserved with rodent SSCs. Furthermore, $THY1^{+}$ cells were $PLZF^{+}$, $GFRA1^{+}$, $NGN3^{+/-}$, and KIT^{-} (CD117), which are also characteristics of mouse SSCs (13,45). Maki et al. used FACS and xenotransplantation and showed that putative Rhesus monkey SSCs express ITGA6 and THY1 (37). The expression of GFRA1, SSEA-4, PLZF, and GPR-125 was detected in these cells, while Nanog expression was low and Kit expression was undetectable. These findings have collectively led to the notion that some SSC properties are conserved in a wide range of mammalian species (10).

For human SSCs, several studies have reported GFRA1, ITGA6, GFR-125, and THY1 as cell-surface markers (30, 36, 39, 46). However, identification of target molecules in these studies relied on morphological/immunophenotypic characteristics or on the ability of human cells to proliferate in a short-term culture; thus, the cells were not identified on the basis of stem cell functional definition. Recently, Izadyar

et al. have shown that human spermatogonia expressing SSEA-4, which also express ITGA6, have a higher repopulation potential after xenotransplantation than non-expressing cells, indicating that SSEA-4 is a putative human SSC marker (11). Interestingly, this study has reported on THY1 expression in cells located in the adluminal compartment of human seminiferous epithelium. Furthermore, ITGB1 was not detected in colonizing human spermatogonia. Since these two antigens are mouse SSC markers, the results highlight apparent differences between human and mouse SSCs.

Interestingly, we observed that the number of colonies carrying 4 cells declined remarkably from 2 to 4 months after transplantation, but that of colonies with 5 or more cells was stable or even showed a trend to increase (Fig. 5). These observations suggest that donor human germ cells did not merely survive after transplantation, but some of them were replenished and/or proliferated, suggesting the presence of long-term self-renewing cells that sustain colonies of human germ cells, although we cannot rule out the possibility that these colonies enlarged simply because committed cells in selected colonies proliferated.

We employed in this study immunomagnetic cell sorting for putative human SSC enrichment, rather than FACS. Although FACS allows for more strict and multi-parameter cell sorting, immunomagnetic cell sorting has practical advantages, which could be beneficial for future clinical applications. First, magnetic cell sorting does not require as many cells as FACS does. Since the source of human germ cells in our study were biopsy specimens, the quantity of available cells was limited. This technical issue should also be an important consideration to clinically apply human SSCs, where harvesting a large number of testis cells is not expected to be a common practice. Second, immunomagnetic cell sorting is rapid and simple and does not require extensive experience for its adequate performance. Thus, this cell-sorting technique can be readily performed in clinical settings.

In this study, we pretreated human testis cells for negative cell sorting using MHC-I prior to CD9-based selection. Since MHC-I is expressed in all nucleated cells but not in spermatogonia, it is expected to be an important antigen to remove somatic cells, including tumorigenic cells contaminating testis cells. In addition, testis cells are highly heterogeneous, which can reduce the effectiveness of immunological cell sorting (47). Therefore, the negative cell sorting using MHC-I as a marker should have an additional advantage for human SSC enrichment.

On this basis, we included the MHC-I negative selection in the current study. Our results (Fig. 4), however, indicated that colony numbers of human germ cells observed in the HLA+ fraction were not significantly different from those in the unsorted fraction, despite a trend of slight decline. We speculate that this was caused by an inherent weakness of immunomagnetic cell sorting for precise separation and a large coefficient of variation of the in vivo transplantation assay. Nonetheless, a significant enrichment of repopulating human germ cells was detected in the HLA^{-} $CD9^{+}$ cells, when compared to HLA^{+} cells (Fig. 4). Thus, although our current data did not clearly demonstrate the effectiveness of HLA-negative selection alone, they did show that CD9 is an effective marker to enrich human testis cells for repopulating germ cells.

Our study adds CD9 to the list of markers for human male germ cells with repopulation potential. Identification of multiple human SSC markers and their degree of SSC enrichment efficiency will be important for biological characterization of these cells and should also provide the versatility in the choice of antigens for cell sorting. This is particularly important in the clinical context of harvesting SSCs for fertility preservation for cancer patients, since different types of tumors express varied markers. This notion is further emphasized by the recent studies reporting that leukemia stem cells evolve and change their immunophenotypic characteristics over time (8,9). Thus, further identification of additional human SSC markers will be beneficial to distinguish SSCs from cancer

stem cells. Future studies should also be directed to determining which marker allows for the most effective human SSC enrichment. These are important issues to be addressed to realize human SSC-based male fertility restoration in clinical settings.

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No competing financial interests exist.

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Chapter 3

Manuscript 2

Preface to Chapter 3

In chapter 2, I added the rodent SSC marker CD9 to the list of markers of human SSCs. Using human testicular tissues, I showed that CD9 is expressed by human male germ cells in the basal compartment of the testicular seminiferous epithelium co-localizing with other SSC markers. Next I used immunomagnetic sorting against CD9 antibody to successfully enrich for human germ cells that repopulated mouse testes for at least 4 months after transplantation. Therefore CD9 can be used in a multi-parameter selection of human SSCs, which may have applicability in the human fertility clinic.

In following chapter, I looked at the correlation between SSC recovery and fertility recovery kinetics after chemotherapy using a mouse model. I posit that the efficiency of male fertility restoration is dependent on the recovery of SSCs after cytotoxic treatment. I further attempted to identify a parameter for monitoring degree of SSC recovery.

The efficiency of male fertility restoration is dependent on the recovery kinetics of spermatogonial stem cells after cytotoxic treatment with busulfan in mice.

Running Title: Stem cell recovery reflects male fertility restoration

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ABSTRACT

Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis and represent a crucial resource for male fertility restoration. It has not been well documented, however, whether the recovery of SSC population size after cytotoxic damage associates with the kinetics of male fertility restoration. We addressed this issue using the mouse as a model. Following single injections of busulfan at 15, 30, or 45 mg/kg into male mice, we examined their ability to sire offspring at different times by natural mating and determined SSC numbers using spermatogonial transplantation. We measured testis physiological parameters (testis weights, sperm counts, serum and intratesticular testosterone levels, and histological assessments of spermatogenic recovery) and quantified the expression of glial-cell-line-derived neurotrophic factor (GDNF) transcripts. Regardless of busulfan doses, fertility was lost within 4 weeks after treatment, while more than 95% of SSCs were lost within 3 days. Fertility and SSC numbers gradually recovered with time, but the recoveries were delayed at higher busulfan doses. Interestingly, SSC numbers reached ~30% of before-treatment levels by 4 weeks prior to the time of fertility restoration, across the dose groups. Sperm counts were ~20% of before-treatment levels at the onset of fertility restoration, regardless of busulfan doses. We detected a significant increase in total GDNF mRNA per testis immediately after busulfan treatment. The loss and restoration of fertility after busulfan treatment are direct consequences of SSC loss and expansion. Our data suggest that there is a threshold in SSC numbers that allows for male fertility restoration and that the testicular somatic environment responds rapidly and temporarily to the loss of spermatogonia, including SSCs, by altering GDNF mRNA levels. This study provides fundamental information to clinically apply SSCs for male fertility restoration in the future.

INTRODUCTION

Spermatogonial stem cells (SSCs) are the stem cells of the male germ line and are the foundation of spermatogenesis. They are present on the basal membrane of the seminiferous tubules in the testis and surrounded by Sertoli cells, which function as an important component of the SSC niche. Recent studies have demonstrated that a Sertoli cell-derived growth factor, glial cell line-derived neurotrophic factor (GDNF), plays a key role to promote SSC survival and self-renewal, thereby stimulating SSC proliferation in vivo and in vitro (1-6). SSCs are detected by their function to regenerate and maintain spermatogenesis. The unequivocal assay to identify SSC function is spermatogonial transplantation (7, 8). In this assay technique, donor testis cells are injected into the seminiferous tubules of a recipient male in which endogenous germ cells have been depleted. SSCs present in the injected cell suspension colonize the recipient seminiferous epithelium and establish colonies of donor-derived spermatogenesis. As each colony is derived from a single SSC (9-11), spermatogonial transplantation allows for the quantification of functional SSCs based on colony numbers.

Clinically, spermatogonial transplantation is expected to provide a powerful tool to preserve and restore male fertility (3).With an increasing survival rate of cancer patients in recent years, infertility caused by anti-cancer treatments has become a significant concern for cancer survivors. One SSC-based strategy to address such a concern is to harvest SSCs before the treatments and autologously transplant them into a patient's testes later. Since SSCs are present in the testis from the time of birth, this strategy should be beneficial to men of any age.

