INVOLVEMENT OF WNT SIGNALLING PATHWAYS IN THE CELL FATE REGULATION OF MOUSE SPERMATOGONIAL STEM CELLS

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A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy (Ph.D.)

Abstract

Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis. These cells have the unique ability to self-renew, to maintain a reserve of undifferentiated germ cells, and differentiate to continuously support long-term spermatogenesis. Thus, SSCs are important cells for male fertility restoration technologies. SSCs are maintained in a specialized microenvironment, in contact with testicular somatic cells and other germ cells. Communication amongst this cell society is believed to balance the SSC fate decision to self-renew or differentiate, to avoid over-proliferation or stem cell depletion. However, the secreted factors mediating this communication network are not completely known. Wnt signalling has been shown to direct cell specification during embryogenesis and regulate the fate decision of various stem cell types. Therefore, in this thesis, I characterize a role for Wnt signalling pathways in SSC fate regulation. In order to study SSCs under defined conditions, I relied on a culture system in which SSCs proliferate as distinct germ cell accumulations, which I term "clusters". In Manuscript 1, I examined this culture in depth and characterized cluster-formation as a reliable in vitro assay for SSC activity. I showed that clusters can be clonally-derived attesting to an ability to quantify cluster-forming cells by counting cluster numbers. Furthermore, I determined that cluster numbers correlated with SSC numbers, as determined by spermatogonial transplantation. Thus, cluster-formation appears to be an in vitro attribute of SSC activity. Clusters are composed of both SSCs and differentiated germ cells indicating that self-renewal and differentiation events occur in vitro. Importantly, cluster formation and SSC maintenance in vitro requires the use of embryonic fibroblasts as a feeder cell layer. Therefore, I screened these feeder cells for potential factors that might support SSC self-renewal and I detected the expression of various Wnt molecules. In Manuscript 2, I defined Wnt5a as a paracrine factor that supports SSC self-renewal. I found that Wnt5a activates a non-canonical Wnt/c-jun-N-terminal kinase pathway and blocks cell death in vitro. Additionally, I detected Wnt5a in testicular somatic cells and Wnt5a receptors on clusters and SSCs, indicating that Wnt5a may act directly on SSCs in

vivo. Interestingly, using a transgenic reporter mouse line to detect canonical Wnt (β-catenin) signalling, I observed a subset of β-catenin signalling cells in clusters. Transplantation of these β-catenin signalling cells showed they did not have SSC activity but appear to be committed progenitor cells. In Manuscript 3, I found that the addition of Wnt3a to culture specifically activated the β-catenin pathway in a subset of cluster cells, which showed reduced SSC activity indicating they were committed progenitors. Under feeder-free conditions, Wnt3a induced cluster formation, which indirectly led to an increase in SSC maintenance. Thus, these results provide evidence that committed progenitors can contribute to the niche regulation of SSCs. Finally, in a preliminary study I performed gene expression profiling comparing β-catenin signalling to non-signalling cells to identify molecular differences in committed cells. I identified Delta-like homolog 1 as a potential factor that could induce SSC differentiation, indicating that committed cells have the capacity to regulate SSC fate. Therefore, this thesis work characterizes a role for Wnt pathways in the communication between SSCs and the testicular niche microenvironment, which properly regulates the SSC fate decision.

Résumé

L'intégrité de la spermatogenèse repose sur l'autorenouvellement et la différenciation des cellules souches germinales (CSGs). Les CSGs assurent le maintient du processus continu de différenciation, des cellules souches aux spermatozoïdes, qui prend place dans le testicule tout au long de la vie de l'individu. Les CSGs sont donc indispensables à la restauration de la fertilité masculine. Dans le testicule, les CSGs coexistent avec d'autres cellules, germinales et somatiques, et les interactions entre ces différents types cellulaires sont essentielles au développement d'un microenvironnement propice à la maintenance des CSGs et à l'induction de la spermatogenèse. La coordination des communications intercellulaires survenant au sein de ce microenvironnement (niche) est cruciale pour la détermination de la destinée cellulaire de CSGs et donc pour l'établissement d'un équilibre entre leur autorenouvellement et leur différenciation afin de prévenir leur hyperprolifération ou une réduction de fertilité. Les signaux cellulaires spécifiques impliqués dans ce réseau de communication intercellulaire régulant la destinée cellulaire des CSGs demeurent par contre méconnus. Le rôle de la voie de signalisation Wnt dans la spécification cellulaire au cours de l'embryogénèse est déjà bien établi. La voie de signalisation Wnt est également impliquée dans la régulation de la destinée cellulaire de plusieurs types de cellules souches. Afin de déterminer le rôle de la voie de signalisation Wnt dans la régulation de la destinée cellulaire des CSGs, j'ai utilisé un système de culture défini dans lequel les CSGs prolifèrent et forment des agrégats de cellules germinales isolés et distincts. J'ai tout d'abord établi la validité de ce système de culture pour l'évaluation de l'activité biologique des CSGs en démontrant que les agrégats forment par dérivation clonale, attestant ainsi que le nombre d'agrégats reflète le nombre initial de cellules ayant le potentiel de former des agrégats. J'ai également démontré une corrélation entre le nombre d'agrégats et le nombre de CSGs en utilisant une méthode de transplantation testiculaire de cellules spermatogoniales. Le potentiel de formation d'agrégats *in vitro* apparaît donc comme une fonction intrinsèque des CSGs. Les agrégats sont constitués non seulement de CSGs mais aussi de cellules germinales

différenciées, démontrant la capacité d'autorenouvellement et de différenciation des CSGs *in vitro*. Puisque la maintenance des CSGs et la formation d'agrégats *in vitro* requièrent la présence d'une couche nourricière de fibroblastes embryonnaires, j'ai analysé les facteurs produits par ces fibroblastes et identifié plusieurs molécules de la famille Wnt susceptibles de jouer un rôle dans la régulation de l'autorenouvellement des CSGs. Mes résultats démontrent que Wnt5a agit comme facteur de signalisation intercellulaire de type paracrine stimulant l'autorenouvellement des CSGs. Wnt5a active la voie de signalisation non canonique Wnt/c-jun-N-terminal kinase et inhibe l'apoptose *in vitro*. De plus, j'ai trouvé que Wnt5a est présent dans les cellules somatiques testiculaires alors que les récepteurs pour Wnt5a sont exprimés par les agrégats et les CSGs, suggérant un rôle pour Wnt5a dans la régulation des CSGs *in vivo*. Par le biais de l'utilisation d'une lignée transgénique rapportant l'activité de la voie de signalisation canonique Wnt (β-caténine), j'ai détecté l'activation de la voie β-caténine dans un sous-ensemble de cellules présentes dans les agrégats. La transplantation testiculaire de ces cellules a révélé que ces cellules ne sont pas des CSGs mais plutôt des progéniteurs spermatogoniaux. J'ai également démontré que l'addition de Wnt3a en culture active la voie de signalisation β-caténine spécifiquement dans les progéniteurs spermatogoniaux. En l'absence de couche nourricière de fibroblastes, Wnt3a induit la formation d'agrégats et promeut la maintenance des CSGs. Ces résultats suggèrent donc que les progéniteurs spermatogoniaux contribuent à la régulation de la niche nécessaire à la maintenance des CSGs. Finalement, j'ai effectué une analyse par micropuce ADN afin de comparer l'expression génique entre les cellules positives et négatives pour l'activation de signalisation β-caténine afin d'identifié des gènes spécifiques pour les progéniteurs spermatogoniaux. Collectivement, mes résultats caractérisent le rôle des molécules de la famille Wnt dans la communication intercellulaire qui survient entre les CSGs et autres cellules avoisinantes présentes dans la niche testiculaire impliqué dans la régulation de la destinée cellulaire des CSGs.

Acknowledgments

Just as spermatogonial stem cells rely on the support from extrinsic factors, so too does a graduate student rely on the contributions from a network of external sources.
Therefore, I would like to acknowledge those who have contributed to this body of work.

Thank you to my supervisor and mentor, Makoto Nagano, for giving me the opportunity to pursue study in his lab. Your knowledge, advice, and love of the natural world have moulded my approach to research and given me a better appreciation for science.

I would also like to acknowledge the help from the members of my lab, in particular, Xiangfan Zhang, also Kevin Ebata, Khaled Zohni, and Sabrina Sicilia. Special thanks also to Cassandre Labelle-Dumais for translation of the abstract.

I would like to thank my thesis committee members: Drs. Jacquetta Trasler, Suhad Ali, and Riaz Farookhi, in particular, Riaz, for your stimulating conversations and advice.

Thank you to my parents, Larry and Theresa Yeh, and my family for your unconditional love and encouragement and for continuously challenging me to strive for my best.

Thank you to my friends, both past and present. In particular, Christine Sykas: my best friend, my better half, and my partner in stupid. Thank you for always motivating me to be a better person.

I would also like to acknowledge my numerous sources of funding: the Canadian Institutes of Health Research, McGill University, the Research Institutes of the McGill University Health Centre, the Stem Cell Network, the International Society for Stem Cell Research, the Réseau Québécois en Reproduction, and my parents.

And finally I would like to acknowledge the many scores of animal subjects who made the ultimate sacrifice by surrendering their masculinity; their losses contributed greatly to my research with spermatogonial stem cells. Hopefully, the results from their numbers will one day benefit individuals, whose genetic guns are loaded with blanks.

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APPENDIX I: GENE EXPRESSION DATASET
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Abbreviations

A

A_s: A_{single}
A_{pr}: A_{paired}
A_{al}: $A_{aligned}$
Akt: Protein kinase B
AMH: Anti-Müllerian hormone
ANOVA: Analysis of variance
APC: Adenomatous polyposis coli

B

Bax: Bcl2-associated X protein
Bcl2: B-cell lymphoma 2
Bcl6b: B-cell CLL/lymphoma 6 member B
BMP: Bone morphogenetic protein
BSA: Bovine serum albumin

C

CaMKII: Calmodulin-dependent protein kinase II
Cdc42: Cell division cycle 42
Cer1: Cerberus 1
CFU-S: Colony-forming unit-spleen
CKI: Casein kinase 1
CSF1: Colony stimulating factor 1

D

DKK1: Dickkopf-1
DLK1: Delta-like homolog 1
DMEM: Dulbecco Modified Eagle Medium
dpc: Days post-coitum
dpp: Days post-partum
Dsh: Dishevelled

E

EDTA: Ethylenediaminetetraacetic acid
EGC: Embryonic germ cell
EGF: Epidermal growth factor
Erm: ETS-related molecule
ESC: Embryonic stem cell

F

FACS: Fluorescent-activated cell sorting
FBS: Fetal bovine serum
FDG: Fluorescein di-β-D-galactopyranoside
FGF2: Fibroblast growth factor 2
Foxo1: Forkhead box O1
Fzd: Frizzled

G

Gapdh: Glyceraldehyde 3-phosphate dehydrogenase
GDNF: Glial cell-line derived neurotrophic factor
GFP: Green fluorescent protein
GFRα1: GDNF family receptor alpha 1
GSC: Germline stem cell
GSK3: Glycogen synthase kinase 3

H

HBSS: Hanks Balanced Salt Solution
hop: Hopscotch

HSC: Hematopoietic stem cell
HTS: High-throughput screening

I **ICM:** Inner cell mass
IG-DMR: Intergenic differentially methylated region
iPSC: Induced pluripotent stem cell
ISC: Intestinal stem cell

J **JAK:** Janus kinase
JNK: c-Jun N-terminal kinase

L **Lhx1:** LIM homeobox gene 1
LIF: Leukemia inhibitory factor
LRP: Low density lipoprotein receptor-related protein

M **MAPK:** Mitogen-activated protein kinase
MEMα: Minimum Essential Medium alpha
MHC: Major histocompatibility complex

N **NCAM:** Neural cell adhesion molecule
Ngn3: Neurogenin 3
Nkd1: Naked cuticle 1
NSC: Neural stem cell

O **Oct4:** Octomer-binding transcription factor 4

P **PBS:** Phosphate buffered saline
PCP: Planar cell polarity
PGC: Primordial germ cell
PI: Propridium iodide
PI3K: Phosphoinositide 3-kinase
PKC: Protein kinase C
Plzf: Promyelocytic leukemia zinc finger
Pten: Phosphatase and tensin homolog
Ptn: Pleiotrophin
PTX: Pertussis toxin

R **RA:** Retinoic acid
Ror2: Receptor tyrosine kinase orphan receptor 2
RSPO1: R-Spondin1
RT-PCR: Reverse transcription polymerase chain reaction

S **SDS-PAGE:** Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM: Standard error of the mean
SF: Steel factor
sFRP1: Secreted frizzled-related protein-1
shRNA: short hairpin RNA
siRNA: short interfering RNA
Sl: Steel locus
Src: v-Src Avian sarcoma viral oncogene
Sry: Sex-determining region Y
SSC: Spermatogonial stem cell

STAT: Signal transducer and activator of transcription
Stra8: Stimulated by retinoic acid 8
STO: SIM derived thioguanine and oubain resistant
SVZ: Subventricular zone

T **Taf4b:** TATA box binding protein associated factor 4b
TCF/LEF: T-cell factor/lymphoid enhancer factor
TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling

U **Upd:** Unpaired

V **VEGF:** Vascular endothelial growth factor

X **X-gal:** 5-bromo-4-chloro-3-indolyl β-D-galactoside

Publications

Published Peer-Reviewed

1. Boyer A, **Yeh JR**, Zhang X, Paquet M, Gaudin A, Nagano MC, Boerboom D; CTNNB1 signalling in Sertoli cells downregulates spermatogonial stem cell activity via WNT4. *PLoS One;* 7(1):e29764 (2012)

2. **Yeh JR**, Zhang X, Nagano MC; Wnt5a is a germ-cell extrinsic factor that promotes mouse spermatogonial stem cell renewal *Journal of Cell Science* 124:2357-2366 (2011)

3. Ebata KT, **Yeh JR**, Zhang X, Nagano MC; Soluble growth factors stimulate spermatogonial stem cell division that maintain a stem cell pool and produce progenitors in vitro. *Experimental Cell Research;* 317(10):1319-1329 (2011)

4. **Yeh JR**, Zhang X, Nagano MC; Establishment of a Short-Term In Vitro Assay for Mouse Spermatogonial Stem Cells. *Biology of Reproduction*; 77(5):897-904 (2007)

Published Review Articles

1. **Yeh JR** and Nagano MC. Spermatogonial stem cell biomarkers: improved outcomes of spermatogonial transplantation in male fertility restoration? *Expert Review of Molecular Diagnostics*; 9(2):109-14 (2009)

2. Ebata KT, **Yeh JR**, Zhang X, Nagano MC; The application of biomarkers of spermatogonial stem cells for restoring male fertility. *Disease Markers;* 24(4-5):267-76 (2008)

Published Book Chapters

1. M.C. Nagano and **J.R. Yeh**; *The Cluster-Forming Activity Assay: A Short-Term In Vitro Method to Analyze the Activity of Mouse Spermatogonial Stem Cells.* In *Male Germline Stem Cells: Developmental and Regenerative Potential*, (eds K.E. Orwig and B.P. Hermann), pp.125-134, New York: Humana Press (2011)

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1. **Yeh JR**, Zhang X, Nagano MC; Indirect effects of Wnt3a/β-catenin signalling support mouse spermatogonial stem cells in vitro.

2. Niles KM, **Yeh JR**, Landry M, Nagano MC, Trasler JM; Haploinsufficiency of the Paternal-Effect gene Dnmt3L Results in Transient DNA Hypomethylation in the Male Germ Line

Contribution of authors

1. **Yeh JR**, Zhang X, Nagano MC; Wnt5a is a germ-cell extrinsic factor that promotes mouse spermatogonial stem cell renewal *Journal of Cell Science* 124:2357-2366 (2011)
   Contributions: I performed all experiments, except for immunofluorescent staining of phosphor-JNK in clusters and seminiferous tubules and TCF/LEF-*lacZ*/Fzd3 double staining, which were performed by MC Nagano. I conducted all data analyses, designed experimental scheme and wrote the manuscript in collaboration with MC Nagano. X Zhang performed all transplantations.

2. **Yeh JR**, Zhang X, Nagano MC; Establishment of a Short-Term In Vitro Assay for Mouse Spermatogonial Stem Cells. *Biology of Reproduction*; 77(5):897-904 (2007)
   Contributions: I performed all experiments for this study. I also conducted all data analyses, designed experimental scheme and wrote the manuscript in collaboration with MC Nagano. X Zhang performed all transplantations.

3. **Yeh JR**, Zhang X, Nagano MC; Indirect effects of Wnt3a/β-catenin signalling support mouse spermatogonial stem cells in vitro. [In Preparation]
   Contributions: I performed all experiments for this study. I also conducted all data analyses, designed experimental scheme and wrote the manuscript in collaboration with MC Nagano. X Zhang performed all transplantations.

4. Ebata KT, **Yeh JR**, Zhang X, Nagano MC; Soluble growth factors stimulate spermatogonial stem cell division that maintain a stem cell pool and produce progenitors in vitro. *Experimental Cell Research;* 317(10):1319-1329 (2011)
   Contributions: I performed flow cytometry experiments examining the expression of GDNF receptors on germ cell clusters and STO feeder cells. I also assisted in the preparation of the manuscript.

5. Boyer A, **Yeh JR**, Zhang X, Paquet M, Gaudin A, Nagano MC, Boerboom D; CTNNB1 signalling in Sertoli cells downregulates spermatogonial stem cell activity via WNT4. *PLoS One;* 7(1):e29764 (2012)
   Contributions: I performed all in vitro experiments examining the effect of Wnt4 on germ cell apoptosis and cell cycling. I also assisted in the preparation of the manuscript.

6. Niles KM, **Yeh JR**, Landry M, Nagano MC, Trasler JM; Haploinsufficiency of the Paternal-Effect gene Dnmt3L Results in Transient DNA Hypomethylation in the Male Germ Line [In Preparation]
   Contributions: I assisted in the in vitro germ cell amplification and recovery for DNA methylation analysis. I also assisted in the preparation of the manuscript.

CHAPTER 1

INTRODUCTION
BACKGROUND
RATIONALE & HYPOTHESIS

Introduction

Regeneration is a remarkable physiological process that allows for tissue repair or continuous cell production. Several invertebrates such as planarian flatworms and Hydra can participate extensively in whole body regeneration, while some vertebrates such as salamanders can regenerate lost appendages. These regeneration events occur through the dedifferentiation of somatic tissues to form precursor cells that proliferate to form the nascent specialized cells of the regenerated organ [1]. Mammals have seemingly lost most whole-organ regeneration ability, however, they possess self-renewing systems such as the epidermis, hematopoiesis, and spermatogenesis, which undergo continuous cell turnover and have the ability for recovery following injury. These regeneration events are not believed to involve dedifferentiation, but rather rely on the activation of pre-existing undifferentiated cells, called stem cells. In general, stem cells are regarded as primitive cells responsible for supporting a tissue or organ. Stem cells can support a system by self-renewing to maintain a cell reserve and by contributing daughter cells allowing for continuous cell turnover for the life of the animal. Over time, stem cells have been identified from numerous embryonic and somatic tissues as well as from germ cells.

Being sexual reproducers, mammals produce male and female gametes, sperm and oocytes, which combine their haploid genomes to generate diploid progeny. In the female, the number of oocytes is determined at birth. However, in the male, sperm is produced continuously through the process of spermatogenesis, involving genetic and morphological changes to gametes. Spermatogenesis is well-timed and coordinated in such a manner that sperm is continually produced at a steady rate. In addition to being continuous, spermatogenesis is also prolific; human males produce an estimated 1000 sperm per heartbeat [2]. This aggressive sperm production strategy alludes to the presence of a stem cell population that supports spermatogenesis: The spermatogonial stem cells. Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis and represent the most undifferentiated germ cells in the hierarchy of sperm formation. SSCs are functionally defined by their ability to self-renew, thereby maintaining a reserve

of stem cells, and to give rise to differentiated germ cells that enter into the spermatogenic cycle. SSCs divide slowly and contribute cells fated for differentiation regularly. As germ cells progress through the steps of differentiation, they mitotically divide which increases their numbers exponentially; from one SSC, theoretically 4096 sperm cells can be generated after one spermatogenic cycle. Therefore, in this way SSCs are responsible for continuous and prolific sperm production during spermatogenesis. Thus, SSCs must maintain a balance between the production of sperm and self-renewal to avoid potential sub-fertility or stem cell depletion. This fate decision of SSCs to self-renew and differentiate is believed to be supported through interactions with somatic cells and, potentially, differentiated germ cells. Previous work has identified a handful of extrinsic factors from these niche-forming somatic cells that can influence the cell fate of SSCs. In turn, these extrinsic factors can stimulate the downstream expression of intrinsic factors that maintain stem cell regeneration ability or mediate differentiation. However, in general the extrinsic factors and intrinsic mediators that are important to maintain SSC activity or push differentiation remain largely unknown. A greater understanding of these mechanisms is tantamount to understanding SSC behaviour and crucial for effective manipulation of these powerful cells.

Why study SSCs? SSCs share attributes and signalling mechanisms with other tissue-specific stem cells [3]. Functional assays exist to study SSC activity [4,5] and in contrast to many other stem cell types, SSC differentiation is unidirectional thereby simplifying their study. Therefore, SSCs may act as a model system to study fundamental questions in stem cell biology. Secondly, SSCs are unique in that they are the only adult stem cells that can transmit genetic information to future generations [6]. Therefore, SSCs may benefit the development of novel approaches in animal transgenesis and genetic screening/therapies to infertility and disease. Finally, human male infertility has become a major health and social concern worldwide and is increasingly realized as cancer survival rates improve. It is estimated that the prevalence of cancer survivors among young adults is 1 in 250 persons [7]. With improving cancer

survival rates male infertility is expected to escalate because anti-cancer treatments greatly affect spermatogenesis [8]. Indeed, an estimated 50% of leukemia survivors become infertile following treatment [9]. As a result, restoration of fertility is and will increasingly become an important quality of life issue for cancer survivors. Currently, semen cyropreservation is the only option to preserve male fertility through assisted reproductive technologies. However, this strategy is not applicable to prepubertal patients who at the time of anti-cancer treatments do not produce mature sperm. One potential solution may be through allogenic SSC transplantation. A general scheme of this strategy involves the collection of SSCs from a patient's testis biopsy prior to anti-cancer therapies, followed by cryopreservation of the SSCs, and transplantation back into the testis once the patient is clear of malignancy. Because of the ability for SSCs to propagate and support spermatogenesis, transplanted SSCs could allow for the natural recovery of fertility. However, effective fertility restoration may require expansion of SSC numbers. Therefore, the identification and understanding of the mechanisms that regulate SSC fate decision will be critical for the efficient and safe use of SSCs in a clinical setting.

In summary, to realize the long-term potential of SSCs it will be important to understand the biology of SSCs; this involves a better understanding of the cell fate mechanisms regulating the balance between self-renewal, differentiation, and death. Similar to other adult stem cells, SSCs are believed to be supported in a specialized niche microenvironment. Therefore, the aim of this thesis is to investigate how communication within this environment can influence the balance between self-renewal, differentiation, and death of mouse SSCs. In particular, I focus on the role of Wnt signalling pathways, which have been implicated in the fate decision process of numerous stem cell types but have not been thoroughly explored in SSC regulation and spermatogenesis.

Part 1: Stem Cells and Male Gametogenesis

What are stem cells?

Stem cells are functionally defined by their unique ability to both self-renew and give rise to differentiated cell types. There exist many different stem cell types, each of which can support a particular tissue (Fig. 1). Many of these tissues exhibit continuous turnover or extensive repair mechanisms which rapidly deplete cell numbers. Thus, tissue-specific (adult) stem cells are necessary to replenish these tissues. These stem cells are often multipotent, that is they have the ability to contribute cells that can form the specific lineages of a given tissue. To date, adult stem cells have been isolated from the bone marrow, epidermis, intestine, brain, and testis, among others [10].

The origin of these stem cell types can all be traced back to a more primitive stage, starting with the fertilized egg. In adult mammals, male and female gametes are necessary instruments of sexual reproduction. These gametes, sperm and oocytes, fuse to form a zygote that has the ability to develop into a new organism, thereby ensuring the continuation of the germ line. From the zygote stage to the 16-cell embryo, each cell (or blastomere) retains the ability to generate the requisite embryonic and extraembryonic tissues to produce a new organism and are thus referred to as totipotent cells (Fig. 1). Evidence of totipotency comes in the form of identical twins, which are generated from the splitting of the early embryo.

As the embryo develops into a many-celled blastocyst, the cells of the inner cell mass (ICM) can be placed into culture to generate colonies of embryonic stem cells (ESCs). These stem cells are pluripotent, that is, they retain the ability to differentiate into any of the fetal or adult tissues of the three germ layers (endoderm, mesoderm, ectoderm) and thus form the whole body of an organism. However, in contrast to totipotent blastomeres, ESCs can only form the embryo proper and not extraembryonic tissues.

In contrast to other adult stem cells, which are derived from the somatic cells of the three primordial germ layers, SSCs are formed from an embryonic germ cell precursor called the primordial germ cell (PGC). PGCs are specified

early in development and exist transiently in the embryo until they contact the cells of the developing gonad and become irreversibly committed to the germ cell lineage. Interestingly, in the presence of growth factors: leukemia inhibitory factor (LIF), Steel factor (SF), and fibroblast growth factor 2 (FGF2), PGCs can also form colonies of pluripotent cells called embryonic germ cells (EGCs) (Fig. 1) [11,12]. EGCs, similar to ESCs, show the ability to form the tissues of the three germ layers and germ cells. Therefore, PGCs appear to retain a level of pluripotency, while other developing cells fated to the somatic lineages have lost this ability. Specification of PGCs in the developing embryo is an important process, ensuring that germ cells are generated to maintain the passing of genes to subsequent generations.

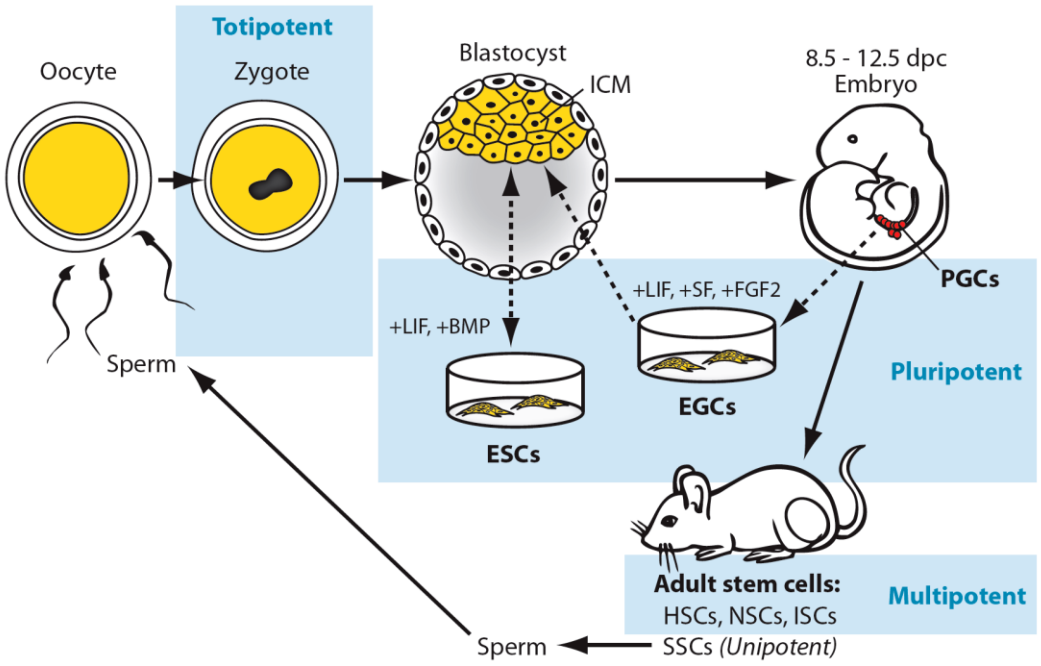

Figure 1: Appearance of various stem cell types in the mouse.

The oocyte is fertilized by sperm to form the totipotent zygote that can produce all cells to generate a new organism. At the blastocyst stage, the inner cell mass (ICM) can be placed in culture to form pluripotent embryonic stem cells (ESCs), which show the potential to generate cells of the three germ layers of the embryo proper. Germ cells are specified as primordial germ cells (PGCs), which can also be induced to form pluripotent embryonic germ cells (EGCs) that are similar in

potential to ESCs. Following birth, self-renewing tissues in the organism rely on tissue-specific (adult) stem cells to replenish continuous cell turnover. These include hematopoietic stem cells (HSCs), neural stem cells (NSCs), and intestinal stem cells (ISCs). These cells are often multipotent as they form the specific lineages of a particular tissue. SSCs are unipotent as they produce only one lineage, sperm, which is vital for the propagation of the species.

Embryonic germ cell development

Primordial germ cell specification

The process of germ lineage specification between species can occur through different mechanisms. However, the end result of this process is similar, that is, repression from a somatic lineage fate. In Drosophila, germ cells in the embryo are established at the posterior pole of the developing embryo. These cells are designated germ cells by the presence of specialized protein and RNA factors, concentrated at the posterior pole of the oocyte, termed "germ plasm". Cells forming at the posterior pole incorporate this germ plasm and become fated to become PGCs [13]. In *Caenorhabditis elegans*, germ plasm is interspersed throughout the oocyte and early zygote. As the embryo divides, the germ plasm is partitioned through a series of asymmetric divisions into one daughter cell, the P4 cell, which is destined to become the single PGC of the worm [13].

How does germ plasm specify the germ lineage? One trait common to both Drosophila and *C. elegans* germ plasm is the presence of factors that lead to transcriptional repression that protect against the expression of somatic differentiation genes. In Drosophila, these factors include the peptide Pgc, and repressors Nanos and Pumilo, which lead to transcriptional silencing. Similarly, PIE-1 is the key repressor in the germ plasm of *C. elegans* [13].

In contrast, mammalian germ lineage specification does not involve a germ plasm. However, the concept is conserved; germ cell establishment occurs through the active repression of the somatic differentiation program. Mouse germ

line specification starts around 5.5 days post-coitum (dpc). Single cell lineage analyses have demonstrated that cells from the proximal region of the epiblast (at the junction with the extraembryonic ectoderm) are the origin of PGCs [14]. To induce PGC specification, the extraembryonic ectoderm begins to express the bone morphogenetic proteins (BMPs), BMP4 and BMP8b [15]. BMPs are expressed uniformly by the extraembryonic ectoderm but inhibitory communication by Cer1, Lefty, and Dkk1, from the visceral endoderm results in only the cells at the posterior proximal end of the epiblast receiving enough BMP signal to inhibit the somatic program and specify as PGCs (Fig. 2). Furthermore, Wnt3 is required by epiblast cells for responsiveness to BMP signals [16].

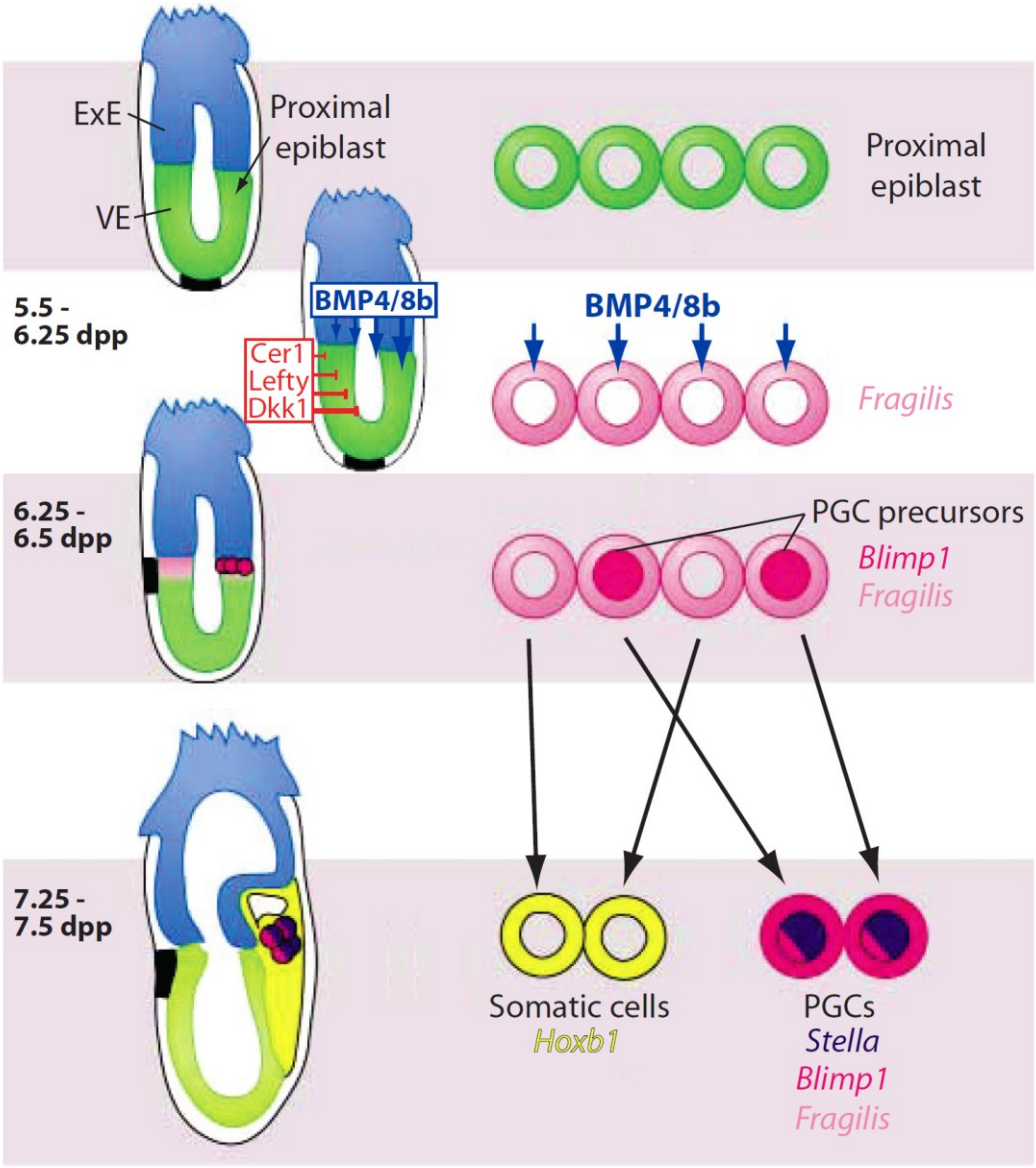

Figure 2: PGC specification during mouse development.

Inductive signalling from the extraembryonic endoderm (ExE) and inhibitory signalling from the visceral endoderm (VE), lead to the specification of PGCs from cells in the proximal epiblast. The cells of the proximal epiblast show the gradual expression of various markers indicating PGC specification, represented by the circles on the right. Figure adapted from Hayashi [17] with copyright © by The American Association for the Advancement of Science.

In the prospective PGC population at 6.25 dpc, BMP4 and 8b induce expression of the transmembrane protein Fragilis/IFITM1 [15,18]. By 6.5 – 7.0 dpc, a small cluster of approximately 40 cells expressing high levels of Fragilis begin to express Dppa3 (Stella), the first marker of developing PGCs , as well as tissue non-specific alkaline phosphatase (Tnap) and stage-specific antigen 1 (Ssea1). Cells expressing low levels of Fragilis do not subsequently express Stella but rather start to express somatic homeobox genes (Hoxa1, Hoxb1, Lim1, and Evx1) and eventually go on to contribute to the extraembryonic mesoderm and allantois (Fig. 2) [18].

Intrinsically, how do BMPs repress the somatic programme and initiate germ cell specification? BMP signalling leads to the expression of Prdm1 (Blimp1), a zinc-finger transcriptional repressor that appears to be the main inhibitor of the somatic program. Blimp1 expression is crucial for germ cell specification; loss of Blimp1 leads to inconsistent expression of Stella and the expression of somatic homeobox genes [18]. Another important transcriptional repressor is Prdm14, which is co-expressed with Blimp1 and whose absence can also lead to a loss of PGCs and sterility [19]. Therefore, germ line specification involves the active repression of genes that promote a somatic cell fate. In contrast to species that use “germ plasm” to repress a somatic programme and establish the germ cell lineage, mammalian germ cell specification relies on induction by extrinsic signals.

Between 7.25 dpc and 12.5 dpc approximately 40 cells are specified as nascent PGCs. These cells undergo rapid mitosis and proliferate to 25,000 cells as they migrate from the base of the allantois into the genital ridges (starting from 10.5 dpc) [20]. PGCs proliferate until 12.5 dpc in both male and female embryos. To this point, PGCs are identical in both male and female embryos and retain the ability to form EGCs [11,12]. After 12.5 – 13.5 dpc, PGC interaction with the somatic cells of the sexually divergent gonad leads to PGC commitment to a respective male or female germ cell fate. In the female gonad, PGCs differentiate into oogonia and enter meiosis arresting at the diplotene stage. Oocytes remain meiotically arrested in diplotene stage until ovulation. On the other hand, PGCs in

the male mitotically arrest at G_0/G_1 and remain arrested until birth when they resume mitotic divisions to establish the SSC population. Work into the mechanisms of this differentiation has determined that factors from the differentiating gonad are responsible for the sex-specific differentiation of PGCs.

Sexual differentiation

PGCs are bipotential, demonstrated by the fact that XY germ cells can develop as oocytes in female embryos while conversely XX germ cells develop as prospermatogonia when cultured with male urogenital ridges [21]. PGCs remain uncommitted until 12.5 dpc in males and 13.5 dpc in females, after the germ cells come into contact and respond to the sexually differentiated gonadal environment.

In the female, retinoic acid (RA), is secreted by the mesonepheros which induces the expression of the gene stimulated by retinoic acid 8 (Stra8) in female germ cells. This appears to promote entry of female germ cells into meiosis [22]. In contrast, the male gonad expresses Cyp26b1, which encodes an enzyme that metabolizes RA [23]. Cyp26b1 is expressed by Sertoli cells and appears to be important to keep RA levels low in the embryonic testes; $Cyp26b1^{-/-}$ embryonic testes show Stra8 expression in germ cells and the presence of meiotic germ cells [24]. Therefore, the sexual commitment of germ cells relies on external signals from the surrounding sexually differentiating gonad.

What factors are involved in the sexual specification of the gonad? The developing gonad is considered sexually indifferent until 10.5 dpc when sex-determining region Y (Sry), a Y chromosome-linked gene, is expressed in the XY gonad [25,26]. In the XX gonad, Sry is not present leading to differentiation of the supporting somatic lineage into granulosa cells, which surround the germ cells forming the primordial follicles, while the germ cells differentiate into oocytes. Therefore, Sry is the crucial testis-determining gene that directs the gonad to differentiate into a testis; knockout of Sry in XY mutant mice leads to formation of an ovary [27]. However, ovary-inducing factors have also been defined. In particular, the Wnt signaling molecules Wnt4 and R-Spondin1 (RSPO1) have been demonstrated to be required for female sex differentiation. $Wnt4^{-/-}$ females

show aspects of female-to-male sex reversal, even in the absence of SRY, including Müllerian duct regression, Leydig cell formation, and the degeneration of oocytes. Testis development is unaffected in Wnt4 mutant males [28]. The loss of Wnt4 leads to Sox9 and Fgf9 expression, indicating that Wnt signalling is necessary to actively downregulate the expression of testis-determining genes [29,30].

Sry expression in the XY gonad leads to the upregulation of the transcription factor Sox9. Both Sry and Sox9 are necessary to differentiate the bipotential somatic supporting cell lineage into testis-specific Sertoli cells [26]. Sertoli cells are a major somatic cell population in the testis that are responsible for regulating spermatogenesis. Sertoli cells also express Fgf9, which act to maintain Sox9 expression [30,31]. The Sertoli cells then masculinise other cell types in the gonad and mesonephros to stimulate testis differentiation. Thus, Sertoli cells are responsible for triggering male differentiation of the testosterone-producing Leydig cells and peritubular myoid cells that surround the Sertoli and germ cells to form the testis cords. However, the mechanism of how the Sertoli cells masculinise germ cells in the developing testes is not well understood. Nevertheless, germ cells respond to the male gonad environment and differentiate to prospermatogonia, becoming committed to spermatogenesis by 12.5 dpc. These germ cells remain mitotically arrested within the seminiferous cords as the rest of the surrounding testis develops.

Postnatal germ cell development

Testis

The testis is the male organ responsible for the production of sperm. The testis is located in the scrotum outside of the abdominal cavity, as the process of spermatogenesis requires a lower than core body temperature. The testis is in essence a capsule of fibrous extracellular matrix proteins, called the tunica albuginea, encapsulating multiple loops of seminiferous tubules (Fig. 3) [32]. Between seminiferous tubules lies an interstitium composed of an organized mixture of vascular and lymphatic vessels that provide hormones, nutrients,

oxygen, and immune protection to the cells of the testis. This interstitial space is also composed of specialized testosterone-producing Leydig cells. As such, the testis is a major producer of testosterone, which is not only crucial for the process of spermatogenesis but for the development of secondary sex organs such as the penis, epididymis, and prostate. At the boundary between the interstitial space and the seminiferous tubules lies a layer of myoid cells, which are smooth muscle cells believed to aid in the movement of seminiferous fluid through the testis. Furthermore, myoid cells secrete extracellular matrix proteins, such as laminins, collagens, and fibronectins, which form a basement membrane lining the circumference of the seminiferous tubule epithelium (Fig. 3).

The seminiferous tubules loop throughout the testis collecting at a site called the rete, which connects the tubules to the epididymis, and then on to the vas deferens, and urethra. The seminiferous epithelium within the tubules is composed of two cell types: Sertoli cells and germ cells. Germ cells are organized into a radial, multi-layered hierarchy according to their extent of progression through spermatogenesis. Germ cells start spermatogenesis as undifferentiated spermatogonia, which are located along the circumference of the seminiferous tubules (Fig. 3). As they advance into differentiated spermatogonia and later cell types, the germ cells gradually proceed toward the central lumen of the seminiferous tubule. Spermatogenesis is timed such that once the germ cells have reached the lumen they have developed into sperm and are ready to be released into the seminal fluid (See “Spermatogenesis”).

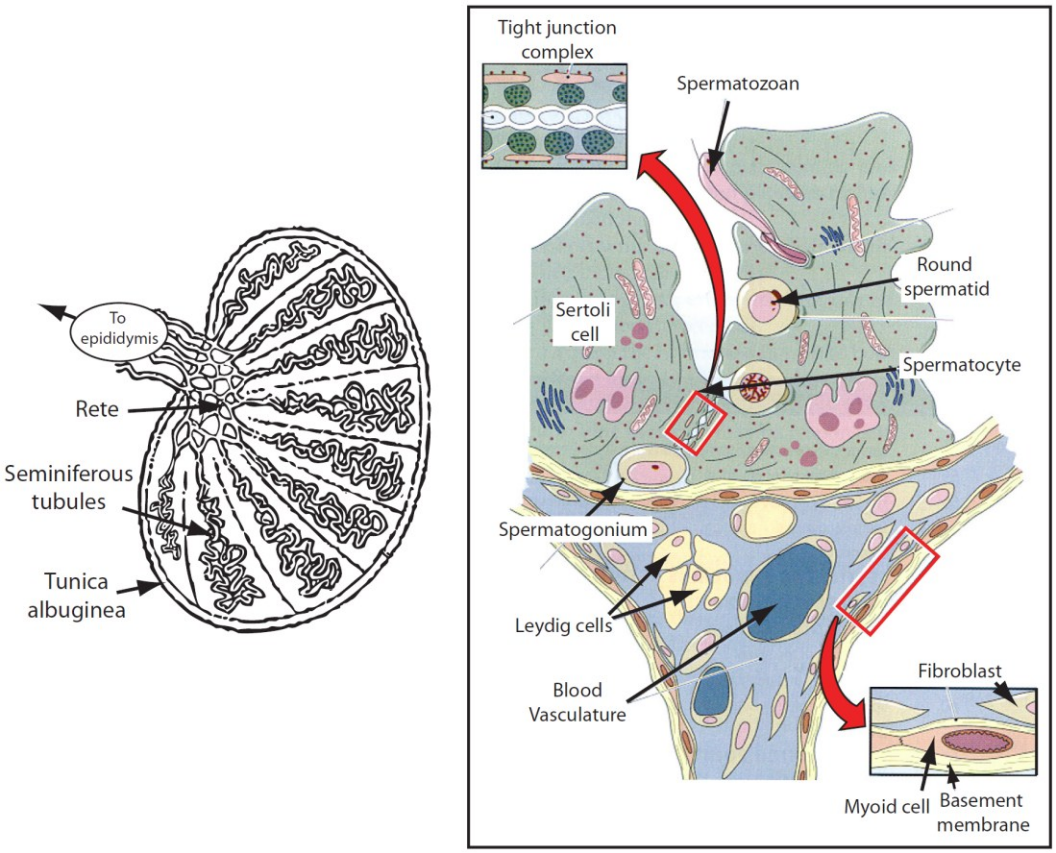

Figure 3: Anatomy of the testis.