Germinal epithelial damage is a recognized consequence of certain chemotherapeutic agents and radiotherapy. This was first reported in 1948 where the absence of spermatogenesis in the seminiferous tubules was observed in 27 of 30 men treated for lymphoma with nitrogen mustard (12). By the late 1960's there were similar reports of testicular toxicity due

to alkylating agents, such as busulfan, chlorambucil, and cyclophosphamide (13)

Busulfan (1,4-butanediol dimethanesulfonate) is often used as a conditioning regimen prior to progenitor cell transplantation for treatment of chronic myelogenous leukemia and can induce prolonged azoospermia (14). It shows cytotoxic effects through the formation of DNA-DNA cross-links, DNA-protein cross-links, and single strand breaks. Busulfan exerts its toxic effects on cells that are at the G_1 phase at the moment of treatment. These cells are killed in the following mitosis, while those that are in S or G_2 phase are killed in the subsequent mitosis (15). In the testis, busulfan preferentially kills spermatogonia of several species, leading to male infertility (16). When administrated to pregnant animals, busulfan produces germ cell-free gonads in the offspring (17, 18).

Bucci and Meistrich (15) showed that the duration of male infertility was dependent on the extent of stem cell depletion that occurred in a dose-dependent manner, using spontaneous regeneration of spermatogenesis after busulfan treatment as a functional measure of SSCs. This observation indicated that SSCs plays critical roles not only to maintain steady-state spermatogenesis but also to restore spermatogenesis and male fertility. Kanatsu-Shinohara et al. used spermatogonial transplantation to quantify SSCs after cytotoxic damage induced by a single dose of busulfan (15 mg/kg) and provided the evidence that SSCs can expand in vivo (1, 2).

These studies indicate that SSC numbers are crucial to confer fertility; however, some important questions still remain to be addressed. First, to what extent does the size of the SSC population need to recover to confer fertility in a male? Second, do the SSC recovery kinetics influence the process of fertility restoration? In addition, does the degree of killing of SSCs affect their expansion during the course of male fertility restoration? Therefore, the linkage between SSC recovery kinetics and the patterns of male fertility restoration has not been well documented. Since SSCs are

expected to be an important resource for male fertility preservation and restoration (3), such a linkage needs to be determined in order to lay a foundation for clinical applications of SSCs. In this study, we aimed at providing direct functional evidence that the recovery of SSCs following cytotoxic damages induced by busulfan reflects the restoration of male fertility using the mouse as a model. We also analyzed multiple physiological parameters related to male reproduction (testis weights, sperm counts, testosterone levels, histological analyses of spermatogenesis, and levels of GDNF transcripts in the testis) in an attempt to identify a parameter that could allow for monitoring the degree of SSC recovery.

MATERIALS AND METHODS

Donor mice and busulfan treatment:

Adult (>6weeks of age) B6ROSA transgenic mice, F1 hybrids of C57BL/6 (B6) and ROSA26 (B6; 129-S-*Gt(ROSA)26Sor*/J, Jackson Laboratory), were used for mating and transplantation experiments. These mice express the LacZ gene ubiquitously, including all types of germ cells (8, 19). Five mice per dose (in mating experiments) and three to five mice per dose (in transplantation experiments) were treated with a single intra-peritoneal injection of 15, 30, or 45 mg/kg of busulfan (Sigma), which was first dissolved in dimethyl sulfoxide (Sigma) before equal volume of distilled water was added to provide the desired final concentration.

In mating experiments, each male was mated with two B6 females for five days every other week, starting from 2 weeks after busulfan injection. Mated females were kept to observe pregnancy.

In transplantation experiments, a single cell suspension of donor cells was prepared using a two-step enzymatic digestion of the testis as described previously (20). The number of cells recovered was determined

using a hemocytometer, and cell viability determined by Trypan blue exclusion, which was 96.3 ± 0.7%.

All animal procedures were approved by the Institutional Animal Care and Use Committee of McGill University.

Recipient mice and transplantation procedure:

Recipient mice were F1 hybrids of 129/SvEv x B6, which are immunocompatible with donor mice. Recipient mice were treated with 50 mg/kg of busulfan at 4 weeks of age to destroy endogenous spermatogenesis and used in spermatogonial transplantation 4 or more weeks later (20). Donor testis cells were resuspended in Dulbecco modified Eagle medium (DMEM) at a concentration of 100×10^6 cells/ml, and 6 to 7 µl of cell suspension were injected into the seminiferous tubules through the rete testis (20). Cells from each donor were transplanted into at least 5 recipient testes. Recipient testes were analyzed for SSC quantification 2 months post-transplantation by staining with 5-bromo-4-chloro-3-indolyl β-galactosidase (X-gal) (20). The number of donor-derived spermatogenic colonies, thus the number of SSCs, was obtained for each donor male as a mean of values collected from all recipient testes used for a specific donor mouse; i.e., although multiple recipients were used, only one number of SSCs was derived for one male treated with each dose of busulfan and at each time point. Results were expressed as numbers of colonies (i.e., functional SSCs) per donor testis, using the number of cells transplanted and that of total cells recovered from a donor testis. The expansion rate of SSCs was calculated by dividing the increase in SSC numbers in a given time interval by the number of days in the same time frame (i.e., cells per day).

Testis weight and sperm count:

Testis weights were measured at the time of euthanasia without removing the tunica. To determine sperm counts, sperm were collected from the cauda epididymis during the course of fertility recovery, as follows. Briefly, the epididymis was clamped in each mouse, and the cauda was dissected and transferred to DMEM supplemented with 0.5% BSA at 37º C. The epididymis was then minced and sperm allowed for dispersing for 5 minutes. Cauda sperm counts per mouse were determined using a hemocytometer. At least 3 mice were analyzed for each time point at each dose.

Histological analyses:

Testes of busulfan-treated mice were fixed in Bouin's solution (Sigma-Aldrich) and embedded in paraffin. Sections of 5 μm thickness were taken in 25- μm section steps (i.e., 1 in every 5 sections was examined) and were stained with haematoxylin and eosin and observed under a light microscope. To determine the proportion of tubules showing spermatogenesis, we counted the number of seminiferous tubule cross-sections with or without spermatogenesis, using the method reported by Kanatsu-Shinohara et al. (1, 2), as follows. Tubule sections were judged to be positive for spermatogenesis when germ cells occupy the basal membrane along the entire tubule circumference and when at least two layers of germ cells are found; otherwise, the sections were identified as negative for spermatogenesis (1, 2). This method can reflect changes in the population of spermatogonia, which are the first cell type that are lost after busulfan treatment and are also regenerated after the loss of spermatogenesis. Then, the proportion of the sections positive for spermatogenesis was recorded. The values for each time point at each dose group were determined in three replicates, in each of which at least 5 sections and an average of 50 tubules/section was examined.

Serum and intratesticular testosterone levels:

To determine serum testosterone levels, 1-2 ml of blood was collected from the jugular veins of donor mice before euthanasia, centrifuged to isolate plasma, and stored at –80° C until analyses. To measure intratesticular testosterone levels, testes were poked with a needle and centrifuged for 30 min at 500 x *g*, and the intratesticular fluid collected was stored at –80° C. Testosterone levels were measured using the Testosterone ELISA kit (Immuno-Biological Laboratories inc, USA, catalog No.: IB79106) according to the manufacturer's instructions.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR):

GDNF mRNA levels were examined using qRT-PCR in testes of mice treated with 15 and 30 mg/kg busulfan. Total RNA was prepared using PicoPure RNA isolation kit (Arcturus) according to the manufacturer's instructions. Complementary DNA was synthesized using Superscript III reverse transcriptase (Invitrogen) with random hexamers. Primer sequence used for transcriptional analysis of GDNF was: (Forward) 5'-TAATGTCCAACTGGGGGTCT -3' and (Reverse) 5'-CGCTTCGAGAAGCCTCTT AC -3'. qPCR was performed with QuantiTec SYBR Green PCR kit (Qiagen) on a Rotogene 6000 (Corbett Research) with the program: 94°C for 15 min followed by 40 cycles of 94°C for 15 seconds/ 58°C for 25 seconds/ 72°C for 35 seconds. Samples were run in triplicate, and the average Ct (threshold cycle) values for GDNF were normalized to those for GAPDH. To generate fair comparisons (see Results), the GDNF transcript levels were normalized to a whole testis level for each time point and in each dose group, using the following formula: (C_t GDNF/C_t GAPDH) x (Total RNA per testis / Total RNA per reaction). Then, the data were expressed relative to the day 0 value.

Statistical analyses:

Statistical analyses were done using t-test or, for multiple comparisons, ANOVA followed by Fisher's Test for Least Significant Difference post hoc test. Significance was determined when $p < 0.05$. All data are expressed as the mean ± SEM.