Left: An illustration of the anatomy of the testis. Right: Illustration of the seminiferous epithelium and adjacent interstitial space. The seminiferous tubules are lined by peritubular myoid cells and a basement membrane of extracellular matrix proteins. Undifferentiated spermatogonia (including SSCs are believed to reside adjacent to this basement membrane). Tight junctions between adjacent Sertoli cells separate the basal and adluminal compartments and create a specialized microenvironment in the seminiferous tubules. Figure adapted from Johnson and Everitt [33] with copyright © by Blackwell Science Ltd.

The other cells comprising the seminiferous epithelium, Sertoli cells, are columnar cells that extend from the basement membrane into the tubule lumen and can interact with germ cells at all stages of spermatogenesis. As mentioned, Sertoli cells are the main regulators of spermatogenesis. Adjacent Sertoli cells are connected to one another through tight-junction complexes, which create a blood-testis barrier and effectively separate the germinal epithelium into two microenvironments: the basal and adluminal compartments (Fig. 3). Sertoli cells regulate the microenvironment of these compartments, in part through the production of germ cell-extrinsic factors that are crucial for survival and development; factors include SF, inhibins, and glial cell-line derived neurotrophic factor (GDNF) [34,35].

Sertoli cells proliferate rapidly after birth coincident with an increase in germ cell numbers. Sertoli cells increase in number until 17 days post-partum (dpp) in mice when they mitotically arrest [36]. Each Sertoli cell is understood to be able to support a finite number of germ cells, therefore, Sertoli cell numbers establish total germ cell numbers. This suggests that a balance is necessary to ensure proper numbers of germ cells for Sertoli cells. Evidence of this concept is found in immature rat testes in which Sertoli cell numbers have been reduced, which leads to a corresponding reduction in germ cells [37]. One manner in which germ cell homeostasis is maintained with Sertoli cells is through the control of germ cell apoptosis. Modulation of the apoptotic regulators Bax and Bcl-2 demonstrate this balance. Knockout of the pro-apoptotic regulator Bax leads to the accumulation of germ cells and a block in spermatogenesis consistent with a failure in apoptosis. However, this accumulation is followed by a catastrophic Bax-independent germ cell loss caused from the imbalance between Sertoli cells and the excessive numbers of spermatogonia [38]. Conversely, overexpression of the pro-survival Bcl-2 produces a similar accumulation to $Bax^{-/-}$ mice [39], indicating that the control of cell survival and death is important to maintain appropriate germ cell numbers. Interactions between Sertoli cells and germ cells are crucial for germ cell survival; for example, SF is secreted and expressed on the membranes of Sertoli cells and can interact with the receptor tyrosine kinase,

c-Kit, on differentiated spermatogonia germ cells. Mice with a mutation in the Steel locus (*Sl*) show a massive loss in differentiated germ cell types, due to apoptosis, resulting in seminiferous tubules with only undifferentiated spermatogonia. Transplantation of the germ cells from *Sl* mice into the seminiferous tubules of wild-type mice result in the transplanted cells being able to complete spermatogenesis [40] indicating that death of differentiated germ cell types in *Sl* mouse testes results from the disruption of communication between Sertoli cells and germ cells. Although SF is demonstrated to regulate differentiated spermatogonia survival, germ cell-extrinsic factors that regulate the cell death and survival of undifferentiated spermatogonia, including SSCs, need to be better explored.

At birth, the testis environment is very different from the adult testes. Somatic cell populations are in a state of expansion and tight junctions have not yet been established between Sertoli cells [41]. Furthermore, the germ cell population (called gonocytes at birth) are located in the center of the seminiferous tubules and have not migrated to the basement membrane, along the circumference of the tubules, to establish the nascent SSC population. It should be noted that not all gonocytes proceed through an undifferentiated spermatogonial stage. Lineage tracing analyses show that a number of gonocytes continue directly to differentiated spermatogonia, which proceed directly into the spermatogenic cycle [42], thereby contributing to the first wave of spermatogenesis. Those gonocytes that do not enter spermatogenic differentiation can remain in the basal layer and mitotically divide to establish the SSC pool.

Spermatogenesis

The hierarchy of spermatogenesis is organized into multiple layers within the seminiferous tubule. SSCs are located among other undifferentiated spermatogonia along the circumference of the basement membrane within the basal layer (Fig. 3). As germ cells proceed through meiotic differentiation they relocate from the basal layer, through the tight junctions between Sertoli cells, and into the adluminal compartment where they differentiate into sperm and are

released into the lumen. Spermatogenesis is a highly synchronized process; the time to complete one spermatogenic cycle, from A_1 spermatogonia to spermatozoa, is consistent among the members of a species: 35 days for mice, 52 days for rats, and 64 days for humans [32]. Previous work has shown that xenotransplantation of rat SSCs into the seminiferous tubules of the mouse leads to a spermatogenic cycle the duration of the rat (52 days) thereby demonstrating that the duration of the spermatogenic cycle is inherent to the germ cells of a particular species [43]. Spermatogenesis is also a continuous process as the entry of undifferentiated spermatogonia into the spermatogenic cycle occurs at specific intervals (every 8.6 days in the mouse). In this way, spermatogenesis appears staggered into stages of differentiation along the length of the seminiferous tubules giving the appearance of a "wave" of spermatogenesis and allowing for the continuous release of sperm [32].

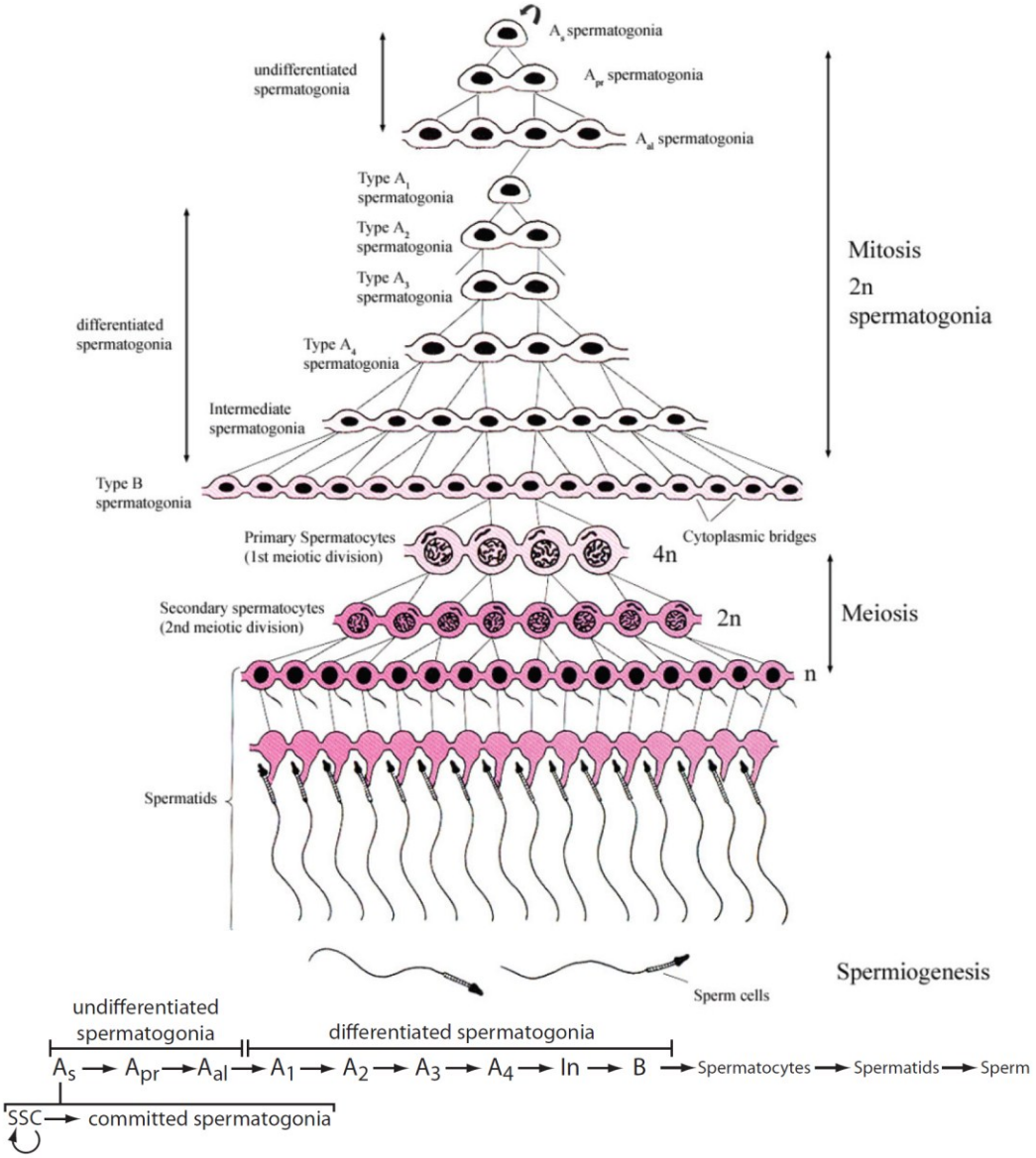

Figure 4: Mouse spermatogenesis.

Top: An illustration of the three phases of spermatogenesis: the mitotic phase, the meiotic phase, and spermiogenesis. Spermatogenesis occurs in a step-wise manner, with differentiation at each step occurring as a synchronized clone of germ cells connected via intercellular bridges. Bottom: The progression of spermatogenesis from undifferentiated spermatogonia to differentiated spermatogonia. SSCs are thought to exist primarily as A_s spermatogonia; SSCs have the unique ability to self-renew and differentiate to produce a cell committed to differentiation. Figure from Fawcett [44], copyright © Chapman & Hall/CRC.

As mentioned, the process of spermatogenesis occurs in one direction with germ cells on the basement membrane moving toward the lumen as they differentiate. In general, spermatogenesis operates in three phases, each with a particular goal: 1) the mitotic phase, which aims to increase the total number of differentiating germ cells; 2) the meiotic phase, which generates genetic diversity through homologous recombination and results in haploid cells; and 3) spermiogenesis, which remodels the germ cells to form the highly specialized sperm architecture (Fig. 4).

The mitotic phase of spermatogenesis occurs strictly in the basal compartment of the seminiferous tubules and deals exclusively with spermatogonia, the earliest cell types in spermatogenesis. The foundation of spermatogenesis is the SSC, which self-renews to maintain a constant supply of germ cells permitting continual production of sperm. Based on morphological study, the earliest spermatogonia are the A_{single} (A_s) spermatogonia located in contact with the basement membrane. SSCs are believed to be A_s spermatogonia however, not every A_s spermatogonia is an SSC (Fig. 4) [45]. As A_s spermatogonia divide, cytokinesis is sometimes incomplete, leading to the presence of an intercellular bridge between the two daughter cells. This intercellular bridge allows the direct communication between a cohort of clones, permitting their simultaneous division and differentiation. Furthermore, this incomplete division demarcates a differentiation event and intuitively these cells are designated A_{paired} (A_{pr}) spermatogonia. Further divisions lead to the formation of chains of spermatogonia connected to one another by intercellular bridges. These $A_{aligned}$ (A_{al}) spermatogonia are typically found as chains of 4 to 16 interconnected cells. Up until this point, all spermatogonia are referred to as "undifferentiated" spermatogonia [32,45], however, the term undifferentiated and the ability to act as an SSC should not be confused. It is unknown at what point spermatogonia lose SSC regenerative ability (Fig. 4). While it is suggested that SSCs are only found as A_s spermatogonia, morphological studies have observed odd-numbered clones of A_{al} spermatogonia from fractionation of individual cells. Following injury, these individual A_{al} spermatogonia have been suggested to act

as SSCs [46]. More recent studies provide evidence that $A_{pr} – A_{al}$ spermatogonia may retain SSC regenerative ability [47] (See "How is SSC activity maintained?"). Furthermore, it has even been suggested that cells past the undifferentiated spermatogonia stage retain a level of SSC activity [48]. Thus, the mediators of early commitment to differentiation (i.e. the inability to act as an SSC) are poorly understood and it is unknown at what point spermatogonia have irreversibly committed to differentiation.

Clones of A_{al} spermatogonia simultaneously form the first "differentiated" type spermatogonia: type A_1 spermatogonia, which represent the first cell type that enters into the spermatogenic cycle (Fig. 4). There is no cell division of A_{al} cells forming A_1 spermatogonia but rather they are termed A_1 spermatogonia at a set time to coincide with the timing of the spermatogenic cycle (every 8.6 days in the mouse). The A_1 spermatogonia subsequently undergo a series of organized divisions that lead to the production of A_2, A_3, A_4, intermediate, and finally type B spermatogonia [32]. Because each cell division effectively doubles cell number, from A_s to A_{al} approximately 16 cells can be generated. It is unclear how many A_{al} spermatogonia enter into the spermatogenic cycle as A_1 although chains of 16 A_1 spermatogonia have been reported previously indicating their origin from a single SSC [32]. Therefore, in theory the mitotic phase can generate approximately 512 cells from one SSC ($A_s \rightarrow$ B spermatogonia: 9 mitotic divisions).

Type B spermatogonia are the final spermatogonia type and divide to form the primary spermatocyte population. These preleptone spermatocytes must then move their way from the basal compartment, through the tight junctions and into the adluminal compartment of the seminiferous tubule where they enter the meiotic phase. At the first meiotic division, each primary spermatocyte divides to form two secondary spermatocytes which subsequently divide to split the chromosomes and form haploid round spermatids. The major physical remodelling that occurs during spermatogenesis takes place in the final stage called spermiogenesis. Round spermatids form elongated spermatids. Finally, a tail and midpiece forms for propulsion and the acrosome develops, which can

penetrate the oocyte, leading to a mature spermatozoan. At this point, the intercellular bridges rupture and the cells are finally released into the lumen of the tubule to be mixed into seminal fluid and ejaculated.

In total, from A_1 spermatogonia until sperm, 9 cell divisions occur including 2 during meiosis. Assuming 16 cells are formed from A_s until A_{al}, and this cohort of 16 cells can enter the spermatogenic cycle, up to 4096 sperm can be hypothetically produced from one SSC. Therefore, from one SSC commitment event potentially a great number of differentiated cells can be generated. Thus, tight regulation at the SSC level can be an important consideration to produce appropriate numbers of sperm.

Summary of Part 1

Sexual reproduction involves the combination of a sperm and oocyte to form an embryo, which develops into viable progeny. Genetic information is passed from generation to generation through these gametes, making germ cells the most important cells for the propagation of a species. To reach a point of sexual maturity, these progeny require systems to repair injury and replenish cell turnover. As a result, these systems of regeneration rely on stem cells for support, that is, cells that can self-renew and differentiate. Spermatogenesis is the process by which sperm is produced and this process relies on a stem cell to replenish its continuous cell production. These stem cells (SSCs) originate from PGCs in the embryo and are localized to the testis in adults. Spermatogenesis is a highly synchronized process involving the amplification of germ cells, the splitting of chromosomes via meiosis to generate genetic diversity, and modifications to produce specialized sperm cells. SSCs are the stem germ cell in the process of spermatogenesis, from which all differentiated germ cells originate. SSCs can self-renew to maintain a germ cell pool able to support the continuous production of sperm. A great number of sperm can be produced from the commitment of one SSC, indicating that tight regulation of SSC self-renewal and differentiation is important from proper spermatogenesis. In the testis, spermatogenesis relies on communication between germ cells and somatic cells, in particular the Sertoli

cells, which create an environment in which germ cells can develop. Sertoli cells are also believed to constitute the SSC niche in the testis, supporting SSCs through extrinsic factors. Thus, a great deal of work focuses on the identity of these factors that promote SSC proliferation, differentiation, and survival.

Part 2: Spermatogonial Stem Cell (SSC) Biology

Spermatogenesis relies on SSCs to replenish cells continuously lost through differentiation to sperm and to maintain an SSC pool such that this replenishment can continue for the lifetime of the individual. Fundamental to this process is a balance between maintaining SSC numbers and contributing differentiated progenitor cells to avoid sub-fertility. Regulation of this balance, between SSC self-renewal and commitment to differentiation, or cell fate decision, is an important focus of SSC biology, in particular, the communication within the surrounding SSC environment that regulates this decision. However, in order to study SSCs it is first important to define how they are detected.

Detecting SSC activity

Classical studies of SSCs focused on morphological analyses to identify these rare cells [49,50]. In general, these studies identify A_s spermatogonia along the basement membrane of the seminiferous tubule with a characteristic dominating nucleus and small amounts of darkly staining heterochromatin. However, definitive identification of SSCs based on this subjective criteria is difficult since their morphology is virtually identical to other undifferentiated (A_s – A_{al}) and differentiated spermatogonia types (A_1 onward) [32]. Furthermore, whereas the appearance of differentiated spermatogonia to sperm is timed to appear at specific stages of the spermatogenic cycle, undifferentiated spermatogonia are present asynchronously with all stages of spermatogenesis.

Functional transplantation assay

Stem cells are defined by functional criteria therefore their unequivocal detection requires an assay that can examine this functionality; both long-term self-renewal potential as well as the production of terminally differentiated cell types. Cell transplantation is one such assay, involving the injection of a cell suspension containing stem cells into a stem cell-depleted site in a recipient animal and evaluating the extent of regeneration.

The first reported functional transplantation assay attempted to identify stem cells of the hematopoietic system. In 1961, Till and McCulloch [51] transplanted isolated bone marrow cells from donor mice into lethally irradiated mice, thereby resulting in the long-term regeneration of hematopoiesis. They observed that colonies of cells formed in the recipient animal spleen, which they called colony-forming units-spleen (CFU-S). CFU-S could be excised, digested into single cells and re-transplanted into additional recipients to form secondary CFU-S, thereby demonstrating self-renewal of colony-forming cells. Differentiated cells from various hematopoietic lineages were found in CFU-S. Furthermore, they found that CFU-S were clonally-derived demonstrating that these differentiated cells could stem from the proliferation of one cell [52]. Thus, CFU-S appeared to present a means to functionally quantify self-renewal and differentiation of the bone marrow. Interestingly, CFU-S-forming cells are actually a more committed myeloid progenitor. In 1990, bone marrow cells depleted of CFU-S were found to contain long-term hematopoietic stem cells (HSCs), while cells selected for CFU-S appeared to be depleted in hematopoietic reconstitution ability compared to unselected bone marrow cells [53].

To identify HSCs (i.e. stem cells that support both myeloid and lymphoid lineages) a competitive repopulation assay is used. Transplanted donor bone marrow cells are injected into lethally irradiated recipient mice along with a competing HSC population. The donor cells must home/colonize their niche in the bone marrow and then proliferate and differentiate to continually form both myeloid and lymphoid cells for the life time of the animal. To detect the function of HSCs, the percent contribution of the transplanted HSC population to the

resulting blood lineages is compared to the functionally standard competitor donor cells. Competitive HSC transplantation allows recipient survival in the absence of adequate numbers of hematopoietic cells. For example, competition permits limited dilution assays, which aim to determine the stem cell activity of individual clones in a population of cells and have allowed for the discovery of cell surface markers that can virtually purify HSCs [54,55]. In addition to HSCs, functional transplantation assays have also been described for the epidermis, mammary gland stem cells, and intestinal stem cells [56].

SSCs are responsible for the production of one type of differentiated cell: sperm. Therefore, in the case of SSCs, the development of a functional assay requires the demonstration of long-term and continuous production of sperm. This functional assay was first described in 1994 by Brinster et al. [4,5]. By transplanting isolated testes cells from a transgenic reporter mouse expressing the *lacZ* transgene in post-meiotic germ cells into wild-type mouse testes, Brinster et al. were able to observe distinct segments or "colonies" of donor mouse-derived spermatogenesis in recipient seminiferous tubules (Fig. 5). Long-term self-renewal was demonstrated by the fact that spermatogenesis was observed 3 – 4 months after transplantation; since one round of mouse spermatogenesis takes 35 days, this implies approximately 3 rounds of spermatogenesis. Donor cells established morphologically normal spermatogenesis with all stages of germ cell differentiation present. Importantly, recipient animals were able to sire progeny harbouring the *lacZ* transgene demonstrating that donor-derived spermatogenesis can produce functional sperm [4].

Allogeneic transplantation has been performed in various ungulates and monkeys, while autogeneic transplantation has been performed in humans [57,58,59,60,61]. However, to more practically study SSCs in these species, xenogeneic transplantation into immune-compromised mouse testes is a possibility. Successful transplantation of other non-rodent species has resulted in complete spermatogenesis; although, hamster spermatogenesis arrested at the round spermatid stage [43,62]. Spermatogenesis is also reported from rabbit, dog, monkey, and human testes cells transplanted into mouse testes but appear to be

arrested as spermatogonial chains due to an apparent incompatibility with the mouse somatic environment [63,64,65]. However, spermatogonia appear to survive long-term along the basal layer, suggesting that SSCs from "higher" mammals may be able to colonize the mouse testes.

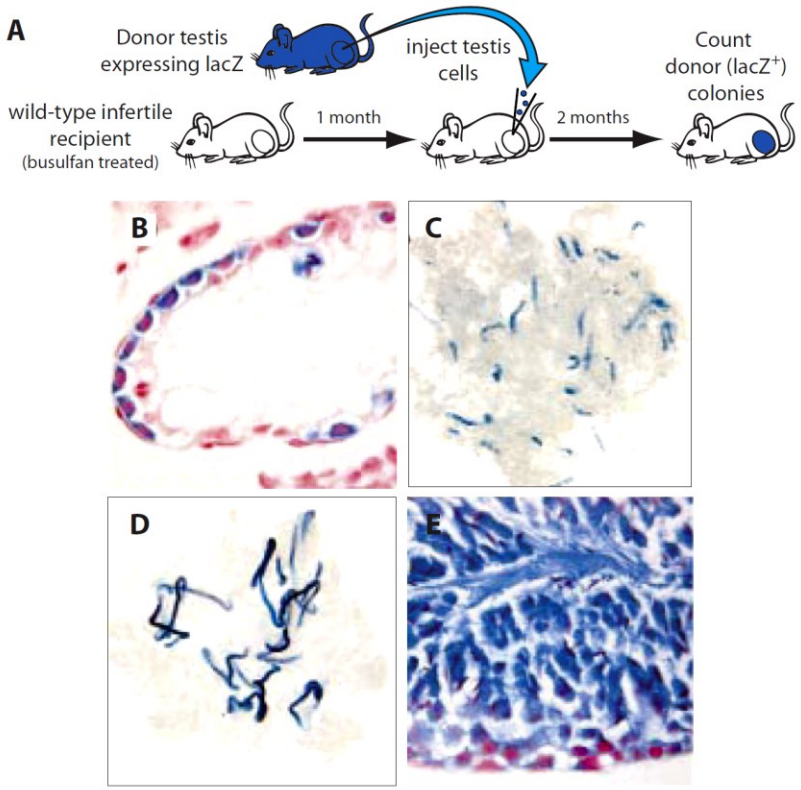

Figure 5: Spermatogonial transplantation assay to detect SSC activity

(A) Scheme of spermatogonial transplantation. Donor mouse testis cells, expressing a reporter marker, can be injected into an infertile recipient mouse testis. Two months later, transplanted testes are stained to observe colonies of regenerated spermatogenesis. (**B**) Cross-section of a recipient seminiferous tubule 1 month following transplantation. $LacZ^+$ donor cells are found along the basal compartment. (**C-D**) Appearance of wholemount recipient testes 1 month (C) and 2 months (D) following transplantation; SSC activity appears as distinct colonies of regenerated spermatogenesis. (**E**) Cross-section of a recipient seminiferous tubule 2 months following transplantation shows complete spermatogenesis. Figure adapted from Nagano et al. [66].

Further studies have characterized the kinetics of spermatogonial transplantation. Because the SSC niche is understood to be adjacent to the basement membrane, along the circumference of the seminiferous tubules, transplanted SSCs have to travel retrograde to spermatogenic differentiation, that is, from the lumen toward the basement membrane. The manner in which this retrograde migration occurs is not well known. Interestingly, 1 week following transplantation donor cells are seen along the circumference of the seminiferous tubules suggesting that this migration or "homing" is a relatively rapid event [66]. By 1 month following transplantation, spermatogenic colonies are observable, composed of a monolayer of spermatogonia spread along the basement membrane (Fig. 5). By 2 months post-transplantation, all germ cell types are present in colonies, including sperm. Interestingly, colony numbers do not change on average between 1 and 4 months after transplantation [66]. Therefore, recipient testes are typically analyzed at 2 months post-transplantation once mature sperm is detected.

The number of cells injected into a testis correlates to the number of colonies that form suggesting that colonies are clonally derived [67]. Zhang et al. [68] demonstrated this trait by transplanting the combined isolated testes cells from two mouse strains expressing differing genetic marks into wild-type recipient mouse testes. Two months post-transplantation, donor-derived colonies were observed. Whole colonies were cut out from recipient seminiferous tubules and individually genotyped to determine whether all cells from a colony were derived from either one genotype (clonal) or composed of both genotypes (non-clonal). Zhang et al. observed that colonies were composed exclusively of cells from one or the other genotype demonstrating that colonies are clonally derived. Work by Kanatsu-Shinohara et al. [69] further confirmed this assertion; they used a lentivirus to randomly insert a GFP construct into individual SSCs in culture and subsequently transplanted these individually marked SSCs into recipient testes. Following spermatogenesis regeneration, each individual colony was excised, digested, and Northern blotting was used to determine the random integration sites found in the cells of the colony. Each colony was shown to be exclusively

composed of cells derived from one independently-marked SSC. Therefore, these results collectively demonstrate that one colony arises from one SSC, meaning that SSCs can be quantified simply by counting colony numbers.

However, the transplantation assay does not allow the quantification of absolute SSC numbers. The main reason for this is due to the frequency of SSC homing, that is, the percent likelihood a transplanted SSC can migrate from the lumen to the basement membrane, survive, and regenerate a colony of spermatogenesis. Because definitive markers to prospectively identify SSCs have not been characterized, Nagano [70] investigated the homing efficiency of SSCs using a serial transplantation approach. This study determined that 12% of transplanted SSCs are able to migrate to their niche and regenerate a spermatogenic colony. From this work, absolute SSC numbers were calculated to be 3000 per testis, comprising 0.01% of total cells in the testis [70]. Prepubertal mouse SSCs have an identical homing efficiency to those of the adult, but SSC concentration is increased due to the absence of differentiated germ cell types [71].

It has been shown that cell-surface markers on SSCs are crucial for the attachment and survival involved in the colonization process. Work by Kanatsu-Shinohara et al. [72] has demonstrated that the germ cell- or Sertoli cell-specific knockout of β1-integrin, a cell surface SSC marker and receptor for laminin proteins, leads to a potential defect in attachment to the niche and the death of all transplanted cells, including SSCs. Therefore, SSC homing appears to be a complex, multi-faceted process, involving crucial steps in attachment, migration, survival, and proliferation. As a result, counting colonies does not represent absolute SSC numbers because colony numbers are the readout of only a small, but relatively consistent, percentage of transplanted SSCs (which can be considered “Functional SSCs”). Nevertheless, the establishment of the transplantation assay was a revolution in studies with SSCs, allowing the unequivocal, quantitative examination of SSCs based on their strict functional definition.

Sphere-forming assays

Not every stem cell population has the benefit of a well-defined transplantation assay. Rather, research with particular stem cells relies on in vitro culture to assay for long-term self-renewal and differentiation capability. One of the best examples is the neurosphere assay for neural stem cells (NSCs). Reynolds and Weiss [73] first described a culture condition in which cells from the subventricular zone (SVZ) of the brain were plated with epidermal growth factor (EGF) in serum-free medium under non-adherent conditions. Under these conditions a minor population of cells were able to proliferate rapidly to form floating spheres of up to 1000 cells. Spheres could be cultured over multiple passage generations demonstrating the presence of self-renewing cells [73]. Furthermore, when plated onto an adherent substrate in the presence of FBS and depletion of EGF, spheres could be differentiated into both neurons and glial cells at any time of long-term culture, demonstrating the retention of multipotentiality in these self-renewing cells. Thus, the use of the neurosphere assay has permitted the study of NSC biology in the absence of a transplantation assay. In particular, neurospheres have been useful in the identification of NSCs from the SVZ. Using this assay a population of GFAP+, CD133+ expressing SVZ cells have been identified, which are believed to constitute the NSC population; almost 80% of these cells show neurosphere-formation ability [74].

Limited dilution and single cell analysis show that neurospheres can be clonally derived attesting to the quantitativeness of this assay [75]. However based on surface markers, it has been determined that NSCs are a minority population in neurospheres and that most cells are actually transit amplifying cells [76]. Furthermore, some populations of these phenotypic transit amplifying cells can give rise to neurospheres, even though their self-renewing activity is limited, suggesting that the number of neurospheres may overestimate NSC numbers [75,77]. Direct comparison of the neurosphere assay with a functional transplantation assay would be the best manner to determine the faithfulness of this in vitro assay. However unlike SSCs, NSCs do not have the benefit of a transplantation assay.

In general, sphere-forming assays such as the neurosphere assay have been used as a facile, retrospective assay to investigate the biology of stem cells from various tissues including the mammary gland, pancreas, and prostate [78,79,80]. In addition, sphere-formation has been useful in the study of proliferating cancers and tumour-forming stem cells [81,82]. Therefore, sphere-formation assays provide a useful and simple tool for assaying stem cell potential in vitro, which can be employed to study the intrinsic and extrinsic molecular regulators governing stem cell renewal and differentiation.

Prospective SSC marker identification

While functional assays identify stem cell activity according to their strict definition, they are limited by the fact that they are retrospective analyses. What this means is that the readout of such assays can show that a stem cell *was* present by the result of its activity, but does not allow the prospective identification of stem cells and hence their study in real-time. Prospective identification may seem like an oxymoron since stem cells are regarded by their function rather than by physical traits. However, work with HSCs has demonstrated that using a multi-step approach it is possible to purify an almost homogeneous population of HSCs. Using antibodies against markers expressed on HSCs (such as Sca-1, c-Kit, Thy1, or CD150) in combination with antibodies against markers on differentiated cells, not expressed on HSCs, (such as CD34 and blood lineage markers) it is possible to isolate cells and transplant them into recipient animals to show HSC functional regenerative ability at a ratio of 1:2 – 1:5 cells isolated [54,55].

Stem cell enrichment using immunological cell separation methods against cell surface molecules has been performed extensively. Two methods are currently in wide use: Immunomagnetic cell separation and fluorescent-activated cell sorting (FACS). Immunomagnetic methods involve incubation of a cell population with antibodies conjugated to small paramagnetic beads. Cells expressing a particular cell surface marker will be bound by antibody and coated with magnetic beads, while those cells that are marker-negative will be absent of beads. These cells are then exposed to a magnetic field causing the separation of

these two populations; the bead-coated cells, attracted to the magnetic field, would adhere to the walls of the tube, while the bead-absent cells would remain in suspension and could be recovered or removed. This technique is quick and technically simple but leads to a crude cell isolation and is considered to be more suitable for the removal of bulk stem cell-negative populations or for the facile enrichment of stem/progenitor cells where absolute purity is not a concern.

For more specific or targeted cell isolation, many rely on a FACS approach. As with all immunological techniques, the principle is similar to immunomagnetic methods, however, FACS requires that antibodies are conjugated to excitable fluorescent molecules (fluorochromes). FACS also requires a flow cytometer; a machine that passes cells through a laser pulse, which excites the fluorochromes and can detect the light given off by the excited fluorochrome. Cells subsequently pass through a charged electromagnetic field that can direct individual cells into separate tubes for collection. Therefore, in contrast to the use of a simple magnetic field that can crudely separate bulk numbers of cells at a time, FACS can rapidly query individual cells as to whether they are bound by fluorochrome-conjugated antibodies or are not, and separate these individual cells accordingly. Furthermore, a flow cytometer can accommodate the use of multiple lasers and detectors simultaneously, allowing multi-dimensional cell separation based on multiple cell surface markers. Additionally, FACS can also be toggled to examine a given cell population based on cell size and complexity, cell cycle phase, or apoptosis/viability. However, FACS is an expensive and laborious technique requiring the use of specialized equipment and technically trained operators.

Enrichment of SSCs based on cell surface molecules has not been as effective as that for HSCs. Several cell surface markers for mammalian SSCs have been identified (Table 1), using immunological cell separation techniques such as those involving magnetic beads or flow cytometry coupled to spermatogonial transplantation. The caveat is that these markers are also expressed on other undifferentiated and/or differentiating spermatogonia and thus,

definitive markers for SSCs, which can identify SSCs with a high fidelity, are unknown.

Table 1: Cell surface markers examined for mammalian SSCs

Positive Markers	References	Negative Markers	References
α6 – integrin	[83,84]	αV – integrin	[83]
β1 – integrin	[72,84]	c-Kit	[3,83]
Thy-1 (CD90)	[3]	CD45	[3]
CD9	[85]	CD34	[3]
CD24	[3]	EPCAM	[86]
E-cadherin	[86]	Sca-1	[3]
GFRα1[a]	[71]	MHC-I	[3]
GPR125	[87]		

[a] expressed on prepubertal mouse SSCs; GFRα1$^+$ cells from adult testis were not enriched for SSCs

The initial identification of mouse SSC surface markers arose from the thought that SSC reside in a niche along the basal layer of the seminiferous tubules in direct contact with the basement membrane composed of extracellular matrix proteins, such as collagens, fibronectins, and laminin. On this basis, α6- or β1-integrin, which together make a receptor of laminin, were the first markers identified on mouse SSCs [83]. In this study, immunomagnetic separation was used to isolate cells expressing these integrins from testes of adult mice. The α6- and β1-integrin-positive fractions were transplanted into recipient mice and displayed greater regenerative activity compared to unsorted cells, thereby confirming the identity of these cell-surface molecules as SSC markers. Likewise, a similar methodology has been employed to identify the expression of other markers, including CD90 (Thy1), CD9, CD24, and GPR125 on SSCs [3,85,87]. On this same note, the surface molecules αV-integrin, c-kit, Sca-1, CD34, CD45,

and MHC-I are not found on SSCs and are thus stem-cell negative markers [3]. The use of these negative markers for SSCs facilitates the depletion of undesired, non-SSCs from a testis cell population. For instance, SSCs can be enriched using cell sorting based on MHC-I and CD45 to eliminate leukemic cells from a testis sample [88]. The difference in marker expression between SSCs and committed spermatogonia can also offer insight into surface phenotype changes as SSC differentiate. Recently, expression of the epithelial cell adhesion molecule (EPCAM) has shown to be increased as SSCs differentiate to committed progenitor spermatogonia [86]. EPCAM was previously shown to be an effective marker to isolate rat SSCs [89]. However in the mouse, EPCAM-selected cells show increased expression of markers on committed spermatogonia (such as c-kit) and SSCs were found to be restricted to a population of $CD9^{+}$ $EPCAM^{low/-}$ cells in the testis. Suppression of EPCAM appeared to decrease differentiated progenitor cell numbers in vitro suggesting an importance for EPCAM in their survival or proliferation [86]. Therefore, this report suggests that EPCAM is dynamically expressed as SSCs differentiate, which might be useful to study the early regulators of SSC commitment to differentiation.

The effectiveness of SSC enrichment can be improved when positive and negative markers are employed in tandem. Selection of a Thy-1^{+} MHC-I^{-} Kit^{-} cell population using flow cytometry from adult mouse cryptorchid testes resulted in cells highly enriched for SSC activity; a cell population containing an estimated 1 SSC in 15 sorted cells was achievable from cryptorchid testes [3].

In contrast to surface markers, undifferentiated spermatogonia, including SSCs, have been documented to selectively express intracellular markers. These markers include the POU-domain octomer-binding transcription factor 4 (Oct4), promyelocytic leukemia zinc finger (Plzf), and B-cell CLL/lymphoma 6 member B (Bcl6b) and each have demonstrated importance in maintaining the intrinsic program of SSC activity (See "How is SSC identity maintained?: Germ cell intrinsic regulators"). However, these intracellular factors are not effective markers for live SSC isolation because immunological selection cannot be achieved against intracellular targets without damaging the cells.

How is SSC identity maintained?

Adult stem cells are localized to specific regions in the body. It is believed that in these regions stem cells reside in a dedicated microenvironment called the niche. The niche is believed to supply extracellular matrix support and growth factors, which allow for the self-renewal and maintenance of the stem cell population. The niches of various stem cells have been described previously, for example, in the bone marrow reside HSCs and mesenchymal stem cells. ISCs are found in the intestinal crypts, while the hair follicle niche is in the bulge region of the hair follicle [90].

Mouse SSCs are also believed to reside in a niche microenvironment in the testis. As mentioned, SSCs are localized to the basal layer of the seminiferous tubule. Supporting the SSCs are the Sertoli cells and the extracellular matrix proteins of the basement membrane, while Leydig cells, myoid cells, and differentiated germ cells are also thought to contribute. These numerous cell populations are thought to communicate with one another and support SSCs through secreted factors extrinsic to SSCs. These extrinsic factors can in turn promote intracellular mediators in SSCs that are important to maintain their function. To understand how these cell-cell interactions might influence SSC behaviour, it might be beneficial to first discuss the niche interactions in Drosophila testes.

Drosophila germline stem cell niche

The Drosophila testis is constructed as a polarized tube; one end opening into the ejaculatory duct and, the other, closed end housing the germline stem cell (GSC) niche. In this distal tip of the testis reside specialized somatic cells called the hub cells, which form the GSC niche and to which GSCs are tightly attached (Fig. 6). Each GSC is also surrounded by two somatic stem cells. Attachment to the hub cells is critical to maintain GSC identity; cell division out of the niche leads to differentiation [91]. Asymmetric GSC division can result from one daughter that remains attached to the hub and another that loses contact. Consequently, the daughter that remains attached maintains GSC identity while

the other enters spermatogenesis. Similarly, somatic stem cells asymmetrically divide leading to one daughter cell remaining by the hub and another that remains associated with the differentiating germ cells throughout spermatogenesis.

The hub maintains GSC identity by secreting extrinsic factors, for example, the BMP homolog Unpaired (Upd). Upd binds to its receptor Domeless on GSCs and activates the Janus kinase – signal transducer and activator of transcription (JAK-STAT) cascade in GSCs via the JAK homolog hopscotch (hop) and Stat92E [92,93]. Although the downstream target genes of this signalling cascade remain to be addressed, loss of JAK-STAT signalling in mutant hop or Stat92E male flies leads to GSC differentiation and depletion of the GSC pool. Conversely, ectopic expression of Upd results in unrestricted self-renewal and the abnormal accumulation of germ cells. These accumulating cells appear to be GSCs since they express the intrinsic GSC transcription factor escargot [92]. Therefore, JAK-STAT signalling promotes Drosophila GSC self-renewal.

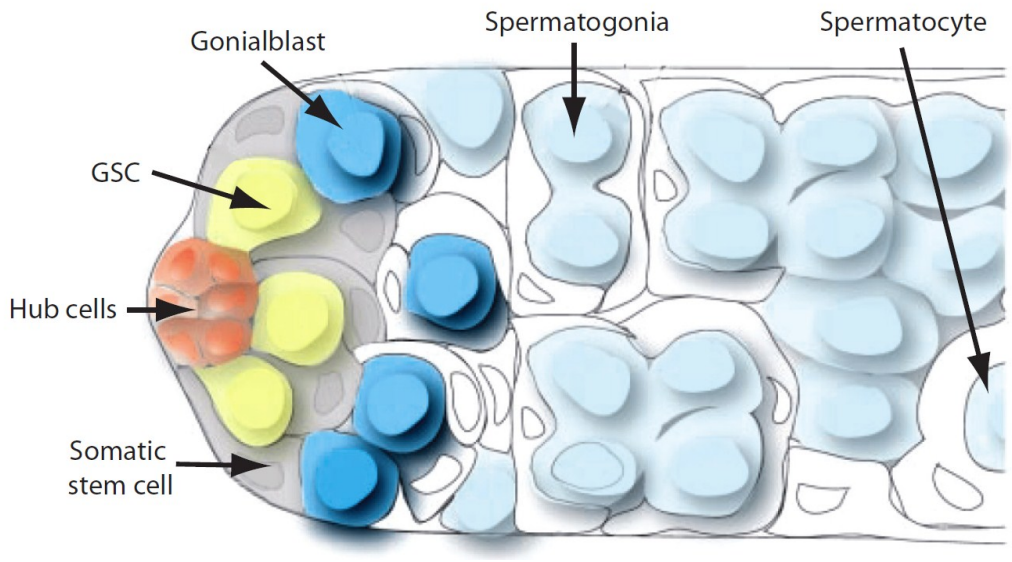

Figure 6: Drosophila male GSC niche

Figure adapted from Tulina and Matunis [93] with copyright © by The American Association for the Advancement of Science.

Differentiated germ cells that lose contact with the hub and exit the niche become gonialblasts, the first stage of differentiation. Subsequent incomplete mitotic cell divisions form 2-cell, 4-cell, 8-cell, and 16-cell cysts, which then enter meiosis to form spermatocytes and finally haploid sperm. Interestingly, using a temperature-sensitive Stat92E allele, GSC self-renewal and differentiation can be reversibly controlled. Though a shift in temperature (from 18°C to 29°C) Stat92E becomes defective leading to a loss in JAK/STAT signalling and a selective loss of GSCs from the hub. However, a switch back to 18°C restores JAK/STAT signalling and allows for a concomitant recovery of GSCs at the hub. These nascent GSCs arise from the dedifferentiation of differentiated germ cells; cells up to the 8 – 16 spermatogonial cyst stage have the capacity to contact the hub and revert to a GSC fate [94]. This indicates that the point of irreversible commitment in Drosophila spermatogenesis does not occur until a relatively late stage of spermatogenesis, that is, just prior to meiosis.

Mouse SSC niche

As opposed to the simple unidirectional tube that is the Drosophila testis, the mouse testis is composed of multiple seminiferous tubules. The mouse SSC niche does not rely on hub cells that can control the cells in contact to act as GSCs. Rather, the mouse environment is contributed to by several cell types. Sertoli cells are the main supporting cells that provide crucial factors for SSC maintenance. Moreover, Leydig cells and myoid cells are speculated to produce factors such as colony stimulating factor 1 (CSF1), which was shown to improve the rate of SSC expansion in vitro [95]. Additionally, the extracellular matrix proteins of the basement membrane can promote SSC survival [83], suggesting that numerous factors contribute to SSC regulation.

Because a definitive SSC marker has not been identified, determining the location of the mouse SSC niche is difficult. A whole testes analysis of fluorescently-labelled undifferentiated spermatogonia show an unequal distribution of these spermatogonia to sections of tubules in proximity to the interstitium, in particular to blood vessels [96]. Real-time imaging of seminiferous

tubules show that differentiating spermatogonial chains migrate away from sites adjacent to the interstitial space, linked to blood vessels, suggesting that these sites harbour undifferentiated spermatogonia [96]. Although this study only examined undifferentiated spermatogonia, these findings suggest that SSCs may reside near the vascular network innervating the testis and may also rely on factors from the blood stream. Finally, SSCs exist in close association with differentiated germ cells raising the possibility that they too might contribute factors to regulate SSC fate.

SSC extrinsic factors

What are factors known to influence SSC fate? Sertoli cells have been shown to produce a variety of factors that support spermatogenesis and regulate SSC fate decision. Glial cell-line derived neurotrophic factor (GDNF) is the best characterized factor that has been shown to support SSCs. GDNF is a distinct member of the transforming growth factor β superfamily, has well characterized roles in the survival and differentiation of several neural cell types [97,98], and is important to direct ureteric branching during kidney morphogenesis [99]. GDNF signals through a single pass transmembrane receptor tyrosine kinase, c-Ret. To signal through c-Ret GDNF first binds to a GPI-linked receptor, GDNF family receptor α 1 (GFRα1). A GDNF dimer binds two molecules of GFRα1 which in turn recruit two molecules of c-Ret (Fig. 7). Upon stimulation the two c-Ret molecules transphosphorylate their intracellular domains [100]. GDNF can also bind a soluble form of GFRα1 and activate c-Ret transactivation in motor neurons [101]. Therefore, soluble GFRα1 does not act as a competitive inhibitor to GDNF signalling but rather can present GDNF to c-Ret expressing cells thereby potentiating its signalling. GDNF has also been shown to signal through the neural cell adhesion molecule (NCAM) complex and activate Fyn, a Src-family tyrosine kinase. However, NCAM appears to be absent from the cell surface of SSCs at all postnatal stages [71]. Nevertheless, GDNF can signal through multiple receptors.

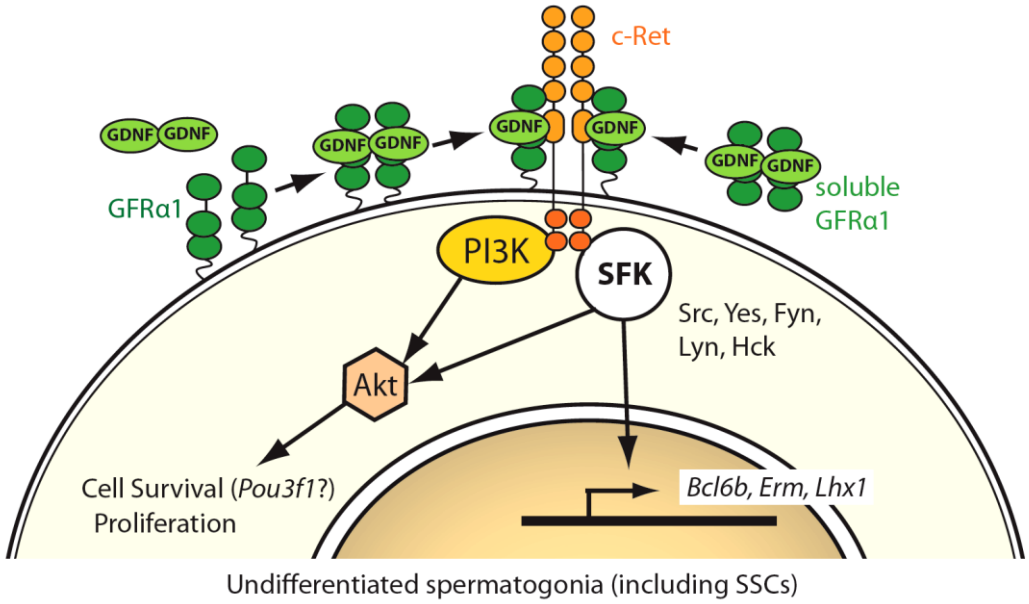

Figure 7: Proposed GDNF signalling mechanism in undifferentiated spermatogonia.

Binding of GDNF to membrane-bound or soluble GFRα1 leads to the recruitment of c-Ret and downstream activation of PI3K/Akt and Src-family kinase cascades. Figure adapted from Sariola and Saarma [100] and Oatley et al. [102].

The first indication of a role for GDNF in SSC renewal was through GDNF loss-of-function and overexpression mutant mice. $GDNF^{-/-}$ mutant mice are neonatal lethal and present without discernable defects in spermatogenesis [35]. GFRα1- and c-Ret-null mutants present similar phenotypes [103,104]. GDNF-heterozygous males, on the other hand, are viable and fertile. However, $GDNF^{+/-}$ males show disrupted spermatogenesis in a number of tubules eventually leading to a Sertoli cell-only phenotype in adult mice [35]. On the other hand, mutant mice overexpressing GDNF show normal spermatogenesis at birth but eventually presented with the accumulation of excessive numbers of undifferentiated spermatogonia, resembling seminomas [35]. These accumulations were mitotically active and the cells did not show the expression of the differentiated spermatogonial marker, c-kit, suggesting that all cells were

undifferentiated spermatogonia. Eventually, these accumulations degenerated resulting in tubular atrophy, perhaps due to the abnormal balance between numbers of germ cells and Sertoli cells. By 10 weeks of age, only spermatogonia were found along the basal layer. To examine if GDNF affects SSCs as a germ cell extrinsic factor, GDNF was overexpressed exclusively in Sertoli cells, using an in vivo electroporation technique to transfect a GDNF-expression vector [105]. Again, undifferentiated spermatogonia aggregations were present in these testes; transplantation of these aggregations into infertile recipient mouse testes led to increased colony formation demonstrating that GDNF is an important extrinsic factor that promotes SSC proliferation [105].

GDNF signalling appears to mediate the self-renewal and proliferation of SSCs through multiple intracellular mediators. Treatment of cultured SSCs, with pharmacological inhibitors against Phosphoinositide 3-kinase (PI3K) or Akt, results in increased cell death and a loss of SSC maintenance in vitro (Fig. 7). However, transfection of active-Akt into cultured SSCs did not result in SSC proliferation in the absence of GDNF, and thus the activation of Akt does not fully mimic the complete effect of GDNF and appears to be important for SSC survival rather than self-renewal [106]. GDNF signalling also recruits Src family kinases (Src, Yes, Lyn, Fyn, Hck); treatment with inhibitors against Src family kinases leads to a reduction in SSC maintenance in vitro without a concomitant increase in cell death [102,106,107]. Therefore, Src family kinase signalling appears to promote the self-renewal of SSCs (Fig. 7). Furthermore, Src family kinase signalling appear to mediate self-renewal in part by the upregulation of important transcription factors (Bcl6b, Erm, Lhx1) which are believed to have roles in SSC maintenance (See "Germ Cell Intrinsic Factors") [102,108]. Finally, GDNF appears to activate the *Ras* proto-oncogene downstream of Src family kinase signalling. Transfection of a constitutively active form of H-Ras into cultured SSCs maintained SSCs in vitro in the absence of growth factors (GDNF, FGF2, and EGF). Interestingly, inhibitors against PI3K/Akt signalling abrogated the SSC maintenance effect of H-Ras-active cultures indicating that PI3K/Akt may promote SSC survival downstream or independent of H-Ras. Additionally,

CyclinD2 and CyclinE were found to be upregulated by Ras signalling; transfection of constitutively active isoforms of CyclinD2/E led to long-term SSC maintenance in vitro in the absence of growth factors, while the same activation of CyclinD1 or CyclinD3 did not maintain SSCs [109]. Therefore, GDNF signalling operates through several intracellular mediators, which induce important intrinsic factors that support the self-renewal and survival of SSCs.

In addition to GDNF, other factors have been implicated as niche factors. Fibroblast growth factor 2 (FGF2) is produced by Sertoli cells and has been shown to promote SSC maintenance in vitro [110]. However, a recent report shows that FGF2 can activate PI3K/Akt and ERK leading to increased activity of mTORC1 in $Plzf^{-/-}$ mouse germ cells [111]. This mTORC1 over-activity results in a loss in SSC activity highlighting the importance for balance in mitogenic signals. Another factor, colony stimulating factor 1 (CSF-1), appears to be expressed by Leydig cells and myoid cells in the testis and its addition to SSCs in vitro leads to increased expansion of these cells [95]. Moreover, insulin growth factor 1 (IGF-1) expression has been detected in testicular Leydig cells and its addition leads to increased SSCs numbers in vitro [112]. Interestingly, IGF-1 also results in the increased emergence and maintenance of pluripotent ES-like colonies from neonatal mouse testis culture [113]. Other factors such as epidermal growth factor (EGF) and Wnt3a have shown some evidence to promote SSC maintenance in vitro. EGF is well documented to stimulate sphere-formation in stem cell cultures, including neurosphere culture, and has been used in the long-term culture of SSCs [114,115]. Wnt3a has been shown to stimulate the proliferation of a spermatogonial cell line in vitro [116]. However, it has not been determined whether these two factors are expressed in the testis making the relevance of these two factors, to the in vivo niche environment, unclear.

In contrast to factors that promote self-renewal, Sertoli cell factors such as BMP4 and Activin A appear to promote differentiation in vitro [110]. However, in general niche-derived factors that promote the early stages of SSC differentiation are virtually unknown.

SSC intrinsic regulators

Signalling by extrinsic factors on SSCs can lead to the expression of various intrinsic regulators that are necessary for proper SSC function. Studies with mouse SSCs have thus far identified a network of intracellular factors that appear to be important to maintain SSCs. Through loss-of-function studies, the transcription factors, Oct4, Plzf, and TATA box binding protein-associated factor 4b (Taf4b) have been found to be important for SSC self-renewal. Oct4 is a POU domain homeobox transcription factor that is expressed in the cells of the epiblast and is a well-defined regulator of pluripotency [117]. Oct4 is expressed in gonocytes and its expression is restricted to spermatogonia in adult testis (Fig. 8) [118]. An Oct4-GFP reporter faithfully labels undifferentiated spermatogonia in prepubertal mouse testes [119]. Transplantation of these Oct-GFP^{+} c-Kit^{-} spermatogonia show a significant increase in SSC numbers compared to Oct4-GFP^{+} c-Kit^{+} cells demonstrating Oct4 expression in SSCs and c-Kit expression of differentiated progenitor cells. Furthermore, lentiviral-mediated short hairpin (sh)RNA knockdown of Oct4 in SSC culture followed by transplantation results in the inability to regenerate spermatogenesis and a loss of SSC maintenance in the testis demonstrating importance for Oct4 in SSCs [120]. Interestingly, Oct4 does not appear to be regulated by GDNF in vitro and short interfering (si)RNA-mediated transient knockdown of Oct4 in SSC culture did not result in a significant reduction in SSC numbers in vitro [121]. Therefore, these results suggest that while this transcriptional factor is important for SSCs in the testis, its contribution in vitro to the intrinsic network supporting SSC function is debatable.

Plzf is expressed in undifferentiated spermatogonia (Fig. 8) and its expression has been shown to lead to the expression of Redd1, which is an important inhibitor of mTORC1 over-activation and maintenance of SSCs [111]. Plzf$^{-/-}$ mutant male mice show degenerated tubules and an absence of spermatogonia along the basal layer leading to complete abolishment of developing germ cells and ultimately resulting in infertility [122,123]. Functionally, SSCs from Plzf$^{-/-}$ mice transplanted into wildtype recipient mouse testes fail to reconstitute spermatogenesis. Moreover, reciprocal transplantation of

wildtype SSCs into *luxoid* mutant mice, which contain a nonsense mutation in the locus containing Plzf, permitted donor-derived spermatogenic colony formation suggesting that the loss of Plzf is an intrinsic defect in SSC function [122,123].

Finally, Taf4b is expressed in spermatogonia and spermatids and Taf4b$^{-/-}$ males are reported to become infertile by 3 months of age and eventually show a loss of all germ cells by 8 months [124]. Transplantation of Taf4b$^{-/-}$ testis cells into wildtype testes has not been reported so it is unknown if Taf4b is important for SSC regenerative ability. However, transplantation of wildtype SSCs into Taf4b$^{-/-}$ resulted in donor-derived spermatogenesis, which suggests that testicular somatic cells are functional in Taf4b$^{-/-}$ animals and germ cell loss may be due to an intrinsic defect in Taf4b$^{-/-}$ SSCs.

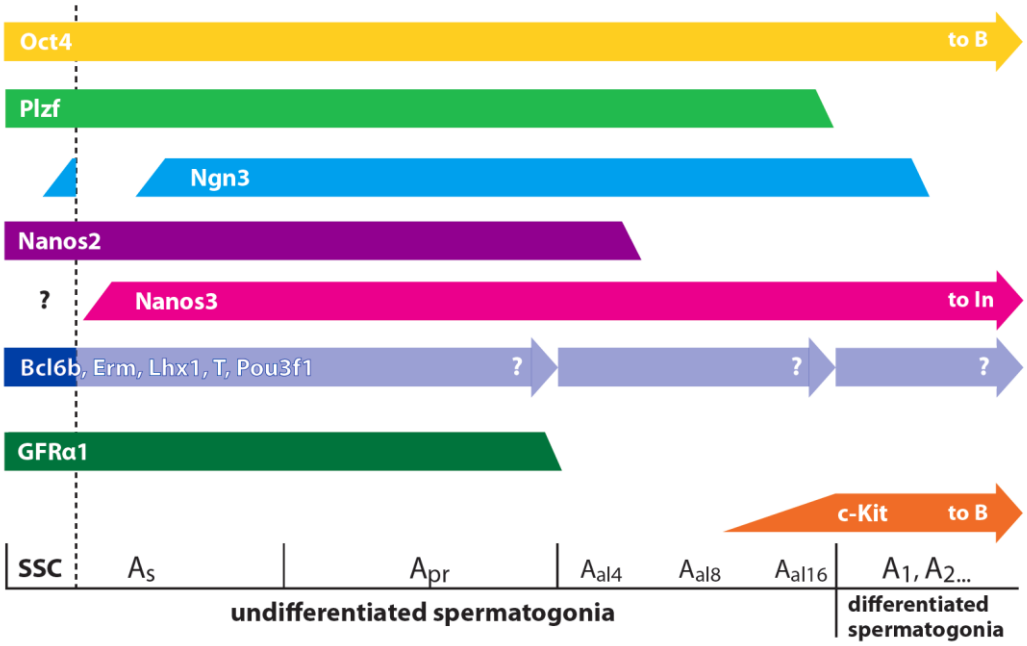

Figure 8: Expression pattern of various intrinsic and cell-surface markers for SSCs.

Numerous intrinsic markers are expressed in SSCs and throughout other undifferentiated spermatogonial cell types. Ngn3 is found to be expressed in a subset of SSCs but is believed to be predominantly expressed in committing spermatogonia. Nanos3 expression in SSCs has not been demonstrated. The cell types expressing Bcl6b, Erm, Lhx, T, or Pou3f1 have not been determined extensively.

GDNF promotes SSC survival and proliferation; therefore, investigation into the identification of GDNF-regulated genes, from cultured SSCs, has reported the expression of transcription factors that might mediate SSC maintenance, such as Bcl6b, Erm, Lhx1 [108]. Using siRNA targeting, each of these transcription factors were transiently reduced independently in SSC culture. These cultures were subsequently transplanted into recipient mice testes to examine SSC activity. Independent reduction of Bcl6b, Erm, or Lhx1 led to a significant reduction in SSC numbers demonstrating that these factors are each important to maintain SSC/progenitor cells in vitro [102,108]. In vivo, expression

of each factor was localized to spermatogonia in testis sections (Fig. 8). However, Bcl6b and Lhx1 also appeared in spermatids, while Erm was also localized to Sertoli cells suggesting that these factors may have diverse roles in spermatogenesis [102,125]. $Bcl6b^{-/-}$ null mutant mice are fertile but males sire lower numbers of pups per litter. Histological analysis of $Bcl6b^{-/-}$ adult testes show the presence of abnormal seminiferous tubules, with ~24% of tubules presenting either degenerated stages of spermatogenesis or a Sertoli cell-only phenotype, suggesting an importance for Bcl6b in maintaining SSCs in the testis [108]. In contrast, $Erm^{-/-}$ null mutants show dramatic infertility; spermatogonia are lost during the first wave of spermatogenesis resulting in seminiferous tubules devoid of germ cells [125].

Further study has shown that another POU domain transcription factor similar to Oct4, called Pou3f1, is upregulated downstream of GDNF-mediated PI3K/Akt signalling [121]. Inhibition of Pou3f1 in SSC culture, using siRNA, led to an increase in apoptosis and transplantation results showed a loss in SSC maintenance in vitro. Therefore, as opposed to Oct4, Pou3f1 appears to be important for the in vitro survival of SSCs [121]. Additionally, Brachyury (T) is implicated in in vitro SSC maintenance. Gene expression profiling on Erm-silenced cultures has shown that expression of T is downstream of Erm [126]. Again, siRNA against T in SSC culture followed by spermatogonial transplantation has demonstrated that T can be considered yet another factor that can comprise the core intrinsic network mediating SSC maintenance in vitro and potentially in vivo.

Recently, Nanos2 and Nanos3 have been detected in the spermatogonia of adult mice testes. These proteins are zinc-finger RNA binding proteins and are homologous to Drosophila Nanos, which is an important component in germ plasm involved in repression of the somatic differentiation programme and PGC specification. Nanos2 is expressed specifically in A_s and A_{pr} spermatogonia and transplantation of Nanos2-expressing cells into recipient mouse testes demonstrate expression of Nanos2 in SSCs (Fig. 8) [127]. Conditional knockout of Nanos2 leads to a gradual defect in spermatogenesis eventually resulting in a

Sertoli-cell only phenotype in most tubules. In comparison, conditional Nanos2 overexpression appeared to block differentiation and increased numbers of $Plzf^{+}$ spermatogonia further demonstrating its role in maintaining SSCs in the testes [127]. Nanos3 is detected primarily in undifferentiated spermatogonia but Nanos3-expressing cells have yet to be transplanted to functionally demonstrate expression in SSCs. Nanos3 expression corresponds strongly with $GFR\alpha1^{-}$ $Neurogenin3^{+}$ $A_{pr} - A_{al}$ spermatogonia (Fig. 8), which suggests that it may support more differentiated spermatogonia than SSCs [128].

Neurogenin 3 (Ngn3) is another transcription factor seemingly expressed in SSCs; transplantation of $Ngn3\text{-}GFP^{+}$ cells into recipient testes leads to colony formation demonstrating that Ngn3-expressing cells have SSC regenerative ability. However, Ngn3 has been observed to be highly expressed in $A_{pr} - A_{al}$ undifferentiated spermatogonia indicating that this factor may be expressed as SSCs differentiate (Fig. 8) (See "When is the point of irreversible commitment to differentiation?") [47].

Intrinsic factors that mediate SSC differentiation have not been well characterized. Recent work has implicated a role for STAT3 in SSC differentiation. Stable knockdown by shRNA against STAT3 in SSC cultures in vitro and subsequent transplantation of these cultures into recipient testes showed that chains of spermatogonia could develop in recipient testes but were arrested at A_{al} spermatogonia; chains longer than 16 cells were not observed in recipient testes [129]. Because JAK/STAT signalling has been described to mediate Drosophila GSC self-renewal, these results serve to demonstrate that signalling pathways between Drosophila and mouse are not evolutionarily conserved.

Recent work has also demonstrated a role for the Forkhead box family (Foxo) of transcription factors in both SSC maintenance and differentiation. Triple knockout of Foxo1, Foxo3, and Foxo4 specifically in germ cells results in some tubules completely devoid of germ cells, while others show a complete spermatogonial arrest, which is suggestive of a role in spermatogenic differentiation [130]. Tubules with germ cells show the absence of multilayer spermatogenesis and the only germ cells present in tubules appear to be

spermatogonia. Furthermore, Foxo1 function appears to be inhibited by PI3K/Akt as conditional knockout of the PI3K inhibitor, Phosphatase and tensin homolog (Pten), or the PI3K/Akt mediator, 3-phosphoinositide-dependent protein kinase (Pdk1), leads to a loss or constitutive activation of Foxo1, respectively [130]. Foxo1 expression is restricted to undifferentiated spermatogonia (A_s to A_{al} spermatogonia) and cytoplasmic to nuclear migration of Foxo1, starting at 3 dpp, appears to be required for the expression of c-kit [130] arguing for the importance of the Foxos in the transition from undifferentiated to differentiated spermatogonia.

When is the point of irreversible commitment to differentiation?

In Drosophila testes, it is observed that upon selective GSC depletion, germ cells up to the 16-cell cyst stage have the ability to revert to GSCs if they migrate back to the niche and contact with the hub cells [94]. Past this stage, it has not been demonstrated that the ability to revert back to GSCs is retained. In mouse spermatogenesis, the point of irreversible commitment is unknown. While it is generally accepted that SSCs constitute a population of A_s cells, it has been suggested that more differentiated spermatogonia can contribute to long-term spermatogenesis regeneration [46]. Using a tamoxifen pulse-chase system to selectively label Ngn3-expressing spermatogonia, Nakagawa et al. [47] observed that most Ngn3-labelled undifferentiated spermatogonia are transit amplifying cells that contribute to only one round of spermatogenesis before exhaustion. This is not surprising since Ngn3-expression is most robust in $A_{pr} - A_{al}$ spermatogonia [47]. However, transplantation of these labelled cells into an SSC-depleted animal led to the formation of spermatogenic colonies showing long-term spermatogenesis regeneration. This suggests that, while $Ngn3^+$ cells may be transit amplifying cells during steady state spermatogenesis they appear to retain the ability to revert to an SSC fate when injected into depleted testes. Another study has suggested that A_{pr} and A_{al} spermatogonia can also give rise to long-term SSCs by fragmenting off the chains of spermatogonia [131]. Moreover, work with spermatogonia in vitro show that c-Kit^+ cells, normally associated with

differentiated spermatogonia, show comparable SSC activity as c-Kit^{-} cells [132]. This work and others [48] posit that differentiating spermatogonia populations, previously believed to be committed to differentiation, might still retain the ability to revert to and support steady-state spermatogenesis as an SSC. In general, these results seem to suggest that commitment takes place gradually and some Ngn3^{+} or c-Kit^{+} cells may still retain a degree of SSC activity. It will be interesting to examine if there is a correlation between the expression levels of particular markers (e.g. Ngn3) and the degree of commitment.

SSC culture

Study of SSCs in vivo is challenging due to their rarity in the testis and lack of prospective markers for their effective identification. Studying genetic mutant animals is one means to investigate the mediators of SSC fate decision. However, within the testis SSCs remain inaccessible and difficult to screen or experimentally manipulate directly. Therefore, the ability to culture SSCs in vitro may circumvent these limitations. Attempts to culture SSCs have replicated conditions found in the niche, such as the use of extrinsic factors. In 1998, it was first demonstrated, using the transplantation assay, that functional SSCs could survive in culture [110]. Screening of different somatic cell lines, for use as feeder cells, demonstrated that several cell lines facilitated in vitro SSC survival, including SIM derived thioguanine and oubain resistant (STO) mouse embryonic fibroblasts, which maintained SSC activity for 4 months [110]. In another study, SSC cultures were transfected with a retroviral vector carrying a lacZ transgene and subsequently transplanted into recipient testes [6]. Because a retrovirus requires cell division to stably-integrate its genome into a host cell, if SSCs are proliferating in culture then they can incorporate the transgene and produce lacZ-expressing progeny following transplantation. Indeed, transplanted testes showed spermatogenic colonies that were positive for lacZ demonstrating that SSCs were proliferating, albeit slowly in vitro [6]. Screening of various growth factors demonstrated SSC maintenance could be improved. In particular, the addition of GDNF appeared to improve SSC maintenance. Building off the findings of these

studies, culture conditions in which SSCs could proliferate long-term were developed [112,114]. These studies used embryonic fibroblasts as a feeder cell layer and GDNF as the primary growth factor to promote self-renewal. Importantly, these studies required the elimination of most testicular somatic cells prior to plating, either through immunomagnetic separation or differential adhering plating and these studies utilized culturing media devoid of serum, which was demonstrated to be generally detrimental for SSC maintenance [112].

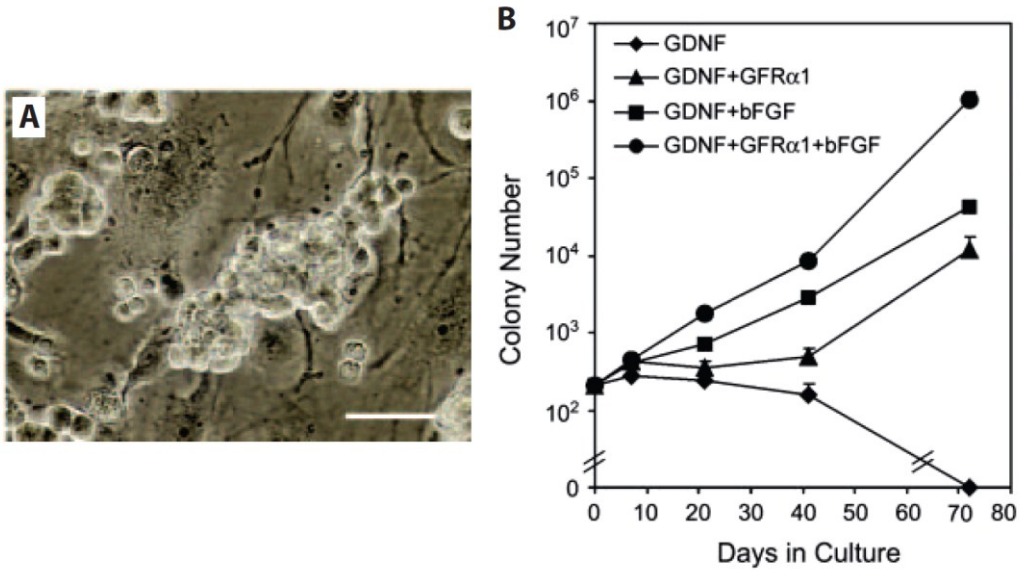

Figure 9: SSCs can be amplified long-term in culture.

(**A**) Photomicrograph of a germ cell "cluster" composed of SSCs and differentiated cells cultured on a feeder cell layer of mouse embryonic fibroblasts. (**B**) SSCs can be expanded over an extended duration in the presence of growth factors GDNF and bFGF (FGF2). Figure adapted from Kubota et al. [112] with copyright © by The National Academy of Science.

Under these conditions, SSCs can be expanded indefinitely and without significant changes in genetic and epigenetic properties [133]. In these cultures, SSCs form distinct cell accumulations that remain in very tight contact with one another and loosely adhered with the STO feeder cell layer. In this thesis, I term these accumulations "clusters" (Fig. 9). These clusters are reminiscent of the accumulations of undifferentiated spermatogonia in GDNF-overexpressing mouse testes [35]. Marker analyses of these aggregations have demonstrated that various SSC markers such as Plzf, Oct4, α_6-integrin, and c-Ret are homogeneously expressed suggesting that all cells are undifferentiated spermatogonia [112]. Furthermore, transplantation of clusters shows that SSCs can be expanded 5000-fold over 70 days, indicating self-renewal occurs in vitro. However, transplantation of these aggregations into recipient testes has shown that SSCs only comprise ~1 – 3% of cluster cells [112,115,134]. Therefore, these germ cell aggregations appear to be composed of a community of SSCs and more differentiated spermatogonia and are thus functionally heterogeneous.

This community formation presents the possibility that SSCs and committed cells may be communicating reciprocally in a manner like in the testis. The interaction between SSCs and committed cells, specifically, if differentiated cells contribute to the niche regulation of SSCs, has not yet been explored in spermatogenesis. Lee et al. [109] have demonstrated that H-Ras is activated by GDNF and can stimulate CyclinD2/E upregulation in clusters. Interestingly, constitutive activation of Ras-CyclinD2E can result in cluster formation and SSC maintenance in the absence of GDNF, suggesting that Ras-cyclinD2E pushes SSC self-renewal. However, stably transfected constitutive active Ras-CyclinD2E SSCs could regenerate spermatogenesis, when transplanted into recipient testes, indicating that these SSCs retain the ability to fully differentiate [109]. This indicates Ras signalling may simply stimulate cell proliferation prior to the decision to self-renew or differentiate. Interestingly, the SSC concentration was not enriched in constitutive active Ras-CyclinD2E clusters (1 – 2.7%) [112,115,134]. The fact that SSC concentration in clusters is identical across numerous studies raises the possibility that an aspect of fate decision regulation of

SSCs may be decided through communication with committed cells. It will be interesting to discover markers to isolate committed cells from SSCs and examine potential mediators of this communication.

Because SSC self-renewal and differentiation events appear to occur continuously in vitro, this system may be useful to identify potential extrinsic factors that may shift the balance of SSC fate decision under well-defined conditions. However, the presence of feeder cells remains necessary for SSC maintenance, in the absence of serum. Therefore, examination of the contribution from feeder cells to SSC maintenance may present novel factors that can regulate SSC fate.

One intriguing finding from SSC culture was the emergence of pluripotent cell colonies with similar properties to ESC or EGC colonies. The appearance of these pluripotent cells was first reported by Kanatsu-Shinohara et al. [135] from neonatal mice testes culture and were later reported derived from adult mouse testicular cells [87]. These pluripotent colonies can be differentiated into cells of the three germ layers and have also been demonstrated to contribute to chimera formation when injected into developing blastocysts, demonstrating their genuine pluripotentiality. Thus, SSC culture shows promise as a potential, non-controversial source for patient-specific pluripotent ES-like cells (at least for males). However, one issue is that it is unknown how these pluripotent cells form in culture, specifically, if they transit from SSCs or other cluster cells.

Summary of Part 2

Stem cells are defined by their ability to self-renew and differentiate and as such, their detection requires an assay that examines this functional activity. Stem cells such as HSCs rely on a cell transplantation assay in which the readout is the long-term regeneration of a stem cell tissue. Other stem cells, such as NSCs, do not have the benefit of a transplantation assay but instead rely on in vitro "sphere-forming" assays. These sphere assays can quantify the presence of sphere-forming cells based on the appearance of clonally-derived spheres. Stem cell spheres are distinct aggregations of stem cells and differentiated progenitor

cells indicating that early events of self-renewal and differentiation can occur in these cell communities.

Work with SSCs has relied on a transplantation assay involving the injection of SSCs into infertile recipient mouse seminiferous tubules. As with other stem cells, the readout is regeneration, which is visualized as segments or colonies of donor-derived spermatogenesis. These colonies are clonally derived indicating that SSCs can be quantified by counting colony numbers. An important consideration is that this quantitative assay is a retrospective analysis, which does not allow for the study for the prospective identification of SSCs. This assay has allowed for SSC marker discovery; using antibody-based selection coupled to spermatogonial transplantation, the cell surface phenotype of SSCs has been explored. SSCs express markers such as Thy1 and α6-integrin and do not express c-Kit, MHC-I, and Sca1. However, this marker profile is shared with other non-SSC differentiated progenitor spermatogonia. Therefore, definitive markers have not been identified to purify SSCs.

This inability to prospectively identify SSCs has hampered the study of their niche regulation. SSC reside along the basement membrane of the seminiferous tubules in close communication with Sertoli cells, differentiated germ cells, and ECM, but because SSCs cannot be prospectively identified, the exact location of the niche and which cells actually contribute is not known. Sertoli cells are crucial to support spermatogenesis and are generally understood to support SSCs. Several factors have been identified from Sertoli cells that can influence SSC proliferation, such as GDNF. This growth factor subsequently promotes expression of various intrinsic factors that maintain SSC function. However, GDNF does not appear to promote expression of certain important SSC regulators, such as Oct4, indicating other extrinsic factors must contribute to SSC regulation. Similarly, the factors defining the switch from SSC to differentiated cell are not known.

Therefore, current issues that should be addressed to progress research with SSCs:

1) Markers that identify/purify SSCs are unknown.
2) Novel SSC niche factors that promote self-renewal, survival, or differentiation (i.e. SSC fate decision) need to be characterized.
3) The intrinsic regulatory network maintaining SSC function or promoting the switch to differentiation is not completely defined.

Using GDNF and FGF2, SSCs can be expanded continuously in vitro. This culture system uses well defined conditions but an embryonic fibroblast feeder cell layer is required, indicating unknown factors are necessary to maintain SSCs. Like other stem cells, SSCs expand as distinct aggregations of stem cells and differentiated cells (called clusters). Because self-renewal and differentiation events occur within clusters, this culture might represent an ideal means to study the cell fate mechanism of SSCs.

Part 3: Wnt Signalling

Work with Wnts began when Drosophila Wingless and the mouse mammary tumor virus insertion site, Int1, were identified as homologous genes. Hence, the name "Wnt" is a portmanteau derived from a combination of Wingless and Int1 [136]. Subsequently, other components related to Wnt signalling in Drosophila were found to be conserved in mammals: components such as the transmembrane receptor Frizzled, co-receptor Arrow/low density lipoprotein receptor-related protein, Dishevelled, and Armadillo/β-catenin. As a result, the discovery of these components collectively generated the framework for what would become known as the canonical (β-catenin) pathway. Later studies identified additional "non-canonical" Wnt pathways that can involve heterotrimeric G proteins, intracellular Ca^{+2}, or various protein kinase cascades.

Wnt proteins are secreted signalling glycoproteins which are hydrophobic due in part to a post-translational palmitoylation modification necessary for signalling [137]. Currently, nineteen Wnts have been identified in mammals, which have been characterized into either "canonical/β-catenin" or "non-canonical" signalling classes, designated according to the pathway they are classically deemed to activate. The ability for different Wnts to stimulate different pathways was first noticed when particular Wnts were found to transform a mouse mammary cell line and induce secondary axis formation in Xenopus embryos [138,139]. These became known as the transforming or β-catenin class Wnts and include Wnt1, Wnt2b, and Wnt3a. On the other hand, non-canonical class Wnts did not transform mammary cells, did not induce a secondary axis in Xenopus embryos, and were later shown to be involved in convergent-extension movements during Zebrafish embryogenesis [140]. This class includes Wnt4, Wnt5a, and Wnt11. Recent work has shown that the pathway activated (i.e. β-catenin or non-canonical cascades) is not exclusively intrinsic to a particular Wnt but rather to the Wnt and the available receptors on a particular cell [141]. For example, Wnt5a, a prototypical non-canonical signalling Wnt, has been shown to induce the canonical pathway upon the overexpression of Fzd4 and LRP5, whereas a non-canonical mechanism is activated when the single pass

transmembrane receptor tyrosine kinase orphan receptor 2 (Ror2) is expressed [141]. Additionally, although Wnt5a is deemed a non-canonical class Wnt, it can also induce secondary axis formation when Fzd5 is overexpressed [142]. Furthermore, another prototypical non-canonical Wnt, Wnt11, is required for convergent-extension movements during Xenopus gastrulation but also works to initiate axis formation through the β-catenin pathway [143].

Wnt signalling pathways

Canonical (β-catenin) pathway

The β-catenin pathway is understood as follows: Wnt molecules bind to the receptor, Frizzled (Fzd), and co-receptor low density lipoprotein receptor-related protein (LRP) at the cell surface. Fzd are seven transmembrane receptors with a long N-terminal extension containing a cysteine-rich domain, to which Wnt proteins bind. Currently, ten Fzd proteins have been identified in mice, however, Wnts have not been demonstrated to show a binding preference to any one particular Fzd [144]. Normally, in the absence of Wnt stimulation, levels of the transcriptional regulator, β-catenin, are kept to a minimum in the cytoplasm through continuous proteasome-mediated degradation (Fig. 10). Under these conditions, β-catenin is reciprocally phosphorylated by the serine-threonine kinases, casein kinase 1 (CKI) and glycogen synthase kinase 3 (GSK3) [145]. The scaffolding proteins Axin and adenomatous polyposis coli (APC) are also involved and together these proteins (CKI, GSK3, Axin, and APC) make up a β-catenin degradation complex that phosphorylates β-catenin and allows it to be recognized by βTrCp, targeted for ubiquitination, and degraded. Binding of Wnt to the Fzd/LRP receptor complex recruits CKI and GSK3 to the cytoplasmic tail of Fzd and LRP [146]. The kinases phosphorylate these sites, which in turn bind Axin at the cell membrane. Furthermore, signalling through Fzd/LRP activates the intracellular mediator Dishevelled (Dsh), which can also inactivate action of GSK3 [144]. Thus, activation of the canonical pathway disrupts action of the β-catenin degradation complex permitting β-catenin to accumulate in the cytoplasm (Fig. 10). As cytoplasmic β-catenin accumulates it migrates into the nucleus and

interacts with the zinc-finger transcriptional factor, T-cell factor/lymphoid enhancer factor (TCF/LEF). There are four TCF/LEF family members, which appear to act non-redundantly and bind to specific responsive elements in the genome. Transcription is normally repressed through interaction of TCF/LEF with a variety of transcriptional repressors [147]. Binding of β-catenin displaces these repressive binding partners from TCF/LEF and drives transcription of target genes.

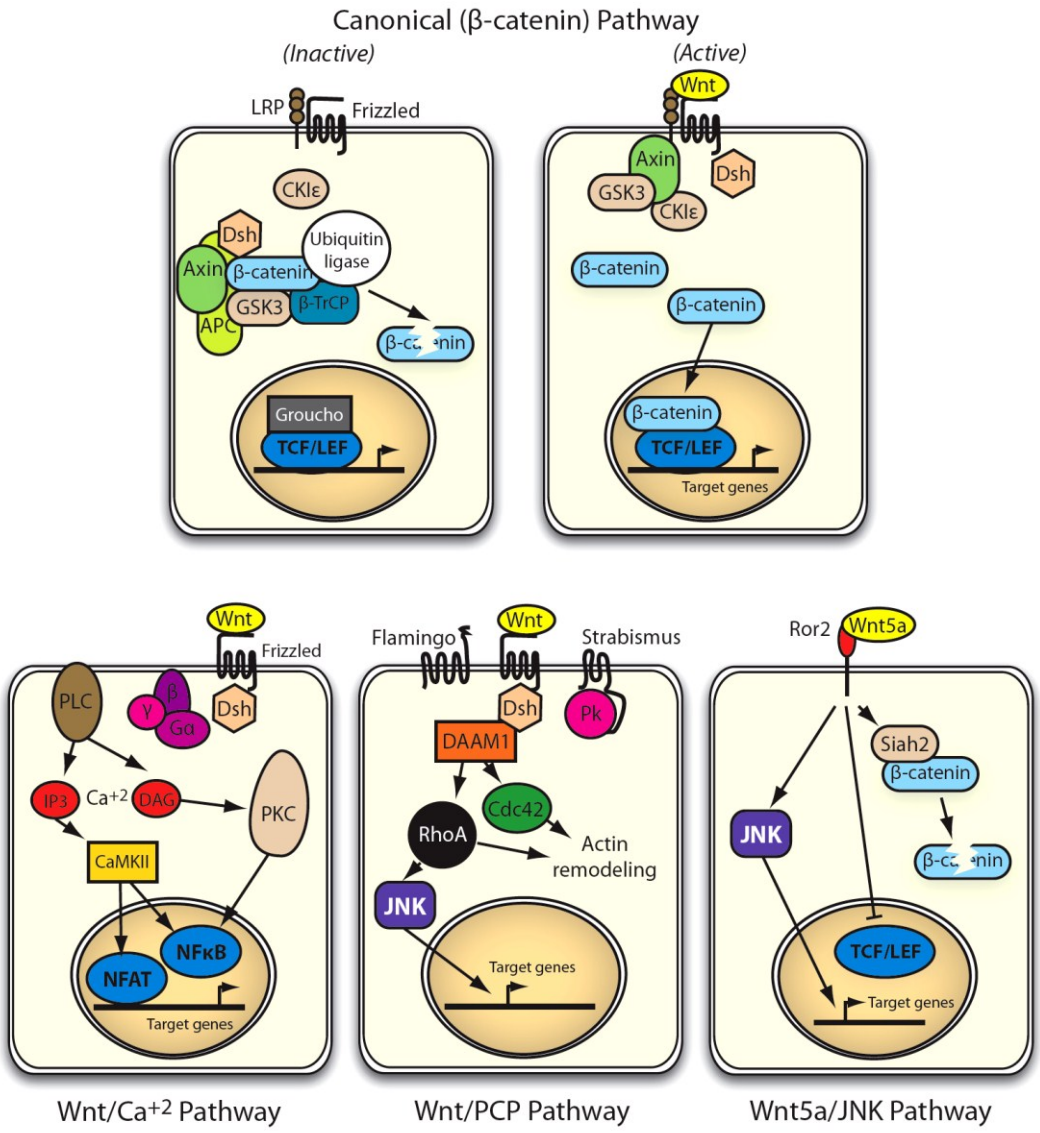

Figure 10: Canonical and non-canonical Wnt signalling pathways.
Figure adapted from Miller [148].

Non-canonical pathways

Wnts have also been shown to activate other signalling cascades. These non-canonical pathways are diverse, activating a variety of intracellular mediators. First indications of the presence of non-canonical pathways arose from the observations that Wnt5a or Wnt11 could increase intracellular Ca^{+2} levels in Xenopus embryos without an increase in nuclear β-catenin [149,150]. Later studies showed that binding of Wnts to Fzd could signal through heterotrimeric G proteins leading to the release of intracellular Ca^{+2}. This signalling also results in the activation of PKC, CaMKII, and the transcription factor nuclear factor of activated T-cells, NFAT [151].

Another non-canonical pathway can activate effects similar to Drosophila planar cell polarity (PCP), which is responsible for the generation of a uniformly oriented cell population such as in patterning the orientation of Drosophila body and wing hairs and the ommatidiae of the eye [152]. This Wnt/PCP pathway has been implicated in the convergent-extension movements that result in the lengthening of the embryo. Activation of this pathway involves Fzd and Dsh as well as the proteins Flamingo, Strabismus, and Prickle. These proteins then stimulate the small G proteins Rho and Rho-associated kinase, ROCK, subsequently resulting in cell polarity by showing unequal distribution of proteins on the cell surface [152]. Wnt can also induce polarity in individual cells. Polarization of the microtubule cytoskeleton and directed cell migration has been shown to result from Wnt5a and appears to operate through a complex composed of Cdc42, Par6, and an atypical PKC [153].

Non-canonical pathways can also converge on the mitrogen-activated protein kinase (MAPK) family member c-Jun N-terminal kinase (JNK), which can then phosphorylate c-Jun within its transactivation domain. JNK activation has been reported in response to Wnt/PCP signalling and Wnt/JNK signalling has also been reported important for convergent-extension movements [152,154]. Recent reports have also implicated the activation of JNK downstream of the receptor tyrosine kinase, Ror2, instead of the typical receptor Fzd. Wnt5a is the best characterized to interact with Ror2; Wnt5a and Ror2-deficient mice exhibit

similar developmental abnormalities reflecting their role in many of the same non-canonical pathways [155]. Wnt/JNK signalling has been implicated in cell proliferation, differentiation, and survival [156]. Moreover, Wnt/JNK signalling has been shown to mediate microtubule polarity and cell migration in cultured fibroblasts via Ror2 [157]. This signalling appears to operate through an atypical PKC suggesting the possibility that it may interact with the Cdc42/Par6/PKC complex to mediate polarity.

The β-catenin and non-canonical pathways have also been shown to interact with one another as non-canonical cascades can inhibit β-catenin signalling. However, the mechanism by which this inhibition occurs is debatable. TCF can be phosphorylated by the MAPK-related protein kinase NLK/Nemo leading to a reduced binding efficiency of β-catenin/TCF to target genes [158]. In contrast, β-catenin can be phosphorylated and targeted for degradation by the protein kinase, Siah2, through a GSK3-independent mechanism [159]. Thus, stimulation of non-canonical pathways may have an opposing effect on the β-catenin pathway in addition to driving transcription of target genes.

Wnt signalling in embryogenesis and cancer

Wnt signalling is found in a wide range of processes and affects cell proliferation, differentiation, and cell fate specification. As a result, this signalling has been implicated in embryogenesis, stem cell regulation, and its disregulation is found in many cancers. Wnt proteins and their respective signalling play important roles in cell specification between equivalent cells during embryogenesis. During PGC specification, Wnt3 is expressed in the posterior proximal epiblast where it is necessary for cells destined to become PGCs to respond to BMP signalling [16]. As mentioned, Wnt4 and RSPO1 are necessary to induce the female differentiation pathway in the bipotential gonad [28]. Loss of Wnt4 or RSPO1 leads to a partial female to male phenotype, even in the absence of testis-determining factors such as Sry. Wnt7a is important for dosalization and the anterior-posterior patterning of developing limbs. $Wnt7a^{-/-}$ mutant females present with abnormal oviducts and uterus, which result from improper

differentiation of the Müllerian duct [160]. In wild-type males, the Müllerian duct is regressed due to the secretion of AMH from Sertoli cells. Instead, the Wolffian duct develops into the epididymis, vas deferens, and seminal vesicle. Interestingly, Wnt7a$^{-/-}$ males show retention of the Müllerian duct due to a loss in AMH receptor expression in Müllerian duct cells [160]. Furthermore, noncanonical Wnt pathways are also involved in proper development. In Wnt5a$^{-/-}$ mutants, defects in lung morphogenesis result and truncated limb malformations are observed [155]. Furthermore, abnormal development of the genital tubercle is noted. Fertility of these mice is unknown due to the perinatal lethality of these mutants. However, it has been reported that abnormal testes may develop as small testis containing fewer than normal seminiferous cords are observed in 15.5 dpp Wnt5a-/- mutant animals [161]. Furthermore, the knockout of Wnt5a results in a defect in PGC migration to the genital ridges, which results in reduced germ cell numbers in both sexes [162].