RESULTS

We first determined the profile of fertility loss and recovery over time after a single injection of busulfan at 15, 30, or 45 mg/kg (Fig. 1). Five mice were used for each dose-group and the fertility capacity was determined as the number of males that sired pups in each dose-group and at each time point. Results showed that all males were fertile across groups at 2 weeks after busulfan injection. At 3 weeks, all males still remained fertile in the 15 mg/kg group, whereas four and two out of five males sired pups in the 30 and 45 mg/kg groups, respectively. By 4 weeks, all mice lost fertility regardless of busulfan doses. Following a period of infertility, all males regained fertility by 12 weeks and 26 weeks in the 15 and 30 mg/kg groups, respectively, and remained fertile for at least the next 6 weeks until the mating was terminated. In the 45 mg/kg group, three out of the five mice regained fertility by 30 weeks, while the other two remained infertile until the end of the study (36 weeks).

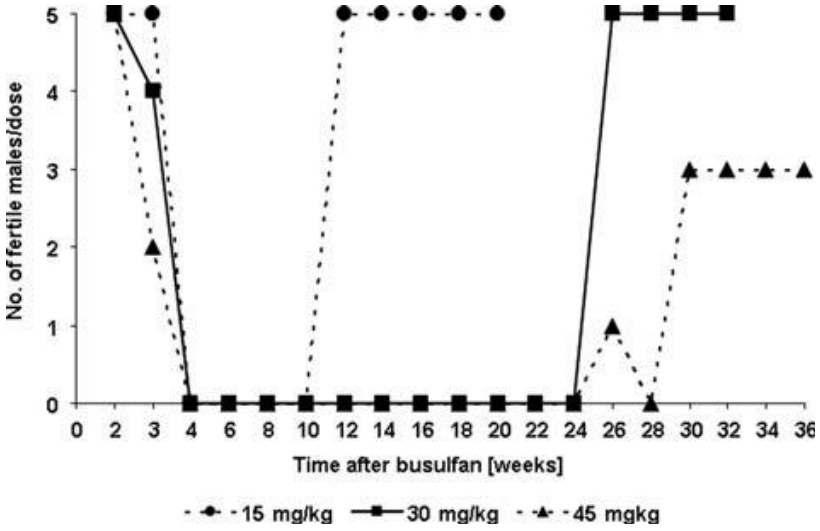
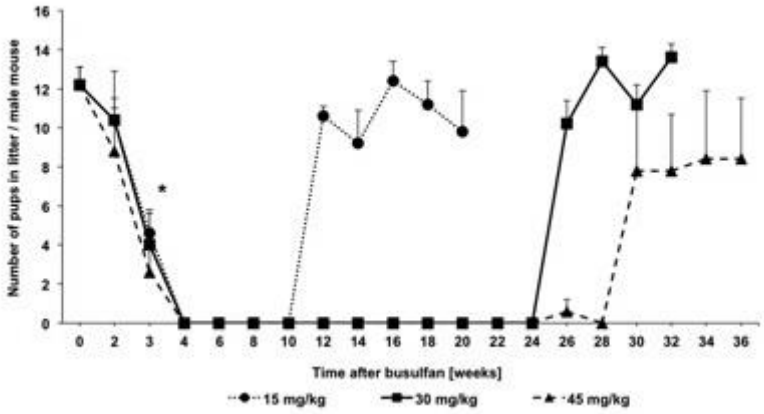


Figure 1 : Number of fertile males after treatment with 15, 30, or 45 mg/kg of busulfan. Five mice were treated per dose and each mouse was mated with 2 females for 5 days. Note that all mice lose fertility by 4 weeks post treatment, regardless of busulfan doses. After treatment with 15 and 30 mg/kg of busulfan, all mice regain fertility at 12 and 26 weeks, respectively, and remain so for at least another 6 weeks. After treatment with 45 mg/kg busulfan, 3 out of 5 mice regain fertility at 30 weeks while the other two do not by at least 36 weeks.

The changes in the litter size were similar to those of male fertility (Supplementary Fig. 1 vs. Fig. 1). When male fertility was restored at 12, 26 and 30 weeks in the 15, 30 and 45 mg/kg groups, respectively, the litter size showed no significant differences compared to that observed before busulfan treatment. Male and female offspring in the first litter born at the time of fertility recovery were kept in all busulfan-dose groups and examined for their fertility. No defects were observed in their gross appearance during postnatal development. When mated with wild-type mice, all male and female offspring produced pups with a normal litter size, indicating that SSCs recovering after busulfan treatment were capable of generating functional gametes and offspring with normal fertility.



Supplementary Figure 1 : Litter sizes after busulfan treatments at 15, 30, and 45 mg/kg. Litter sizes dropped significantly 3 weeks after treatment and were restored to normal levels at 12, 26, and 30 weeks after treatment with 15, 30, and 45 mg/kg of busulfan, respectively. Asterisk indicates significant differences from pretreatment values.

To quantify functional SSCs during the fertility recovery period, male mice were injected with 15, 30, or 45 mg/kg of busulfan, and each was used as a donor for the spermatogonial transplantation assay. As shown in Fig. 2A, nearly all SSCs were lost by day 3 after busulfan treatment. On day 3, SSC numbers per testis were 4%, 0.8%, and 0.4% of the pretreatment level (i.e., determined on day 0) after 15, 30, and 45 mg/kg busulfan, respectively; a significantly higher number of SSCs was detected with the 15 mg/kg group than with the others (Fig. 2B). Thereafter, SSC numbers gradually increased (Fig. 2A), and in the 15 mg/kg group, they reached 28.6% of the pretreatment level by 8 weeks and 70.5% by the time of fertility recovery (12 weeks). In the 30 mg/kg group, 28.5% of SSC restoration was observed by 22 weeks, while it reached 81% of the pretreatment level by the time of fertility recovery (26 weeks). Similarly, in the 45 mg/kg group, 32.7% and 77.7% of SSC were restored by 26 weeks and by the time of fertility recovery (30 weeks), respectively.

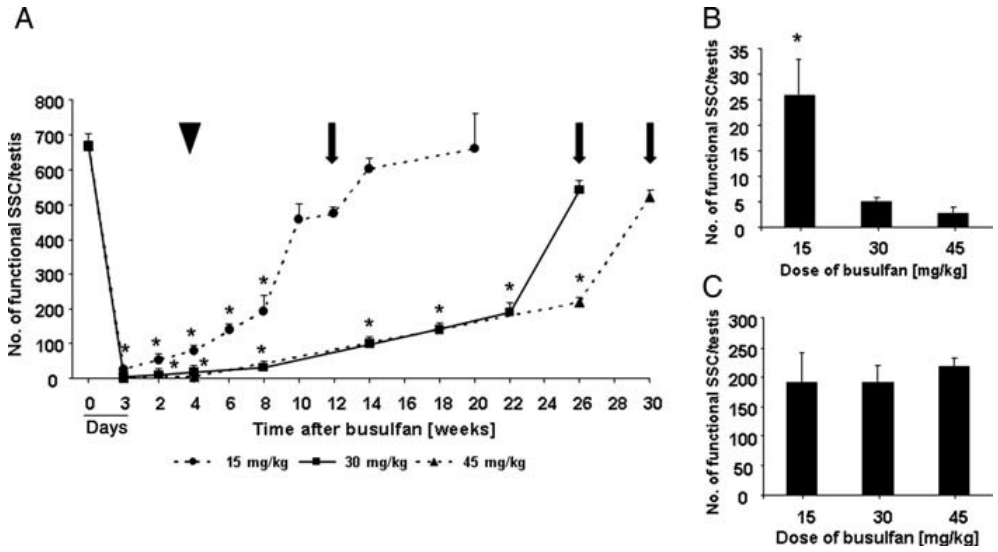


Figure 2: SSC numbers determined by spermatogonial transplantation after treatment with busulfan at 15, 30, and 45mg/kg. (**A**) Kinetics of SSC recovery during the study periods. The timing of fertility loss and restoration is indicated by arrowhead and arrow, respectively. Most SSCs are lost after treatment with busulfan (95%, 99.2%, and 99.6% at 15, 30, and 45 mg/kg busulfan, respectively) but their numbers increase gradually with time. At the onset of fertility recovery, 70-80% of pretreatment levels are present (472, 542, and 520 in 15, 30, and 45mg/kg busulfan at 12, 26, and 30 weeks, respectively) with no significant difference detected among the three groups. Five donors were used in control (day 0), at day 3 and 12 weeks at 15mg/kg, and day 3 at 30 mg/kg, while four donors at 14 weeks at 15 mg/kg, 8 and 18 weeks at 30 mg/kg, and day 3 at 45 mg/kg. Three donors were used in all others. (**B**) Three days after busulfan treatment, significantly higher SSC numbers are detected with the 15 mg/kg group than the others. (**C**) Four weeks before fertility recovery, SSC numbers reach ~30% of pretreatment levels regardless of busulfan doses. No significance is detected across the groups. Asterisks (A and B) indicate significant differences from pretreatment values.