Functional redundancy might also confound interpretation of the mutant phenotype of single Wnts. For example, a Wnt1/Wnt3a double knockout has revealed a requirement for β-catenin signalling in the developing neural tube, which was not observed in the respective Wnt1 or Wnt3a single knockout animals [163].

In adult animals, misregulation of the β-catenin pathway can result in cancers. The best known example is familial adenamatous polyposis, represented by mice that have a truncation mutation in the gene encoding APC [90,144]. This results in the inability to target β-catenin for destruction and hence constitutive activation of the β-catenin pathway. Furthermore, mutations that lead to the stabilization of β-catenin have also been found in human colorectal neoplasias and melanomas, while mutations in the human AXIN gene, leading to constitutive activation of the β-catenin pathway, have been observed in hepatocellular carcinomas [164,165,166]. Interestingly, it has been shown that heterozygous Wnt5a$^{+/-}$ mice develop B cell lymphomas and chronic myeloid leukemias [167]. From this study, it was gleaned that Wnt5a can normally act as a tumour suppressor, signalling through the Wnt/Ca^{+2} pathway to inhibit B cell

overproliferation [167]. Therefore, these results highlight the importance of Wnt signalling regulation and emphasize the antagonistic role Wnt5a noncanonical signalling plays with the β-catenin pathway.

Wnt signalling in stem cell fate

Given the involvement of Wnt signalling in cell specification and communication during development it is not surprising that this signalling has also been implicated in the fate regulation of various tissue-specific stem cells. One of the first stem cell populations to demonstrate a role for Wnt signalling was intestinal stem cells (ISCs), which support the epithelial population of crypts and villi in the gut. $TCF4^{-/-}$ neonatal mutant mice present with a complete absence of the crypt compartment [168]. ISCs have been localized specifically to the crypt indicating that Wnt signalling through TCF4 is required to maintain the stem cell population. Further studies have identified Wnt3, secreted by the supporting Paneth cells, as the Wnt responsible for β-catenin pathway induction in ISCs [169].

Following these studies in mouse intestine, Wnt signalling was implicated in the niche regulation of other stem cells. Wnts have been detected in the niche in the bone marrow and not surprisingly, HSCs have been shown to respond to Wnt signals. Functionally, Wnt3a has been demonstrated to stimulate the expansion of phenotypic HSCs in vitro, which further suggests that activation of the β-catenin pathway drives proliferation of stem cell populations. However, constitutive activation of β-catenin does not expand HSCs [170]. Furthermore, conditional knock-out of β-catenin does not lead to a loss in hematopoiesis [171], which calls into question the role of β-catenin signalling in HSC regulation. Treatment with Wnt5a appears to benefit HSC colonization; Wnt5a activates a non-canonical Wnt mechanism and leads to increased HSC colonization resulting from the induction of quiescence, thereby demonstrating that Wnts can have diverse effects on a particular cell population [172].

A role for Wnts in NSC regulation has yet to be fully understood. β-Catenin signalling is linked to stem cell expansion in the embryonic nervous

system. However, in the adult brain, β-catenin signalling does not appear to be active during homeostasis. NSCs reside in the subventricular zone (SVZ), a thin layer of cells on the lateral side of the lateral ventricles of the mammalian brain. SVZ cells, isolated from β-catenin$^{-/-}$ mutant mice, are not depleted in NSCs indicating that this signalling is not crucial for normal NSC maintenance in the brain [173]. However, β-catenin signalling was shown to become active following the onset of injury in the brain, that is, when NSCs are provoked to undergo increased symmetric division [174]. Thus, the effect of Wnt signalling pathways on stem cell fate regulation is diverse and appears to be type specific.

Wnt signalling in the testis

Given the involvement of Wnt signalling in other self-renewing tissues, it is possible that this signalling may be present in the testis and play a role in spermatogenesis. However, to date virtually no definitive role has been characterized in the testis. Early studies examining Wnt signalling in the testis detected Wnt1 expression in the round spermatid stage. Attempts at determining a role for Wnt1 in spermatogenesis involved the expression of anti-sense RNA, against Wnt1, in transgenic mice. However, no abnormality in spermatogenesis was observable [175,176]. In contrast to work with germ cells, recent studies have shown that constitutive β-catenin signalling in Sertoli cells leads to a functional deficiency in their ability to support spermatogenesis. Using a constitutively active form of β-catenin under the control of the AMH receptor promoter, several groups have shown that these mutant mice become gradually infertile through the deterioration of seminiferous tubules and germ cell loss indicating that suppression of β-catenin signalling in Sertoli cells is necessary for proper spermatogenesis [177,178]. Furthermore, accumulations of somatic cells were observed to develop in the testes of these mutant mice. Sustained activation of the β-catenin pathway in PTEN null-mutant animals led to testicular cancers demonstrating that proper regulation and expression of the Wnt pathway is important to avoid improper cell growth [179].

Previous biochemical studies have identified the expression of multiple Wnt genes in the testis, in particular, Wnt5a gene expression has been detected in the Sertoli cell population [180,181,182]. In the seminiferous epithelium, the expression of crucial Wnt pathway mediators such as GSK3 and Dsh, and the expression of Wnt signalling antagonists naked cuticle 1 (Nkd1) and dickkopf-like 1 appear to be expressed in a germ cell type-specific manner [183,184,185,186]. This cell-specific distribution of Wnt signalling regulators further indicates that a balance between Wnt signalling expression in the testis might be an important consideration for proper spermatogenesis.

Summary of Part 3

Wnt proteins are an evolutionarily conserved family of secreted signalling molecules. Wnts signal through a complex of transmembrane receptors including Fzd and LRP5/6, although recently Wnts have been demonstrated to signal through other receptors, such as Ror2. Depending on receptor availability Wnts can activate two classes of signalling, termed canonical (β-catenin) and non-canonical pathways. The β-catenin pathway is the best characterized pathway and involves the intracellular mediators GSK3, β-catenin, and the TCF/LEF family of transcription factors. In the absence of Wnt stimulation, intracellular β-catenin levels are kept low due to targeting by GSK3 for degradation. Activation of this pathway leads to the inhibition of GSK3, thereby allowing β-catenin to accumulate and associate with TCF/LEF, driving transcription of target genes. In contrast, non-canonical pathways are diverse stimulating mediators such as G-proteins, CaMKII, and JNK. Importantly, the non-canonical pathways have been shown to antagonize the β-catenin pathway.

Wnt signalling is important for proper embryo development. Postnatally, disregulation of Wnt signalling is implicated in certain cancers. Perhaps not surprisingly, Wnt signalling has been implicated in the fate decision control of various stem cell types. β-Catenin pathways have shown the ability to support stem cell self-renewal. However more recently, roles for non-canonical Wnt pathways have been characterized in stem cell regulation. In the testis, several

Wnts and signalling components have been detected but to date, no role for Wnt signalling in spermatogenesis has been characterized. Given the expression of Wnts and signalling components in germ cells and Sertoli cells (i.e. cells that are believed to construct the SSC niche) the possibility exists that Wnt signalling pathways may be involved in the communication network that regulates the SSC fate decision.

Rationale and Hypothesis

SSCs are the foundation of spermatogenesis, the process by which sperm is generated. These cells have the unique ability to maintain a stem cell reserve of undifferentiated cells, while also contributing cells into the spermatogenic cycle. This ability to either self-renew or differentiate to sperm is the essence of what defines a SSC and is a major focus of research into SSC biology. SSCs exist in a specialized environment composed of testicular somatic cells and germ cells and it is believed that communication between these cell populations and SSCs influence SSC fate. Extrinsic factors mediate this complex communication and regulate this SSC fate decision. However, the identity of these extrinsic factors and signalling pathways involved in SSC regulation is not completely understood.

Wnt signalling pathways have demonstrated importance in the cell proliferation and specification of embryonic cells during development. Not surprisingly, these signalling pathways have also been implicated in the fate regulation of various stem cell populations. Previous studies have reported the expression of various Wnt genes and signalling regulators in the testis, in particular, Wnts have been localized to somatic cells in the testes, including Sertoli cells, suggesting that Wnt signalling may contribute to SSC fate regulation.

In this thesis, I hypothesize that Wnt signalling pathways participate in SSC niche regulation by influencing the SSC fate decision.

Studies with SSCs utilize a classical transplantation assay which allows for the retrospective, quantitative identification of SSCs based on their strict functional definition. However, this assay has disadvantages; it is time-consuming and labour-intensive, requiring two months to acquire results. In lieu of a transplantation assay, NSC detection relies on a simple, in vitro neurosphere-forming assay. Similarly, SSCs can form sphere-like aggregates in culture, termed "clusters". Therefore in the same manner the neurosphere assay was defined to facilitate the in vitro study of NSCs, in Chapter 2, I characterized a short-term in vitro assay for SSCs based on cluster-formation activity. Clusters were observed to be clonally-derived and their numbers correlated directly with the results of the

functional transplantation assay, demonstrating that in vitro cluster-formation activity is a quantitative measure of SSC activity.

Clusters are known to be composed of SSCs and differentiated cells indicating that self-renewal, survival, and early differentiation events are all occurring in vitro. Therefore, the cluster community is a good model to investigate the role of Wnt signalling pathways in SSC fate. Because SSC culture requires the presence of a feeder cell layer, I screened these cells for novel SSC extrinsic factors. In Chapter 3, I identified Wnt5a as a novel factor that promotes SSC survival. This chapter demonstrated that the effects of Wnt5a are through a non-canonical Wnt5a/JNK mechanism. Notably, while Wnt5a was in the supporting feeder cells in vitro, it was also detected in Sertoli cells in vivo alluding to a functional role in the testis environment. Additionally in Chapter 3, I document the presence of β-catenin signalling cells within the cluster community. These cells did not have SSC regenerative ability, indicating that functionally heterogeneous populations in clusters appear to respond differently to particular Wnts.

Given β-catenin signalling was not associated with SSC activity; in Chapter 4, I examined how Wnt3a stimulation of β-catenin cells might influence SSC fate. Wnt3a stimulated β-catenin signalling, in a subset of cluster cells but this population contained fewer SSCs indicating that β-catenin signalling cells are a more committed progenitor. Interestingly, the addition of Wnt3a led to improved SSC maintenance. This coincided with increased cluster-formation, leading me to speculate that committed cells might communicate with SSCs in the cluster and influence their fate. Thus, committed cells may be a constituent of the SSC niche.

Finally, in a Supplementary Chapter, I examined how these non-SSC committed progenitor cells might influence SSCs using a genome-wide comparison of β-catenin signalling and non-signalling cluster cells. Several targets were identified including Dlk1, which appeared to induce SSC differentiation.

In summary, this body of work demonstrates that Wnt signalling pathways are involved in the complex communication regulating SSC fate. The results presented here imply that different Wnt signalling pathways interact and help balance proper SSC fate regulation. Furthermore, my study presents new technologies to study and manipulate SSCs, provides novel markers to prospectively identify SSCs, and strongly suggests that SSC daughter cells can contribute directly to the niche regulation of SSCs.

CHAPTER 2

MANUSCRIPT I

Preface to Chapter 2

Studying SSCs in the testis is difficult due to their rarity and the lack of definitive markers to distinguish SSCs from other testicular cells. As a result, the study of SSCs would benefit greatly if these cells could be assessed under well-defined conditions. Therefore, in my studies I utilize an in vitro SSC culture system to dissect the mechanisms governing the SSC fate decision (i.e. the decision to self-renew or differentiate). Because this SSC culture system is used extensively in this thesis, I start by characterizing the behavior of SSCs in vitro.

Similar to how NSCs form neurospheres in vitro, SSCs can be expanded in vitro as distinct cell communities, which I termed “clusters”. Neurospheres can be continually sub-fractionated and differentiated to form neural cell types. These attributes functionally imply the retention of NSCs in vitro. However, committed progenitor cells have also shown the ability to form neurospheres [77]. Therefore, to unequivocally detect stem cell activity in neurospheres a regenerative transplantation assay should be used. Unfortunately a transplantation assay does not exist for NSCs so the relation of neurosphere-formation to definitive NSCs remains unknown. As mentioned, SSC clusters can also be continually sub-fractionated demonstrating long-term self-renewal. However, in contrast to neurospheres, transplantation of clusters into SSC-depleted testes is possible to unequivocally demonstrate the retention of SSC regenerative ability in vitro.

In the following Chapter, I examine the correlation between cluster-formation ability in vitro and colony-formation ability using the transplantation assay. From this data, I establish whether cluster formation represents SSC activity and present evidence for the use of our SSC culture system as a short-term functional assay for SSC activity.

Establishment of a short-term in vitro assay for mouse spermatogonial stem cells

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Biology of Reproduction 77(5): 897-904, (2007)

Short title: In vitro spermatogonial stem cell assay

Summary: Spermatogonial stem cells can be reliably detected using an in vitro cluster-forming assay

Financial support: CIHR (MOP-49444) and FRSQ (Bourse de career)

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Abstract

Spermatogonial stem cells (SSCs) are responsible for life-long daily production of male gametes and for transmission of genetic information to the next generation. An unequivocal detection of SSCs has relied on spermatogonial transplantation, in which functional SSCs are analyzed qualitatively and quantitatively based on their regenerative capacity. However, this technique involves some significant limitations. For example, it is a time-consuming procedure, as data acquisition requires at least 8 weeks after transplantation. It is also laborious, requiring microinjection of target cells into the seminiferous tubules of individual testes. Donor-recipient immunocompatibility for successful transplantation and large variance in data obtained represent further limitations of the technique. In this study, we provide the evidence that a recently developed SSC culture system can be employed as a reliable, short-term in vitro assay for SSCs. In this system, donor cells generate three dimensional structures of aggregated germ cells in vitro, termed "clusters", within 6 days. We show that each cluster originates from a single cell; thus, by counting clusters, "cluster-forming cells" can be quantified. We also detect a strong linear correlation between the numbers of clusters and SSCs over extended culture periods; therefore, cluster numbers faithfully reflect SSC numbers. These results indicate that by simply counting the number of clusters, functional SSCs can readily be detected within 1 week in a semi-quantitative manner. The faithfulness of this in vitro assay to the transplantation assay was further confirmed under two experimental situations. This in vitro "cluster-formation assay" provides a reliable short-term technique to detect SSCs.

Introduction

Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis, a process that produces numerous spermatozoa throughout life after puberty [187]. The presence of SSCs allows regeneration of spermatogenesis and restoration of male fertility following various testicular insults, such as cancer chemotherapy. Compared to other stem cell types, SSCs are unique, since they are dispensable for the life of an individual, but critical for the continuation and evolution of the species.

Since stem cells are defined by their function to self-renew and differentiate, thereby regenerating and maintaining a complete adult tissue [188], the detection of SSCs has relied on an in vivo functional assay, spermatogonial transplantation [5]. In this technique, a single cell suspension prepared from donor testes is injected into the seminiferous tubules of infertile recipient testes, leading to donor-derived spermatogenesis reconstitution. Because injected non-stem germ cells lack the potential of continuous self-renewal, these cells are eliminated through differentiation or death over time. Thus, spermatogonial transplantation selectively allows only SSCs to re-establish and maintain long-term, donor-derived spermatogenesis in recipient testes [5,66], and represents the unequivocal assay of functional SSCs.

Spermatogonial transplantation is also an effective method to quantify SSCs. Following transplantation, donor-derived spermatogenesis is regenerated in the form of distinctive colonies along the recipient seminiferous tubules [66]. Recent studies have convincingly shown that each colony arises from a single stem cell [68,69]. Therefore, by simply counting the number of colonies, the number of functional SSCs can be determined. As such, spermatogonial transplantation is a powerful technique to qualitatively and quantitatively analyze functional SSCs that successfully regenerate spermatogenesis after transplantation.

Although this in vivo transplantation technique has been essential to study SSCs, it is not without limitations. Perhaps, the greatest problem is its time-consuming nature; the preparation of recipients requires 4 or more weeks, and the

transplantation results are obtained at least 8 weeks after transplantation, at which time spermatogenic regeneration is completed [66]. In addition, the microinjection of donor cells into recipient seminiferous tubules is a laborious procedure, and transplantation experiments require careful planning to ensure the immunocompatibility between donors and recipients. It is also of note that the data obtained using this in vivo assay show large variance, requiring a large number of recipient testes to obtain consistent results.

These problems can be circumvented if a reliable, short-term in vitro assay is established. For example, stem cells of the central nervous system can be quantified in vitro using the neurosphere assay [73,189]. In this assay, neual stem cells (NSCs) actively divide under a chemically defined culture condition, which results in formation of three-dimensional floating spheroid cell clusters, called neurospheres. NSCs are then quantified by counting the number of neurospheres [190]. However, such an in vitro assay system has not been developed for SSCs.

Recently, it has become possible to culture SSCs long-term, during which these stem cells can be expanded extensively [112,115]. In this system, donor testis cells are cultured on a feeder layer with glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2) in a serum-free or -reduced medium [112,115]. Under this condition, donor cells form groups of aggregated germ cells on the feeder layer within 1 week, which are termed “clusters” to distinguish them from “colonies” established in recipient testes after transplantation. The transplantation of clusters results in regeneration of spermatogenesis, confirming that clusters contain SSCs [112,115]. Furthermore, SSCs can be maintained in vitro virtually indefinitely by serially passaging clusters [133].

Since germ cell clusters show a distinct three-dimensional structure, it may be possible to quantitatively analyze SSCs in vitro by counting clusters, a procedure analogous to the neurosphere assay for NSCs. To this end, two important criteria need to be addressed. Since quantification based on cluster number can only be valid if clusters are clonal, the first criterion is whether each cluster is clonally generated by a single “cluster-forming cell”. The second

criterion is whether, over an extended period of time, the number of clusters directly correlates with the number of spermatogenic colonies established by SSCs after transplantation. This issue is known to be one of the caveats in the neurosphere assay. Since a transplantation assay to detect the regenerative activity of NSCs is not possible in the central nervous system, the fidelity of the neurosphere assay relies on the long-term self-renewal activity of sphere cells, which is detected by proliferation kinetics of spheres over prolonged passaging periods [75,77]. However, since committed progenitors can also form spheres, the proliferation kinetics of neurospheres can differ from those of NSCs [77,191]. In contrast, long-term proliferation kinetics of germ cell clusters can be directly correlated with those of SSCs by comparing cluster numbers with SSC numbers determined by transplantation. Nonetheless, a correlation between these two parameters has not been demonstrated clearly.

In this study, we present the evidence that the SSC culture system satisfies both of these criteria and that functional SSCs can therefore be semi-quantitatively determined by simply counting the number of clusters formed in vitro within one week. Furthermore, we demonstrate that the results of SSC quantification are nearly identical between the in vitro assay and the transplantation assay under experimental conditions. Importantly, this in vitro assay overcomes most of the problems encountered with the transplantation assay. Thus, the development of this reliable short-term in vitro assay provides a powerful technique in SSC investigation and should facilitate the progress of this research field.

Materials and Methods

Donor Mice and Cell Preparation

Donor mice were the F_1 progeny of C57BL/6 (B6) females and B6.129S7-*Gtrosa26Sor* (designated ROSA26; The Jackson Laboratory) males. These mice were designated B6/ROSA. ROSA26 mice express the *Escherichia coli lacZ* transgene in virtually all cell types, including all types of postnatal male germ cells, allowing the discrimination of donor cells from recipient cells after

transplantation in vivo and from feeder cells in culture [66,192]. In some experiments, B6 mice were also used as donors. Donor cells were obtained from 6- to 8-day-old pup testes, and a single-cell suspension was prepared using a two-step enzymatic digestion as previously described [193,194]. All animal procedures were approved by the Institutional Animal Care and Use Committee of McGill University.

Immunomagnetic Cell Sorting

A single suspension of testis cells were passed through a 40 μm mesh to remove undigested testis fragments and were subsequently enriched for SSCs through immunomagnetic cell sorting, using a double selection protocol as described with modifications [71]. Briefly, 6×10^6 testis cells were resuspended in 1 ml Dulbecco Modified Eagle Medium (DMEM) supplemented with 1 % fetal bovine serum (FBS). These cells were first incubated with primary antibodies against two cell surface molecules demonstrated to be expressed on non-SSCs in the testis (negative markers) [3,71]: biotin conjugated anti-α_V-integrin antibody (BD Biosciences, clone RMV-7) and rat anti-β2-microglobulin antibody (BD Biosciences, clone Ly-m11). Antibodies were incubated at a concentration of 5 μg/ml each at 4°C for 30 min with gentle agitation. Unbound primary antibodies were removed by washing twice with phosphate-buffered saline (PBS) supplemented with 1 % FBS, and the cells were subsequently resuspended in 1 ml DMEM with 1 % FBS. Secondary antibodies conjugated to magnetic beads were then added: M450 anti-rat antibody and M280 streptavidin (Dynal), at 20 μl each per 1 ml cell suspension. Secondary antibodies were allowed to react for 30 min at 4°C with gentle agitation. The antibody-bound fraction was sorted using a Magnetic Particle Concentrator (Dynal) for 5 min. The antibody non-bound, α_V -integrin-negative; β2-microglobulin-negative fraction was subsequently resuspended in 1 ml DMEM with 1 % FBS and incubated with biotinylated anti-Thy1.2 antibody (BD Biosciences, clone 52-2.1); an antibody against a cell-surface molecule demonstrated to be a positive marker for SSCs [195]. This antibody was reacted for 30 min at 4°C with gentle agitation and at a

concentration of 5 μg/ml. Unbound antibody was removed by two washes of PBS with 1 % FBS; then, M280 streptavidin was added at 5 μl per 1 ml cell suspension. Sorting was accomplished using the Magnetic Particle Concentrator for 1 min. The antibody-bound, Thy1.2-positive; α_V-integrin-negative; β2-microglobulin-negative fraction constituted ~3 % of initial pup testis cells in both B6 and B6/ROSA mice, and was enriched 6-fold for pup SSCs versus unselected control testis cells in B6/ROSA mice. These SSC-enriched cells were used for all experiments unless otherwise indicated.

Cell Culture and Cluster Analysis

Testis cells sorted using immunomagnetic cell sorting were cultured on a feeder layer of STO (SIM mouse embryo-derived thioguanine and ouabain resistant) embryonic fibroblasts [110]. STO feeder cells were mitotically inactivated after mitomycin C treatment, and seeded in a 24-well tissue culture plate at 5×10^4 cells/cm^2 in 1 ml medium. Sorted testis cells were placed on the STO feeder layer at 1.25×10^4 cells/cm^2, unless otherwise indicated. The SSC culture medium was composed of Minimum Essential Medium α (Invitrogen), with 0.2 % BSA (Sigma), and supplements, as described previously [112]. Growth factors used were recombinant human GDNF (R & D Systems), recombinant rat GFRα1 (R & D Systems), and FGF2 (Invitrogen) at 40 ng/ml, 300 ng/ml, and 1 ng/ml, respectively [112]. Medium was changed every 3 – 4 days, and all cultures were digested with 0.25 % trypsin-EDTA and subcultured at a 1:2 – 1:4 dilution every 6 – 7 days. All cultures were maintained at 37°C in a humidified atmosphere of 5 % carbon dioxide.

For cluster quantification, cultures were fixed with 0.5 % gluteraldehyde and reacted with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) to distinguish B6/ROSA-derived cells from the STO feeder layer and B6-derived cells. All clusters in a well were visually counted. Data were obtained from 2 – 4 experiments, and two wells of a 24-well culture plate were counted in each experiment. Data are expressed as mean ± SEM. Significance was determined

using ANOVA followed by Fisher test for Least Significant Difference or Student *t*-test.

To generate single cell cultures, individual clusters from established clusters (> five passages) were picked using a flame polished Pasteur pipette connected via tubing to a mouth piece. Isolated clusters were gently digested to single cells with 0.05 % trypsin-EDTA in PBS and subsequently placed at a clonal concentration in three 96-well culture plates for visual inspection. Each well containing only one cell was transferred to one 96-well plate on STO feeder cells with growth factors and cultured for 7 days.

Recipient Mice and Transplantation Analysis

Recipients were 129/SvEv × B6 F_1 hybrid mice. To deplete endogenous spermatogenesis, recipient mice were injected i.p. with busulfan (50 mg/kg body weight) at 4 weeks of age [193,194]. Approximately 4 – 6 weeks after busulfan treatment, these mice were used as recipients for transplantation. The transplantation assay was used to quantify SSCs in donor testis cell preparations and in a population of cultured cells. For SSC quantification in donor testis cell preparations, SSC-enriched cells were resuspended at $1 – 1.2 \times 10^6$ cells/ml, and injected into recipient seminiferous tubules through the efferent duct [193,194]. Cultured cells were collected using 0.25 % trypsin-EDTA, resuspended at $1 – 1.2 \times 10^6$ cells/ml, and transplanted as above. For SSC quantification, recipient testes were harvested 2 mo. after transplantation, and stained with X-gal to visually count the colonies of donor-derived spermatogenesis; the number of colonies indicates that of SSCs [68]. Data were obtained from 2 – 4 experiments, involving at least 10 recipient testes, and expressed as mean ± SEM. Significance was determined using ANOVA followed by Fisher test for Least Significant Difference or Student *t*-test.

Hypotonic Treatment

Approximately 10^5 sorted pup testis cells were incubated in 1 ml of 20mM Tris-HCl buffer (hypotonic solution, pH 7.0) for 5 or 20 min at room temperature

[196,197]. The hypotonic treatment was terminated by adding 9 ml of Hanks Balanced Salt Solution (HBSS). The cells in the control group were incubated in HBSS, without hypotonic treatment. Each of these groups was next divided into two groups; one group cultured with growth factors to determine cluster number, and the other group transplanted into recipient mice to quantify SSCs. Data were collected from 4 experiments; 2 – 7 recipient testes and 2 wells of a 24-well culture plate per experiment. To quantify the survival of all the cells in the seminiferous tubules following hypotonic conditions, a single cell suspension of unselected B6/ROSA pup tubules were treated with hypotonic solution as above. Trypan blue exclusion and the total cell recovery were used to determine the number of viable cells. Experiments were conducted 4 times and data are expressed as the mean percentage ± SEM versus an untreated control. Significance was determined through ANOVA followed by Fisher test for Least Significant Difference.

Results

Each cluster is derived from a single cluster-forming cell

Testis cells were obtained from B6/ROSA transgenic mice (6 – 8 days old) and enriched for SSCs in the Thy1^{+} α_V-integrin^{-} β2-microglobulin^{-} fraction using two-step immunomagnetic cell sorting (see Materials and Methods, "sorted cells" hereafter) [3,71]. Sorted cells were cultured on a STO feeder layer in the presence of GDNF, soluble GFRα1, and FGF2, which are required for long-term maintenance and expansion of SSCs in vitro [112]; this growth factor cocktail is designated herein as "growth factors". As reported previously [112], clusters of donor cells developed by the sixth day of culture (Fig. 1A); no clusters appeared without growth factors. These clusters were typically non-spherical, but rather appeared as globular three dimensional structures attached to the feeder layer. We define a cluster as a group of at least 6 cells. Occasionally, chains of cells were found, which are indicative of differentiating germ cells [49,198], and they were not counted as clusters (Fig. 1B).

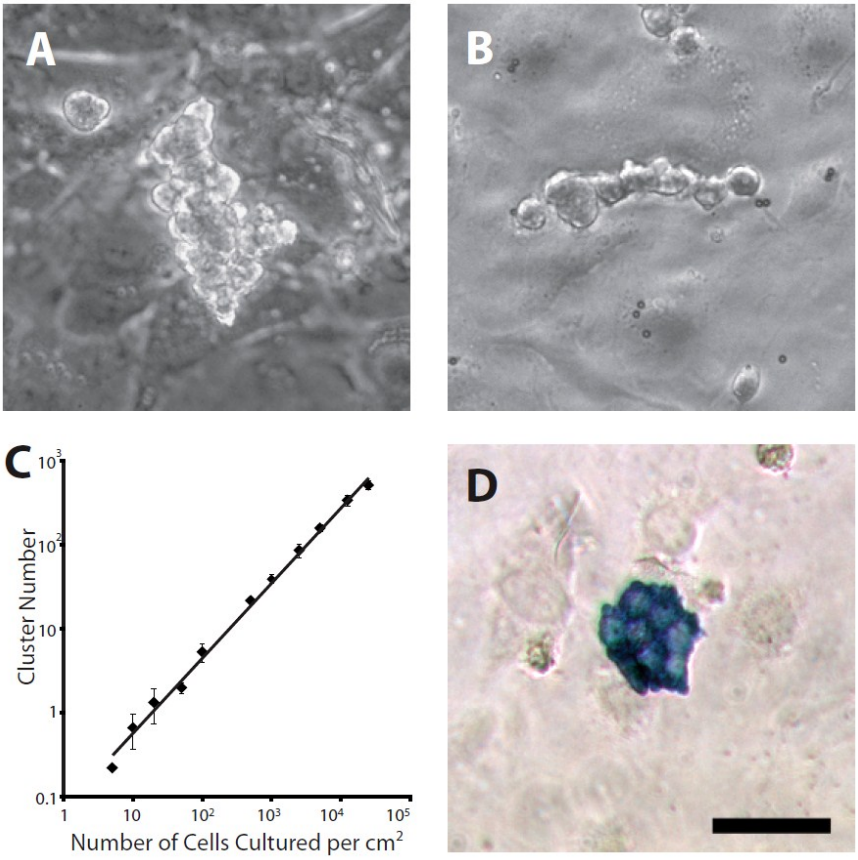

Figure 1: The number of clusters correlates linearly with that of cells placed in culture.

A) Appearance of a cluster after 6 days in culture. Clusters form distinctive three-dimensional structures on feeder cells. **B)** Appearance of a chain of cells typical of differentiating male germ cells after 6 days in culture. These cell-chains are not counted as clusters. **C)** Comparison of the cluster number to the initial number of cells seeded in culture. Following immunomagnetic cell sorting, sorted cells were seeded at varied densities. Clusters were counted after 6 days. A strong linear correlation is detected between the two parameters. Mean ± SEM (n = 6 per group). **D)** Appearance of a cluster derived from a single B6/ROSA cell. The cluster cells stain positively for β-galactosidase, distinguishing them from feeder cells. Scale bar: 50 μm (A, B, D).

We first investigated whether or not a cluster arises from one cell. To this end, sorted testis cells from B6/ROSA mice were first seeded in a limiting dilution, ranging from 5 to 25,000 cells/cm^2 (Fig. 1C). After 6 days of culture, the number of clusters was visually determined and correlated with the number of cells seeded. The results clearly showed a positive linear relationship between these two parameters, suggesting that each cluster is derived from one "cluster-forming cell".

To confirm that individual cells can form germ-cell clusters, single cell cultures were prepared from established cluster cells (> five passages). In these experiments, individual clusters were isolated from feeder cells using gentle aspiration and digested to single cells. These cluster cells were first placed at a clonal concentration into 96-well culture plates, and wells containing one cell were visually identified. The contents of single cell-containing wells were transferred onto STO feeders, in individual wells, with growth factors. After one week, we observed a germ cell cluster (Fig. 1D), at a frequency of 1 per 40 cells seeded. These results demonstrate that a single cell can form a cluster.

To substantiate these results and detect the occurrence of polyclonal clusters, a cell chimerism experiment was performed. In this experiment, sorted cells derived from B6/ROSA mice was mixed at a 1:1 ratio with those from wild-type C57BL/6 (B6) mice at increasing total cell densities and cultured for 6 days in cluster-forming conditions (Fig. 2). Subsequently, the clusters that formed were incubated with X-gal to distinguish B6/ROSA cells from B6 cells. Three cluster staining patterns emerged: clusters comprised of β-galactosidase-positive cells alone (i.e., B6/ROSA origin, Fig. 2A bottom cluster), β-galactosidase-negative cells alone (B6 origin, Fig. 2A top cluster indicated by arrow), and both (mixture of the two, Fig. 2B). The data showed that most clusters produced were either of B6/ROSA origin or B6 origin, and not a mixture of both at all cell densities examined, further suggesting that the majority of clusters are exclusively of one genotype (Fig. 2C).

These results collectively indicate that each germ-cell cluster generated in vitro arises from one "cluster-forming cell", and therefore, the number of clusters corresponds to the number of functional cluster-forming cells in vitro.

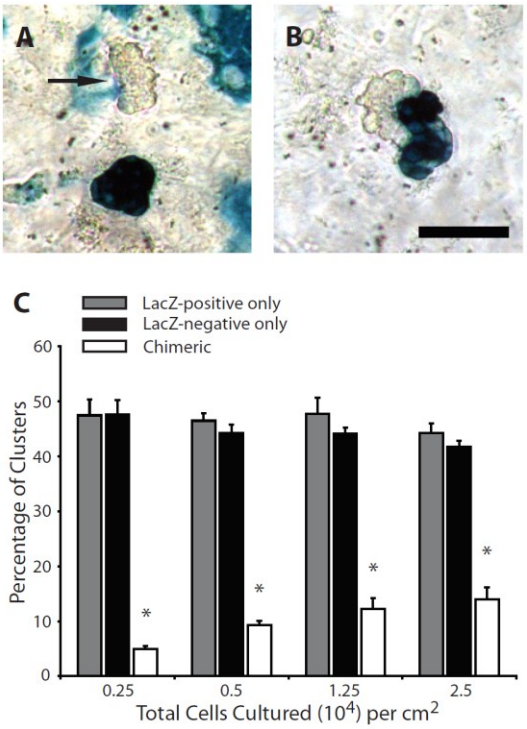

Figure 2: Cluster chimerism assay to examine the clonality of clusters. Following immunomagnetic cell sorting, sorted cells from wild-type B6 mice and transgenic B6/ROSA mice were cultured at a 1:1 ratio. **A)** Clusters showing a single genotype. A B6-only cluster with no LacZ-staining (top cluster indicated by arrow) and a B6/ROSA-only cluster with LacZ-staining (bottom cluster) are seen. Non-cluster cells derived from transgenic B6/ROSA mice stain positively for LacZ expression. **B)** A cluster showing two genotypes (chimeric) composed of both B6 and B6/ROSA cells. **C)** Percentages of single genotype and chimeric clusters. A 1:1-mixture of B6 and B6/ROSA cells was seeded at varied densities. X-axis indicates the number of total cells seeded (B6 + B6/ROSA). Numbers of B6/ROSA clusters and B6 clusters are found at a statistically similar frequency at all cell densities examined. Chimeric clusters are always a significantly minor population, (asterisks, $p < 0.001$), representing 4.9 – 14 % of total clusters. Scale bar: 50 μm (A, B).

Cluster numbers correlate linearly with colony numbers

We next compared the proliferative properties of cluster-forming cells to those of SSCs. After one week of culture, $1.63 \pm 0.16 \times 10^4$ (n = 8) clusters were generated per 10^6 sorted cells initially placed in culture. When germ-cell clusters were enzymatically dispersed to single cells on day 6 or 7 in vitro and subcultured on a fresh STO feeder layer, secondary clusters formed. Following serial passaging, the number of clusters exponentially increased over a long time. As shown in Fig. 3A, the increase of cluster number was 40,000-fold in 12 weeks (11 passaging generations). Since one cluster arises from one cluster-forming cell, this result indicates that cluster-forming cells are long-term self-renewing cells and proliferate with a population-doubling time of 5.5 days (84 days/$\log_2$40,000) under the culture condition used.

To determine the SSC activity present in vitro, we transplanted cultured cells into recipient testes at various time points during the 12-week culture, which resulted in the production of colonies of regenerated spermatogenesis (Fig. 3B, C); colony numbers were determined 2 months after transplantation. We observed that the number of colonies increased exponentially from $2.91 \pm 0.38 \times 10^3$ (n = 24) per 10^6 sorted cells initially placed in culture to $1.09 \pm 0.32 \times 10^8$ (n = 10) after 12 weeks. Thus, SSCs proliferated approximately 40,000-fold in vitro over the 12-week span, which is near identical to the expansion kinetics of cluster-forming cells. Importantly, the proliferation kinetics of cluster-forming cells paralleled with those of SSCs, as no difference was detected between both regression coefficients (Fig. 3A). These results demonstrate that cluster numbers correlate linearly with colony numbers throughout the long-term multiple passaging period and that the number of clusters detected within a week in culture faithfully reflects the number of SSCs that are determined 2 months after transplantation. These results suggest that SSCs can be measured using this "cluster-forming assay" by counting the number of clusters established in vitro. Although it is not clear at present if every cluster implies a stem cell (See Discussion), we calculate on average that each cluster represents 1.2 SSCs (Fig. 3D), assuming a homing efficiency of 12% [70]. This ratio remains relatively

constant throughout long-term SSC culture, reflecting the correlation between cluster and colony number and further demonstrating that this in vitro assay allows a semi-quantitative detection of SSCs.

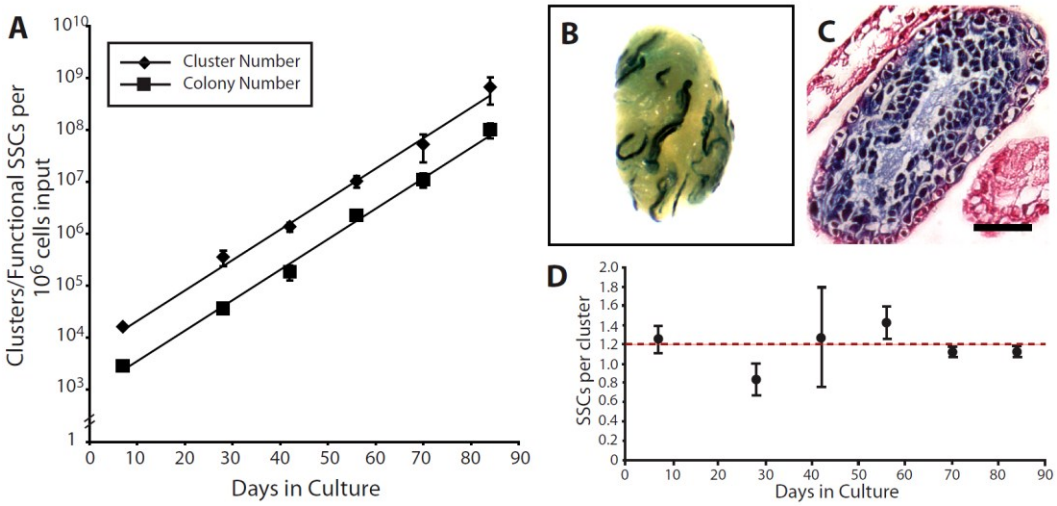

Figure 3: Cluster-forming cells are capable of long-term self-renewal and proliferate in parallel with SSCs.

A) Correlation analysis between cluster number and colony number over a long-term culture scheme for 12 weeks (11 passaging generations). Shown are numbers of colonies and clusters per 10^6 cells originally seeded in culture. **B)** Appearance of a recipient testis 2 months after transplantation of clusters. Each blue segment of seminiferous tubule represents a donor-derived spermatogenic colony. **C)** Paraffin section of a colony derived from cluster cells. Regeneration of spermatogenesis is seen, indicating stem cell activity of cluster cells. (**D**) Calculation of number of SSCs per cluster over the course of long-term culture. On average, 1.2 SSCs per cluster is calculated (represented by dashed line). Scale bar: 0.2 mm (A), 30 µm (B).

The results of the cluster-forming assay faithfully reflect those of the transplantation assay under experimental conditions.

To further verify the fidelity of the cluster-forming assay, we next applied it in two experimental conditions, and compared the results with those obtained using the transplantation assay. First, we cultured sorted cells without growth factors, and measured the stem cell activity remaining in culture using both the transplantation assay and the cluster-forming assay (Fig. 4A); sorted cells were obtained from B6/ROSA pup testes. The transplantation assay was done at days 0 (no culture), 2, and 5 in vitro, and colonies were counted 2 months later (Fig. 4A). At each time point, these cells were also passaged onto fresh STO feeder cells and cultured with growth factors to generate clusters; then, the number of clusters was determined 6 days after passaging. We expected that SSC number declines with time due to the lack of growth factors.

The data shown in Fig. 4B indicate that both assays gave rise to similar results. Compared to day 0, the number of colonies and clusters was 1.2- and 1.1-fold higher at days 2 and 5, respectively. However, no significant difference was detected with either assay. Therefore, these findings demonstrate that the results of the cluster-forming assay faithfully recapitulate those of the transplantation assay in a semi-quantitative manner and that despite the absence of growth factors, SSC activity or cluster-forming activity does not decline during the initial 5 days in culture of sorted cells.

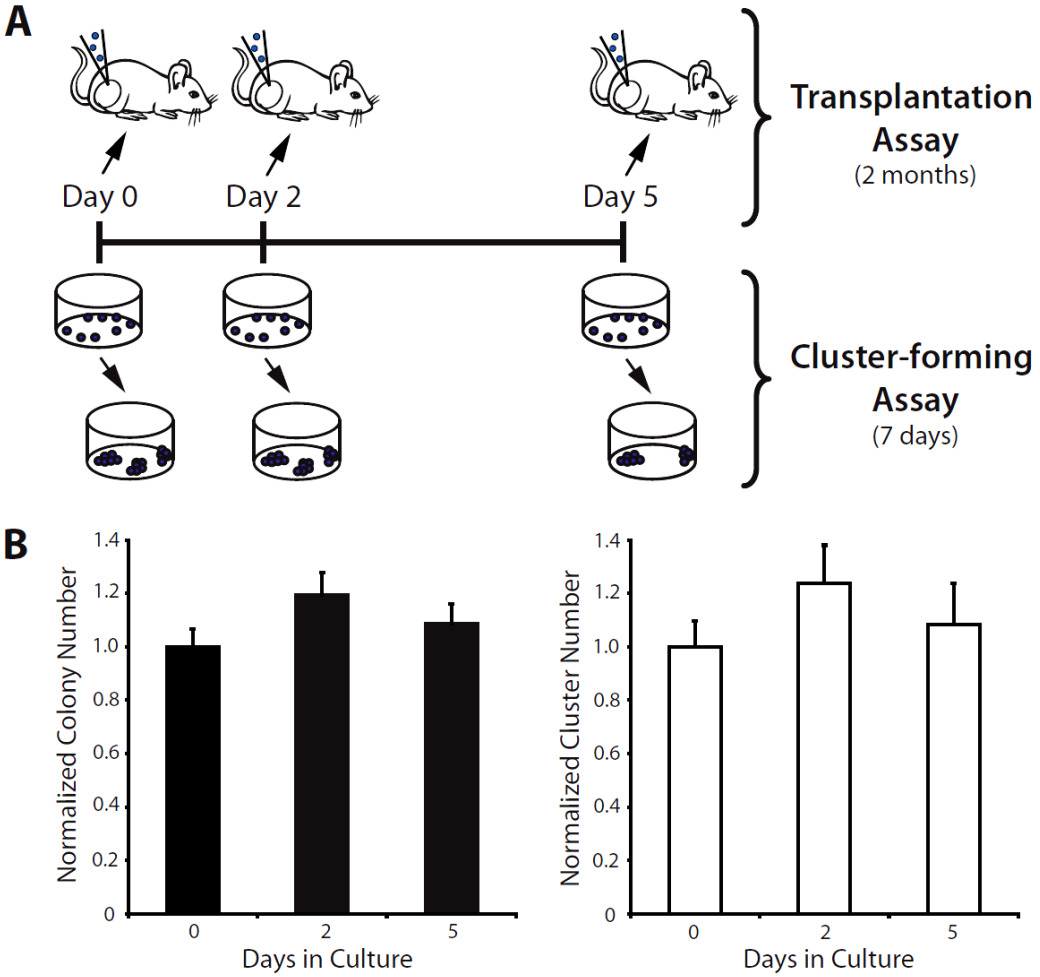

Figure 4: Effect of growth factor absence on maintenance of spermatogonial stem cells.