Interestingly, these data indicated that regardless of busulfan doses, SSC numbers reached approximately 30% of the pretreatment level 4 weeks before the time of fertility recovery, while the numbers were 70 to 80 % at the time of fertility recovery; no significant differences were detected in both times among the dose groups (Fig 2A,C). From these data, we calculated that SSCs expanded at 3 cells/day in the 15 mg/kg group and 1.2 cells/day in the 30 and 45 mg/kg groups until 4 weeks prior to fertility recovery, when SSC numbers reached 30% of the pretreatment level. Once this level of recovery was achieved, the SSC expansion accelerated to 10-12.5 cells/day in all groups until the time of fertility recovery.

Since we noted a correlation between kinetics of SSC recovery and those of fertility restoration, we examined if there is a physiological parameter that allows us to monitor SSC recovery kinetics and thus, male fertility restoration, without using spermatogonial transplantation. To this end, we first assessed testis weights and sperm counts. Testis weights declined after busulfan treatment to 50% of the pretreatment level by the time of fertility loss (4 weeks) in the 15 and 30 mg/kg groups (Fig. 3A). Thereafter, the weights gradually increased in the 15 mg/kg groups but decreased further in the 30 mg/kg group until 8 weeks before initiating recovery. In the 45 mg/kg group, testis weights were 36% of control level at 4 weeks and also at 26 weeks. At the time of fertility recovery, animals in the 15 and 30 mg/kg groups showed a full recovery of testis weight whereas those in the 45 mg/kg group reached 73% of pretreatment level (Fig. 3A), which was significantly lower than in the other two groups (Fig. 3B).

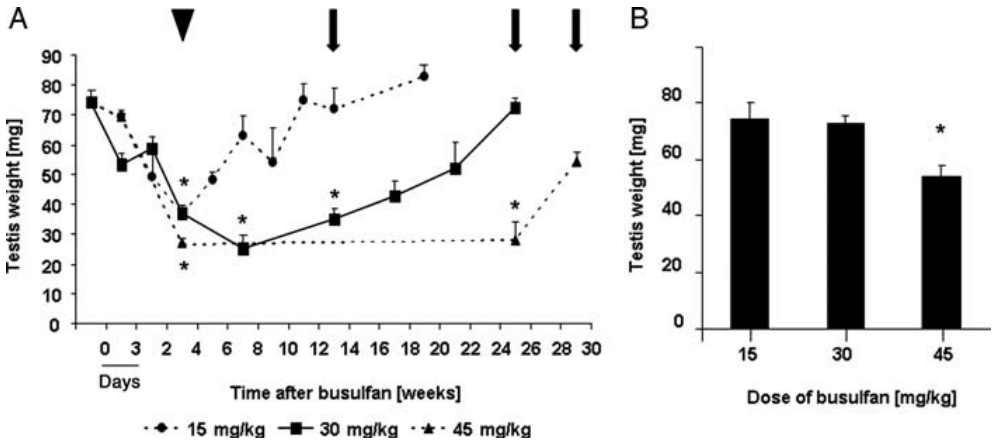


Figure 3 : Testis weights after busulfan treatments at 15, 30, and 45 mg/kg. (**A**) Time course of loss and recovery of testis weights. Asterisks indicate significant differences from pretreatment values. The timing of fertility loss and restoration is indicated by arrowhead and arrow, respectively. (**B**) Testis weights at the time of fertility recovery in each dose group. The values are significantly lower in the 45 mg/kg group compared to the other two groups, indicated by asterisks.

We collected sperm from the caudal part of the epididymis, as described in Material and Methods. Sperm counts declined until 4 weeks after busulfan treatment in all groups (Fig. 4A). At this time point, sperm counts were 8.8% of the pretreatment level in the 15 mg/kg group, and further declines were observed with higher busulfan doses (2.3% of pretreatment level in the 30 mg/kg group and undetectable levels at 45 mg/kg groups). Later, sperm counts increased with time in all groups. At the time of fertility recovery, sperm counts were ~20 % of the pretreatment level regardless of busulfan doses. Although there was a trend at the time of fertility restoration that mice treated with 30 and 45 mg/kg busulfan produced somewhat lower numbers of sperm than those treated with 15 mg/kg busulfan, we did not detect significant differences (Fig. 4B; $p \leq 0.136$ in 15 vs. 30 mg/kg and $p \leq 0.061$ in 15 vs. 45 mg/kg). These results

suggest that ~20% of normal sperm counts may represent a threshold value that confers the fertility to a male mouse.

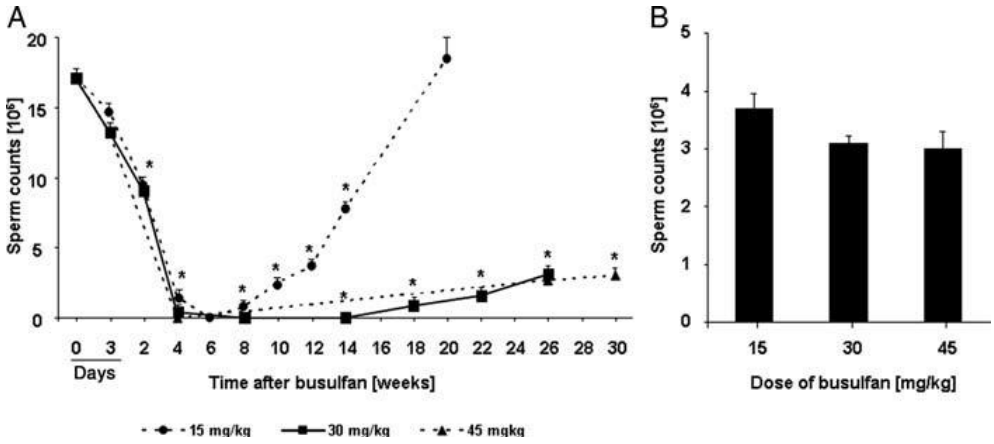


Figure 4 : **Sperm counts after busulfan treatments at 15, 30, and 45 mg/kg.** (**A**)Time course of loss and recovery of sperm counts. Asterisks indicate significant differences from pretreatment values. (**B**) At the time of fertility recovery, sperm counts are ~20% of pretreatment levels in all dose groups and show no significant differences.

Although the recovery kinetics of testis weights and sperm counts appeared to correspond to those of SSCs generally, we did not observe a clear shift in the kinetics in both parameters as we did with SSC recovery.

To evaluate the relationship between the recovery of SSC numbers and that of spermatogenesis, we analyzed the histology of the seminiferous tubules in paraffin sections (Fig. 5). Consistent with the results of past studies (15, 21), we observed that cells in the basal compartment of the seminiferous epithelium were the first to disappear by 3 days after busulfan treatment, regardless of busulfan doses (Fig. 5A,B). By 4 weeks, a significant number of the tubule sections lost germ cells in all treatment groups (Fig. 5 C,D). Thereafter, the epithelium was reconstituted gradually and continuously with time (Fig. 5E-H).

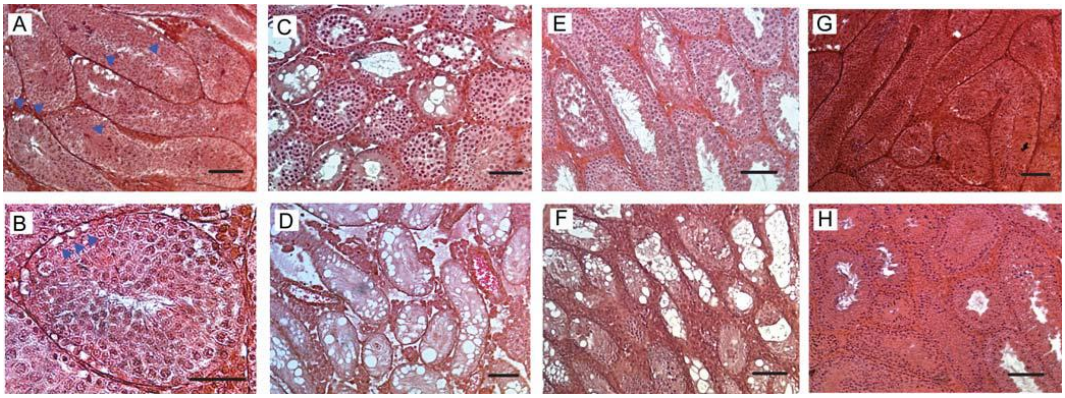


Figure 5 : The structural recovery of the seminiferous epithelium after busulfan treatment as determined in histology. Paraffin sections are stained with haematoxylin and eosin. (**A, B**) Cells in the basal compartment of the seminiferous epithelium are the first to disappear by 3 days; some are depicted by blue arrow heads. A low magnification after 15 mg/kg busulfan is shown in (A) and a higher magnification after 30 mg/kg busulfan in (B). (**C, D**) By 4 weeks, germ cell loss is seen in significant numbers of tubule sections, and damage is more evident with a higher dose of busulfan (C at 15 mg/kg vs. D at 30 mg/kg). (**E, F**) By 8 weeks after treatment, greater reconstitution of the epithelium is seen at 15 mg/kg (E), compared to 30 mg/kg (F). (**G, H**) At the time of fertility recovery, a majority of tubules show spermatogenesis (G at 15 mg/kg, H at 45 mg/kg). Observations derived with busulfan treatment at 45 mg/kg are similar to those derived with 30 mg/kg. Scale bars = 70 μm (A, E, G and H), 50 μm (B) and 100 μm (C, D and F).

To give a quantitative measure to the qualitative observations described above, we determined the proportion of seminiferous tubules with spermatogenesis compared to pretreatment values, as defined by Kanatsu-Shinohara et al. (2) (see Materials and Methods). As shown in Fig. 6, the proportion continuously declined by 4 weeks to 24% of pretreatment levels in the 15 mg/kg group and to 14% in the 30 and 45 mg/kg groups. Thereafter, it increased until the time of fertility recovery

where complete spermatogenesis was observed in ~70% and ~90% of tubule sections at 4 weeks before and at the time of fertility recovery, respectively, across the dose groups.

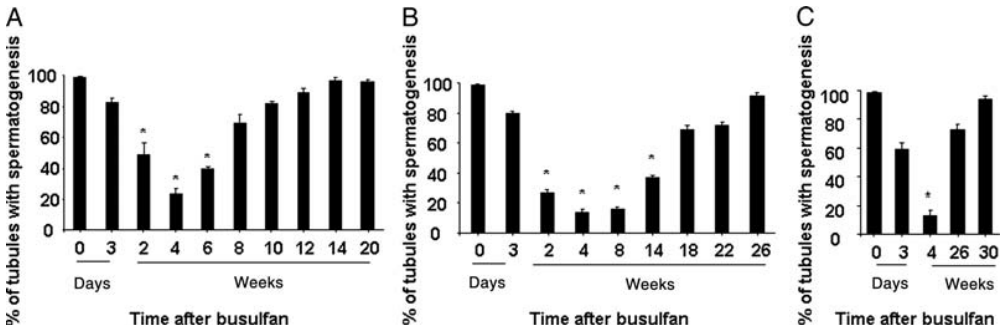


Figure 6 : Quantitative measurements of reconstitution of the seminiferous epithelium, as observed in Figure 5. The proportion of tubules with spermatogenesis was measured using the method of Kanatsu-Shinohara et al. (2), as described in Materials and Methods. Scores after treatment with15 mg/kg of busulfan are presented in **A**, those with 30 mg/kg in **B**, and with 45 mg/kg in **C**. Asterisks indicate significant differences from pretreatment values.

The morphological parameters examined above (testis weights, sperm counts, and testis histology) can be affected by testosterone. We thus assessed levels of serum and intra-testicular testosterone in the mice treated with 15 or 30 mg/kg busulfan. The results showed that both levels remained constant throughout the study period (Fig. 7). These results are consistent with previous reports (15, 22), that busulfan treatment does not alter significantly the steroidogenic environment in mice. Our data thus confirm that testosterone does not affect the restoration of male fertility and the kinetics of SSC recovery in mice.

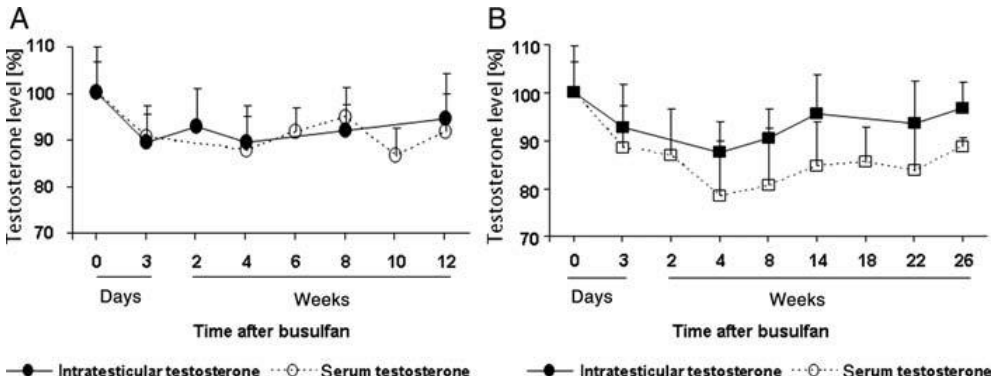
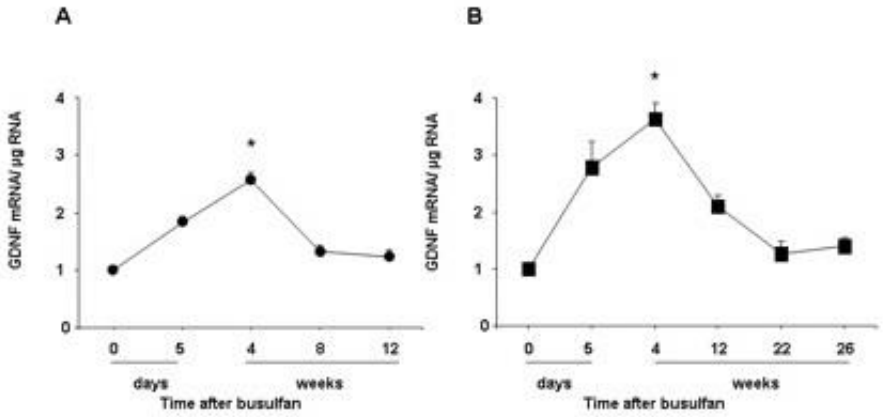


Figure 7 : Intratesticular and serum testosterone levels after busulfan treatments at 15 mg/kg (**A**) and 30mg/kg (**B**). Data are represented as percentages of pretreatment levels, compared to which no significant differences are detected for both levels and in both dose groups.

Since GDNF, which is expressed by Sertoli cells in the testis, is known to be a critical paracrine factor that promotes SSC self-renewal, we measured its mRNA levels using qRT-PCR after 15 and 30 mg/kg busulfan treatments. The expression levels were initially measured in comparison to those of a house-keeping gene (GAPDH). As shown in Supplementary Figure 2, the relative abundance of GDNF transcripts increased up to 4 weeks after transplantation, during which germ cells are gradually lost (Figs. 5 and 6), and returned thereafter to the pretreatment level.



Supplementary Figure 2 : GDNF mRNA expression levels per microgram total RNA used in one reaction of RT-PCR after busulfan treatments at 15 mg/kg (**A**) and 30 mg/kg (**B**), measured by qRT-PCR and expressed as relative to pretreatment levels (day 0). GDNF transcript levels increase up to 4 weeks after busulfan treatment and return to the pretreatment levels thereafter in both dose groups. Asterisks mark significant differences.

However, busulfan is known to eliminate germ cells but not affect testicular somatic cells numbers, including Sertoli cells (15); therefore, our data of GDNF transcripts may be biased because of a fluctuation of the Sertoli cell concentration in a testis when germ cell numbers decline or increase during the study periods. To circumvent this problem, we normalized the qRT-PCR results to total levels of GDNF transcripts in an entire testis (see Materials and Methods). Since Sertoli cell numbers have been reported to not change after busulfan treatment (15), this normalization should provide more accurate analyses.

Total GDNF mRNA levels per testis increased significantly by day 5 after treatment in both dose-groups (1.23-fold ± 0.03 and 1.81-fold ± 0.09 at 15 and 30 mg/kg busulfan, respectively vs. the pretreatment level) (Fig.

8). By 4 weeks and afterwards, the mRNA level showed no significant difference compared to the pretreatment level.

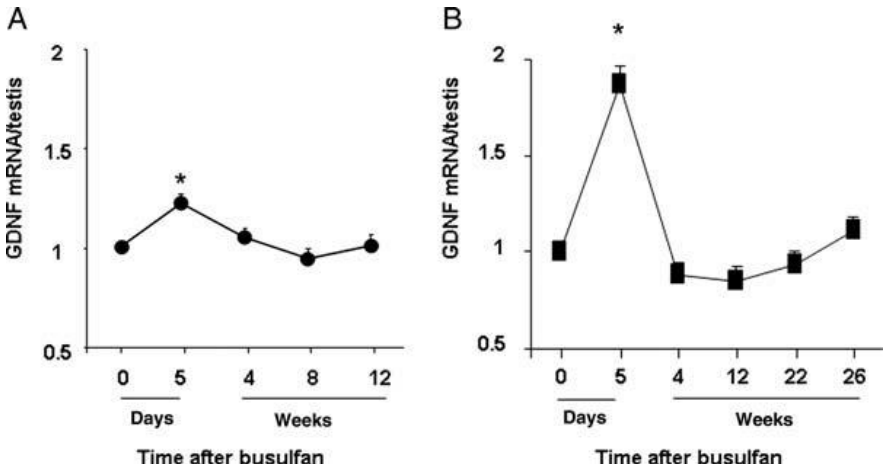


Figure 8 : GDNF mRNA expression levels per testis after busulfan treatments at 15 mg/kg (**A**) and 30 mg/kg (**B**), measured by qRT-PCR and expressed as relative to pretreatment levels (day 0). GDNF mRNA levels increase significantly in both dose groups only at day 5 after treatment, compared to pretreatment levels. Asterisks mark significant differences.