A) Experimental scheme. Sorted cells are cultured on feeder cells in the absence of growth factors for 2 and 5 days; no clusters emerge during these initial culture periods. At each time, cells are transplanted into recipient testes as well as cultured in the presence of growth factors to induce cluster formation. **B)** Quantification of colonies (left) and clusters (right) using the transplantation and cluster-forming assays, respectively. Both assays produce near identical results, and indicate no change in the stem-cell or cluster-forming activity over 5 days. Data are normalized based on the day-0 values for each assay. On each day examined, 20 – 25 recipient testes were used for the transplantation assay, and 8 replicates for the cluster-forming assay.

In the second experiment, we used spermatogonial transplantation and the cluster-forming assay to measure the number of SSCs and cluster-forming cells following hypotonic treatment of sorted cells. Hypotonic lysis has been widely used to selectively eliminate germ cells from heterogeneous mixtures of the seminiferous epithelium. Accordingly, hypotonic treatment has been used to prepare Sertoli cell-only cultures [196,197]. However, it has not been demonstrated if SSCs are also eliminated by this treatment. Therefore, we used hypotonic treatment as a means to potentially reduce SSC and cluster-forming cell numbers. Following immunomagnetic cell sorting, B6/ROSA pup testis cells were treated or untreated with a hypotonic solution for 5 or 20 min. Then, cells were transplanted into recipient testes as well as cultured to induce cluster formation. Colony numbers were determined 2 months after transplantation, while cluster numbers were assessed 6 days after culture.

As shown in Fig. 5A and B, both assays detected no significant decrease in colony and cluster numbers after a 5-min exposure. In contrast, a 50 %-decrease was detected in both numbers after treatment for 20 min. These results further demonstrate that cluster numbers are representative of colony numbers. A 50%-survival of SSCs after a 20-min exposure to hypotonic solution was somewhat surprising, because the solution presents such a harsh environment to the cells. Thus, we examined the survival kinetics of whole testes cells in hypotonic solution, and compared them to those of SSCs. As shown in Fig. 5C, a significant proportion of the total testis cells died by 5 min, and only 33.4 % survived after 20 min. Since SSCs did not decrease at 5 min and 50 % of them survived for 20 min, these results indicate that SSCs are more resistant to a hypotonic environment and survive the conditions better than non-SSC testis cells.

The results under these two experimental conditions collectively demonstrate that the number of clusters faithfully reflect the number of spermatogenic colonies established after transplantation.

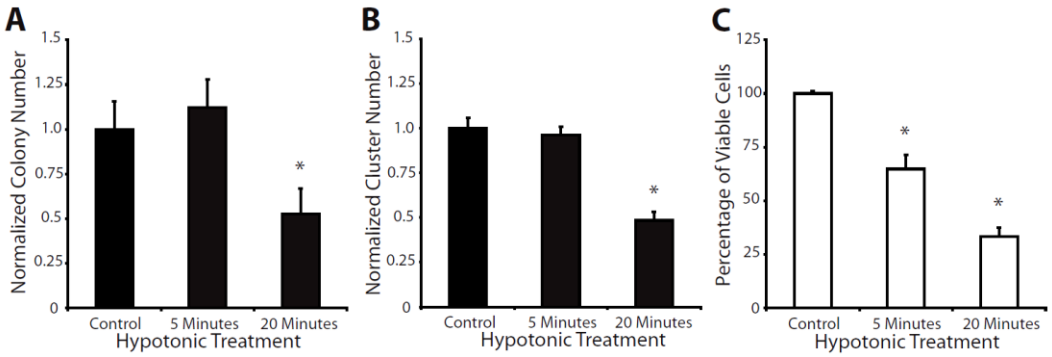

Figure 5: Effect of hypotonic conditions on colony and cluster formation. SSC quantification after treatment under hypotonic conditions, measured by the transplantation assay **(A)** and by the cluster-forming assay **(B)**. Data are normalized to an untreated control (mean ± SEM). Both assays give near identical results. No reduction in SSC numbers is seen after a 5-min treatment. After 20 min of hypotonic treatment, both colony numbers (52.5 ± 14.2 %, n = 21) and cluster numbers (48.4 ± 4.8 %, n = 8) significantly decline ($p \leq 0.029$ and < 0.001, respectively). **C)** Effects of hypotonic conditions on whole testis cell populations. Numbers of viable cells are normalized to an untreated control. Surviving cells decline to 64.8 ± 6.5 % (n = 12) at 5 min, and 33.4 ± 4.0 % (n = 12) at 20 min, both values significantly lower than control ($p < 0.001$).

Discussion

In this study, we have shown that a recently established SSC culture system [112] allows us to reliably detect functional SSCs within one week in a semi-quantitative manner. We established this in vitro SSC assay, the cluster-forming assay, based on two important stem cell characteristics: clonogenicity and long-term self-renewal ability, and confirmed the faithfulness of this assay to the definitive SSC assay, spermatogonial transplantation, using two experimental conditions. The data presented in Figs. 1 – 3 demonstrate that most clusters originate from a single cluster-forming cell (clonogenecity), and that the cluster-forming activity is sustained for at least 12 weeks during 11 passaging generations

(long-term self-renewal). Significantly, this robust self-renewal ability of clusters is clearly in parallel with that of definitive SSCs, which was measured using the in vivo transplantation assay (Fig. 3). These results therefore indicate that we can now faithfully detect and quantify functional SSCs by simply counting the number of clusters formed in vitro within 1 week. A similar methodology has been used in the neurosphere assay for NSCs [189,190], except that this method is not directly associated with regenerative ability of stem cells because a transplantation assay is not feasible for the central nervous system. In contrast, we have demonstrated that the results of the short-term in vitro assay directly correlate with the results of spermatogonial transplantation, the unequivocal functional assay for SSCs.

The advantage of the in vitro cluster-forming assay is fivefold. First, as described above, the assay can be completed in a short period of time (6 days). Second, the procedure is simpler than transplantation, allowing greater ease of use. Since spermatogonial transplantation is a laborious procedure, it has been technically demanding to compare five or more experimental conditions at once. The in vitro assay overcomes this limitation. Third, while immunocompatibility between donors and recipients is required for a successful transplantation, it is not the issue in the in vitro cluster-forming assay. In this context, fourth, the in vitro assay can be used for SSCs derived from wild-type mice. As shown in Fig. 2, we found that when donor B6 and B6/ROSA cells were co-cultured at a 1:1 ratio, the number of clusters with a single genotype was similar for either cell population, indicating that the frequency of SSCs is equivalent in B6 and B6/ROSA cells. In the transplantation assay, a quantification of SSCs derived from wild-type mice has been technically demanding, since the lack of genetic labels makes it difficult to distinguish donor-derived spermatogenesis from endogenous spermatogenesis of a recipient. As such, the cluster-forming assay now allows us to circumvent this problem. However, the application of the cluster-forming assay to varied strains of mice may be necessary to evaluate the versatility of this in vitro assay. Finally, this in vitro assay appears to involve less variation in data, compared to the in vivo transplantation assay. Based on the data presented in Figs. 4 and 5, we

calculate that the coefficient of variation is 2-fold higher with the transplantation assay than with the cluster-forming assay; the former gives 50.1 % of the coefficient, while the latter, 25.2 %. This lower level of variance should provide more consistency in data acquisition and justify the reduction of sample numbers.

Three disadvantages can be found in the cluster-forming assay. First, to induce cluster formation, donor testis cells need to be sorted for SSC enrichment. This is probably because without cell sorting, contaminating somatic cells interfere with the action of SSCs in vitro in the presence of growth factors. This disadvantage, however, is restricted to primary cells freshly prepared from testes and it is not a significant problem when established clusters are used to study biological properties of SSCs in vitro. Second, SSC detection using this in vitro technique is still retrospective, as it is with spermatogonial transplantation. Third, the cluster-forming assay does not entirely replace the transplantation assay for SSC detection. Since spermatogenesis cannot currently be reconstituted in vitro, the cluster-forming assay is not directly based on the regenerative activity of stem cells, and thus, is not an unequivocal stem cell assay on its own.

In this context, it should also be noted that counting cluster numbers does not present SSC numbers in an absolute manner. For example, it cannot be convincingly determined that there are 100 SSCs when we observe 100 clusters. This is because the quantification of SSCs in absolute numbers is currently not possible using a biological assay. In the cluster-forming assay, cluster chimerism at higher cell seeding densities can lead to underestimation of cluster numbers. Additionally, cluster-forming cells may die or be physically lost when re-plating cells to induce secondary clusters. Similarly, in the transplantation assay, the proportion of SSCs that survive transplantation and colonize recipient testes (seeding efficiency) has not been determined unequivocally [70,199,200]. At present, therefore, cautions are necessary to correlate cluster numbers and SSCs directly in absolute values. Considerations aside, our calculation based on the results shown in Fig. 3 estimates that each cluster houses 1.2 SSCs, when we assume a 12 % seeding efficiency after transplantation [70,199]. This result indicates that SSCs are actually a minority population in clusters and that most

cells comprising a cluster are differentiated progenitor cells. This ratio of SSCs to progenitor cells remained relatively consistent throughout the course of long-term culture. Furthermore, the percentage of SSCs to total cells in culture has been reported to be relatively consistent (1 – 3 %) over various studies [109,112,115], suggesting that a balance between SSCs and progenitor cells, within the cluster community, might regulate ideal SSC numbers. Therefore, maintaining this balance may explain why colony numbers correlate directly to cluster numbers. Further study is necessary to examine the regulation of this balance of SSC self-renewal and differentiation in the cluster environment. Nevertheless, the cluster-forming assay clearly has significant advantages as described above and this in vitro assay should now provide a powerful assay tool in SSC research, particularly when used in combination with spermatogonial transplantation to substantiate the results of the cluster-forming assay.

We applied both in vitro and in vivo SSC assays in two experimental conditions, and in either case, both assays gave rise to near identical results, further attesting to the fidelity of the cluster-forming assay. In one experiment, we quantified SSCs following a culture without growth factors, which are required for long-term SSC maintenance and expansion in vitro [112]. The results showed that the number of SSCs remaining for 5 days in culture was similar, irrespective of the factor presence. This was contrary to our expectation that the lack of growth factors would reduce the number of surviving SSCs. However, this experiment was short-term and done using cells freshly obtained from donor testes. Since in vitro SSC expansion is exponential, and SSCs eventually disappear over time without growth factors in vitro [112] (Fig. 3), the effect of factors may be difficult to observe at an early stage of culture, and the results may simply imply that the culture condition is sufficient for SSC survival over the short term, despite the lack of growth factors.

In the second experiment, we measured the number of SSCs that had survived a cytotoxic hypotonic environment. The results of both the transplantation and cluster-forming assays showed that SSCs survived well after a 5-min treatment, but half of them died after a 20-min treatment. Interestingly,

although SSC numbers did not change after a 5-min hypotonic treatment, the number of viable total testis cells significantly declined (Fig. 5). After a 20-min treatment, the survival of total testis cells, including somatic cells, was significantly lower than the survival of SSCs measured with the cluster-forming assay (i.e., Fig. 5B vs. C; $p \leq 0.03$); this difference was not detected with the transplantation assay (Fig. 5A vs. C; $p \leq 0.20$), due probably to the large variance in data. Therefore, SSCs appear to be more robust and less susceptible to osmotic changes than non-SSC testis cells and hypotonic treatment may be effective to partially enrich testis cells for SSCs.

In conclusion, this study provides the evidence that the cluster-forming assay reliably and reproducibly detects functional SSCs within one week in a semi-quantitative manner. This efficient and versatile assay should facilitate progress in studies of SSCs in the future.

Acknowledgement

The authors thank Kevin Ebata for his careful reading of this manuscript and suggestions.

CHAPTER 3

MANUSCRIPT II

Preface to Chapter 3

In Chapter 2, I determined that a cluster is an in vitro unit of functional SSC activity; I observed that cluster formation correlates with colony formation and that by counting cluster numbers SSC numbers could be quantified. Interestingly, transplantation of clusters revealed that they are heterogeneously composed of SSCs and differentiated daughter cells, suggesting that self-renewal and early stages of differentiation continually occur in vitro. Therefore, cluster culture appears to be an ideal model to study the regulation of SSC fate decisions.

The SSC culture system is well defined and uses the extrinsic factor GDNF to support SSCs long-term. However, SSC culture also requires an embryonic feeder cell layer, which contributes unknown factors that are crucial to maintain SSCs. For example, recall that in Chapter 2 I observed that testis cells could be maintained for 6 days without a loss in SSC activity when cultured on this embryonic feeder cell layer, in the absence of GDNF, further suggesting their importance in maintaining SSCs in vitro.

In the following chapter, I took a candidate approach to identify potential SSC-extrinsic factors by examining targets that might influence SSC fate in vitro. In particular, I focused on Wnt molecules from the embryonic fibroblast feeder cell layer. I posit that these feeder cells provide extrinsic factors that can support SSC self-renewal in vitro and might be an SSC niche factor in the testicular microenvironment.

Wnt5a is a cell-extrinsic factor that supports mouse spermatogonial stem cell self-renewal

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Journal of Cell Science 124:2357-2366, (2011)

Short-title: Wnt5a supports spermatogonial stem cells

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Abstract

The maintenance of spermatogonial stem cells (SSCs) provides the foundation for life-long spermatogenesis. Although glial cell-line derived neurotrophic factor and fibroblast growth factor 2 are critical for SSC self-renewal, recent studies have suggested that other growth factors play important roles in controlling SSC fate. Since β-catenin-dependent Wnt signalling promotes self-renewal of various stem cell types, we hypothesized that this pathway contributes to SSC maintenance. Using transgenic reporter mice for β-catenin-dependent signalling, we found that this signalling was not active in SSCs in vitro and in most spermatogonia in vivo. Nonetheless, a pan-Wnt antagonist significantly reduced SSC activity in vitro, suggesting that some Wnt molecules exist in our serum-free culture system and contribute to SSC maintenance. Here we report that Wnt5a promotes SSC activity. We found that Wnt5a-expressing fibroblasts better supported SSC activity than those not expressing Wnt5a in culture, and recombinant Wnt5a stimulated SSC maintenance. Furthermore, Wnt5a promoted SSC survival in the absence of feeder cells, and this effect was abolished by inhibiting the c-jun N-terminal kinase cascade. In addition, Wnt5a blocked β-catenin-dependent signalling. We detected the expression of Wnt5a and potential Wnt5a receptors by Sertoli cells and stem/progenitor spermatogonia, respectively. These results indicate that Wnt5a is a cell-extrinsic factor that supports SSC self-renewal through β-catenin-independent mechanisms.

Introduction

Spermatogonial stem cells (SSCs) are a rare population in testes, but are responsible for life-long sperm production. These cells are present on the basement membrane of the seminiferous tubules of the testis. Recent studies suggest that SSC activity is regulated in a microenvironment that is composed of Sertoli cells, the extracellular matrix, and the vasculature network [84,96,201,202,203]. SSC self-renewal is known to be stimulated by glial-cell-line-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2), which are both expressed by Sertoli cells in testes [202]. In vitro, GDNF and FGF2 allow for long-term SSC maintenance and expansion [112,202], attesting to their importance as cell-extrinsic effectors of SSC activity. However, recent studies have indicated that colony-stimulating factor 1 (CSF-1) promotes SSC self-renewal in vitro as a cell-extrinsic factor [95,204], demonstrating that other factors are involved in controlling SSC activity.

Wnt proteins are lipid modified, secreted morphogens that control a variety of cell processes such as embryogenesis, cell proliferation and differentiation, and stem cell regulation [90,144]. In the testis, several Wnt ligands and signalling components are known to be expressed [176,180,205], but no clear role during spermatogenesis has been identified. In this study, we examined the potential involvement of Wnt signalling in regulating SSC self-renewal.

Wnt signalling is mediated by a wide range of intracellular signalling cascades, which are roughly categorized into β-catenin-dependent and independent pathways [144]. In the β-catenin-dependent Wnt pathway, Wnt ligands bind to the receptor, Frizzled (Fzd), and co-receptor, low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) [144]. Activation of this pathway inhibits the action of glycogen synthase kinase-3β (GSK3β) and prevents β-catenin degradation. β-Catenin then translocates to the nucleus and induces target gene expression together with transcription factors of the T cell factor/lymphoid enhancer factor (TCF/LEF) family.

β-Catenin-dependent signalling has been known to promote self-renewal of many stem cell types, including those of embryonic and postnatal origins

[168,206,207,208]. Hence, this pathway was initially regarded as a general promoter of stem cell self-renewal [90]. Recent studies, however, indicate that the action of β-catenin signalling is more complicated. For instance, this signalling directs embryonic and adult progenitors in the skin to differentiate [209] and aging muscle satellite cells and neural crest stem cells toward fibrogenesis [210] and a sensory neural fate [211], respectively. In the germ line, activation of β-catenin signalling is detrimental for development of primordial germ cells [180]. Therefore, the response of stem cells to the β-catenin pathway is cell-type specific.

In contrast, the mechanisms of β-catenin-independent Wnt signalling are diverse and have not been well elucidated. It is known that this signalling can be mediated by G-proteins and an intracellular calcium flux, thereby activating downstream effectors, such as calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC) [212]. β-Catenin-independent signalling also acts through c-jun N-terminal kinases (JNK) to regulate planar cell polarity in Drosophila or convergent-extension movements during Xenopus gastrulation [212]. In mammalian cells, this signalling can control cell polarity and migration through the JNK cascade [153]. In general, the activation of β-catenin-independent pathways is known to inhibit the β-catenin-TCF/LEF pathway [141,213].

Recently, Wnt5a has been reported to control the activity of various stem cell types in a β-catenin-independent manner. For instance, it simulates repopulation potential of hematopoietic stem cells while inhibiting β-catenin signalling [172] and supports multipotentiality of mesenchymal stem cells [214]. Studies using vertebrates and invertebrates have indicated that β-catenin-independent Wnt5a signalling can be mediated by multiple receptors, such as Fzd3, Fzd5, and Fzd7 [142,215,216]. In mammalian cells, it has been shown that Ror2, a receptor tyrosine kinase, can also mediate Wnt5a signalling through JNK signalling [141].

In this study, we initially hypothesized that β-catenin-TCF/LEF signalling (termed “β-catenin pathway” hereafter) promotes SSC maintenance. However, results using SSC culture indicated the contrary. Further in vitro analyses showed

that Wnt5a supported SSC maintenance in a β-catenin-independent manner and enhanced the survival of stem/progenitor spermatogonia. These Wnt5a effects were abolished by the inhibition of JNK signalling. Additionally, we localized Wnt5a expression to Sertoli cells in mouse testes and detected Wnt5a receptors on the cell surface of SSCs. Thus, we identify Wnt5a as a novel extrinsic regulator of SSC self-renewal.

Materials and Methods

Donor Mice

TCF/LEF-*lacZ* mice (from Dr. D. Dufort, McGill University) have CD-1 genetic background and carry the *lacZ* reporter gene driven by the β-catenin-TCF/LEF responsive elements [217,218]. B6ROSA mice are F_1 hybrids of C57BL/6 (B6) and ROSA26 (B6;129S-*Gt(ROSA)26Sor*/J) mice, which express the *lacZ* gene ubiquitously [192]. B6GFP mice (C57BL/6-Tg(CAG-EGFP)1Osb/J) express GFP ubiquitously. Oct4GFP mice (GOF-18ΔPEOct4/GFP) were generated as Yoshimizu et al. [119] by Dr. J. Trasler (McGill University) and express GFP specifically in spermatogonia up to ~7 days of age. Experimental cryptorchidism and orchidopexy were induced as described previously [219]. Animal procedures were approved by the Animal Care and Use Committee of McGill University.

Recipient Mice and Spermatogonial Transplantation

Recipient mice were prepared and spermatogonial transplantation was performed as in [134]. Recipients for B6ROSA donor cells were 129/SvEv × B6 F_1 hybrids, and those for TCF/LEF-*lacZ* cells, Ncr nu/nu mice (Taconic). Recipient testes were analyzed for SSC quantification following staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) 2 months post-transplantation.

Cell Cultures

SSC cultures were generated using Thy1-positive testis cells of 6 – 8 days post-partum (dpp) mice, as described previously [134]. Cultures were maintained with a STO feeder layer [112,134] and "growth factors": i.e., GDNF (20 ng/ml),

GFRα1 (75 ng/ml), and FGF2 (1 ng/ml). All in vitro experiments, except those where indicated, were conducted using established cluster cultures (i.e. > 5 passages) on STO feeders with 40 ng/ml GDNF, 300 ng/ml GFRα1, and 1 ng/ml FGF2. The day of cell seeding was designated as day 0. In one series of experiments, L cells or L cells stably transfected with Wnt5a [218] (from Dr. K. Kuroda, University of Ottawa) were used as feeders. For short-term feeder-free cultures, culture plates were coated with Matrigel (BD Biosciences), diluted 1:2, and incubated overnight at 4°C.

To activate β-catenin signalling, 5 mM lithium chloride was added to B6ROSA or TCF/LEF-*lacZ* cluster cultures on day 3 (three replicates). On day 6, TCF/LEF-*lacZ* clusters were trypsinized and reacted with X-gal to quantify signalling-positive cells using a hemocytometer. B6ROSA clusters were transplanted into recipient testes to quantify SSCs.

To inhibit Wnt signalling, Dickkopf-1 (Dkk1; R&D Systems) or Secreted frizzled-related protein-1 (sFRP1; R&D Systems) was added on day 0 to TCF/LEF-*lacZ* or B6ROSA cluster cultures and replenished on day 3. On day 6, B6ROSA clusters were quantified visually, and signalling-positive cells in TCF/LEF-*lacZ* clusters, using a hemocytometer. At least three experiments were done. The same experimental schedule was used for competition assays of recombinant Wnt5a (R&D Systems) against sFRP1. Clusters were visually quantified on day 6. Since cluster numbers semi-quantitatively indicate SSC numbers, SSC activity can be measured by this "cluster-formation assay" [134].

To examine inhibition of β-catenin signalling by Wnt5a, TCF/LEF-*lacZ* clusters were cultured for 6 days with 400 ng/ml Wnt5a. β-Catenin-signalling cells were then quantified using a hemocytometer (four experiments). To examine effects of Wnt5a in the absence of feeders, B6ROSA clusters were removed from STO feeder cells by gentle pipetting [108] and trypsinized into single cells. Resulting cells were placed onto Matrigel with Wnt5a, and 4 days later, they were trypsinized and subjected to the cluster-formation assay (passaging onto STO feeder cells with 1:1 split) and to spermatogonial transplantation. Three experiments were performed for the cluster-formation assay and six for

spermatogonial transplantation.

To inhibit Wnt5a intracellular signalling, inhibitors against CaMKII (KN-93; Calbiochem), PKC (GF109203X; Biomol), G-proteins (Pertussis Toxin; Sigma), and JNK (JNK Inhibitor III and SP600125; Calbiochem) were added on day 0 to B6ROSA clusters. On day 3, the inhibitors were withdrawn, and on day 6, clusters were quantified. Inhibitor doses were determined according to previous reports [220,221,222,223,224]. At least three experiments were performed. In one series of experiments, clusters were treated with SP600125 as above; on day 6, they were trypsinized and subjected to the cluster-formation assay in three experiments. In feeder-free cultures on Matrigel, Wnt5a and/or SP600125 was incubated for 4 days, followed by the cluster formation assay in four experiments.

To analyze Wnt5a effects on cell cycle and apoptosis, TCF/LEF-*lacZ* or B6ROSA clusters were cultured feeder-free on Matrigel for 2 or 4 days and subjected to flow cytometry.

Wholemount Testis Staining

TCF/LEF-*lacZ* mouse testes were fixed in 4% paraformaldehyde while adult testes in Bouin's solution and reacted with X-gal overnight. Paraffin-sections (5 μm) were counterstained with nuclear fast red.

Flow Cytometric Analysis and Sorting

Flow cytometric analyses and fluorescent-activated cell sorting (FACS) were done using FACScan and FACSAira, respectively (Beckton Dickinson). All reactions with antibodies (Table 1) were at 4°C for 30 min with gentle agitation. To isolate β-catenin-signalling cells, TCF/LEF-*lacZ* clusters were digested into single cells with trypsin and 1 μg/ml DNase. Cells resuspended at $8 – 9 \times 10^6$ cells/ml in PBS were reacted with 500 μM fluorescein di-β-D-galactopyranoside (FDG, Marker Gene Technologies) in double-distilled H_2O for 1 min at 37°C, following the manufacturer's protocol. Reaction was arrested with 10 mM HEPES and 4% FBS in PBS at 4°C. Cells were resuspended in Modified Eagle Medium (MEM; Invitrogen) with 1% FBS before FACS. Experimental gates were

established using control cells: B6ROSA (positive) and B6 (negative) cluster cells. B6ROSA and B6 cells showed >96% positive and <1% positive, respectively. Data were collected from two experiments.

Cell cycle profiles were examined with B6ROSA cluster cells. Cells were trypsinized and fixed in 70% ethanol, followed by incubation with 40 µg/ml propridium iodide (PI) and 100 µg/ml RNase at 37°C for 30 min. Data were collected from four experiments with 5,000 – 30,000 events collected per sample. To determine G_0 phase cells, B6ROSA cells were fixed in 1% paraformaldehyde for 30 min and 70% ethanol overnight and stained for Ki-67 expression, followed by incubation with PI and RNase. Experimental gates were established from unstained controls. Data were collected from three experiments with 5,000 – 10,000 events collected per sample.

Apoptosis profiles were analyzed with B6ROSA cluster cells by staining with the APO-Direct Apoptosis Detection Kit (BD Biosciences). Three experiments were performed with 5,000 – 10,000 events collected per experimental group.

To determine the expression of Wnt5a receptors on cluster cells, clusters from B6ROSA or B6-GFP mice were removed from STO feeder cells using gentle pipetting and dispersed using a micropipette into single cells for flow cytometric analyses. At least 10,000 events were collected. Data were from three to five experiments per particular receptor, except for integrin-α6, from two experiments.

Fzd3 expression on SSCs was analyzed using testis cells of 3-month-old B6ROSA mice (six testes/experiment). A single cell suspension was separated on a Percoll gradient as in [195]. The SSC-containing fraction was further enriched for SSCs using immunomagnetic cell sorting against β2-microglobulin. Six million cells in 1 ml DMEM were used per sorting. β2-microglobulin-negative cells, representing 5 – 7.8% of total testis cells, were subjected to flow cytometric analyses for Fzd3 and Thy1 expression. Gates were established from primary antibody-omitted negative controls. Data were collected from two experiments; six recipient testes per group per experiment.

Table 1: Antibodies

FACS					
Primary Antibody	**Company**	**Concentration**	**Secondary Antibody**	**Company**	**Concentration**
rat anti-Fzd3	R&D Systems	1-2 μg/10^6 cells	PE- donkey anti-rat IgG	Jackson Immunoresearch	1 μg/10^6 cells
mouse anti-Fzd5	Abcam	5 μg/10^6 cells	Dylight649-donkey anti-mouse	Jackson Immunoresearch	3 μg/10^6 cells
biotinylated goat anti-Fzd7	R&D Systems	10 μg/10^6 cells	streptavidin-AlexaFluor488	Jackson Immunoresearch	1 μg/10^6 cells
rabbit anti-Ror2	Cell Signalling Technologies	5 μg/10^6 cells	FITC- goat anti-rabbit IgG	Jackson Immunoresearch	1 μg/10^6 cells
rat anti-integrin-a6	BD Biosciences	5 μg/10^6cells	PE- donkey anti-rat IgG	Jackson Immunoresearch	2 μg/10^6 cells
biotinylated anti-Thy1 antibody	BD Biosciences	2 μg/10^6 cells	streptavidin-AlexaFluor488	Jackson Immunoresearch	1 μg/10^6 cells
goat anti-Ki-67 antibody	Santa Cruz Technologies	2 μg/10^6 cells	FITC- rabbit anti-goat IgG	Jackson Immunoresearch	1 μg/10^6 cells
Immunomagnetic Sorting					
Primary Antibody	**Company**	**Concentration**	**Secondary Antibody**	**Company**	**Concentration**
anti-mouse β2-microglobulin	BD Biosciences	5 μg/6 x 10^6 cells	Dynabeads sheep anti-mouse IgG	Invitrogen	8 x 10^6 beads/ml
biotinylated anti-Thy1 antibody	BD Biosciences	5 μg/6 x 10^6 cells	Dynabeads M-280 streptavidin	Invitrogen	3.5 x 10^6 beads/ml
Immunofluorescent Staining					
Primary Antibody	**Company**	**Concentration**	**Secondary Antibody**	**Company**	**Concentration**
rabbit anti-β-catenin	Sigma	3.2 mg/ml	FITC- goat anti-rabbit IgG	Jackson Immunoresearch	30 μg/ml
rat anti-Fzd3	R&D Systems	2 μg/ml	FITC- goat anti-rat	Jackson Immunoresearch	15 μg/ml
goat anti-Fzd5	R&D Systems	5 μg/ml	PE- donkey anti-goat	Jackson Immunoresearch	5 μg/ml
biotinylated goat anti-Fzd7	R&D Systems	5 μg/ml	streptavidin-AlexaFluor488	Invitrogen	10 μg/ml
rabbit anti-Ror2	Cell Signalling Technologies	2 μg/ml	Cy3- goat anti-rabbit	Jackson Immunoresearch	4 μg/ml
rabbit anti-phospho-JNK	Abcam	0.1 mg/ml	Dylight549-donkey anti-rabbit IgG	Jackson Immunoresearch	6.3 μg/ml
Immunoblotting					
Primary Antibody	**Company**	**Concentration**	**Secondary Antibody**	**Company**	**Concentration**
rabbit anti-phospho-JNK	Abcam	0.5 μg/ml	peroxidase-goat anti-rabbit IgG	Jackson Immunoresearch	40 pg/ml
rabbit anti-JNK/SAPK	Cell Signalling Technologies	0.2 μg/ml	peroxidase-goat anti-rabbit IgG	Jackson Immunoresearch	40 pg/ml

RNA Isolation and RT-PCR Analysis

Total RNA was extracted using TRIzol (Invitrogen). Complementary DNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen) with random hexamers. Primer sequences are shown in Table 2. Those for Wnt transcripts were as reported in Mohamed et al. [225], for Fzds in Chen et al. [226] and Torday and Rehan [227], for Ror2 in Mikels and Nusse [141], and for LRP5/6 in Stump et al. [228]. PCR was performed using the program: 95°C for 3 min followed by 25 – 30 cycles of 95°C for 30 sec/51.1 – 61.5°C for 30 sec/72°C for 30 sec with a final extension at 72°C for 5 min. Quantitative PCR was performed with QuantiTect 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 sec/60°C for 30 sec/72°C for 35 sec.

Table 2: PCR Primer Sequences

Target	Forward Primer	Reverse Primer
Wnt1	5′-AAATCGCCCAACTTCTGCA-3′	5′-AATACCCAAAGAGGTCACAGC-3′
Wnt2	5′-CGGCCTTTGTTTACGCCATC-3′	5′-TGAATACAGTAGTCTGGAGAA-3′
Wnt2b	5′-TGTACTCTGCGCACCTGCT-3′	5′-TGCACTCACACTGGGTGAC-3′
Wnt3a	5′-ATTGAATTTGGAGGAATGGT-3′	5′-CTTGAAGTACGTGTAACGTG-3′
Wnt5a	5′-TCCTATGAGAGCGCACGCAT-3′	5′-CAGCTTGCCCCGGCTGTTGA-3′
Wnt6	5′-GCACCGAGTGTAAGTGCCAT-3′	5′-GAAGCGGCACAGACAGTTCT-3′
Wnt7b	5′-ACCAAAACTTGCTGGACCAC-3′	5′-ACGTGTTGCACTTGACGAAG-3′
Wnt10a	5′-AAAGTCCCCTACGAGAGCCC-3′	5′-CAGCTTCCGACGGAAAGCTT-3′
Wnt11	5′-GCCATGAAGGCCTGCCGTAG-3′	5′-GATGGTGTGACTGATGGTGG-3′
Fzd1	5′-CAGTTCACTTCCGACAAAGG-3′	5′-AGGTAGGAAGGCACCCTGAG-3′
Fzd2	5′-GGAACTCCTGCGCTACTCAC-3′	5′-GCGCTCACCCAGAAACTTAT-3′
Fzd3	5′-TTTTCCATGGGCGTAGGA-3′	5′-TAACACGGTTCATGCTGGTG-3′
Fzd4	5′-ACAACCACATGTGCATGGAA-3′	5′-TCCTTAGCTGAGCGGCTGTA-3′
Fzd5	5′-GACGCCGAGGTTCTGTGTAT-3′	5′-TGCGCACCTTGTTGTAGAGT-3′
Fzd6	5′-CCCTCGTAAGAGGACACAGC-3′	5′-TTGCAAGATGCAGAAAGTGC-3′
Fzd7	5′-GCCAGACCCACCTTTCACT-3′	5′-CGAACCGTCTCTCCTCTTCTT-3′
Fzd8	5′-AGTACCGGCCTGACGTGG-3′	5′-AGACCTGGGACAATGGCA-3′
Ror2	5′-TGGAACTGTGTGACGTACCC-3′	5′-GCGAGGCCATCAGCTG-3′
LRP5	5'-GATGTGCGGCTAGTGGATG-3'	5'-GCCCGAGATGACAATGTTCT-3'
LRP6	5'-GGGCCGATGCAAAACTTAATT-3'	5'-CTGTGTTGGCTGAAAGCAT-3'
Oct4	5′-CACGAGTGGAAAGCAACTCA-3′	5′-TTCATGTCCTGGGACTCCTC-3′
Plzf	5′-GCAGCTATATTTGCAGTGAG-3′	5′-TCTTGAGTGTGCTCTCATCC-3′
Ngn3	5′-CTCATTGGAGGAATTCCCTG-3′	5′-TTTCCACTAGCACCCACCAC-3′
Kit	5′-TGGGAGTTTCCCAGAAACAG-3′	5′-AAATGGGCACTTGGTTTGAG-3′

In Situ Hybridization

B6ROSA testes were fixed in 4% paraformaldehyde and then in increasing concentrations of sucrose before cryosection at 10 μm. Sections were treated with 20 μg/ml proteinase K (Invitrogen) at 65°C for 10 min and post-fixed in 4% paraformaldehyde. Samples were acetylated in 0.25% acetic anhydride (Fisher) in 0.1M triethanolamine pH 8.0 for 20 min. Hybridization was carried out using a digoxigenin-labeled riboprobe generated from a pGEM32F (Promega) vector carrying a 360 bp PCR fragment containing the Wnt5a coding sequence [229] (from Dr. D. Dufort). Counterstaining was with YOYO-1 (Invitrogen). Images were captured with LSM 510 Meta laser scanning confocal microscope (Zeiss).

Immunostaining

To detect β-catenin expression, adult ROSA26 mouse testes were cryosectioned and fixed in ice-cold MeOH prior to immunodetection. To identify putative Wnt5a receptors, B6ROSA clusters were fixed with 4% paraformaldehyde for 20 min. TCF/LEF-*lacZ* clusters were fixed with 0.5% gluteraldehyde for 5 min and subsequently reacted with X-gal for 6 hr prior to chromogenic immunodetection for Fzd3. Antibodies and their concentrations are listed on Table 1. Hoescht 33342 (Invitrogen) was used for nuclear staining. Primary antibodies were omitted in negative controls. In some cases, image contrast was adjusted using Photoshop CS2 to better reflect our visual observations.

Western Blot Analysis

B6GFP cluster cells were cultured on Matrigel overnight in the absence of growth factors prior to addition of Wnt5a, or Wnt5a and SP600125, or vehicle alone for 2 hr as described previously [230]. Equal amounts of whole-cell lysate from each group were resolved using SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes, and immunoblotting for phospho-JNK or total-JNK levels was performed. The blots were stripped after data acquisition, α-tubulin was immunoblotted and visualized as a loading control. Visualization was

obtained using ECL Plus Western Blotting Detection Kit (GE Healthcare). Quantification of blot intensities was performed using ImageJ software (NIH; Bethesda, MD) as per the developer's protocol. Data were collected from three separate experiments. Antibodies and their concentrations are listed in Table 1.

Data Presentation and Statistics

Data were expressed as mean ± SEM. Numbers of clusters in vitro and SSCs detected with spermatogonial transplantation were indicated as those per cm^2 of growth surface in culture, unless specified otherwise. Significance was determined using Student's *t*-test or ANOVA followed by Fisher's Test for Least Significant Difference. $p < 0.05$ determined significance.

Results

β-Catenin signalling is not active in SSCs.

To examine if β-catenin signalling is involved in controlling SSC activity, we initiated SSC culture using SSC-enriched cells freshly prepared from testes of transgenic reporter mice (TCF/LEF-*lacZ* mice). These mice carry the *lacZ* reporter gene linked to β-catenin-TCF/LEF binding sites, allowing for faithful monitoring of β-catenin signalling activation [217]. The cultured cells formed three-dimensional aggregates of spermatogonia [112,134], termed "clusters" hereafter. *LacZ* expression was observed in a subpopulation of cluster cells (Fig. 1A), demonstrating that the cluster is a functionally heterogeneous cell community, composed of at least two cell types: β-catenin signalling-positive and negative cells.

β-Catenin-signalling cells were not observed on day 1 of culture, but appeared by day 3 and increased in number thereafter (Fig. 1B). A similar increase in signalling cells was seen when established clusters (>5 passages) were used (Fig. 1C). To assess if SSCs are included in β-catenin-signalling or non-signalling cells, we isolated the two cell populations from established TCF/LEF-*lacZ* clusters using a vital fluorescent substrate of β-galctosidase and FACS. All cluster cells were found in Fraction II with a profile of side-scatter^{low} and relative

homogeneity in size, as reported previously [231], and which we confirmed (Fig. 1D, E). Fraction I (side-scatter^{hi} cells) was feeder cells. Fraction II was then separated into β-catenin-signalling and non-signalling cells, Fractions IV and III respectively (Fig. 1F, G). Quantitative PCR analyses for markers of undifferentiated (Oct4, Plzf, Ngn3) and differentiating spermatogonia (c-kit) indicated that both fractions similarly express the markers examined (Fig. 1H). However, there was a trend showing slightly decreased expression of undifferentiated spermatogonial markers in Fraction IV, although no significance was detected. When the SSC activity of each cell population was examined by spermatogonial transplantation, nearly all SSC activity was detected in Fraction III. Virtually none was found in Fraction IV (Fig. 1J, K), which was not due to cell death, since the vast majority of cells in this fraction were viable (Fig. 1I). The low SSC activity in Fraction I was attributed to contamination. These results indicate that β-catenin signalling is not active in SSCs.

Next, we activated β-catenin signalling in cluster cells using lithium chloride, a potent inhibitor of GSK3 [232]. We then quantified β-catenin signalling-positive cells and measured SSC activity by spermatogonial transplantation. The lithium chloride treatment increased numbers of signalling-positive cells by 10-fold but significantly decreased SSC numbers (Fig. 1L). Hence, activation of β-catenin signalling reduced SSC activity in vitro.

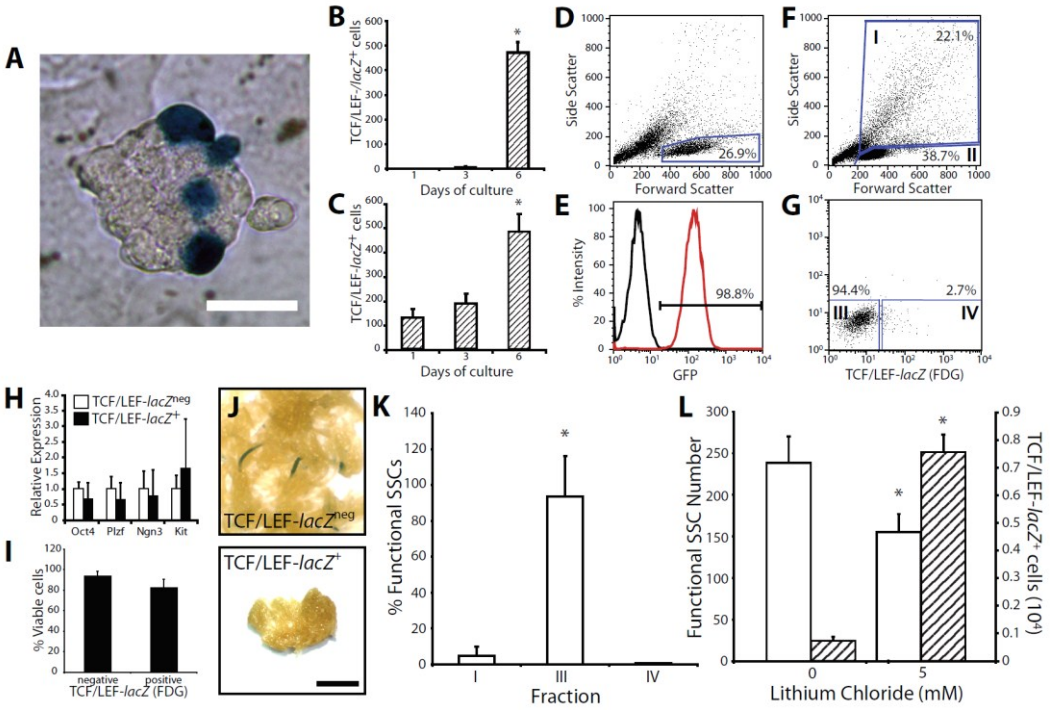

Figure 1: β-Catenin-signalling cells in germ-cell clusters do not have SSC activity.

(**A**) A TCF/LEF-*lacZ* cluster after 6 days in culture. A cluster contains β-catenin-signalling (blue) and non-signalling cells. (**B**) β-Catenin-signalling cells increase in number with time. The cells were derived freshly from testes. (**C**) Emergence of β-catenin-signalling cells in established clusters. (**D-E**) Representative flow cytometric scatter-plot of B6GFP cluster cells shows a distinct, relatively uniform cell population (D); virtually all cells in this gate are B6GFP cluster cells (E). (**F-G**) Sorting cluster cells: (F) Cluster cells are found in Fraction II; (G) Fraction II can be subdivided into β-catenin non-signalling (Fraction III, average; 96.6 ± 3.1%) and signalling cells (Fraction IV, average; 3.3 ± 0.5%). (**H**) Quantitative PCR on β-catenin-non-signalling cells (open bars) and signalling cells (filled bars) examine markers of undifferentiated (Oct4, Plzf, Ngn3) and differentiated spermatogonia (c-kit). A significant difference was not observed. (**I**) Flow cytometric scatter-plot for Annexin-V staining in β-catenin-signalling cells; no difference in cell viability was found. (**J**) Recipient testes 2 months following transplantation of β-catenin-non-signalling (top) or β-catenin-signalling cells (bottom). (**K**) Relative SSC activity found in β-catenin non-signalling (Fraction

III) vs. signalling cells (Fraction IV), measured with spermatogonial transplantation. Almost all SSC activity was found in Fraction III. (**L**) Quantification of SSCs (open bars) and β-catenin-signalling cells (hatched bars) after lithium chloride treatment. Scale bar: 30 μm (A), 0.6 mm (J, top), 4.5 mm (J, bottom).

β-Catenin signalling is activated in differentiating germ cells in vivo.

To examine the activation of β-catenin signalling in vivo, we analyzed the expression of the *lacZ* reporter gene in TCF/LEF-*lacZ* mouse testes at different ages during postnatal development (Fig. 2). In mouse testes, only spermatogonia exist for the first week after birth, meiotic cells appear around 10 dpp, and haploid cells are formed around 18 dpp; the first spermatozoa are found around 35 dpp [233]. β-Catenin-signalling cells were not found until 12 dpp (Fig. 2A-E, G). By 1 month of age and throughout adulthood (Fig. 2F, H), signalling cells became numerous and were observed in the adluminal compartment, where meiotic and haploid cells reside. Occasionally, signalling-positive spermatogonia were found in adult testes (Fig. 2H inset). No reporter activation was detected in the interstitial space. To verify our observations in TCF/LEF-*lacZ* reporter mouse testes, we examined β-catenin protein expression in adult testes using immunofluorescent staining. We detected expression in most cells along the basal layer of the seminiferous epithelium and β-catenin expression was mostly restricted to the membrane and cytoplasm in these cells (Fig. 2K). This finding supports our result that β-catenin signalling is not activated in the cells of the basal layer and is consistent with previous results described in rat testes [234]. We also observed diffuse cytoplasmic or nuclear β-catenin expression in cells of the adluminal compartment. Occasionally, we observed nuclear β-catenin expression in the basal layer (Fig. 2K inset), which corresponds to rare β-catenin signalling cells observed in the basal compartment of the seminiferous epithelium in reporter mouse testes (Fig 2H).

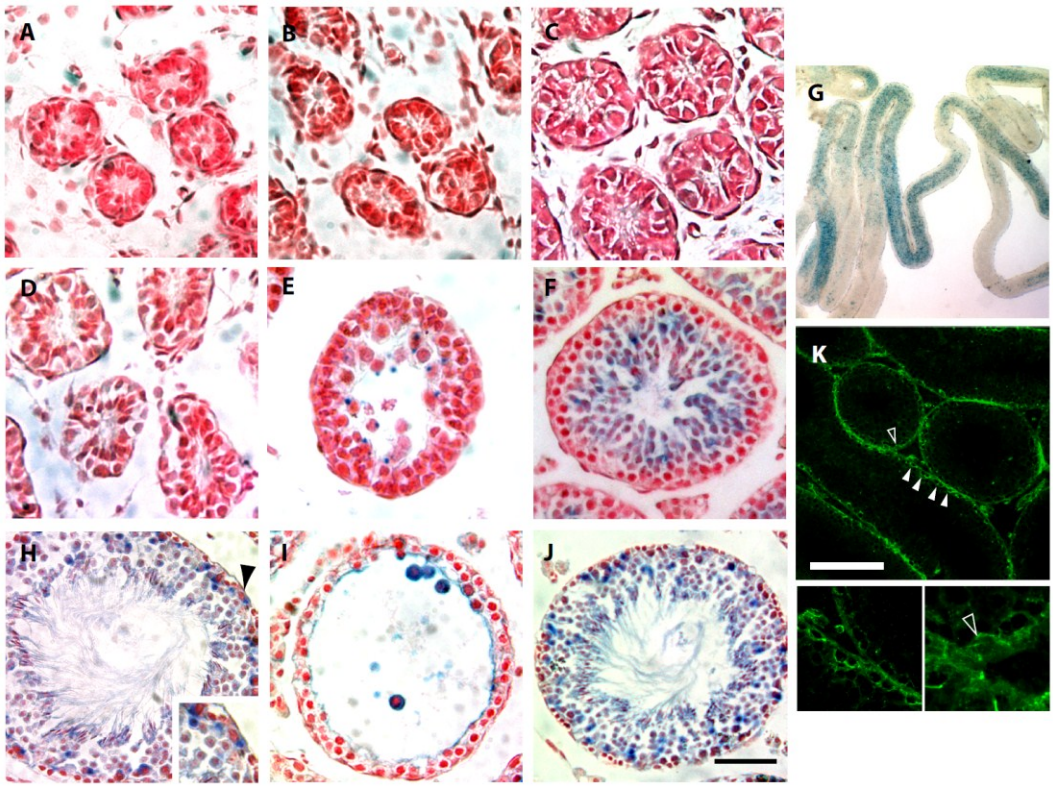

Figure 2: Identification of β-catenin-signalling cells in testes.

(**A-F, H**) Histology of TCF/LEF-*lacZ* mouse testes stained for reporter activation at 0 dpp (A), 3 dpp (B), 5 dpp (C), 7 dpp (D), 12 dpp (E), 30 dpp (F), and 60 dpp (H). β-Catenin-signalling cells are found in the adluminal compartment. In adult testes, signalling cells are observed occasionally in the basal compartment (H, arrowhead & inset). (**G**) Reporter expression in whole-mount seminiferous tubules of the TCF/LEF-*lacZ* mouse at 15 dpp. β-Catenin-signalling cells are seen in distinct segments and are generally not found along the basal layer of the tubule. (**I, J**) Histology of seminiferous tubules one month after experimental cryptorchidism (I) and two months after orchidopexy (J). (**K**) Expression of β-catenin protein in adult mouse testes. Cytoplasmic and membrane expression is observed in the cells along the basal layer (filled arrowheads; bottom left image, higher magnification). Occasionally, nuclear localization of β-catenin is found in basal layer cells (open arrowhead; bottom right image, higher magnification). Scale bar: 20 µm (A-E), 25 µm (F, I), 50 µm (H, J), 0.2 mm (G), 100 µm; 64 µm bottom left image; 32 µm bottom right image (K).

We next examined the pattern of reporter gene activation in TCF/LEF-*lacZ* mouse testes using experimental cryptorchidism and orchidopexy, an in vivo regeneration model of spermatogenesis [235]. In cryptorchid testes, where spermatogenesis is disrupted, reporter expression was virtually abolished (Fig. 2I), even though spermatogonia and Sertoli cells were present. After orchidopexy, which induces spermatogenic regeneration, reporter gene expression was restored in the adluminal compartment (Fig. 2J). These results collectively indicate that β-catenin signalling is activated in differentiating male germ cells but not in most spermatogonia.

Wnt5a promotes SSC maintenance as a cell-extrinsic factor in vitro.

The activation of β-catenin signalling in some cluster cells (Fig. 1) suggests that Wnt ligands are present in our SSC culture system. This is notable, because our culture medium is serum-free and does not contain Wnt ligands [112]. If Wnt ligands exist in the culture they must be expressed by feeder cells and/or cluster cells. To test this, we first cultured clusters derived from β-actin-GFP (B6GFP) mouse cells [236] and separated them from feeder cells using FACS. We examined the expression of Wnt1, Wnt2b, and Wnt3a, all of which are classically categorized into a β-catenin-dependent class of Wnt ligands, as well as those of Wnt5a, Wnt6, and Wnt11, which are often recognized as the β-catenin-independent class [212]. Using RT-PCR, we detected transcripts of Wnt2b, Wnt5a, and Wnt11 in feeder cells, but only Wnt1 transcripts in cluster cells (Fig. 3A).

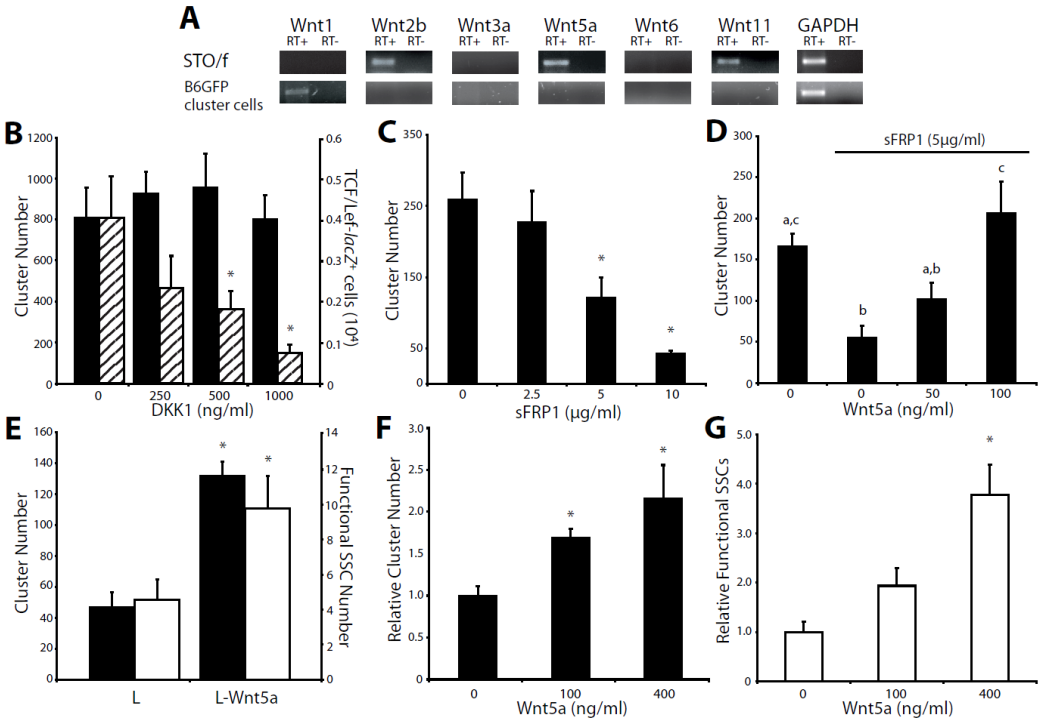

Figure 3: Wnt5a promotes SSC maintenance in vitro.

(**A**) RT-PCR analyses for various Wnt transcripts in feeder cells (STO/f) and cluster cells. (**B**) Cluster numbers (filled bars) and β-catenin-signalling cells (hatched bars) after treatment with Dkk1. Only β-catenin-signalling cells decline in number. (**C**) Quantification of clusters after treatment with sFRP1. Cluster numbers decline dose-dependently. (**D**) Cluster numbers following sFRP1 and Wnt5a treatment. Wnt5a competes against sFRP1 and restores cluster formation. Significance is indicated by different characters. (**E**) Cluster numbers (filled bars) and SSC numbers (open bars) when cultured with stable Wnt5a transfectants (L-Wnt5a) and parental L fibroblasts. Both parameters increase with L-Wnt5a. Results of the cluster-formation (**F**) and transplantation (**G**) assays for SSC quantification after feeder-free culture with Wnt5a. A significant increase in SSC numbers is detected with Wnt5a in both assays. Data are normalized to untreated control values.

We next asked if Wnt ligands affect SSC maintenance by using two soluble antagonists of Wnt signalling, Dickkopf-1 (DKK1) and secreted frizzled-related protein 1 (sFRP1). We used these antagonists, since DKK1 specifically blocks β-catenin signalling by binding to LRP5/6, while sFRP1 inhibits both β-catenin-dependent and independent signalling by binding Wnt ligands [144,153]. When DKK1 was applied to TCF/LEF-*lacZ* cluster cultures, cluster numbers did not change, but numbers of β-catenin signalling cells markedly declined in a dose-dependent manner (Fig. 3B). Since cluster numbers correlate with SSC numbers [134], these results suggest that inhibition of β-catenin signalling did not affect SSC activity. On the other hand, sFRP1 dose-dependently reduced cluster numbers (Fig. 3C). Collectively, these results imply that β-catenin-independent Wnt signalling may be involved in SSC maintenance.

Wnt5a is most often associated with β-catenin-independent signalling and can also block β-catenin signalling [141]. Our data showed that β-catenin signalling was not active in SSCs and that Wnt5a was expressed by feeder cells in our SSC culture. These observations led us to hypothesize that Wnt5a contributes to SSC maintenance in vitro.

To test this, we competitively added recombinant Wnt5a at increasing doses in the presence of sFRP1 and quantified clusters in a 6-day culture (Fig. 3D). We found that Wnt5a dose-dependently restored cluster numbers that had been affected by sFRP1. Next, we cultured clusters on a feeder layer of L fibroblasts stably transfected with Wnt5a (L-Wnt5a) or parental L cells, which lack Wnt5a transcripts (Mohamed et al. [218] and our data). SSC activity was determined by cluster numbers and spermatogonial transplantation. We detected a 2.8-fold increase in cluster numbers with L-Wnt5a feeders (Fig. 3E). Similarly, transplantation results showed a 2.2-fold increase in SSC activity with L-Wnt5a feeders (Fig. 3E), indicating that Wnt5a promotes SSC maintenance as a cell-extrinsic factor in vitro.

Since we employed feeder cells in all the above experiments, Wnt5a could have affected SSC maintenance indirectly through feeder cells. Thus, clusters were removed from feeder cells by gentle pipetting, by which cluster cells are

recovered with a >90% purity (Oatley et al. [108] and our data), and were cultured on Matrigel with or without Wnt5a for 4 days. No clusters emerged under these conditions. SSC activity was then measured by transferring these cells to our standard SSC culture conditions (cluster-formation assay). We found that SSC activity was significantly higher in the presence of Wnt5a (Fig. 3F). Likewise, spermatogonial transplantation showed that Wnt5a increased SSC numbers by 3.8-fold, compared to cultures in its absence (Fig. 3G), demonstrating that SSC maintenance by Wnt5a was not indirect through feeder cells.

β-Catenin-independent signalling mediates Wnt5a action.

When TCF/LEF-*lacZ* clusters were cultured on feeder cells with added Wnt5a for 6 days, the number of β-catenin-signalling cells significantly declined (Fig. 4A), indicating that Wnt5a suppresses β-catenin signalling in clusters and suggesting that Wnt5a affects SSC maintenance in a β-catenin-independent manner. We thus blocked β-catenin-independent pathways using inhibitors of the CaMKII, PKC, G-protein, and JNK cascades and assessed the effects on cluster formation. Cluster cells were exposed to each inhibitor for the first 3 days of culture, and clusters were quantified on day 6. The results showed that while cluster numbers did not change with inhibition of CaMKII, PKC, and G-protein signalling, they declined significantly with inhibition of JNK signalling using a cell-permeable competitive peptide (JNK Inhibitor III) and a small molecule inhibitor (SP600125) in a dose-dependent manner (Fig. 4B, C, filled bars). Further, when clusters treated with SP600125 were passaged and cultured under our standard SSC culture condition, numbers of secondary clusters declined dose-dependently (Fig. 4C, open bars). To eliminate the possibility of indirect effects through feeder cells, we cultured cluster cells on Matrigel with Wnt5a alone or Wnt5a plus a JNK inhibitor and quantified SSCs using spermatogonial transplantation. The data showed that the inhibition of JNK signalling abolished the Wnt5a-induced increase in SSC numbers to control levels (Fig. 4D open bars, S3A). The cluster-formation assay generated similar results (Fig. 4D, filled bars) suggesting that Wnt5a promotes SSC maintenance through the JNK pathway.

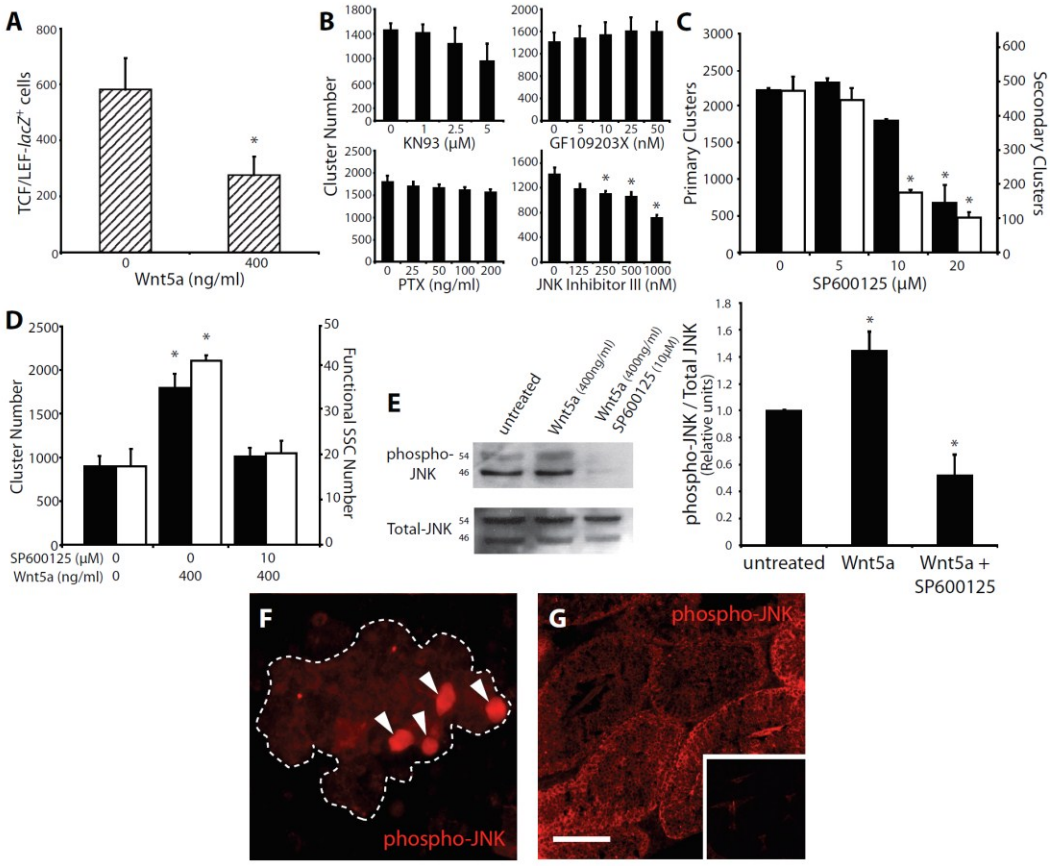

Figure 4: Identification of a potential Wnt5a signalling mechanism in cluster cells.

(**A**) β-Catenin-signalling cells decline in number after Wnt5a treatment. (**B**) Cluster quantification after culture with inhibitors against mediators of β-catenin-independent Wnt5a signalling. KN93 (CamKII inhibitor), GF109203X (PKC inhibitor), and Pertussis toxin (PTX, G-protein inhibitor) do not affect cluster numbers. Only a cell-permeable peptide JNK inhibitor (JNK Inhibitor III) shows significant effects. (**C**) Effects of a JNK inhibitor (SP600125) on cluster-forming cells. Cluster numbers (filled bars) significantly decrease after the treatment. Secondary cluster formation after passaging treated clusters (open bars) also shows a significant loss of cluster-forming cells. (**D**) Analysis of JNK inhibition on Wnt5a-induced SSC maintenance in feeder-free culture. SSC activity was examined using the cluster-formation (filled bars) and transplantation (open bars) assays. JNK inhibition negates the effect of Wnt5a. (**E**) Left: Western blot analysis shows increased phospho-JNK levels upon 2-hr stimulation with Wnt5a

in cluster cells, while treatment with the JNK inhibitor SP600125 reduces these levels. Total JNK levels were generally unaffected. Right: Comparison of phospho-JNK levels, normalized to total JNK levels following treatment as indicated. Wnt5a increased phospho-JNK levels 1.4-fold, while addition of SP600125 reduced levels ~50% below untreated levels. All groups were normalized to an α-tubulin loading control. (**F**) Immunofluorescent staining for phospho-JNK expression in a cluster (cluster demarcated by dotted outline). A subset of cluster cells show increased expression (arrowheads). (**G**) Immunofluorescent staining for phospho-JNK expression in adult testis cryosections. Diffuse expression is observed in the cells of the seminiferous tubule. Intense staining is observed in the cells lining the basal compartment. The inset shows negative control result. Scale bar: 44 μm (F), 100 μm; 220 μm inset (G).

To confirm that Wnt5a activates the JNK cascade, we performed Western blot analysis following short-term feeder-free culture of cluster cells. The data showed that Wnt5a-treatment led to a significant increase in levels of the activated form of JNK (phospho-JNK), despite seemingly high basal levels of phospho-JNK (Fig. 4E). This apparent basal level could be due to insulin in our culture medium [237] . Addition of the inhibitor diminished these phospho-JNK levels below basal levels and to ~90% of activated levels (Fig. 4E). Total JNK levels were unaffected by these treatments. We further examined if JNK activity is found in spermatogonia in vitro and in vivo by assessing the expression of phospho-JNK in clusters and adult testis. Immunofluorescence for phospho-JNK showed diffuse expression throughout all cluster cells with increased intensity restricted to a small subset of cells within clusters (Fig. 4F). A similar pattern of expression was also observed in testis as diffuse phospho-JNK expression throughout the cells of adult seminiferous tubules and increased expression restricted to the cells along the basal compartment (Fig. 4G). Therefore, these results collectively demonstrate that Wnt5a activates JNK in spermatogonia.

Wnt5a inhibits apoptosis through β-catenin-independent signalling.

When cluster cells were cultured feeder-free with Matrigel for 4 days, we observed a trend that more cells were detected in the presence of Wnt5a, compared to untreated controls, although the differences were not statistically significant (Fig. 5A). This suggested that Wnt5a may have affected SSC maintenance by altering cell proliferation and/or survival. Hence, we first analyzed cell cycle profiles of cluster cells cultured on Matrigel with or without Wnt5a for 2 or 4 days, using flow cytometry. The results showed that the majority of cluster cells were in the G_0/G_1 phase and that Wnt5a did not alter the percentage of cycling cells at either time (Fig. 5B). Thus, Wnt5a did not affect the cell cycle profile of cluster cells under these conditions. Since the induction of quiescence is known to support hematopoietic stem cell activity [172], we analyzed cluster cells at the G_0 phase by detecting Ki-67-negative cells in the G_0/G_1 fraction. Flow cytometric analyses indicated that ~13% of G_0/G_1 cells on average were Ki-67$^-$, which was not affected by Wnt5a (Fig. 5C). Hence, Wnt5a apparently does not induce quiescence in cluster cells.

To examine the effect of Wnt5a on cell death, cluster cells were cultured on Matrigel with Wnt5a, and 48 hr later, subjected to TUNEL analysis. Flow cytometric analyses showed that 52.6 ± 9.2% of cluster cells were apoptotic in the absence of Wnt5a in contrast to 21.9 ± 2.3% in its presence (Fig. 5D), demonstrating that Wnt5a inhibits apoptosis of cluster cells.

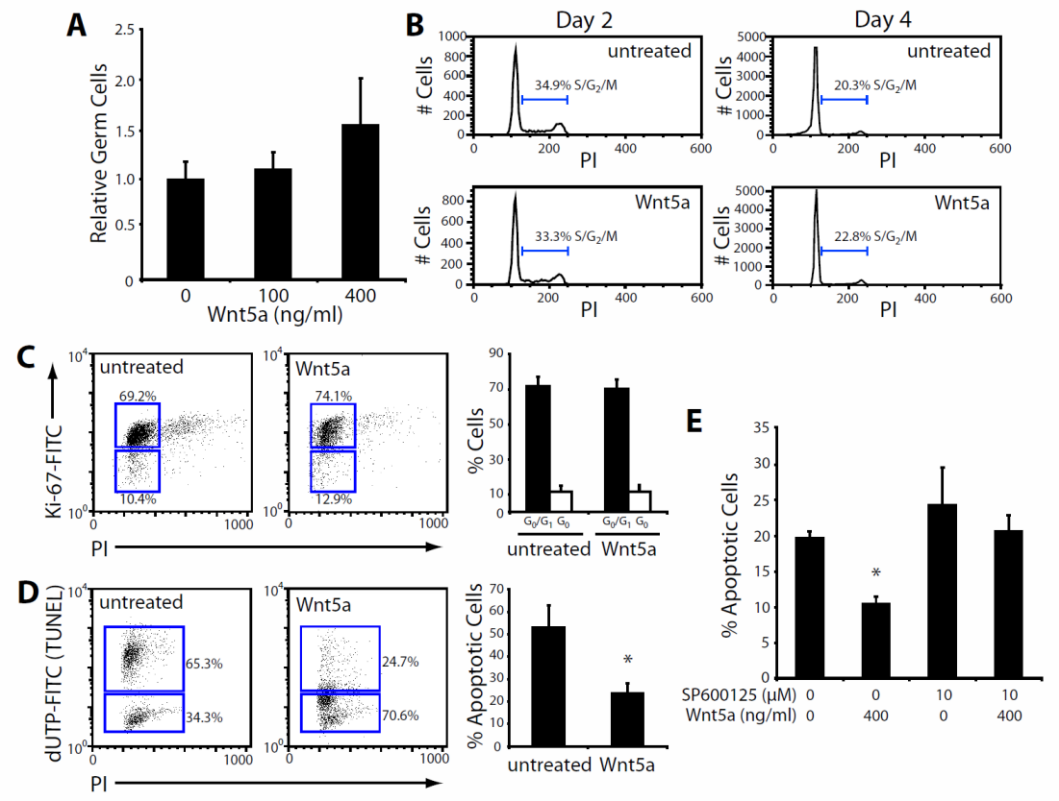

Figure 5: Wnt5a effects on cluster cell proliferation and apoptosis in feeder-free cultures.

(**A**) Cluster cell numbers after feeder-free culture with Wnt5a, normalized to those without Wnt5a. (**B**) Flow cytometric histograms showing cell cycle distribution after control (top) and Wnt5a (bottom) treatments at 2 days (left) and 4 days (right) of culture. No difference in the cycling cell percentage is observed. (**C**) Wnt5a effects on cell quiescence (G_0), as defined by lack of Ki-67 expression in the G0/G1 peak. Representative flow cytometric scatter-plots show quiescent cells in the bottom gate (left panels). The percentage of G0 cells is not affected by Wnt5a (right). (**D**) Apoptotic cell percentages after Wnt5a treatment, determined by TUNEL staining. Representative flow cytometric scatter-plots show apoptotic cells in the upper gate (left panels). Wnt5a significantly reduces apoptosis. (**E**) Analysis of JNK signalling on Wnt5a-induced inhibition of apoptosis, normalized to control values. A JNK inhibitor (SP600125) abolishes the Wnt5a-induced apoptotic inhibition.

Finally, we asked whether Wnt5a inhibits apoptosis through JNK signalling. Clusters were cultured on Matrigel and treated with Wnt5a alone, a JNK inhibitor alone, or both. The proportion of apoptotic cells was determined using TUNEL staining and flow cytometry. Data showed that Wnt5a reduced numbers of apoptotic cells while the inhibition of JNK signalling negated this effect (Fig. 5E). Importantly, the inhibitor alone did not affect apoptosis compared to control. Since the inhibition of JNK signalling in feeder-free cultures abolished the effect of Wnt5a on both SSC activity (Fig. 4D) and cluster cell survival (Fig. 5E), these results suggest that Wnt5a promotes SSC maintenance by inhibiting apoptosis through JNK signalling.

Table 3: RT-PCR analyses for Wnt expression in postnatal mouse testes

	Wnt1	Wnt2	Wnt2b	Wnt3a	Wnt5a	Wnt6	Wnt7b	Wnt10a	Wnt11
0 dpp	-	+	+	-	+	+	-	-	+
10 dpp	-	-	+	-	+	+	-	-	+
21 dpp	+	-	-	-	+	+	-	-	+
Adult (>2mo)	+	-	-	-	+	+	-	-	+
Adult Cryptorchid	-	*n.d.*	+	-	+	-	*n.d.*	*n.d.*	+

n.d.: not determined

Wnt5a is detected in Sertoli cells in testes.

To gain insight into the potential involvement of Wnt5a in regulating SSCs and spermatogonia in vivo, we examined its expression in testes during postnatal development using RT-PCR (Table 3). Wnt5a transcripts were detectable in all developmental stages examined as well as in adult cryptorchid testes (Table 3 and Fig. 6B). Since neonatal and cryptorchid testes contain no meiotic and haploid germ cells, these results suggest that Wnt5a is expressed in somatic cells and/or spermatogonia in mouse testes. To identify the cell types expressing Wnt5a, we employed in situ hybridization in neonatal testes. Wnt5a-expressing cells were observed only in the seminiferous epithelium and clearly in Sertoli cells (Fig. 6A), in good agreement with a previous report that suggested Sertoli cells express Wnt5a in mouse testes [181].Wnt5a transcripts were also detected in two Sertoli cell lines (Fig. 6B). The intimate contact of spermatogonia with Sertoli cells made it difficult to determine the staining in spermatogonia.

Hence, we FACS-purified spermatogonia from testes of transgenic Oct4-GFP mice, which express GFP specifically in spermatogonia at least up to 7 dpp (Yoshimizu et al. [119] and our data). The RT-PCR analyses of GFP^{+} spermatogonia at 6 – 7 dpp showed that Wnt5a transcripts were undetectable in spermatogonia (Fig. 6B). These results indicate that Sertoli cells express Wnt5a in mouse testes.

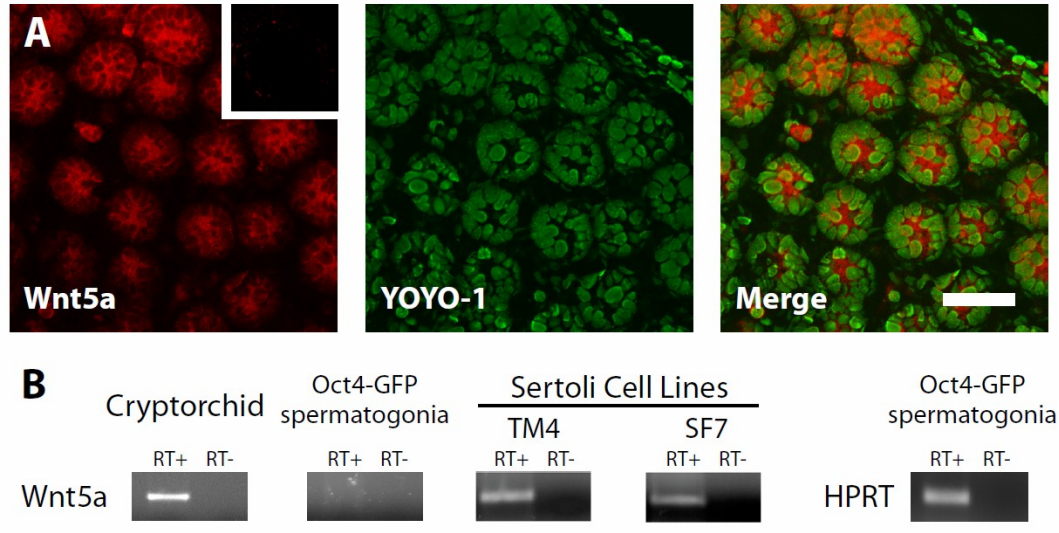

Figure 6: Sertoli cells express Wnt5a.

(**A**) In situ hybridization for Wnt5a transcripts in neonatal testes showing expression in the seminiferous tubules. The inset shows negative control with a sense probe. Scale bar: 25 μm; 330 μm (inset). (**B**) RT-PCR detects Wnt5a expression in seminiferous tubules of cryptorchid testes and Sertoli cell lines, but not in purified spermatogonia.

SSCs express Wnt5a receptors.

If Wnt5a acts on SSCs, Wnt5a receptors must be expressed by SSCs. We thus examined the expression of various Wnt receptors in cluster cells. RT-PCR analyses using FACS-purified B6GFP clusters detected transcripts of Fzd3, Fzd5, Fzd7, and Ror2 (Fig. 7A). Interestingly, all these receptors are known for their ability to transduce β-catenin-independent Wnt5a signalling [141,142,215,216]. We also detected transcripts of LRP5 and LRP6 (Fig. 7A), which mediate β-catenin signalling [238] (see Discussion). To examine the protein expression of Fzd3, Fzd5, Fzd7, and Ror2 in clusters, we used immunofluorescent staining and flow cytometry. Immunostaining showed that all cells in clusters expressed Fzd5, Fzd7, and Ror2 (Fig. 7B). Flow cytometric analyses supported these results; histograms of staining-intensity showed all cluster cells were positively stained, compared to negative controls (Fig. 7B). A similar profile was observed when cluster cells were stained for integrin-α6, an established SSC marker (Fig. 7D). Thus, Fzd5, Fzd7, and Ror2 are expressed by all cluster cells, including SSCs.

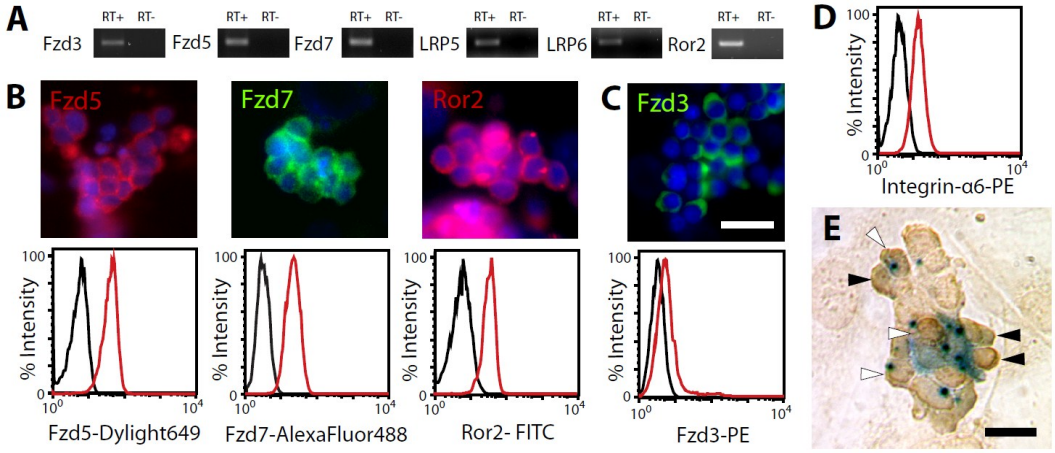

Figure 7: Expression of Wnt5a receptors on SSCs.

(**A**) RT-PCR analysis detects the expression of Wnt receptors Fzd3, Fzd5, Fzd7, and Ror2 and co-receptors LRP5 and LRP6. The expression of Fzd 1, 2, 4, 6, and 8 was undetectable. (**B**) Immunofluorescent staining and flow cytometric histograms for Fzd5, Fzd7, and Ror2 expression on clusters. All cluster cells express these receptors. (**C**) Immunofluorescent staining and flow cytometric

histograms for Fzd3 expression on clusters. Fzd3 expression is apparently heterogeneous. (**D**) Flow cytometric histogram for integrin-α6 expression. (**E**) Germ-cell cluster stained for expression of TCF/LEF-*lacZ* and Fzd3. Fzd3 expression is detected on β-catenin-non-signalling cells (black arrowheads) as well as on β-catenin-signalling cells (white arrowheads). Scale bar: 25 μm (B, C), 25 μm (E).

In contrast, Fzd3 staining was apparently heterogeneous in clusters, as Fzd3-positive and negative cells were observed (Fig. 7C). Double staining for TCF/LEF-*lacZ* activity and Fzd3 expression indicated that Fzd3 expression on cluster cells did not correlate with TCF/LEF-*lacZ* expression (Fig 7E). With flow cytometry, we could not clearly resolve these two populations (Fzd3-negative and positive) (Fig. 7C). Therefore, to determine Fzd3 expression on SSCs, we isolated $Fzd3^+$ cells from adult mouse testes and measured their SSC activity using spermatogonial transplantation. To this end, SSC-enriched testis cells were prepared from B6ROSA adult mice using Percoll-based cell separation [195] (Fig. 8A, B), followed by immunomagnetic cell sorting for β2-microglobulin-negative cells (Fig. 8A). Resulting cells were analyzed for the expression of Fzd3 and Thy1 with flow cytometry. The data showed that on average, 17% of sorted cells were $Thy1^-Fzd3^+$ and 2.1% were $Thy1^+Fzd3^+$; the remainder expressed neither molecule (Fig. 8C). We did not detect a $Thy1^+Fzd3^-$ population, indicating that all $Thy1^+$ cells express Fzd3 in the testis. Spermatogonial transplantation demonstrated that approximately 80% of SSCs were found in the $Thy1^+Fzd3^+$ population, while no SSC activity was detected with $Fzd3^-$ cells (Fig. 8D). Minor SSC activity in $Thy1^-Fzd3^+$ cells likely resulted from contaminating SSCs from the $Thy1^+Fzd3^+$ population. Collectively, these results indicate that SSCs express receptors that are known for their ability to transduce β-catenin-independent Wnt5a signalling.

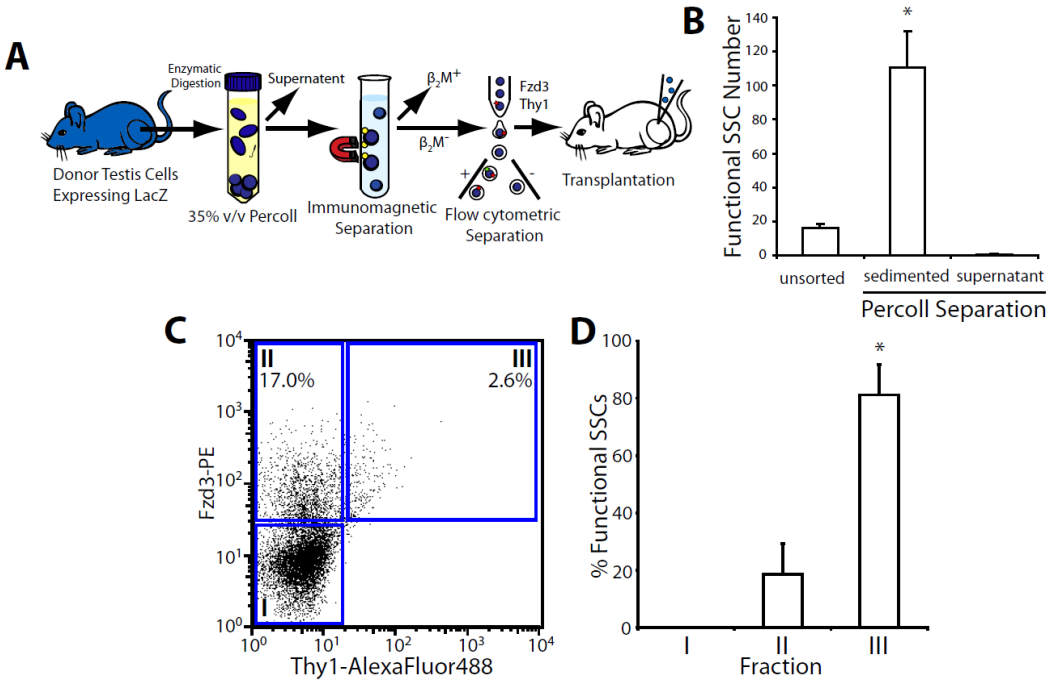

Figure 8: Examination of Fzd3 expression on SSCs.

(**A**) The procedure to enrich adult mouse testis cells for SSCs to examine Fzd3 expression in SSCs. Testis cells are first enriched for SSCs using Percoll, followed by depletion of non-spermatogonial cells (β2-microglobulin, β2M). The resulting cells are sorted for Fzd3 and Thy1 using FACS. SSC activity is measured by spermatogonial transplantation. (**B**) SSC numbers after transplantation of adult testis cells separated using a Percoll-based method. Virtually all SSC activity is found in the sedimented cells. (**C**) A FACS scatter-plot for Fzd3 and Thy1 expression in SSC-enriched cells. (**D**) Transplantation results of FACS-isolated fractions in (C). The majority of SSCs are found in the $Fzd3^{+}Thy1^{+}$ population, indicating Fzd3 expression by SSCs.

Discussion

Wnt signalling participates in multiple developmental processes during embryogenesis, where tight cell-cell communication is critical to generate a properly patterned embryo [144]. This necessity for cell-cell communication is applicable to the biology of stem cells, which intimately interact with their surrounding environment. It may not be surprising therefore that Wnt signalling

has been implicated in the maintenance of various stem cell types. One of the first examples demonstrated was in the mouse intestine where β-catenin-dependent Wnt signalling is essential for homeostasis and tumorigenesis [168]. Furthermore, this pathway is known to promote the in vitro expansion of neural stem cells and hematopoietic stem cells [207,208]. Therefore, classically, β-catenin signalling has been deemed a general regulator of stem cell self-renewal. However, recent evidence has indicated that the pathway can also support stem cell differentiation, and the effects of Wnt signalling on stem cell maintenance appear to be cell-type specific. Our study identifies for the first time that β-catenin-independent signalling mediated by Wnt5a, but not the β-catenin-dependent pathway, can promote SSC activity.

It has been reported that heterogeneity exists in germ-cell clusters [95,120] and that SSCs represent a small subpopulation of cluster cells [133,134]. In this study, we were able to visualize the heterogeneity of cluster cells in functional terms: i.e., activation of β-catenin signalling (Fig. 1A). Since the signalling-positive cells comprised a minority population, we hypothesized that these cells were SSCs. However, our results revealed that cluster cells with activated β-catenin signalling had lost SSC function (Fig. 1). This finding is in line with our in vivo observations (Fig. 2) that most, if not all, spermatogonia did not show active β-catenin signalling, while more differentiated cells did. These results imply that β-catenin signalling may be associated with differentiation during spermatogenesis and that there may be mechanisms that suppress activation of β-catenin signalling in spermatogonia.

Our data indicate that Wnt5a is involved in such a mechanism and promotes SSC maintenance as a cell-extrinsic factor in vitro (Figs. 3, 4). The following evidence supports this conclusion. First, Wnt5a was expressed in feeder cells in vitro. Second, feeder cells expressing Wnt5a supported SSC maintenance better than those that do not express Wnt5a. Third, a pan-Wnt inhibitor, sFRP1, diminished cluster formation, and this effect was competitively overcome by Wnt5a. Fourth, Wnt5a supported significantly more SSCs in feeder-free culture conditions, suggesting that Wnt5a can act directly on SSCs. In addition, we found

that Wnt5a is expressed in Sertoli cells in the seminiferous epithelium, suggesting that Wnt5a may act as an environmental factor for SSC regulation in vivo. Further studies are necessary to address this notion.

We have also found that Wnt5a promotes SSC maintenance by supporting cell survival, and as a possible mechanism of action, we have shown that inhibition of a β-catenin-independent mechanism (i.e., JNK signalling) blocks Wnt5a-mediated SSC maintenance (Fig. 4). In a feeder-free culture condition, Wnt5a did not affect cell cycle but suppressed cell death, and inhibition of JNK signalling abolished the pro-survival effect of Wnt5a (Fig. 5). However, it is possible that Wnt5a may also block differentiation of SSCs and spermatogonia. Our data showing that Wnt5a reduces numbers of β-catenin-signalling-positive cells, which do not have SSC activity, suggests this possibility. Nonetheless, we note that these two populations are composed of undifferentiated spermatogonia and did not differ drastically in expression of the SSC markers examined (Fig. 1). Therefore, it will be important to further characterize β-catenin-positive and negative cluster cells, and gene expression profiling may reveal the difference between these two cell populations at the molecular level.

We observed that Wnt5a significantly increased phospho-JNK levels (Fig. 4E-G), supporting the results of our inhibitor screening experiments (Fig. 4) and apoptosis analyses (Fig. 4, 5). Although this increase was modest (1.4-fold), it may be sufficient to mediate the effects of Wnt5a. Signalling mechanisms other than the JNK cascade could also participate in Wnt5a signal transduction. In this context, Wnt5a is known to activate the PI3K/Akt cascade in human dermal fibroblasts [215]; a cascade of known importance in SSC renewal. Nonetheless, our data identify JNK signalling as a potential candidate mediator of Wnt5a signalling.

Wnt5a has unique characteristics that are potentially important as a cell-extrinsic regulator of SSC function. Wnt5a acts over short distances and can regulate cell polarity. For instance, it contributes to the redistribution of cytoskeletal proteins and surface receptors, including Fzd3, and centrosome reorientation [153,239]. Wnt5a has also been shown to enhance polarization of

melanoma cells toward a chemokine gradient, thus facilitating the cells' response to directional cues [239]. Therefore, it is tempting to speculate that Wnt5a may be involved in the regulation of cell polarity in SSCs, thereby contributing to SSC fate control and their directional localization in the seminiferous epithelium. Since Wnt5a-knockout mice die perinatally [240], in vivo analyses of such Wnt5a actions will require a conditional knockout mouse model, and our study provides the foundation to explore such a research direction.

For Wnt signalling mechanisms in general, it is known that distinct receptor-ligand pairings, but not properties intrinsic to Wnt ligands, determine which signalling pathway is activated [141,212]. Although we detected on SSCs the expression of all receptors that have been reported to mediate β-catenin-independent Wnt5a signalling (Fig. 7, 8), it remains unknown which receptor was responsible for Wnt5a action. In this regard, Ror2 is an interesting target, since Wnt5a-Ror2 interaction has been demonstrated to lead to the activation of JNK signalling [141,155].

A recent study has reported that Wnt3a, which activates β-catenin signalling, stimulates proliferation of a spermatogonial cell line in vitro [116]. We detected LRP5/6 transcripts in clusters (Fig. 7). Since these receptors act as essential receptor subunits that uniquely transduce β-catenin signalling [144], our data suggest that these cells have the machinery to respond to Wnt3a. However, we also found that β-catenin-signalling-positive cells did not possess SSC activity and blocking the function of LRP5/6 by DKK1 did not alter cluster numbers. We and others localized β-catenin expression on the plasma membrane and cytoplasm of most spermatogonia (Fig. 2 and [234]) rather than in the nucleus, the latter of which is a typical consequence of β-catenin signalling activation. Therefore, we speculate that Wnt3a may affect the activity of progenitor spermatogonia, rather than SSCs.