Thus, the data showed that the increase in GDNF mRNA levels coincided with the period of rapid loss of SSCs and spermatogonia after busulfan treatment (Figs. 2 and 5), suggesting that Sertoli cells may respond rapidly and temporarily to the loss of spermatogonia, including SSC, by increasing the expression of GDNF transcripts.

DISCUSSION

In this study, we demonstrated that kinetics of fertility restoration correlated with those of SSC recovery in mice. Importantly, we found that SSCs restored their population size to ~30% of the pretreatment level, regardless of busulfan doses examined, by 4 weeks before males became capable of siring offspring by natural mating. Although the 30% level was

determined based on pretreatment levels, rather than those of age-matched controls, this finding suggests that there may be a threshold in the SSC population size that allows for eventual restoration of male fertility in a defined time frame (~4 weeks after the 30% threshold is reached in mice). Further, SSCs shifted their expansion kinetics around the time of the 30% threshold, and the expansion accelerated once the threshold level was achieved. These results demonstrate that following cytotoxic effects of busulfan, the restoration of male fertility is closely linked to the recovery kinetics of the SSC population size, further emphasizing the fundamental role of SSCs in male reproduction.

It was unexpected to us that there was a shift in SSC expansion kinetics during fertility restoration where SSCs initially increased their numbers more slowly but accelerated later. A previous study showed a linear increase in SSC numbers without a threshold following busulfan treatment, although the dose of busulfan examined was only 15 mg/kg (1, 2). The cause of this discrepancy is unclear, but the difference in strains of donor and recipient mice between the two studies could have contributed to it; the H2 haplotypes of donors and recipients were different in the previous study while they were identical in ours. Nonetheless, it is notable that we observed a near-identical shift of SSC expansion kinetics with all busulfan doses examined. The results thus suggest that even though SSCs do expand following the initial massive reduction of the stem cell pool induced by cytotoxic effects of busulfan, they may commit to differentiation more preferentially until the population size reaches a threshold level; our observation that regeneration of spermatogenesis was seen in 70 – 80% of tubules at the time of the 30% threshold also corresponds to this notion (Figs. 2 and 6). Thereafter, SSCs may accelerate their expansion to regenerate an appropriate size of the stem cell pool to sustain steady-state spermatogenesis.

In this regard, we reported previously that when mouse SSCs derived from 1-week-old pups or adult males with experimental cryptorchidism

were transplanted into recipient testes, more committed daughter cells were produced, compared to when SSCs derived from adult intact testes with steady-state spermatogenesis were transplanted (23). Together with the results of the current study, these observations suggest that even though SSCs continue to proliferate during the study periods, SSCs tend to produce differentiated cells and functional gametes initially at the expense of robust SSC proliferation, compared to the later stages of SSC recovery; i.e., SSC fate decision appears to be skewed towards differentiation until the population size reaches the threshold level. Such a SSC behavior could be beneficial to rapidly produce functional gametes and efficiently achieve male fertility restoration.

Our data of initial SSC killing and physiological parameters are generally in agreement with those reported in the past (1, 2, 15, 21). Contrary to our results, however, Bucci and Meistrich (15) did not observe fertility restoration when the busulfan dose exceeded 28 mg/kg. The difference in mouse strains used may have caused this discrepancy. Nonetheless, we found that more SSCs survived at a lower busulfan dose (15 mg/kg) than at higher doses (30 and 45 mg/kg) (Fig. 2), a trend that was observed in the previous study (15). Interestingly, our data show that a higher survival of SSCs is associated with a greater SSC expansion rate until SSC recovery reaches the 30% threshold (3 cells/day at 15 mg/kg vs. 1.2 cells/day at 30 and 45 mg/kg; Fig. 2A,B). These observations suggest that an initial population size of surviving SSCs may influence a later trajectory of SSC expansion. It is also possible, however, that higher doses of busulfan may have damaged SSCs more significantly, and thus, a longer time was necessary for SSCs to recover. Another possibility is that a high dose of busulfan might have affected some actions of the somatic environment. For example, after treatment with 45 mg/kg busulfan, SSC numbers recovered to a level comparable to those seen in other dose groups at the time of fertility restoration (Fig. 2A), but testis weights were significantly lower (Fig. 3B). Furthermore, the recovery of

sperm counts showed remarkably contrasting kinetics between busulfan doses at 15 mg/kg and 30 and 45 mg/kg (Fig. 4A). Hence, we cannot rule out the possibility that busulfan could affect functions of the somatic environment to support spermatogenic recovery. Further studies are necessary to address these possibilities.

Since the restoration of the SSC population associates with the recovery of male fertility after cytotoxic damage, a physiological parameter that correlates with SSC expansion should provide an approach to monitoring the process of male fertility recovery and could perhaps predict the timing of fertility restoration. In this study, we examined various physiological parameters but were not able to identify those that can faithfully correlate with the recovery of the SSC population, particularly the shift of SSC expansion kinetics. Since GDNF plays a critical role in promoting SSC self-renewal, we reasoned that its transcript levels could reflect SSC behaviors more faithfully. Although the data did not support our reasoning, they showed that the expression of GDNF transcripts was temporarily elevated soon after busulfan treatment (Fig. 8), suggesting that Sertoli cells may promptly respond to the loss of spermatogonia and drive the survival and expansion of SSCs. Notably, a magnitude of increase in GDNF transcripts on day 5 was greater when the busulfan dose was 30 mg/kg than 15 mg/kg (1.2-fold vs. 1.9-fold after 15 and 30 mg/kg, $p \leq 0.0001$) (Fig. 8), raising the possibility that Sertoli cells may have expressed GDNF transcripts according to the degree of damage inflicted on the population of spermatogonia. During SSC expansion periods, however, GDNF levels did not change in both busulfan dose groups. This finding suggests that a basal level of GDNF transcription may be sufficient to provide an environment that is permissive for SSC self-renewal and expansion.

In this regard, O'Shaughnessy and coworkers (22) recently examined mRNA levels of 26 Sertoli cell-specific genes for up to 50 days after germ cell depletion induced by 30 mg/kg busulfan. They reported that the loss of

germ cells led to varied responses of Sertoli cells in gene expression patterns and that most changes were associated with the loss of spermatids. Although GDNF was not included as a target gene of the study, the authors further showed a rapid increase (within 5 days) in expression of five genes by Sertoli cells, namely, Cst9, Shbg, Inhbb, Wnt5a, and Clu. This pattern of gene expression was similar to what we observed with GDNF in the present study. The rapid increase in Wnt5a expression is intriguing, as we have recently shown that WNT5A promotes self-renewal of mouse SSCs, and this effect is exerted in part by stimulating SSC survival (24). As GDNF also promotes SSC survival (25, 26), it appears that the loss of early spermatogonia, including SSCs, may stimulate the activity of Sertoli cells to encourage SSC survival and self-renewal, which could be mediated in part by soluble growth factors, such as GDNF and WNT5A. Further investigations are necessary to address such a possibility.

To analyze GDNF transcript levels, we normalized data of qRT-PCR to the whole testis level, assuming that Sertoli cells do not change in number after busulfan treatment (15). A caution is necessary, however, because it is unknown if the expression of GAPDH, an internal control chosen for our assay, is not affected by busulfan treatment. To overcome the same issue associated with PCR-based quantification of GDNF transcripts in young and old mouse testes, Ryu et al. (27) used the Sertoli cell-specific GATA4 gene as an internal control. While this approach eliminates the necessity to convert data to those at the whole testis level, it is unknown if GATA4 expression remains constant as the mice and Sertoli cells age. It appears, therefore, that assessing the transcript levels by qRT-PCR bears an inherent problem when the number of a given cell type fluctuates in a target organ or tissue. To solve such a problem in the future, it may be necessary to measure the absolute number of target mRNA molecules using synthetic complementary RNA as an internal

control (28). In this regard, it may also be useful to examine GDNF expression at the protein level.

In conclusion, this study provides functional evidence that restoration of male fertility results from that of the SSC population after cytotoxic damage. Our study also proposes that there may be a threshold in the size of the SSC population that is required for the onset of male fertility restoration in a given time frame.

AUTHOR CONTRIBUTIONS

M Nagano, P Chan, and K Zohni designed research; K Zohni and X Zhang performed research; S.L Tan, P Chan, K Zohni, and M Nagano contributed with critical discussion; K Zohni and M Nagano analyzed data and wrote the paper.