Finally, which molecules activate β-catenin signalling in clusters (Fig. 1A)? We detected the transcripts of Wnt2b in feeder cells and those of Wnt1 in cluster cells. A recent DNA microarray analysis has identified the expression of R-Spondin in clusters [108]. All these molecules are known to stimulate β-catenin

signalling [241] and are candidate molecules that activate the signalling in clusters. In this regard, Wnt1 is known to be expressed by spermatids in testes [176]. It is thus not surprising that we observed activation of β-catenin signalling in germ cells in the adluminal compartment. Interestingly, however, β-catenin signalling activation was seen approximately one week before spermatids emerge during postnatal development (Fig. 2). Although the cause of this earlier-than-expected activation is unclear, we note the expression of Wnt2b in testes from the time of birth (Table 3). Combined with the expression of Wnt1 and R-Spondin transcripts detected in cluster cells, these factors could be responsible for the earlier activation of β-catenin signalling in vivo. These observations lead us to suspect that β-catenin-dependent and independent pathways may cross-talk and contribute to coordinated regulation of SSC activity and spermatogenesis.

In summary, our study demonstrates that Wnt5a supports SSC self-renewal in part by promoting their survival. Our results suggest that JNK signalling is at least one of the mediators of this pro-survival effect of Wnt5a in a β-catenin-independent manner. We also found that other Wnt ligands are expressed in feeder cells and in the testis. These observations suggest that although Wnt5a contributes to SSC maintenance, it may represent only one aspect of the complex mechanisms driven by Wnt molecules that control SSC activities and spermatogenesis.

Acknowledgements

The authors thank Drs. D. Dufort, J. Trasler, K. Kuroda, R. Farookhi, and H. Clarke for transgenic mice and reagents. The authors also thank Dr. K. Ebata, C. Park, and J-C. Neel for technical support and Drs. K. Orwig and R. Farookhi for comments on the manuscript.

CHAPTER 4

MANUSCRIPT III

Preface to Chapter 4

In Chapter 2, transplantation of germ cell clusters into recipient mouse testes revealed that these cell communities are functionally heterogeneous populations composed of SSCs and differentiated cells. I estimated that SSC numbers per cluster remained relatively constant indicating that a balance between SSC fate decisions may be important to maintain ideal SSC numbers in a cluster.

In Chapter 3, I identified Wnt5a as an extrinsic factor that promoted SSC survival and self-renewal. Wnt5a mediated its effects through a non-canonical Wnt pathway; in contrast the β-catenin pathway was active in a subset of cluster cells but these cells did not have SSC activity and were deemed committed cells. Therefore, the response to a Wnt signal appears to functionally demarcate SSCs from committed cells in a germ cell cluster. Surprisingly, another study claims that Wnt3a promotes SSC activity through the activation of the β-catenin pathway [116]. This study used a cultured spermatogonial cell-line and by adding Wnt3a to their cultures they stimulated proliferation of these cells. However, the ability of these cells to function as SSCs is unknown. Thus, the effect of Wnt3a on actual SSC activity was not unequivocally demonstrated in this study.

In the following chapter, SSC cluster cells were treated with Wnt3a and transplanted into recipient testes to determine the effect of Wnt3a on SSC activity. Based on my results from Chapter 3, I speculated that Wnt3a might selectively stimulate the proliferation of committed cells through the β-catenin pathway. Because of the close interaction between SSCs and differentiated cells in a cluster, it is possible that the latter population may influence SSC fate. In this way, Wnt3a-mediated stimulation of the β-catenin pathway in committed cells might indirectly affect SSC activity in vitro. This concept of committed cells contributing to SSC niche regulation has not yet been demonstrated.

Indirect effects of Wnt3a/β-catenin signalling support mouse spermatogonial stem cells in vitro.

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Short-title: Wnt3a stimulates spermatogonial progenitors in vitro

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Abstract

Proper regulation of spermatogonial stem cells (SSCs) is crucial for sustaining steady-state spermatogenesis. Previous work has identified several paracrine factors involved in this regulation, in particular, glial cell line-derived neurotrophic factor and fibroblast growth factor 2, which promote long-term SSC self-renewal. Using a SSC culture system, we have recently reported that Wnt5a promotes SSC self-renewal through a non-canonical Wnt mechanism whereas the β-catenin-dependent Wnt pathway is not active in SSCs. In contrast, another study has reported that Wnt3a could stimulate the proliferation of a spermatogonia cell line through the β-catenin-dependent pathway. To reconcile these two contradictory reports, we assessed Wnt3a effects on SSCs, rather than a cell line, in vitro. We observed that Wnt3a induced β-catenin-dependent signalling in a large subset of germ cells and increased SSC numbers. However, further investigation determined that the β-catenin-signalling cell population contained fewer SSCs. The increased maintenance of SSCs by Wnt3a coincided with increased cell cycling in the β-catenin-signalling cells and resulted in germ cell cluster-formation under feeder-free conditions. Therefore, the results of this study demonstrate that Wnt3a selectively stimulates proliferation of progenitors committed to differentiation, which leads to improved germ-cell community formation and results in the indirect support of SSC activity in vitro.

Introduction

Spermatogonial stem cells (SSCs) are the foundation of life-long spermatogenesis. SSCs have the unique ability to maintain the stem cell pool through self-renewing divisions as well as to generate daughter cells committed to differentiation thereby producing mature sperm. This fate decision is believed to be tightly regulated during steady-state spermatogenesis and occurs in a specialized microenvironment, often referred to as the niche, which supports SSCs [201]. The concept of a niche is well demonstrated in the Drosophila testis where somatic cells, hub cells, are located at the distal tip of the testis and are responsible for maintaining the stemness of germ-line stem cells through direct contact [242]. Migration away from contact with hub cells leads to differentiation toward sperm. Interestingly, it has been shown that differentiated germ cells can regain stem cell activity upon homing back to hub cells suggesting the inductive nature of the Drosophila testis niche [94].

Such a defined inductive niche has not been characterized in mammalian testes. Rather, SSCs reside along the basement membrane of the seminiferous tubule in close contact with Sertoli cells, the supporting somatic cells [201]. A great deal of work has been performed to identify how Sertoli cells support SSCs. To date, a handful of factors derived from testicular somatic cells have been demonstrated to influence SSC renewal, in particular glial cell-line derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2), colony stimulating factor 1, and Wnt5a [95,112,202,203]. In contrast, knowledge of factors associated with SSC commitment to differentiation is limited. In addition, SSCs are not only in close contact with Sertoli cells but also with other types of spermatogonia in the niche. Therefore, the possibility exists that non-stem spermatogonia can communicate with SSCs and contribute to the regulation of SSC activity. However, this possibility has not been investigated.

Although mechanisms that control SSC fate are not completely understood, SSCs can be expanded in vitro under well defined conditions. In the presence of GDNF and FGF2, SSCs proliferate in vitro and form distinct accumulations of SSCs and daughter spermatogonia, which we term “clusters”

[112,115,134]. Interestingly, previous work has shown that SSCs are a minority population among cluster cells, and the reported percentages of SSCs in clusters are relatively constant (1 – 3%) across several studies [115,134,202]. These observations raise a possibility that clusters constitute a society of male germ cells in vitro in which cells communicate with one another, thereby regulating the proportion of SSCs in the community [243].

In a previous study, we reported the expression of various Wnts in mouse testes and feeder cells used in SSC cultures [203]. Wnts are a family of lipid-modified, secreted glycoproteins with diverse functions in embryogenesis, tumorigenesis, as well as stem cell proliferation and differentiation [90,144]. In general, Wnt proteins can activate two classes of signalling cascades. The better characterized canonical (β-catenin) pathway involves Wnt stimulation with its receptor Frizzled and co-receptor low density lipoprotein-related protein (LRP) 5/6, ultimately resulting in interaction between β-catenin and members of the T-cell factor/Lymphoid enhancer factor (TCF/LEF) transcription factor family in the nucleus and modulation of target gene transcription. In contrast, non-canonical pathways involve a wide host of mediators that do not act through β-catenin.

Previous studies have demonstrated the importance of Wnt signalling in the niches of various adult stem cell types [244]. In vitro, β-catenin signalling promotes the expansion of phenotypically-defined hematopoietic stem cells (HSCs), neural stem cells (NSCs), and intestinal stem cells (ISCs) [169,207,208]. However, further studies show that HSCs can self-renew in the absence of β-catenin [171] and while constitutive β-catenin-signalling can expand HSCs in vitro, these cells do not retain the functional ability to differentiate into blood lineages [170]. Moreover, β-catenin signalling appears to be dispensable for NSCs and rather supports the adhesion and survival of progenitor cells in neurospheres [173]. Stem cells are defined functionally; therefore, these studies serve to highlight the importance of assessing pathway activation effects on stem cell activity in a functional manner.

Recently, we identified Wnt5a as a novel factor, expressed by feeder cells and Sertoli cells, that supports SSC renewal [203]. Using the functional

transplantation assay for SSCs, we demonstrated that Wnt5a acts through a non-canonical mechanism and promotes SSC survival and self-renewal. Furthermore, the β-catenin pathway was not activated in SSCs, and spermatogonia with active β-catenin signalling had committed to differentiation. In contrast, a study by Golestaneh et al. [116] has shown that Wnt3a stimulates the β-catenin pathway and leads to proliferation of an SV40-transformed spermatogonia cell line. However, the identity of this cell line as SSCs has not been examined functionally; i.e., the ability of these cells to regenerate and maintain spermatogenesis has not been demonstrated.

In an attempt to reconcile these two contrasting results, we set out to functionally assess the effect of Wnt3a on SSCs. We hypothesized that in accordance with our previous work Wnt3a activates β-catenin-TCF/LEF signalling (termed "β-catenin pathway" hereafter) in clusters and stimulates proliferation of non-SSCs in vitro. Surprisingly, our results showed that Wnt3a led to a significant increase in SSC numbers. We also observed that Wnt3a stimulated the β-catenin pathway in a large subset of cluster cells. These results appeared to contradict the findings of our previous study. However, further investigation demonstrated that although Wnt3a produces a large number of β-catenin signalling cells and drives their cell cycle, these cells exhibited markedly reduced levels of SSC activity, suggesting that the increase in SSC numbers occurred in cluster cells in which β-catenin pathway was not activated. In addition, the Wnt3a-induced increase in SSC numbers correlated with the increased formation of cell aggregations and cell-cell associations. Therefore, our study suggests that Wnt3a may regulate SSC activity indirectly by acting on committed daughter cells in vitro.

Materials and Methods

Donor Animals

Homozygous TCF/LEF-*lacZ* mice (from Dr. D. Dufort, McGill University) are on a CD-1 genetic background and carry the *lacZ* reporter gene driven by β-catenin-TCF/LEF responsive elements [217,218]. B6ROSA mice are

F_1 hybrids of C57BL/6 (B6) and ROSA26 mice, which express the *lacZ* gene ubiquitously in virtually all cell types [192]. B6GFP mice (C57BL/6-Tg(CAG-EGFP)1Osb/J; The Jackson Laboratory) express GFP ubiquitously. Animal procedures were approved by the Animal Care and Use Committee of McGill University.

Recipient Animals and Transplantation

Spermatogonial transplantation was performed and recipients were prepared as described previously [203]. Recipient mice for B6ROSA cells were 129/SvEv × B6 F_1 hybrids, and those for TCF/LEF-*lacZ* cells, Ncr nu/nu mice (Taconic). Recipient animals were injected with busulfan i.p. (50 mg/kg for 129/SvEv × B6, 40mg/kg for Ncr nu/nu) at 4 – 6 wk of age to eliminate endogenous spermatogenesis, at least 1 mo prior to transplantation. Donor cells were enzymatically digested to a single cell suspension and injected into the rete testis of recipient mice, to fill the seminiferous tubules. Recipient testes were analyzed for SSC quantification following staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) 2 months post-transplantation. Colony numbers of donor-derived spermatogonesis were visually counted [68] and expressed as colonies/10^6 cells initially placed in culture.

Cell Culture

SSC cultures were established from immunomagnetic selected Thy1-positive testis cells from 7 – 8 days post partum (dpp) mice as described previously [134]. Cultures were maintained with a serum-free MEMα-based medium with supplements on a feeder layer of STO fibroblasts. Media were replenished every 3rd day and clusters were subcultured onto freshly prepared STO feeder cells every 6 – 7 days. Cultures were maintained with "growth factors" consisting of GDNF (20ng/ml), GFRa1 (75ng/ml), and FGF2 (1ng/ml) [112,134]. Experiments were conducted using established cluster cells (> 5 passages), removed from STO feeder cells using gentle pipetting, which results in an isolation of cluster cells at more than 90% purity [203]. Subsequently, clusters

were digested to single cells following treatment with 0.05% trypsin-EDTA. For short-term feeder-free cultures, culture plates were coated with Matrigel (BD Biosciences), diluted 1:2 in serum-free culture media, and incubated overnight at 4°C. The next day, excess Matrigel was removed and the plate was transferred to a 37ºC humidified incubator, 30 min prior to use. For experiments, we used GDNF at 40ng/ml, GFRα1 at 300ng/ml, and FGF2 at 1ng/ml, as in [112]. Recombinant Wnt3a and DKK1 (R&D Systems) were added at concentrations as previously reported [116]. All cultures were maintained at 37ºC in a humidified incubator with 5% CO_2.

To quantify TCF/LEF-*lacZ* expressing cells, clusters derived from reporter mice were stimulated with Wnt3a, reacted with X-gal overnight, and visually counted using a hemocytometer.

Cluster-Formation Analysis

As a short-term semi-quantitative assay for SSC activity, it has been determined that cluster number correlates with functional SSC number [134]. Hence, cluster cells exposed to experimental conditions were subcultured onto STO feeder cells in the presence of growth factors to induce cluster formation. Media were replenished 3 days after initial seeding and by day 6 cluster numbers were ready for quantification. Cluster numbers were determined in one of the following two ways. B6ROSA-derived clusters were reacted with X-gal overnight and cluster numbers were counted visually under a microscope. B6GFP-derived cluster numbers were acquired automatically using an ImageXpress^{MICRO} imaging system (Molecular Devices) as described previously [245]. Three experiments were performed for all cluster formation analyses and the average of at least two wells was recorded for each group per experiment. Cluster numbers were normalized to 10^6 cells placed in culture.

Flow Cytometric Analysis and Sorting

To isolate β-catenin-signalling cell populations following Wnt3a stimulation, TCF/LEF-*lacZ* clusters were reacted with 500 μM fluorescein di-β-

D-galactopyranoside (FDG, Marker Gene Technologies) and sorted as described previously [203]. Experimental gates were established using control cells: B6ROSA (positive) and B6 (negative) cluster cells. For cell cycle analyses, B6ROSA cluster cells were fixed in 70% ethanol at -20ºC overnight followed by incubation with 40 μg/ml propridium iodide (PI) and 100 μg/ml RNase at room temperature. Data were acquired on a FACScan or Accuri C6 Cytometer (Becton Dickinson), from three experiments with at least 10,000 events collected per sample.

Cell Profiler Analysis

To quantify Wnt3a effects on germ cell clustering, B6GFP cluster cells were cultured feeder-free in a 96-well plate at 5 x 10^4 cell/cm^2 from 1 to 4 days in the presence or absence of Wnt3a. The time of cell seeding was deemed Day 0. Cultures were terminated each day from days 1 to 4 and, at each day, were reacted with a DNA dye (DAPI), to assist in the identification of cells. Thirteen representative fluorescent photomicrographs were taken from various points randomly in each culture well. For neighboring analyses we used the Cell Profiler Software Platform (Broad Institute) [246]. To this end, a pipeline was developed that identified individual cells based on their shape and expression of GFP and DAPI. Neighboring cells were defined as cells in physical contact and were identified as individual cells with adjacent cell boundary pixels to generate data on how many cells are associated with a given cell.

Immunofluorescent Staining

B6ROSA cluster cells were cultured on Matrigel as above with Wnt3a (400ng/ml). On day 4, cultures were fixed in 4% paraformaldehyde. Primary antibody used was mouse anti-LRP5/6 (R&D Systems). Secondary antibody used was goat anti-mouse conjugated-R-phycoerythrin (Jackson ImmunoResearch). DAPI was used to visualize cell nuclei.

Statistics

Data were expressed as mean ± SEM. Numbers of clusters in vitro and SSCs detected with spermatogonial transplantation were indicated as those per 10^6 cells placed in culture, unless specified otherwise. Significance was determined using ANOVA followed by Fisher's Test for Least Significant Difference. $p < 0.05$ determined significance.

Results

Wnt3a increases SSC numbers in vitro

A recent study has described the ability of Wnt3a to activate β-catenin signalling and stimulate proliferation in an SV40-transformed spermatogonia cell line [116]. This cell line is immunophenotypically similar to SSCs but has not been functionally tested as SSCs using spermatogonial transplantation. Therefore, whether Wnt3a affects SSCs in a similar manner to this cell line is unknown. To address this, we initially used an in vitro culture system in which SSCs can be expanded. This culture system uses the growth factors GDNF and FGF2 and a layer of mitotically inactivated mouse embryonic fibroblasts (STO cells) as feeder cells [112,134]. Under these conditions, SSCs and their daughters can be expanded over a long period as distinct communities, termed "clusters" (Fig. 1A), of which SSCs comprise the minority population [115,202]. Recombinant Wnt3a was added to B6ROSA clusters for one week and clusters developed similarly under both conditions (Fig. 1A). We transplanted treated clusters into the testes of infertile recipient mice to assess Wnt3a effects on SSC activity. Using this spermatogonial transplantation technique, SSCs normally hidden among the cells of a cluster can be unequivocally detected by their ability to regenerate spermatogenesis. Two months following transplantation, recipient mouse testes were analyzed to count colonies of regenerated spermatogenesis. We observed that Wnt3a led to a 1.4-fold increase in numbers of functional SSCs, but this increase was deemed to be not significantly different (Fig. 1B), leaving the result of Wnt3a on SSC activity somewhat ambiguous.

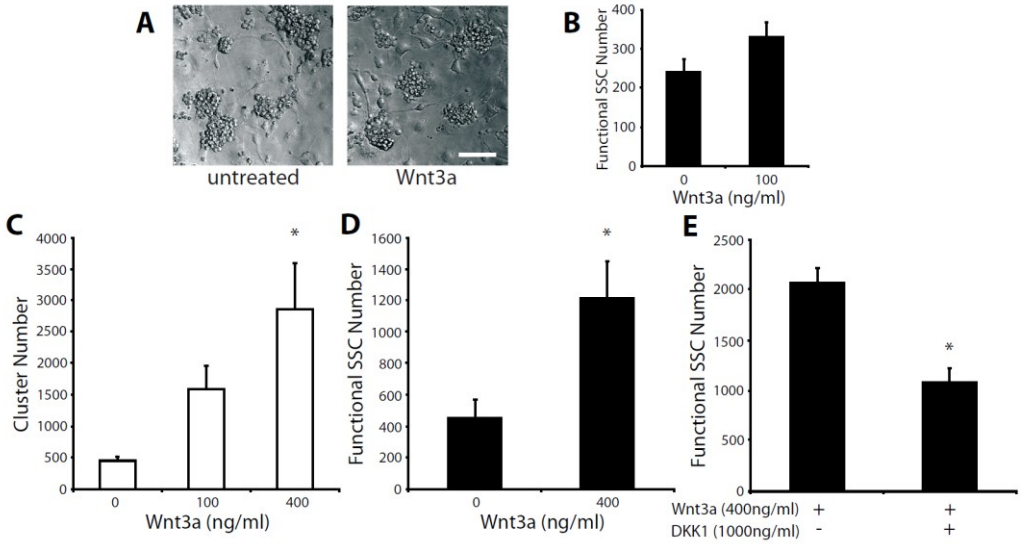

Figure 1: Wnt3a increases SSC numbers under feeder-free conditions.
(**A**) Germ cell clusters (left) and after 6 day treatment with Wnt3a (right) (**B**) Quantification of SSCs following Wnt3a treatment. Results of the cluster-forming (**C**) and transplantation (**D**) assays for SSC quantification, after feeder-free culture with Wnt3a. A significant increase in SSC numbers is detected with Wnt3a treatment by both assays. (**E**) Spermatogonial transplantation results following the addition of the β-catenin signalling inhibitor, DKK1, to Wnt3a-treated cultures. Inhibition of the β-catenin pathway inhibits the effect of Wnt3a on SSC activity. Scale bar: 75 μm.

Previously, we reported the expression of other Wnt molecules by our STO feeder cells. To circumvent potential indirect effects via feeder cells, cluster cells were removed from feeder cells through gentle pipetting and cultured under feeder-less conditions on Matrigel-coated plates for a short duration (4 days) with growth factors and in the presence or absence of Wnt3a. Since our STO feeder cells are critical for cluster formation and SSC proliferation, clusters do not form under these conditions. Following treatment, cluster cells were recovered from feeder-less conditions and cultured back onto feeder cells to induce cluster

formation. The number of clusters that form in this cluster-formation assay correlates to functional SSC numbers, thereby providing a short-term method to quantify SSCs [134]. Interestingly, we observed a dramatic dose-dependent increase in cluster-formation ability with the addition of Wnt3a (Fig. 1C). To verify this Wnt3a effect on functional SSCs, treated cluster cells were subjected to spermatogonial transplantation. We confirmed that the addition of Wnt3a led to a significant 2.7-fold increase in colony numbers (Fig. 1D), demonstrating that Wnt3a promotes SSC activity in vitro. Finally, we added the β-catenin signalling-specific inhibitor Dickkopf1 (DKK1) to Wnt3a-treated cultures and observed a significant decrease in colony numbers (Fig. 1E). Thus, these results indicate that the effects of Wnt3a on SSC activity involve the β-catenin pathway.

Wnt3a activates β-catenin signalling in a subset of cluster cells and reduces SSC activity in these cells.

Previously, we demonstrated that Wnt5a supported SSCs through a non-canonical mechanism, whereas Wnt3a has been reported to act primarily through β-catenin signalling [203]. Immunofluorescent staining for the Wnt co-receptor low density lipoprotein-related protein (LRP) showed that all cluster cells express this protein (Fig. 2A). LRP, in conjunction with the Wnt receptor Frizzled, is necessary to transduce the Wnt/β-catenin signal. Frizzled protein expression has been reported on cluster cells [203]; therefore, all cluster cells appear able to signal through the β-catenin pathway. To confirm if Wnt3a indeed activates the β-catenin signalling pathway in cluster cells, we used established cluster cells derived from transgenic reporter mice (TCF/LEF-*lacZ* mice). These mice carry the *lacZ* reporter gene downstream of TCF/LEF binding sites, allowing faithful monitoring of β-catenin signalling activation [217,218]. TCF/LEF-*lacZ* cluster cells were treated with Wnt3a on Matrigel, and the presence of β-catenin signalling cells were visually quantified after 4 days. On day 1, we detected a dramatic increase in the percentage of β-catenin signalling cells compared to untreated control cluster cells (Wnt3a vs. untreated; 79.0 ± 3.6 vs. 1.8 ± 0.2%) (Fig. 2B, C). Interestingly, the levels of β-catenin signalling activation varied in

these cells with a gradient from moderate to robust lacZ expression (Fig. 2B, C). By day 4 of culture, we noted that this proportion of TCF/LEF-*lacZ* dim and high cells remained constant. We did not detect a significant change in total cell numbers upon Wnt3a stimulation under this feeder-free condition (Fig. 2D). β-Catenin signalling cells remained the majority population on day 4 and were significantly greater in number in Wnt3a-treated culture compared to untreated control culture (Wnt3a vs. untreated; 68.3 ± 2.4 vs. 3.8 ± 0.5%). These results indicate that Wnt3a activates the β-catenin pathway in cluster cells.

Previously, we demonstrated that β-catenin signalling activation led to a loss in SSC activity suggesting that these signalling cells may be committed progenitors [203]. We therefore asked whether Wnt3a-stimulated β-catenin-signalling cells had SSC activity. To this end, TCF/LEF-*lacZ* cluster cells were cultured feeder-free in the presence of Wnt3a. These treated cluster cells were subsequently reacted with a fluorescent vital β-galactosidase substrate (FDG) and then separated via FACS according to staining levels (Fig. 2E, F). Flow cytometric histograms of FDG activity were gated into three fractions, TCF/LEF-*lacZ* signalling negative (42.8 ± 4.6%), dim (38.4 ± 1.7%), and high expressing (18.7 ± 3.4%). The three fractions were isolated and separately transplanted into recipient testes to assess the SSC activity of each population. We observed that TCF/LEF-*lacZ* negative cells were highly enriched in functional SSCs (181.5 ± 30.4 functional SSCs per 10^6 cells), while β-catenin signalling cells had minimal SSC activity, similar to our previous observation (Fig. 2G) [203]. The TCF/LEF-*lacZ*^{dim} population was composed of ~50 functional SSCs per 10^6 cells, while TCF/LEF-*lacZ*^{high} had ~18 functional SSCs per 10^6 cells showing a trend of declining SSC frequency with an increase in β-catenin signalling intensity. Therefore, these results collectively demonstrate that Wnt3a stimulates the β-catenin pathway in a subset of cluster cells but the population of functional SSCs is significantly diminished as β-catenin signalling is activated.

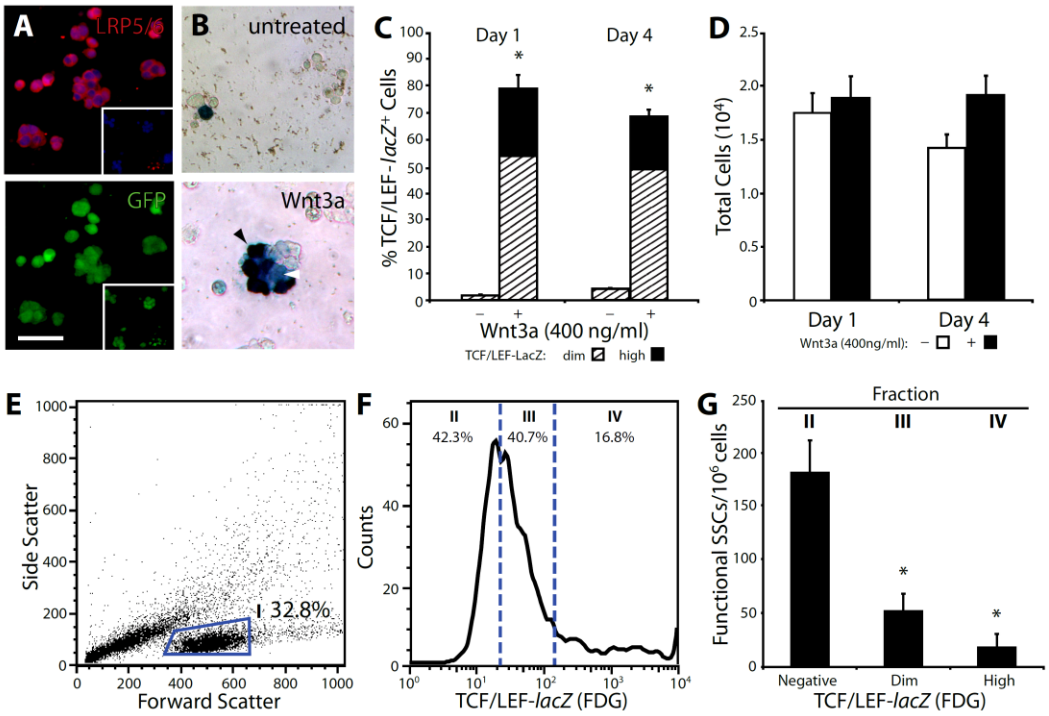

Figure 2: β-Catenin signalling cells increase with Wnt3a but have reduced SSC activity.

(**A**) Immunofluorescent staining for LRP5/6 (top) and corresponding image showing B6GFP-derived cluster cells (bottom) indicates all cluster cells express LRP. (Inset) Representative negative staining control. (**B**) TCF/LEF-*lacZ* cluster cells after 4 days under feeder-free conditions (top) and after Wnt3a treatment (bottom). Three populations emerge: β-Catenin signalling negative, dim (as indicated by white arrowhead), and high (as indicated by black arrowhead). (**C**) Wnt3a increases β-catenin signalling positive cells, however, proportions are maintained throughout the course of culture. (**D**) Total cell numbers do not change significantly upon Wnt3a stimulation. In the absence of Wnt3a, cell numbers recovered appear to decrease slightly. (**E**) Flow cytometric scatter-plot assessing cell morphology. Cluster cells are found in Fraction I. (**F**) A representative FACS histogram sub-dividing Fraction I into β-catenin signalling negative (II), dim (III), and high (IV) fractions. (**G**) Concentration of SSCs in each fraction, measured by spermatogonial transplantation. A decrease in SSC activity is observed with an increase in β-catenin signalling intensity. Asterisks indicate significance from fraction II. Scale bar: (A – B) 50 μm, inset: 100 μm.

Wnt3a stimulates active cycling of β-catenin signalling cells and leads to cluster-like aggregations under feeder-free conditions

Since a previous study reported that proliferation of a spermatogonia cell line was stimulated by Wnt3a [116], we analyzed cell cycle profiles of cluster cells after Wnt3a treatment using propidium iodide staining and flow cytometry. Cluster cells were treated with or without Wnt3a overnight, under feeder-free conditions, and profiles were assessed the next day. We observed that most cluster cells are not actively cycling (G0/G1 phase) (Fig. 3A). However, following Wnt3a treatment we observed a significant 3-fold increase in the percentage of actively cycling cells (in S, G2, & M phase). We next asked whether active cycling is induced equally in both β-catenin signalling and non-signalling cells. To this end, TCF/LEF-*lacZ* clusters were stimulated with Wnt3a and separated via flow cytometry. Cell cycle profile analysis of each fraction showed that the fraction of actively cycling cells was markedly higher in β-catenin signalling cells than in non-signalling cells (negative: 6.7 ± 1.3%, dim: 21.9 ± 1.9%, high: 21.8 ± 1.4%), (Fig. 3B). These results indicate that Wnt3a activates the β-catenin pathway in a select population of cluster cells and drives their cell cycle.

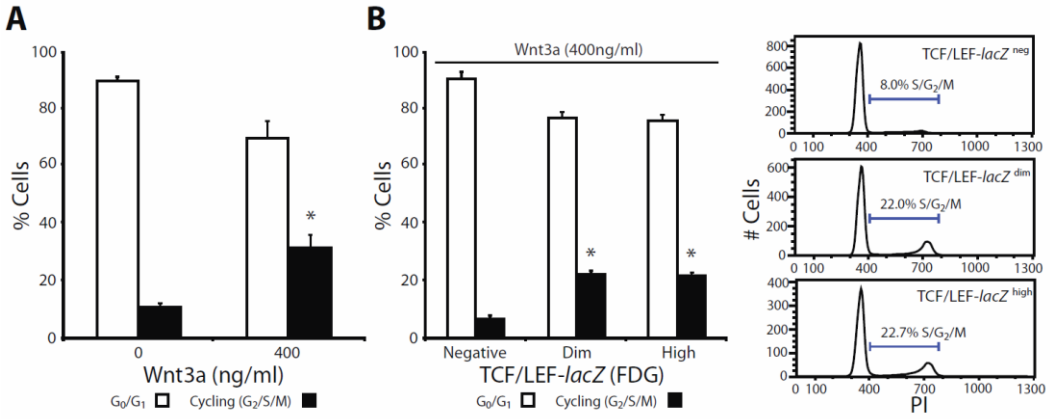

Figure 3: Wnt3a stimulates cluster cell proliferation.

(**A**) Percentage of non-cycling (G0/G1; open bars) and actively cycling (S/G2/M; filled bars) cells following Wnt3a stimulation. (**B**) Percentage of cycling cells in β-catenin signalling populations following Wnt3a stimulation. Representative flow cytometric histograms show β-catenin signalling positive cells are actively cycling after Wnt3a stimulation.

Over the course of our short-term feeder-free culture, we observed that "cluster-like" accumulations formed in the presence of Wnt3a, while in its absence most cells remained as singles or doublets (Fig. 4). Therefore, to quantify whether Wnt3a led to an increase in the frequency of directly-contacted, aggregating cells, we employed Cell Profiler image analysis software (Broad Institute) [246] and monitored the daily growth of B6GFP cluster cells in the presence of Wnt3a, comparing them to untreated controls. We utilized an algorithm that identified individual cells based on GFP expression and the presence of a DNA dye. This algorithm then measured how many adjacent, neighboring cells each cell directly contacts, thereby identifying the frequency of cell aggregation. The results showed that in the absence of Wnt3a, most cells remain with 0 – 1 connected cells (i.e. singles or doublets) throughout the course of culture (Fig. 5A, B). In contrast, Wnt3a-treated cells started as singles or doublets at day 1 of culture, similar to controls. However, the frequency of singles/doublets declined throughout the course of culture until most cells had at least 1 – 2 neighboring cells, while only ~14% of cells are classified as singles by day 4 (Fig. 5C, D). Furthermore, Wnt3a-treated cultures showed 10% of cells with 4 or more neighboring cells by day 4, which represents a significant increase compared to untreated controls where only 5% of cells have 4 or more neighbors. Therefore, these results show that Wnt3a stimulation leads to the formation of cluster-like aggregations under feeder-free conditions.

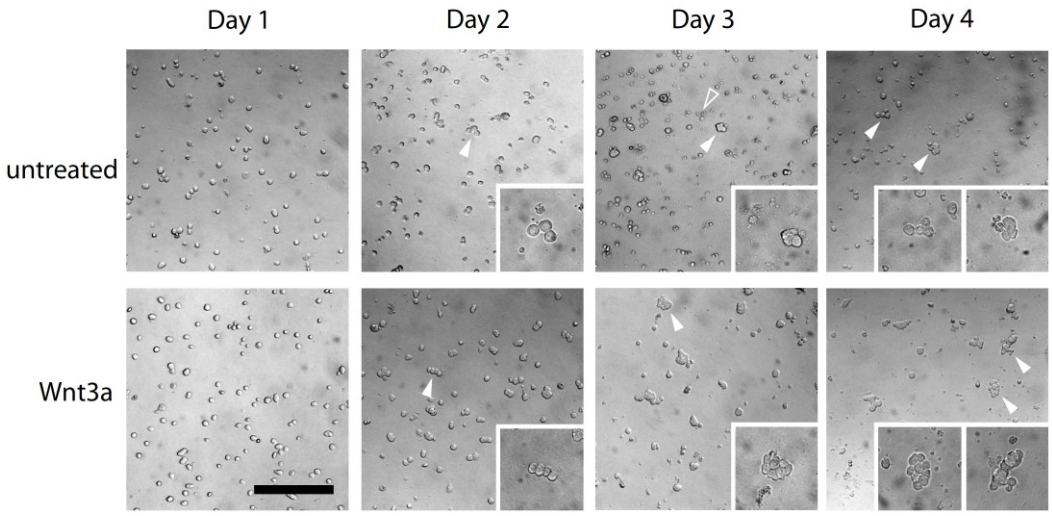

Figure 4: Wnt3a leads to increased "cluster-like" aggregations.
Representative brightfield photomicrographs of cluster cells following control or Wnt3a treatment at each day of culture. At day 1 after seeding, cluster cells are arranged predominantly as singles or doublets under both conditions. At day 2, most cells remain as singles and doublets but longer chains are more easily observable (arrowheads). By day 3, small clumps are easily observable in Wnt3a-treated cultures (arrowhead). In day 3 control cultures, most cells remain as singles but small clumps are occasionally observed (filled arrowhead). Dead or dying cells characterized by ruffled membranes are present (unfilled arrowhead). On day 4, large "cluster-like" cell are found throughout Wnt3a-treated cultures (arrowheads), while control cultures infrequently show small cell accumulations or the occasional cell chain (arrowheads) but lack these large cell accumulations. Insets show higher magnification images. Scale bar: 50 μm, inset: 25 μm

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Finally, we attempted to determine if Wnt3a leads to SSC expansion over the course of 4 day culture, under feeder-free conditions. Using the in vitro cluster-formation assay, we compared the numbers of SSCs at the beginning (day 0) to the end (day 4) of feeder-free culture with or without Wnt3a. From this comparison we can determine if SSC numbers increase, decrease, or are maintained. From day 0 to day 4, SSC numbers increased 1.3-fold with Wnt3a, which was deemed not significant (Fig. 5E). In the absence of Wnt3a cluster-

forming cells decreased dramatically. Therefore, these results indicate that development of cell-cell associations correlates with the maintenance of SSC activity over the course of feeder-free culture.

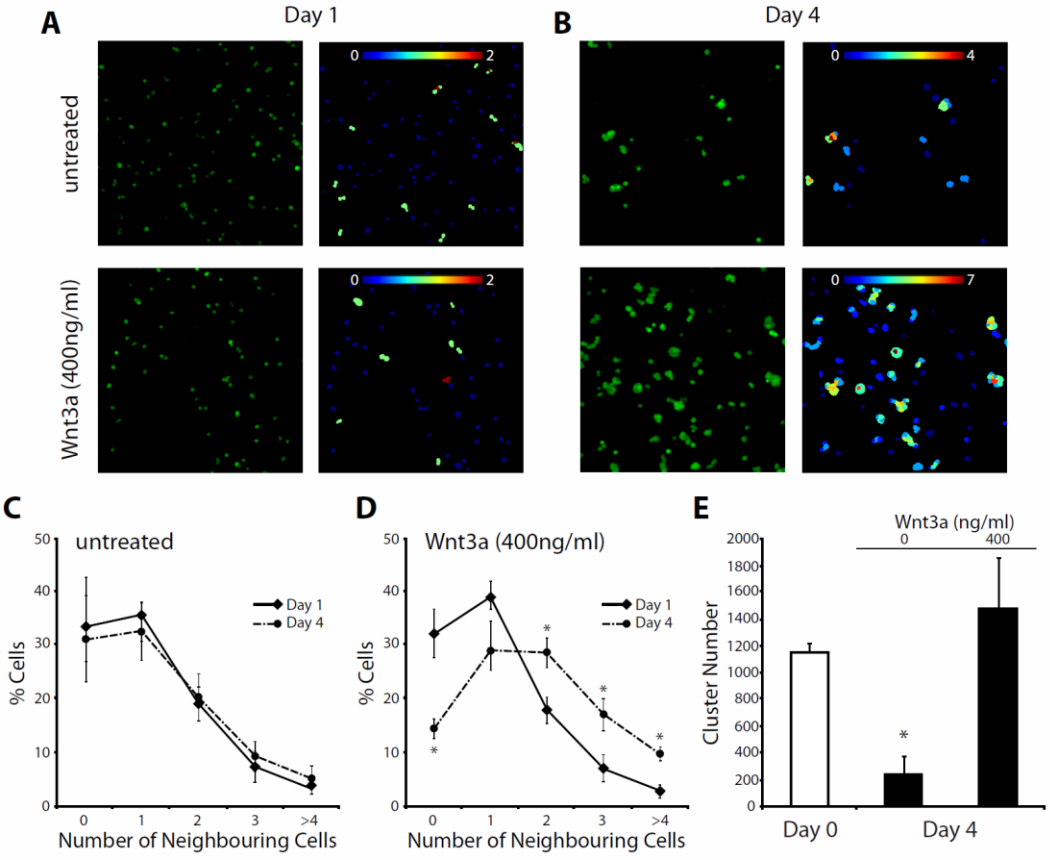

Figure 5: Wnt3a leads to increased cell-cell interactions.

Representative fluorescent photomicrographs (left) and corresponding CellProfiler Image analysis (right) of B6GFP cluster cells following control or Wnt3a treatment after 1 day (**A**) and 4 days (**B**). The colour gradient labels individual cells according to the number of directly-contacted neighbouring cells. Percentage of clustering cells after 1 day (solid line) and 4 days (dashed line) following control (**C**) and Wnt3a treatment (**D**). Wnt3a stimulates cells to cluster, while most cells remain as singles or doublets in controls. (**E**) Cluster-formation assay results showing SSC numbers over the course of feeder-free culture with or without Wnt3a. Increased SSC survival and proliferation is observed with Wnt3a.

Discussion

The results of this study indicate that Wnt3a can activate the β-catenin pathway in a subset of cluster cells but these cells show greatly diminished SSC activity, which is consistent with our previous study [203]. Interestingly, Wnt3a treatment leads to an overall increase in SSC numbers. We observe that β-catenin pathway activation leads to increased cell cycle activity and more robust cell-cell association upon Wnt3a treatment, resulting in an increase in SSC maintenance. Thus, these results collectively suggest that Wnt3a increases SSC numbers indirectly, through cell-cell interactions within the cluster community.

During steady-state spermatogenesis, SSCs are believed to represent a population of the most primitive A_{single} spermatogonia. However, following injury or depletion it has been shown that more differentiated A_{paired} or $A_{aligned}$ spermatogonia may revert to an SSC fate [47,131]. Accordingly, cluster cells expressing the differentiation marker c-kit are observed to retain SSC regenerative ability equal to their c-kit^{-} counterparts [132], raising the possibility that all cells within the cluster community may retain a level of SSC activity. We observed that the majority of functional SSCs were TCF/LEF-*lacZ* non-signalling cells and that SSC activity is lost as activation levels of β-catenin signalling increased (Fig. 2), raising the possibility that cluster cells gradual commitment to differentiation was induced upon activation of this signalling pathway. Therefore, we speculate that activation of β-catenin signalling may artificially force cells toward commitment. Thus, this may become a means to examine the factors mediating differentiation, in particular, the point of irreversible commitment from SSCs. Such a question can be addressed by analyzing global gene expression profiles of the β-catenin signalling and non-signalling populations.

How might Wnt3a stimulation support SSC maintenance? We found that manipulation with Wnt3a led to the formation of germ cell aggregations that resemble clusters, potentially as a result of activated cell cycle in non-SSCs. Interestingly, we note that tissue specific stem cells, which can proliferate ex vivo, have not been maintained alone but are always accompanied by differentiated daughter cells, such as seen in neurospheres and mammospheres [77,247]. Neural

stem cells are reported to represent ~2% of total neurosphere cells [77], which suggests dependence by adult stem cells for cellular cues to maintain stem cell functionality. This dependence is exemplified in ISC cultures, in which single stem cells produce crypt-like organoids composed of interacting ISCs and Paneth cells [169]. Paneth cells are specialized progeny, derived from stem cells, and comprise the ISC niche by producing extrinsic factors to support ISCs. In vivo, a recent study pointed out a potential interplay between the stem cell pool and the progenitor pool to maintain the size of each cell population in balance [248].

Likewise, the formation of a germ cell cluster, which is an integrated community of interacting SSCs and daughter cells, may be an important in vitro phenomenon to support SSCs and may act as an in vitro SSC niche. It has been shown that constitutive activation of Ras-cyclin D2 in germ cells circumvents the need for GDNF and FGF2, yet induces cell division and cluster formation, leading to long-term SSC proliferation [109]. Even in this case, however, SSCs remained to be a minority population, implying that Ras-cyclin D2 stimulated cell divisions resulting in the production of mostly non-SSCs. Importantly, the frequency of SSCs after Ras-cyclin D2 stimulation did not differ from that reported for wild-type SSC clusters [109] which is relatively constant across studies reported thus far (1 – 3%) [115,134,202]. In our case, an SSC concentration was estimated to be 0.7 – 1.7% following Wnt3a stimulation (unpublished data). These results therefore argue that the SSC frequency is controlled to an optimal level in an in vitro germ cell cluster, perhaps through the communication among constituent cells of a cluster community. Thus, it is possible that Wnt3a may maintain SSCs by increasing the number of committed cells thereby facilitating formation of clusters, and accordingly, regulating proper SSC numbers within a population. On this basis, we speculate that SSCs may actively suppress β-catenin signalling, or may not be equipped with the full range of cell-surface or intracellular mediators to transduce this signalling. Further studies are necessary to test this hypothesis of clusters acting as an in vitro SSC niche.

Wnt3a expression is not detected in the testis, implying that these in vitro findings may be the result of functional redundancy with another Wnt. Functional

redundancy has been shown by Wnt1, Wnt3a, or Wnt8 resulting in axis duplication during Xenopus gastrulation [249,250]. In the testis, we have previously detected the expression of classical β-catenin signalling class Wnts, notably Wnt1 and Wnt2b [203]. In vivo, Wnt1 expression is detected starting in the round spermatid stage however a role in spermatogenesis has not been characterized [251]. Wnt2b also has no defined role in spermatogenesis however Wnt2b/β-catenin signalling has been implicated in kidney morphogenesis regulation in conjunction with Wnt11 and GDNF [99,252]. Furthermore, Wnt2b has been shown to induce the proliferation of retinal progenitor cells in the avian eye [253]. In the testis, Wnt2b is detected at birth, is undetectable in adult, but is detected in cryptorchid testes suggesting that it may be expressed by spermatogonia.