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Chapter 4

General discussion and conclusion

General discussion

Although the incidence of cancers commonly diagnosed in the adolescent and young adult population, such as acute lymphocytic leukemia, Hodgkin's and non-Hodgkin's lymphoma, and cancer of the testis (1), has been increasing, the chances of long-term remission and cure in these patients and surviving rates are remarkably improving with the use of cytotoxic regimens. Unfortunately, many of these cytotoxic regimens are known to be gonadotoxic causing fertility impairment with azoospermia as the outcome (2). Currently, sperm banking by cryopreservation is the only option feasible to preserve fertility for post-pubertal boys and men (3), but not for children who have not entered spermarche and are unable to produce mature sperm. Furthermore, cryopreserved sperm represent a finite source of gametes. After cytotoxic treatment, regeneration of spermatogenesis and restoration of fertility depend on surviving SSCs to resume their dual function in self-renewal and replenishment of the stem cell pool as well as that of differentiated germ cells. Since SSCs are present in the testis from the time of birth, there is potential for utilizing SSCs to preserve and restore the fertility of patients of any age undergoing sterilizing anticancer therapies.

The findings in this thesis are a step toward developing SSC-based, safe and reliable technology in fertility preservation. First, my work adds CD9 to the list of markers capable of enriching for human germ male cells with repopulation potential. Second, it provides direct functional evidence that the restoration of SSC population after cytotoxic damage results in male fertility restoration and, suggests a threshold for the size of SSC population required for the onset of male fertility restoration.

The proposed strategy of SSC-based male fertility restoration suggests the following paradigm: SSCs are harvested from patient testicular biopsy prior to treatment, cryopreserved, and transplanted to

patient testes after cure in order to regenerate spermatogenesis. Major concerns for this paradigm are i) the risk to transfer tumor cells leading to relapse of the oncological disease and, ii) potentially inefficient restoration of spermatogenesis. Although these concerns have been over come in mice (4, 5), we still need to addressed them in human to lay the foundation to translate this success in animal models to clinics (6).

Purification of an adult stem cell population has not been achieved yet in any tissue-resident stem cells. Nevertheless, a near homologous population of hematopoietic stem cells (HSCs) can be obtained using a multistep cell sorting. Virtually in all human HSC purification methods, the first step is to eliminate red blood cells. The second step is to eliminate committed progenitor cells of various lineages using lineage-specific markers and negative selection. Antibodies against lineage-associated antigens (e.g., CD2, CD3, CD4, CD7, and CD8 for the T-cell lineage, CD11b, CD14, and CD15 for the myeloid lineage, CD19 and CD20 for the B- lymphoid lineage, CD56 for natural killer cell antigen, and Glycophorin A for the erythroid lineage) are conjugated to the same fluorophore simplifying the negative selection. The third step is to enrich for HSCs expressing the CD34 antigen. CD34+ cells are isolated based on subsequent labeling with antibodies directed against antigens characteristic for the major types of hematopoietic stem and myeloid progenitor cells, including long-term HSC (LT-HSC), short-term HSC (ST-HSC), common myeloid progenitors (CMP), granulocyte-monocyte progenitors (GMP), and megakaryocyte-erythrocyte progenitors (MEP) including CD34, CD38, CD90 (Thy-1), CD123 (IL3Rα), and CD45RA (7-9).

Similarly, I expect that multiple efficient human SSC markers will be required to design antigen-based isolation/enrichment strategies for any clinical SSC-based transplantation therapy. The absence of such a list of markers makes human SSC isolation using immunological cell separation methods against cell surface molecules not as effective as with human HSCs. As a consequence, a major concern for the clinical use of SSCs

transplantation is the risk to transfer tumor cells leading to relapse of the oncological disease. Jahnukainen et al. showed that in a rat model, as few as 20 leukemic cells injected into the testis can induce leukemia (10). Moreover, studies using one marker for positive selection and one marker for negative selection with MACS or FACS failed to completely deplete testicular tissue from malignant cells (11). It should be worthwhile to study further the efficiency of immunological sorting following the use of multiple antibodies for different cell surface antigens.

It is possible, however, that some cancer cells also express markers shown to be human SSCs markers. For example Thy1 (CD90), which was shown to be a marker of rodent and rhesus monkey SSCs, is also expressed in murine breast (12), human glioblastoma (13) and hepatic cancer stem cells (CSCs) (14). Collectively these data demonstrate the importance of my work adding CD9 antigen to molecules shown to be expressed by human SSCs and thus expanding the list of markers to provide the versatility in the choice of antigens for cell sorting. In the following paragraphs, I will discuss some of these issues in relation to my study presented in Chapter II.

Izadyar et al. recently showed that stage-specific embryonic antigen-4 (SSEA-4) is a marker of putative human SSCs (15). They transplanted testicular SSEA-4+ cells sorted using MACS and unsorted cells, and reported 40 to 50-folds enrichment of human SSCs in the SSEA-4+ population. In order to identify human cells colonizing mouse testes one month after transplantation, they used human nuclear protein (HPN) antibody conjugated to a flurorescein fluorophore. As non-germ cells can colonize testes after transplantation (16,17), and as HPN antibody will identify all types of transplanted human cells, Izadyar et al. further had to co-stain HPN+ cells in mouse testes with markers expressed on human spermatogonia. Indeed after staining HPN + cells with ITGA6, only a quarter of HPN+ cells were ITGA6+, and they reported that “about 75% of

the SSCs that have integrated have yet to be characterized with a surface marker that is feasible with flow cytometry". In my work, I used MAGEA4 as a sole marker able to identify only human spermatogenic cells at early stages in the testes of recipient nude mice. The rationale for this was that the MAGEA4 antibody shows the reactivity to the entire population of human spermatogonia, to which SSCs belong. Moreover, the antibody has a species specificity (not react to mouse testis cells), which I confirmed for the first time in this study. This allowed for species and cell-type specific identification of colonizing donor cells as human germ cells. MAGEA4 was previously used as a marker of human spermatogonia in several studies (18,19). Yakirevich et al. did a meticulous computerized image analysis of testicular biopsy from patients with non-obstructive azoospermia and obstructive azoospermia stained using anti- MAGEA4 antibody and concluded that it is a useful marker for the detection and quantitation of human spermatogonia in the histopathological evaluation infertile men (20).

Interestingly, Izadyar et al. reported that 88.3% of SSEA-4+ cells were ITGA6+ (15). In my work, I showed that 88% of CD9+ were ITGA6+. It would be interesting in the future to examine the co-localization of SSEA-4 and CD9 on human SSCs and investigate if SSEA4+ and CD9+ cells are identical or are separate cell populations that share a ITGA6+ cell population at a similar proportion.

Very recently, Hermann et al reported successful autologous transplantation of busulfan-treated non-human primate SSCs with the production of functional sperm capable of fertilizing 81 of 85 oocytes (93%) in vitro, which led to the development of pre-implantation embryos (21). Several issues are of importance in this study. First, it demonstrates the transplanted SSCs can produce functional sperm in primates. Second, It shows that the testicular environment in primates is competent to support full spermatogenesis after cytotoxic treatment. Note that in Hermann et al. study, an average of 88×10^6 cryopreserved viable cells/ml

were injected per recipient testis. In my work, testicular biopsy of 67 mg per patient gave a mean cell recovery of 5.54 x 10^6 viable cells (5.8 x 10^6 cells, 95.5% cell viability). After cryopreservation, the number of viable cells decreased sharply as cell viability decreased to 58%. Similar decrease in number of viable cells was seen in Hermann et al. study, who reported a significant decrease of cell viability of thawed cells compared to fresh cells (58% vs. 94.6%) (22). With clinics around the globe already cryopreserving testicular tissues for boys to be used in restoring fertility (23), a balance between two factors should be kept:

a. Cells are harmed after tissue freezing with sharp decline in the number of viable cells. This decrease can jeopardized the success of a SSC-based surgical strategy especially when in-vitro expansion is not feasible.

b. The priority after cytotoxic therapy is to normally restore spermatogenesis from testicular tissues and thus spontaneous recovery of fertility. A large biopsy especially in a child can leave him short of enough surviving spermatogonia.

It is therefore critical to balance the size of testicular biopsy recovered from patients prior to anti-cancer therapy that will give a sufficient number of SSCs to be transplanted without any additional manipulations, and the size of remaining testicular tissue that will keep the testis potentially functional.

In many cases, the size of testicular biopsy is expected to not give enough SSCs to fully repopulate the testis after transplantation making in-vitro propagation necessary to obtain adequate numbers of cells and to potentially increase the concentration of SSCs in the cell preparation to be injected. Sadri-Ardekani et al. isolated human testicular germ cells from six patients and cultured these cells for up to 15 weeks (24). He et al. cultured human GFRA1+ cells spermatogonia sorted using MACS over 2 weeks and detected a 5-fold increase in the cell number after a 14-day culture

(19). With the availability of a human SSCs culture system, it would be interesting to assess CD9+ cells proliferation in vitro to determine their phenotypic characteristics, to identify new markers by comparing it later with other possible SSC markers.

Successful SSC transplantation depends directly on the availability of niches in the recipient testis for occupancy by donor cells. In animal studies, therefore, recipient testes devoid of endogenous spermatogenesis were prepared either after cytotoxic treatment or genetic mutation. In chapter 2, I used nude mice as recipients for human testis cell transplantation, as their T- cells are defective. To destroy the endogenous spermatogenesis in these mice, I treated them with 40 mg/kg of busulfan 6 weeks before transplantation, since this busulfan dose is near lethal due to myelo-suppressive effects with decreased erythrocytes and leucocytes as the lethality was reported to be 5% (16, 19, 21, 22, 25, 26). In my work, few treated mice actually died or had to be euthanized before or after transplantation, circumventing from the loss of recipient mice when availability of human testis samples was limited.

Another alternative to nude mice might be W/W^{v} (WBB6F1/J-$Kit^{W}/Kit^{W\text{-}v}$)(27) or the *Juvenile spermatogonial deletion (jsd)* locus mutant mice (28, 29). W/W^{v} mice lack endogenous germ cells due to a mutation in the c-kit tyrosine kinase gene normally expressed on germ cells. This mutation does not affect Sertoli cells and their seminiferous tubules provide a suitable environment for regeneration of spermatogenesis (30). *Jsd* mutant mice complete the first round of spermatogenesis but fail to proceed with subsequent rounds. When *jsd* germ cells were transplanted into the testis of wild-type recipients, they colonized the recipient seminiferous tubules but failed to complete spermatogenesis. On the other hand when wild-type germ cells were transplanted to *jsd* mutant testis, they re-established complete spermatogenesis in the recipient testis (31). These results indicate that *jsd* mutation causes a germ cell specific defect.

Thus, these mutant strains can provide a normal somatic environment for spermatogenic regeneration wihtout pretreatment with gonadotoxic agents, such as busulfan.

A major obstacle to use these mice will be the risk of immune rejection. An option to overcome this risk is to induce immunological tolerance toward transplanted human SSCs using costimulation blockade. Grinnemo et al treated C57BL/6 mice with a course of three costimulatory receptor-blocking agents; cytoptoxic T-lymphocyte-associated antige-4 (CTLA4)-Ig, anti-CD4 ligand (anti-CD40L), and anti-lymphocytic function-associated antigen 1 (anti-LFA1) and showed immunological tolerance of treated mice to transplanted human HSCs injected under the testes capsule (32).

It is logical to assume that a sufficient number of SSCs needs to be transplanted to efficiently induce spontaneous fertility recovery; if numbers of transplanted SSCs are small, spermatogenesis may be regenerated but the number of sperm generated is not large enough to allow for conception through intercourse (33). In Chapter 3 of this thesis, I determined the kinetics of SSC recovery after cytotoxic damage in a mouse model using the transplantation assay and provided functional evidence that the restoration of male fertility is dependent on the restoration of SSC population after cytotoxic damage. Importantly, I determined using this model the size of the SSC population that needs to be recovered to allow for restoration of spermatogenesis and male fertility in a defined time frame. In my work, I determined 30% of the original SSC population size to be a threshold for the SSC population in mice to achieve recovery of spontaneous fertility in 4 weeks. Notice that, before reaching this threshold, SSCs showed slow expansion of their numbers and accelerated their expansion rate dramatically only thereafter reaching. This threshold value corresponded with 70-80% of seminiferous tubules showing complete spermatogenesis and fertility restored 4 weeks later.

Yet this 30% threshold has to be challenged. Future experiments should investigate fertility recovery and SSCs recovery kinetics after transplanting various numbers of SSCs per mouse testis (e.g 10%, 30%, 50%) into the testis of an infertile recipient mouse and determined the duration required for fertility restoration. If such a threshold is evolutionarily conserved and human fertility is also related to a threshold level of SSC population size, then, my study may provide fundamental and quantitative reference points that could be applied in clinical situations. The data I generated may also give foundation in the future to evaluate or monitor spontaneous recovery of male fertility in cancer survivors.

Although my attempt to identify physiological parameters related to male reproduction that could be used to monitor the degree of SSC recovery did not yield a definite parameter, my work provides basic information to be used in future studies. In Chapter 3, I determined the change in the pattern of GDNF expression after germ cell loss. Interestingly, O'Shaughnessy et al reported on the change in mRNA levels of 26 different genes expressed specifically by the Sertoli cells in response to ablation of germ cells (34). This study did not include GDNF as a target gene, the results showed that mRNA of Cst9, Shbg, Inhbb, Wnt5a, and Clu increased 5 days after germ cells depletion (34), which is a similar pattern that I observed with GDNF. This response coincided with the period of rapid loss of SSCs after treatment with similar dose of busulfan (30 mg/kg). Very recently, Caires et al. treated mice with 40 mg/kg of busulfan and determined mRNA GDNF expression in treated testes compared to control, and in agreement with my results, they showed a 11-fold increase in mRNA level of GDNF compared to control after germ cell loss (35).

These results suggest that loss of spermatogonia, including SSCs, stimulates Sertoli cells to produce factors important for establishing an environment for SSC expansion and self-renewal. Future studies should

investigate this hypothesis. One approach is to examine the level of GDNF expression in vitro when Sertoli cells are co-cultured with different numbers of SSC-enriched germ cells. However, the signal that stimulates Sertoli cells might not be a mere presence of germ cells, but rather the loss of germ cells that were already interacting with Sertoli cells. Then, we may need to produce transgenic SSCs for which we can control their death. For instance, we can deliver a doxcyclin-inducible pro-apoptotic caspse transgene (e.g., caspase 3) into SSCs using lentiviral vectors. Then, such SSCs may be useful to kill only germ cells, sparing Sertoli cells, in this experimental scheme.

In this context, I used busulfan to deplete testes of endogenous spermatogenesis (Chapter III). Although busulfan has been shown not to affect Sertoli cell numbers in mice (26), their functionality remains a concern. Recently, Savitt et al. generated mice carrying a single amino acid mutation (V805A) in Ret, the signaling subunit of the GDNF receptor. This mutation does not affect normal GDNF signaling but blocks it when the ATP competitive inhibitor, NA-PP1, is added, resulting in loss of primitive/undifferentiated spermatogonia (36). When GDNF signaling in germ cells was restored by omitting NA-PP1, spermatogonia proliferated. These mutant mice can be used to induce germ cell loss in a systematic manner in the experimental scheme proposed above. At the same time, it would also be interesting to determine the expression of mRNA GDNF in Sertoli cells during SSCs recovery and study if a significant increase in GDNF expression will be seen with the loss of germ cells. As such, this mutant mouse strain can be a good candidate to address the co-dependence of spermatogonia and Sertoli cells.

Conclusion

The spermatogenic lineage in males is maintained by a pool of SSCs going through self-renewal or differentiation divisions. These cells are

present since birth and are expected to play an important role in preservation of fertility in cancer patient survivors. Following successful application in animal models, cryopreserving SSCs harvested from cancer patients prior to cancer therapy and transplanting them after cure to patient's testes is thought to restore fertility in these patients.

I note two major concerns to be addressed to realize this approach; the risk of re-transplanting tumor cells and the inefficient restoration of spermatogenesis after SSC transplantation. Decontaminating transplanted SSCs from malignant cells will require cell selection with surface markers capable of identifying and isolating SSCs (positive selection) and malignant cells of different lineages (negative selection). To date no proven single marker is known to purify SSCs and thus the need for multiple markers is mandatory. In chapter 2, I added CD9, a known marker of rodents SSC, to the list of markers of human SSCs. I showed that CD9 is expressed by human male germ cells in the basal compartment of the testicular seminiferous epithelium co-localizing with other SSCs markers. Next I used immunomagnetic sorting against CD9 antibody to successfully enrich for human germ cells that repopulated mouse testes for at least 4 months after transplant. CD9 can be used in a multiparameter selection of human SSCs, which may have applicability in the human fertility clinic. The efficiency of SSCs transplantation is associated with the number of stem cells injected.

In chapter 3, I used a mouse model and determined 30% of the original SSCs population size to be the threshold required to confer fertility. With the speculated small number of SSCs collected from testicular biopsy, this work set a value that will ensure a sufficient number of SSCs reaching the basement membrane of testicular seminiferous tubules. As I hypothesized, my results demonstrated that the kinetics of fertility restoration is correlated with those of SSC recovery. This is the first functional evidence on this correlation. Finally, GDNF level significantly increased shortly after germ cell loss. This suggests that Sertoli cells

sensed the loss of early spermatogonia and responded through the increase in GDNF expression to encourage SSC survival and self-renewal. These results indicate that spermatogonia play important role in regulation of Sertoli cells activity.

In closing, this body of work presents a new marker to identify human SSCs, provides functional proof on correlation of SSCs and fertility recovery kinetics, and determines a novel threshold of SSCs population size to confer fertility after transplantation.

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