In summary, this study demonstrates that Wnt3a activates the β-catenin pathway in committed progenitor cells and suggest that progenitors in turn promote SSC maintenance. We observed that Wnt3a stimulated cell division of β-catenin signalling cells and stimulated community formation between SSCs and daughter cells, resulting in SSC maintenance. Therefore, these findings lead us to propose that differentiated daughter cells may be capable of contributing to the SSC regulatory function of the niche.

SUPPLEMENTARY CHAPTER

PRELIMINARY MANUSCRIPT

Preface to the Supplementary Chapter

In Chapter 2, I observed that germ cell clusters are composed of SSCs and differentiated cells. Notably, the number of SSCs per cluster is calculated to be relatively constant throughout long-term culture suggesting that SSC numbers are somehow balanced among the cluster society.

In Chapter 3, I was able to visualize the heterogeneity of clusters as a canonical (β-catenin) signalling cell population and a non-signalling population. Transplantation of these two distinct populations showed that β-catenin signalling cells were virtually absent of SSC activity. Marker analysis of this population showed that they express markers of undifferentiated spermatogonia, suggesting that β-catenin signalling cells are committed progenitor cells.

In Chapter 4, I observed that cell cycle stimulation of the β-catenin committed cell population led to cluster-formation, that is, increased cell-cell interactions between SSCs and differentiated cells, resulting in the indirect support of SSC maintenance. Collectively with the results of Chapter 2, these findings indicate that communication among the cluster cell community may influence SSC regulation. However, it is unknown what factors are involved in regulating the balance of SSC fate decisions.

Therefore, in the following Supplementary Chapter, I performed global gene expression profiling, comparing the β-catenin signalling vs. non-signalling cell populations from Chapter 3, to investigate how these two populations differed at the molecular level. Interestingly, this dataset may also serve to identify potential targets provided by committed cells that might regulate SSC fate balance within the cluster community.

Delta-like homolog 1 (DLK1) negatively regulates mouse spermatogonial stem cell maintenance

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Short-title: DLK1 induces spermatogonial stem cell differentiation

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Abstract

A proper balance between spermatogonial stem cell (SSC) self-renewal and differentiation is important to maintain ideal SSC numbers during spermatogenesis. Several paracrine factors are known to support SSC self-renewal, while factors that can promote SSC commitment to differentiation are virtually unknown. This is due in part to the inability to prospectively identify SSCs from committed cells. Thus, it is not currently possible to study how these two cells populations differ at the molecular level. Using a SSC culture system, we have previously reported that SSCs do not signal through the β-catenin pathway but rather, this signalling is active in non-SSC committed cells. Therefore, in this system we can isolate committed progenitor cells from SSCs based on their response to β-catenin signalling activation. In this preliminary study, we screened the gene expression profiles of these two populations. We identified potential targets upregulated in β-catenin signalling committed cells, which might influence the SSC fate decision. One target in particular, Delta-like homolog 1 (Dlk1), was found to be upregulated 8-fold in β-catenin signalling cells. The addition of recombinant DLK1 to feeder-free SSC culture led to a significant decrease in SSC numbers without a concomitant change in total cell numbers or cell death, suggesting that DLK1 promotes SSC differentiation. Therefore, this preliminary study identifies a means in which committed cells may influence SSC numbers within the germ-cell cluster community. Furthermore, the dataset presented here provides potentially novel target markers to prospectively identify SSCs from other committed cells.

Introduction

Spermatogonial stem cells (SSCs) are responsible for long-term sperm production by their unique ability to self-renew and continually contribute germ cells to spermatogenesis. In the testis, SSCs are maintained in a niche environment of Sertoli cells, extracellular matrix proteins, and differentiated germ cells [84,201]. Numerous Sertoli cell factors such as glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2), and Wnt5a have been demonstrated to support SSC self-renewal [202,203]. In contrast, factors from differentiated germ cells that can influence the SSC fate decision to self-renew or differentiate are completely unknown.

SSCs are a rare population in the testis, making their study difficult in vivo [70]. This problem is further exacerbated by the lack of definitive markers identifying SSCs. Therefore, the study of SSCs benefits from the development of in vitro culture systems [112,114]. SSCs can be expanded long-term ex vivo within three-dimensional communities of spermatogonia (termed "clusters") [203]. Previous work has demonstrated that SSCs comprise only a minority population in a cluster community with most cells in a cluster believed to be more differentiated progenitors [203]. These differentiated germ cells have been shown to support SSCs by forming an in vitro niche within the cluster community (Chapter 4). Therefore, a thorough examination of the factors provided by SSC daughter cells may offer a clue as to how these cells promote SSC self-renewal and differentiation.

We have determined that cluster cells can be functionally separated according to their response to the canonical Wnt pathway [203]. In the canonical (β-catenin) pathway, the β-catenin protein binds to T-cell factor/Lymphoid enhancer factor (TCF/LEF) family transcription factors and drives expression of target genes [144]. Using transgenic reporter mice (TCF/LEF-*lacZ* mice), which carry the *lacZ* reporter gene under control of β-catenin-TCF/LEF binding sites [217], we demonstrated that SSCs are non-β-catenin signalling, while β-catenin signalling cells are not SSCs [203].

In this study, we took a global gene expression approach, comparing β-catenin signalling and non-signalling cells, to identify potential targets that might regulate the proliferation and differentiation of SSCs. We observed that one target in particular, Delta-like homolog 1 (Dlk1), was expressed greater than 8-fold by β-catenin signalling cells. Dlk1, also known as preadipocyte factor 1 (Pref1), is part of the imprinted Dlk1-Dio3 gene cluster on mouse chromosome 12 and encodes a transmembrane glycoprotein similar to the Delta/Notch/Serrate family of signalling molecules [254]. DLK1 can also be secreted and is not believed to participate in the Notch signalling pathway. Dlk1 is expressed at high levels in embryonic tissues [255,256] and has been demonstrated previously to maintain adipocyte precursor cell populations and inhibit differentiation [257]. Moreover, DLK1 is required for postnatal neurogenesis; secreted DLK1 from the niche-forming astrocytes appears to stimulate self-renewal of neural stem cells (NSCs) in the subventricular zone (SVZ) of the brain [258]. Therefore, we hypothesize that Dlk1 might be a factor expressed by progenitor cells that influence SSC fate within the cluster population. Addition of recombinant DLK1 to SSC culture led to a significant decrease in SSC activity, without a concomitant change in cell numbers or apoptosis, suggesting that DLK1 induces SSC differentiation. Therefore, these preliminary results suggest that factors preferentially expressed by progenitor cells can influence SSC fate.

Materials and Methods

Donor Animals

TCF/LEF-*lacZ* mice (from Dr. D. Dufort, McGill University) are on a CD-1 genetic background and carry the *lacZ* reporter gene driven by β-catenin-TCF/LEF responsive elements [217]. B6ROSA mice are F_1 hybrids of C57BL/6 (B6) and ROSA26 mice, which express the *lacZ* gene. B6GFP mice (C57BL/6-Tg(CAG-EGFP)1Osb/J; The Jackson Laboratory) express GFP ubiquitously. Animal procedures were approved by the Animal Care and Use Committee of McGill University.

Recipient Animals and Transplantation

Spermatogonial transplantation was performed and recipients were prepared as in [134]. Recipient mice for B6ROSA cells were 129/SvEv × B6 F_1 hybrids, and those for TCF/LEF-*lacZ* cells were Ncr nu/nu mice (Taconic). Recipient testes were analyzed for SSC quantification following staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) 2 months post-transplantation.

Cell Culture and Cluster Formation Assay

SSC cultures were generated from immunomagnetic selected Thy1-positive testis cells from 6 – 8 days post partum (dpp) mice as described previously [134]. Cultures were maintained on a feeder layer of STO fibroblasts with "growth factors" consisting of GDNF (20ng/ml), GFRα1 (75ng/ml), and FGF2 (1ng/ml). Under experimental conditions, growth factors were used at different concentrations: GDNF (40ng/ml), GFRa1 (300ng/ml), and FGF2 (1ng/ml). For short-term feeder-free cultures, culture plates were coated with Matrigel (BD Biosciences), diluted 1:2, and incubated overnight at 4°C, as in [203].
To examine the effect of DLK1, B6GFP clusters were flushed off feeder cells with gentle pipetting, which isolates clusters cells with over 90% purity [203]. Cluster cells were cultured under feeder-free conditions with recombinant DLK1 (Enzo Life Sciences, Switzerland) and growth factors for 3 – 4 days. Cultures were subsequently transplanted into recipient testes to quantify SSC number.

Flow Cytometric Analysis

Flow cytometric analyses and sorting were performed using an Accuri C6 and BD FACSAria (BD Biosciences), respectively. To distinguish β-catenin-signalling cells, TCF/LEF-*lacZ* clusters were reacted with 500 μM fluorescein di-β-D-galactopyranoside (FDG, Marker Gene Technologies) as described previously [203]. Experimental gates were established using control cells: B6ROSA (positive) and B6 (negative) cluster cells. Data were collected from three independent cell lines.

To determine the extent of apoptosis after DLK1 treatment, B6ROSA cluster cells were cultured for 4 days under feeder-free conditions on Matrigel. Cells were collected on day 4 and stained with the APO-Direct Apoptosis Detection Kit (BD Biosciences), as per the manufacturer protocol. Data were collected from three experiments and at least 10,000 were collected per group.

Microarray

TCF/LEF-*lacZ* clusters were trypsinized to single cells on day 6, resuspended at 10^7 cells/ml in PBS, and reacted with 500 μM fluorescein di-β-D-galactopyranoside (FDG, Marker Gene Technologies) in double-distilled H_2O for 1 min at 37°C, following the manufacturer's protocol and as described previously [203,259]. Two biological replicates, using cluster cells derived from different animals, were prepared. Four to six separate FACS collections, for each individual cell line, were performed over time, cryopreserved, and pooled to collect enough total RNA for the microarray procedure. Total RNA was extracted using the Picopure RNA Extraction kit (Molecular Probes). RNA quality was examined with an Agilent Bioanalyzer and an RNA Integrity Number of 9.0 out of 10 was deemed acceptable. From each sample, 1.5μg of total RNA underwent one-round amplification using the TotalPrep RNA Amplification kit (Ambion) before cDNA synthesis and in vitro transcription to biotinylated cRNA. Samples were prepared and hybridized to a MouseWG-6 v2 Expression BeadChip (Illumina) according to the Illumina protocol. Three replicates for each TCF/LEF-*lacZ* negative and positive population were collected, totalling six samples on one chip. All processing, hybridization, and chip scanning was performed by The McGill University and Génome Québec Innovation Centre.

Illumina data files were analyzed by FlexArray 1.6 (McGill University) to normalize and quantify expression levels of each probe set. Raw signal intensity values were normalized using a Variance-Stabilizing Transformation algorithm designed for the Illumina platform. Probe intensity above background levels was used to calculate detection as presence or absence. All probes deemed absent in any two replicates were excluded, which led to a reduction from 45,000 to 21,328

probes. To visualize the general trends of expression over the two cell populations, principal components analysis (PCA) was used. PCA is a statistical method to reduce the dimensionality of a large number of variables whilst retaining the variance of the data and can identify problem samples that cluster away from replicate samples. PCA showed that between two experimental replicates, TCF/LEF-*lacZ*-negative and positive samples clustered separately. However, one replicate pair clustered independently of the others and was therefore omitted from further analysis. Paired SAM statistical analysis was employed to quantify probe expression changes between both cluster cell populations. From this gene list, genes that showed fold changes greater than a minimum threshold >2-fold increase or decrease were deemed "differentially expressed" between TCF/LEF-*lacZ*-negative and positive cell populations.

Quantitative Real-time Polymerase Chain Reaction Analysis

Total RNA was extracted using the Picopure RNA Extraction Kit (Molecular Probes). Complementary DNA was synthesized with Superscript III Reverse Transcriptase (Invitrogen) with random hexamers. Quantitative PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) on a Rotogene 6000 (Corbett Research) with the following program: 94°C for 15 min followed by 40 cycles of 94°C for 15 sec/60°C for 30 sec/72°C for 35 sec. Primers against the specified targets were generated using Primer3 software [259] and were as follows: Dlk1 5' TGTGACCCCCAGTATGGATT, 3' CCAGGGGCAGTTACACACTT; Ptn 5' CCTTCCTGGCATTGATTTTC, 3' ACTCCACTGCCATTCTCCAC; Gapdh 5' TGTGTCCGTCGTGGATCTGA, 3' CCTGCTTCACCACCTTCTTGA.

Statistics

Data were expressed as mean ± SEM. Colony numbers were indicated as those per 10^6 cells placed in culture, unless specified otherwise. Significance was determined using Student's t-test or ANOVA followed by Fisher's Test for Least Significant Difference. $p < 0.05$ determined significance.

Results and Discussion

Previously, we demonstrated that germ cell clusters are functionally heterogeneous according to their response to Wnt/β-catenin signalling [203]. Using a reporter mouse line that faithfully indicated β-catenin signalling activation (TCF/LEF-*lacZ*) [217], we isolated β-catenin signalling and non-signalling cells and demonstrated that SSCs are non-signalling cells, while β-catenin signalling cells appear to be differentiated progenitors. Therefore, we first asked how β-catenin signalling and non-signalling cells differed on the molecular level. To this end, we compared global gene expression profiles between β-catenin-signalling and non-signalling TCF/LEF-*lacZ* cluster cells using the Illumina Expression BeadChip platform. TCF/LEF-*lacZ* mice clusters were cultured for 6 days followed by reaction with a fluorescent vital *lacZ* stain and separation of signalling and non-signalling cell populations using FACS, as described previously. β-Catenin signalling cells are a minority population [203], therefore, 4 – 6 separate FACS collections from each individual cell line were performed over time and pooled.

Previously reported targets of β-catenin signalling were found to be moderately upregulated attesting to the activation of this pathway, such as Tcf4, Fgf7, WISP1, WISP2, and Wif. We found that the expression of several SSC cell surface markers (GFRa1, CD9) and transcriptional regulators (Oct4, Plzf, Sall4, Ngn3) were detected equally between β-catenin-signalling and non-signalling cells, in accordance with previous results [203], thereby attesting to the relative similarity between these two cell populations. Surprisingly, the known SSC surface marker Thy1 was observed to be up-regulated in β-catenin-signalling cells (2.5-fold), while c-Kit, normally associated with differentiating spermatogonia, was marginally up-regulated (1.5-fold) [3].

Table 1: Gene expression comparison between TCF/LEF-*lacZ*$^{+}$ and TCF/LEF-*lacZ*^{neg} cells.

Category	Gene	Gene Name	Fold Change*
Secreted Factors	Ptn	Pleiotrophin	10.915
	Dlk1	Delta-like homolog 1	9.350, 8.345
	Sparc	Secreted protein, acidic, cysteine rich	5.550
	Ctgf	Connective tissue growth factor	3.686, 3.115
	Bmp1	Bone morphogenetic protein 1	2.450
	Fgf7	Fibroblast growth factor 7	2.357
Transcription Factors	FosB	FBJ murine osteosarcoma viral oncogne	3.407
	Elk3	ETS-domain protein	2.532
	Ets2	v-ETS avian erythoblastosis virus E26 oncogene	2.500
	NfIB	Nuclear factor I/B	2.136
	Tcf4	T-cell factor 4	2.103
Cell Adhesion	Vcam1	Vascular cell adhesion molecule 1	5.673
	Cldn5	Claudin 5	3.952
	Esam	Endothelial cell adhesion molecule	3.824
	Cdh5	Cadherin 5 (VE-cadherin)	2.546
	Icam2	Intercellular adhesion molecule 2	2.042
Extracellular Matrix	Col4a1	Collagen type IV, alpha 1	7.128
	Col4a2	Collagen type IV, alpha 2	5.466
	Fbln2	Fibulin 2	4.772

* Upregulated gene expression in TCF/LEF-*lacZ*$^{+}$ vs. TCF/LEF-*lacZ*^{neg} cells

Genes expressed 2-fold or greater in β-catenin-signalling cells compared to non-signalling cells, were filtered resulting in the identification of 119 unique targets (Table 1 and Appendix A). We note that no genes were found to be downregulated greater than 2-fold in β-catenin signalling cells under these parameters. We identified the upregulation of several targets that can affect proliferation and differentiation and could potentially influence SSC fate. One target in particular, Dlk1, was expressed greater than 8-fold in β-catenin signalling cells. Dlk1 is expressed at high levels in embryonic tissues and is a demonstrated secreted NSC niche factor that is required for NSC maintenance in the SVZ [255,258]. Therefore, we hypothesized that Dlk1 might influence SSC fate and focused on this target for further study.

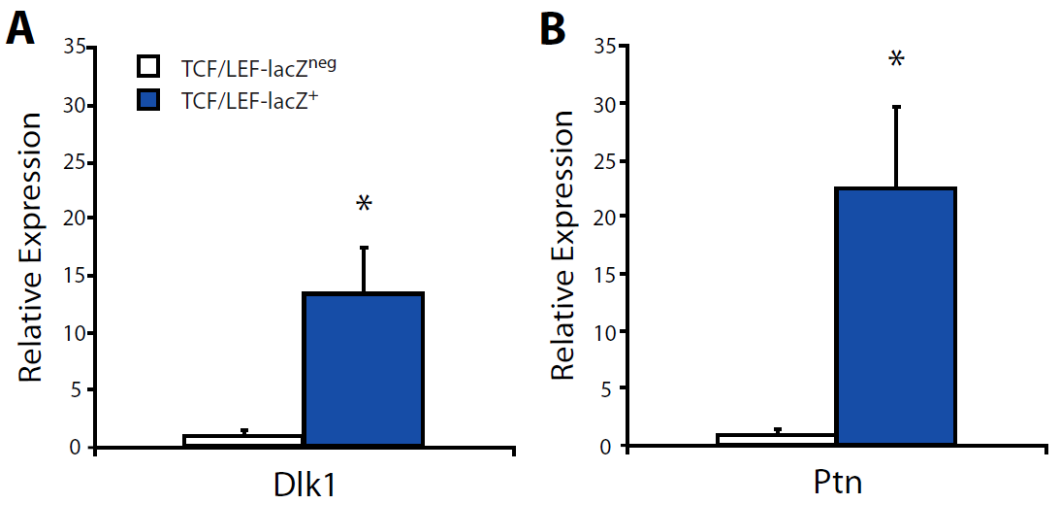

Figure 1: Quantitative RT-PCR validation of targets in β-catenin signalling cells.

(**A-B**) Quantitative RT-PCR results confirming upregulated expression of Dlk1 (A) and Ptn (B) in β-catenin-signalling cells vs non-signalling cells.

Using quantitative RT-PCR, we confirmed the up-regulation of Dlk1 in β-catenin signalling cells compared to non-signalling cells (Fig. 1A). To examine a potential biological effect of DLK1 on SSC activity, we added recombinant DLK1 to B6GFP clusters cultured under feeder-free conditions on Matrigel. We observed that 4-day culture with recombinant DLK1 led to a dose-dependent increasing trend in total cluster cell numbers, although this increase was deemed to be not significant (Fig. 2A). We next examined whether our perceived increase in cluster cells reflected an increase in SSC activity. To this end, B6ROSA clusters were cultured with recombinant DLK1 for 4-days, as above, and subsequently trypsinized and transplanted into recipient testes to examine functional SSC activity. The results of the transplantation assay showed that DLK1 led to a significant 56% decrease in functional SSC numbers compared to an untreated control demonstrating that DLK1 does not support an SSC fate in vitro, but rather may induce death or differentiation (Fig. 2B).

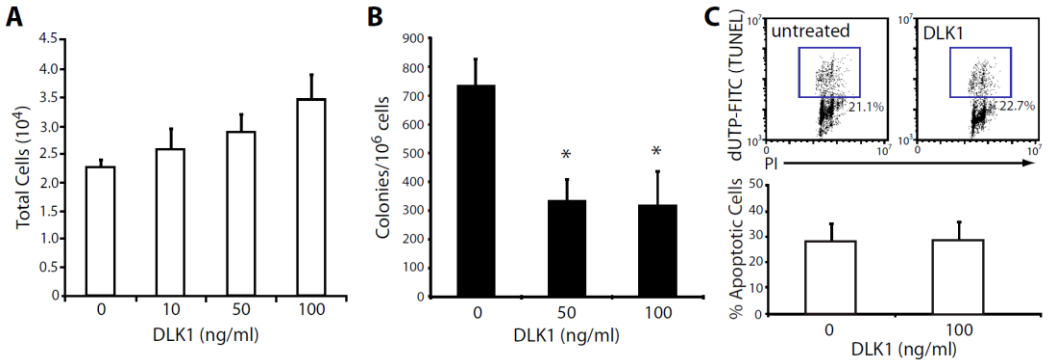

Figure 2: DLK1 effects on SSC activity and apoptosis in feeder-free cultures
(**A**) Total recovered cell numbers following the addition of DLK1 in feeder-free conditions shows an increasing non-significant trend. (**B**) Transplantation results of cluster cells following the addition of DLK1 in feeder-free conditions demonstrates that DLK1 leads to a loss in SSC activity. (**C**) Representative flow cytometric scatter-plots show apoptotic cells in the gate (top panels). Apoptotic cell percentages after DLK1 treatment, determined by TUNEL staining, shows that DLK1 addition does not affect apoptosis.

These results collectively demonstrate that the heterogeneous cell populations that comprise a cluster community can potentially communicate within the community to regulate SSCs. Although this preliminary study focuses on a potential role for Dlk1 in SSC differentiation, our microarray data also identifies other candidates that might influence SSC fate. Pleiotrophin (Ptn), a secreted growth factor, is one such candidate and was upregulated 10-fold in β-catenin signalling cells. Ptn is expressed in the brain and testis and has been demonstrated to promote HSC expansion in vitro and regeneration in vivo through PI3K signalling [260]. Interestingly, dominant-negative Ptn mutant mice are sterile owing to massive apoptosis of spermatogonia during postnatal development [261]. Quantitative RT-PCR results confirmed increased expression of Ptn in β-catenin signalling cells (Fig. 1B), thus, Ptn is another strong candidate regulator of SSC activity in the cluster environment and testis. We noted other secreted candidates of interest

upregulated in β-catenin signalling cells, including Secreted protein, acidic, cysteine-rich (Sparc)/osteonectin (5.5-fold), Connective tissue growth factor (Ctgf; 3.5-fold upregulation) and Bone morphogenic protein 1 (Bmp1; 2.5-fold). Interestingly, each of these secreted factors has been implicated in the synthesis or maintenance of extracellular matrix [262,263,264]. We also observed the upregulation of collagen proteins, Col4a1 and Col4a2 (7-fold and 5.5-fold, respectively), both of which are important components of basement membrane, and Fibulin 2 (Fbln2; 4.7-fold), which has been shown to interact with laminin proteins. In the testis, basement membrane proteins contribute to the SSC niche [84] suggesting that β-catenin signalling cells might support SSCs in part by contributing extracellular matrix proteins to an in vitro niche.

β-Catenin signalling cells have been demonstrated to be non-SSCs but their identity remains elusive. Our results show that these cells express various spermatogonial genes similarly to non-signalling cells, implying these cells are undifferentiated spermatogonia [203]. However, the increased expression of Dlk1 may offer a clue to their loss of SSCs ability. Dlk1 is part of a conserved imprinted gene cluster that includes three paternally-expressed protein-coding genes (Dlk1, Rtl1, Dio3) and maternally expressed microRNAs, small nucleolar RNAs (snoRNAs) and the noncoding gene, Gtl2 [254,265]. Recently, it has been shown that a loss of imprinting at the Dlk1-Gtl2 intergenic differentially methylated region (IG-DMR) leads to biallelic expression of Dlk1 by astrocytes in the SVZ of the brain [258]. Biallelic expression coincided with a switch to secreted DLK1 and this signalling was crucial to maintain NSCs and neurogenesis. In the male germ line, this imprinted locus is methylated by the Dnmt3 family of de novo DNA methyltransferases. Interestingly, the germ cell-specific knockout of Dnmt3a or Dnmt3L leads to spermatogenic arrest and the presence of only a few spermatogonia by 11 wk of age indicating that the establishment and maintenance of methylation imprints are important for spermatogenesis progression [266,267]. As such, our observed increase in Dlk1 expression in β-catenin signalling cells could be attributed to changes in methylation and/or biallelic expression of Dlk1. These changes in Dlk1

expression may represent a switch in commitment to spermatogonial differentiation, hence an inability for β-catenin signalling cells to display functional SSC activity. These observations collectively lead us to speculate that β-catenin signalling cells may represent an early point of SSC commitment to differentiation. A detailed examination of the methylation status of these DMRs between β-catenin signalling and non-signalling cells may offer insight into the consequences of increased expression of Dlk1.

Another intriguing possibility to explain the identity of Dlk1-expressing β-catenin signalling cells is that they have exited from SSCs and entered a transition stage where they can acquire pluripotency potential. It has been demonstrated that pluripotent ESC-like cells can arise from neonatal and adult germ cell culture [87,135] but their cell-of-origin is currently unknown. Recently, it has been shown that the degree of activation of the Dlk1-Dio3 region positively correlated with an increased level of pluripotency in induced pluripotent stem cells (iPSCs) [268,269]. Increased expression of Gtl2, the snoRNA Rian, Dlk1, and microRNAs was indicative of a particular iPSC clone being more closely related to authentic ESCs and correlated with a more complete reprogramming. Increased expression of Gtl2, Rian, and Dlk1 was reported to be due to both histone modifications and a reduction in DNA methylation [268,269], indicating that demethylation may occur at some point in transition to acquiring pluripotency. Therefore, the possibility exists that β-catenin signalling cells are spermatogonia with the capability of transitioning to pluripotent cells. Although purely speculative, it may be interesting to isolate β-catenin signalling cells and expose them to culture conditions optimal to form ES or EG cells.

In this preliminary study, we identified novel genes that are upregulated in committed, non-stem germ cells within the cluster community using a global gene expression approach. Our data further showed that committed cells have the capacity to communicate and potentially maintain ideal SSC numbers in a cluster by promoting the differentiation of SSCs through Dlk1.

CHAPTER 5

GENERAL DISCUSSION & CONCLUSION

General Discussion

The unique ability of SSCs to self-renew and continuously support spermatogenesis relies on external cues, such as extrinsic factors from the niche, for communication to balance this cell fate decision. Several factors have been identified that support renewal, however the mechanism of SSC maintenance is far from being completely understood. Similarly, factors and pathways that are associated with SSC commitment to differentiation are almost completely unknown. One major issue continues to be the fact that markers, which prospectively identify SSCs, have not been characterized. The discovery of these markers will facilitate the real-time study of SSCs, potentially offering clues to the mechanisms underlying SSC fate regulation.

In this thesis, I investigated the fate decision mechanism of SSCs, specifically examining the role of Wnt signalling in self-renewal and commitment to differentiation. I determined that Wnt signalling pathways are involved in SSC survival and communication with daughter germ cells. Thus, this body of work will have an impact on understanding communication within the testis in particular as it pertains to SSC fate regulation. Furthermore, the data in this thesis work presents potential target surface markers to definitively identify SSCs. This work will further benefit studies of human SSCs as well as their effectiveness in a clinical application.

Because SSCs can only be defined functionally, their study requires an assay that examines this functionality: spermatogonial transplantation. However, this assay is labour-intensive and time consuming, therefore, I first developed a short-term in vitro assay for SSCs (Chapter 2). This assay utilizes a culture system in which SSCs are maintained long-term within distinct aggregations, called clusters. In my cluster-formation assay, SSC activity is measured by simply counting cluster numbers. I found that clusters are composed of both SSCs and differentiated cells, indicating that self-renewal and differentiation events can be studied in vitro. Therefore, using this short-term cluster-formation assay, I identified Wnt5a as a novel extrinsic factor that supported SSC self-renewal (Chapter 3). Cluster formation and SSC maintenance in vitro requires a feeder cell

layer. I identified Wnt5a expression in these cells and I found that Wnt5a activated a non-canonical pathway in cluster cells, which blocked cell death. In contrast, activation of the canonical (β-catenin) pathway was found in cells lacking SSC regenerative ability. Thus, SSCs do not appear to rely on the β-catenin pathway for their maintenance. However, surprisingly, addition of Wnt3a to SSC cultures activated the β-catenin pathway but also increased overall SSC numbers (Chapter 4). Further investigation showed that Wnt3a activated the β-catenin pathway specifically in non-SSC differentiated cells, which promoted the formation of germ cell communities of SSCs and daughter cells, i.e. clusters. Community formation led to increased maintenance of SSCs (Chapter 4), suggesting that cell-cell communication between cells in the cluster environment can influence SSC fate. Finally, gene expression analysis was conducted, which presents potential targets that might mediate this communication (Supplemental Chapter).

Currently, one focus of work with SSCs is on the identification of extrinsic factors and SSC intrinsic regulators associated with the SSC ground state. Another focus is elucidating how SSCs commit to differentiation. A major problem is the fact that SSCs cannot be prospectively identified. Therefore, several key questions should be addressed to further progress into SSC research, such as: 1) what factors comprise the testis niche environment and what intracellular regulators do they activate in SSCs? 2) What mechanisms are involved as SSCs commit to differentiation? And 3) what surface markers identify SSCs? Furthermore, SSC work in animal models will lead to study in humans as a potential fertility restoration therapy. Therefore, the results of this thesis justify several research directions addressing these questions.

Defining Wnt5a as an extrinsic niche factor in the testis

Currently, a handful of extrinsic factors have been implicated in the regulation of SSC fate, including GDNF, FGF2, CSF1, BMP4, and Activin A [95,102,106,110]. Chapter 3 demonstrates that Wnt5a promotes germ cell survival in vitro, thereby adding this factor to this list. However, GDNF is the only factor

that has been thoroughly challenged in genetic mutant mice to be a definitive extrinsic regulator of SSC proliferation in the testis [35,105]. Given that Wnt5a is detected in Sertoli cells [182,203], a subsequent research direction would be to functionally examine the role of Wnt5a signalling in the testis.

Before exploring experimental approaches, it might be beneficial to first speculate how Wnt5a signalling might influence SSCs in vivo. In Chapter 3, I found that Wnt5a promoted germ cell survival in vitro by inhibiting apoptosis, thereby maintaining SSCs. Interestingly, the control of apoptosis during postnatal development and steady-state spermatogenesis is an important mechanism to maintain appropriate numbers of all germ cell types. A loss in apoptosis regulation may have profound effects on spermatogenesis and fertility, as observed in mice mutant for Bcl2 or Bax, which mediate survival or apoptosis respectively [38,39]. Mice deficient of the pro-apoptotic gene Bax show excessive accumulation of spermatogonia by 2 weeks of age indicating that Bax is a major promoter of apoptosis during the first round of spermatogenesis [38]. By the same logic, forced expression of the pro-survival gene Bcl2 in spermatogonia also leads to germ cell accumulation in all seminiferous tubules, by 4 weeks of age [39]. The accumulating germ cells are reported to be arrested as spermatogonia indicating a block in differentiation. By 7 weeks of age, most seminiferous tubules were devoid of germ cells, except for a few spermatogonia indicating the catastrophic loss of germ cells. Interestingly, by 6 months of age spermatogenesis had partially recovered in Bcl2-expressing mice, with about one-third of the tubules containing sperm, suggesting that the proper balance of Bcl2 is crucial for the first round of spermatogenesis. Disruption of another pro-survival factor, Bcl-w, does not affect the first round of spermatogenesis; mutant mice testes appear morphologically normal up to 4 weeks of age. However, by 8 weeks numerous degenerating germ cells appeared and by 52 weeks almost no germ cells were discernible [270], indicating a proper balance of Bcl-w appears to be important for steady-state spermatogenesis.

What extrinsic factors regulate cell survival and death? The receptor tyrosine kinase, c-Kit, is expressed on differentiated (A_{al} to B) spermatogonia,

while its ligand, SF, is secreted by and expressed on the membranes of Sertoli cells. Mice with a mutation in the Steel locus (*Sl*) show cell death of all germ cells from differentiated spermatogonia and later, resulting in seminiferous tubules of only undifferentiated spermatogonia. Transplantation of the undifferentiated spermatogonia from *Sl* mice into the seminiferous tubules of wild-type mice show donor derived spermatogenesis, indicating that SSCs are present in *Sl* mice [40]. Therefore, it is evident that SF is necessary for the cell survival of differentiated spermatogonia but not undifferentiated spermatogonia, including SSCs. In terms of extrinsic factors that promote the survival of undifferentiated spermatogonia, GDNF has demonstrated to support SSC maintenance in vitro, in part through promoting their survival [102]

Given my in vitro findings in Chapter 3, the possibility exists that Wnt5a is important for survival of undifferentiated spermatogonia during spermatogenesis and in response to injury. I hypothesize that Wnt5a is a pro-survival spermatogonial factor and that disruption of Wnt5a signalling in adult and developing testes may lead to SSC and germ cell depletion. Homozygous Wnt5a knockout mice are neonatal lethal so postnatal spermatogenesis cannot be studied in these mice [155]. Furthermore, Wnt5a is upregulated at 12.5 dpc in the male embryonic gonad and its expression is important for PGC migration; reduced numbers of germ cells reach the genital ridges in $Wnt5a^{-/-}$ mice [162]. Other embryonic gonad defects have been reported in $Wnt5a^{-/-}$ male mice including abnormal transabdominal descent resulting in cryptorchidism [161,162]. Therefore, conditional mutagenesis might be an approach to circumvent the lethality of Wnt5a-knockout mice. To date a floxed Wnt5a mouse has not yet been reported. For this approach, it would be important to first establish the cell types expressing Wnt5a in the testis. Results from Chapter 3 show that Wnt5a expression is most likely restricted to Sertoli cells in neonatal testes. However, to confirm the cell types that express Wnt5a during postnatal testis development, in situ hybridization for Wnt5a expression at various postnatal ages could be a first step. To target Wnt5a expression in Sertoli cells, floxed Wnt5a mice can be mated with Cre-expressing mice under the control of the anti-Müllerian hormone (AMH)

receptor gene. Under control of this promoter, Cre-specific expression in Sertoli cells has been demonstrated in previous reports [177]. In this way, Wnt5a can be selectively targeted in the SSC niche and SSC survival can be assessed over the course of postnatal development and into steady-state spermatogenesis. It should be noted that AMH is expressed by embryonic Sertoli cells but in situ hybridization for Wnt5a revealed that it did not colocalize with AMH-expressing Sertoli cells in 13.5 dpc embryonic gonads [162]. Nonetheless, $Wnt5a^{flox/flox}$ mice are expected to be viable and the selective knockout of this gene in Sertoli cells will reveal a role for Wnt5a in the survival of SSCs and help define it as an SSC factor in the testis.

The possibility exists that the effects of Wnt5a on SSC survival may only be detectable during recovery following injury, for example, from that caused by chemotherapy. Previous work has shown that expression of Wnt5a transcripts are upregulated in Sertoli cells in response to busulfan treatment [181,182], raising the possibility that it may play an important role in germ cell survival or recovery. Therefore, busulfan treatment of $Wnt5a^{flox/flox}$ mice may reveal defects in SSC response to injury and recovery of spermatogenesis. Moreover, as a positive approach the overexpression of Wnt5a in Sertoli cells or injections of recombinant Wnt5a into mice prior to busulfan treatment may be a means to improve SSC survival following chemotherapeutic treatment. Recently, it was shown that the kinetics of male fertility restoration after treatment with busulfan correlates with SSC recovery in mice; greater SSC survival immediately following busulfan treatment resulted in more robust SSC proliferation [271], meaning that a subtle improvement in SSC survival after busulfan treatment can manifest as a more rapid recovery time to fertility. Therefore, the fertility recovery kinetics of Wnt5a-null and Wnt5a-injected mice following busulfan treatment should be assessed by mating each male with a new set of female mice on a weekly basis.

Another way to examine how a loss in Wnt5a signalling affects SSC survival in the testis would be to knockdown putative Wnt5a receptors from SSCs. Based on the results presented in Chapter 3, potential Wnt5a receptors expressed on SSCs are Fzd3, 5, 7, and Ror2. Fzd3 and Ror2 mutant animals have

been described as neonatal lethal as they show defects in either central nervous system development or somitogenesis, respectively [155,272]. Interestingly, Ror2 knockout mice are observed to mimic the phenotype of Wnt5a knockout mice, including abnormally short limbs and dysplastic genitalia, attesting to the faithfulness of this receptor to mediate Wnt5a signalling [155]. Both $Fzd3^{-/-}$ and $Ror2^{-/-}$ animals have no reported defect in spermatogenesis or testis development. Because both of these genetic mutants die at birth, SSCs can be harvested from their testes at birth and transplanted into wildtype recipient testes to assess their ability to colonize and regenerate spermatogenesis. As for Fzd5 and Fzd7; Fzd5 loss-of-function mutant mice are embryonic lethal due to a yolk sac defect [273], while Fzd7 knockout mice have not yet been generated. Therefore, one potential method to knockdown the expressions of Fzd5 and Fzd7 from SSCs would be to use RNAi against these receptors in cluster cells. In this way, these Fzd5- or Fzd7-knockdown SSCs can be transplanted into recipient testes to determine if they can colonize and regenerate spermatogenesis. Of course, the possibility exists that the Wnt receptors expressed on SSCs might show functional redundancy to mediate the Wnt5a signal. Therefore, the use of RNAi in cluster cells might be a handy approach to knockdown the expression of multiple Wnt receptors on SSCs.

Wnt5a was not the only prototypical non-canonical Wnt detected in vitro and in the testis. Wnt6 and Wnt11 were both robustly detected throughout all postnatal ages suggesting that they might be important spermatogenesis factors. However, their biological function in testes remains unknown. $Wnt6^{-/-}$ mice do not show an abnormal phenotype [274]. Wnt11 is required for convergent-extension movements during Zebrafish and Xenopus gastrulation [140,275]. This effect does not involve β-catenin signalling but rather acts through the Wnt/PCP pathway to regulate cytoskeletal rearrangements and polarized cell movements during vertebrate gastrulation. Similar to Wnt5a, Wnt11 has also shown the ability to activate the JNK cascade [276]. $Wnt11^{-/-}$ mice are neonatal lethal, dying by 2 dpp [99]. These mutant mice present with defects in cardiogenesis and ureteric duct formation in the developing kidney. Interestingly, Wnt11 has been

characterized to coordinate with GDNF to regulate ureteric morphogenesis during embryonic kidney development [99]. During this process, Wnt11 is expressed in the growing ureteric epithelium where it promotes the expression of GDNF from the adjacent mesenchyme, which in turn stimulates growth of the ureteric epithelium. In the absence of Wnt11, GDNF expression is significantly reduced, while conversely Wnt11 expression is reduced in the absence of GDNF signalling. Therefore, reciprocal interaction between Wnt11 and GDNF acts in a feedback manner to ensure the controlled development of the kidney. In the testis, Wnt11 expression is detected throughout postnatal development and in cryptorchid testes implying that Wnt11 may be a somatic or spermatogonia-expressed factor (Chapter 3). Therefore, because Wnt11 may be expressed by Sertoli cells, an approach similar to my study of Wnt5a can be used to assess a potential role of Wnt11 in SSC maintenance. Moreover, in the event Wnt11 is expressed by spermatogonia, the possibility exists that Wnt11 may act to communicate with other germ cells, including SSCs, or with Sertoli cells to potentially regulate their expression of secreted factors, such as GDNF.

Identifying factors that promote SSC commitment to differentiation

Another fundamental question regarding SSCs is: what mechanisms define SSC differentiation? That is, at what point can a cell no longer act as an SSC but rather is fated to differentiate? Currently, a handful of factors important for the differentiation of spermatogonia have been characterized. For example, c-Kit signalling is important for the proliferation and survival of differentiating type A spermatogonia and in general c-Kit^{+} cells in the testis do not show SSC ability [3]. More recent work has shown dynamic changes in EPCAM expression resulting in upregulated expression of EPCAM as SSCs differentiate [86]. Transient knockdown of EPCAM in clusters reduces differentiated cell numbers in vitro but a role in the testis has not been explored. In terms of intracellular mediators of differentiation, knockdown of the intracellular mediator STAT3 results in an inability to differentiate past Apr – Aal spermatogonia stage [129], while the germ cell-specific knockout of the Foxo family of transcription factors results in the

catastrophic loss of differentiated germ cell types in the testis [130]. However, because SSCs cannot be prospectively identified currently from the earliest committed cells, it is unknown what factors mediate early commitment from SSCs.

In Chapter 3, I observed that β-catenin signalling cluster cells had virtually no SSC activity, indicating that activation of this signalling might represent the loss of SSC ability. Moreover, when I added Wnt3a, I observed that β-catenin signalling was active in a subset of cluster cells, TCF/LEF-*lacZ* dim and high cells, which had less SSC activity again indicating that they were non-SSC progenitor cells. Therefore, it is possible that by adding Wnt3a and activating the β-catenin pathway in a subset of cluster cells, Wnt3a might artificially push these cells into differentiation.

As the global gene expression analysis in my Supplemental Chapter showed, all cells in a cluster are transcriptionally similar. However, subtle differences in gene expression were detectable between TCF/LEF-*lacZ*^{neg} and TCF/LEF-*lacZ*$^{+}$ populations. Several transcription factors were observed to be upregulated in TCF/LEF-*lacZ*$^{+}$ cells, including, v-Fos ongene b (FosB; 3.4-fold), retinol binding protein 1 (Rbp1; 2.7-fold), an ETS-related transcription factor (Ets2; 2.5-fold), and Tcf4; 2.1-fold.

In the same manner, a global gene expression analysis may detect subtle differences between Wnt3a-stimulated TCF/LEF-*lacZ* negative, dim, and high populations. Through this discovery approach, it may be possible to identify potential mediators associated with early commitment away from SSCs. This dataset can be compared to that found in the Supplemental Chapter to identify common targets. Once these potential targets of early differentiation are identified, they should be each transiently silenced, using siRNA in clusters, and after some time in vitro these clusters can then be transplanted into recipient testes. This strategy will determine what effect loss of these mediators has on differentiated cells in vitro; similar to the study of EPCAM on differentiated cells [86], if silencing these mediators blocks differentiation then I would expect that the concentration of SSCs in a cluster will increase. As another approach,

potential mediators can be stably knocked-down in cluster cells using shRNA delivered by lentiviral vectors and these cluster cells can be then transplanted into recipient testes. Similar to the report identifying STAT3 in SSC differentiation [129] if the loss of target mediators leads to an inability for SSCs to commit to differentiation, spermatogenesis will not proceed and colonies of spermatogenesis will not form. Suppression of differentiation factors should not affect SSC ability to maintain in the niche. Therefore, these cells should present as individual A_s spermatogonia located along basal layer of the tubule and can be identified in wholemount tubules or in histological sections.

Finally, as a positive approach, potential mediators of differentiation can be expressed in cluster cells to see if they result in a loss of SSC activity. To this end, target mediators can be placed under the control of the EF-1α promoter for constitutive expression and transfected into clusters. In this way, it will be interesting to determine the factors that comprise the core regulators of the earliest SSC commitment steps.

Discovery of novel SSC cell-surface markers

To date, definitive markers distinguishing SSCs from other undifferentiated spermatogonia have not been characterized. Proteins expressed on the cell surface of SSCs have been described but these are also expressed on other non-SSC spermatogonia. Clusters are undifferentiated spermatogonia and my gene expression data, comparing TCF/LEF-*lacZ* signalling and non-signalling cells, demonstrates that all cells in a cluster are quite similar at the gene expression level. However, subtle differences are noted and may reflect subtle differences between the cell-surface phenotypes of SSCs and differentiated cells. Distinguishing SSCs from the earliest committed cells might finally lead to the ability to purify SSCs.

From the data present in my Supplementary Chapter and Appendix I, several targets were found, upregulated in the non-SSC TCF/LEF-*lacZ*$^+$ population, which demarcates committed cells. For example, Ly6a (Sca1; 5.1-fold)) is an HSC marker that has been shown to be a negative marker of SSCs

from cryptorchid testis [3]. Ifitm3 (4.0-fold) is similar to Fragilis, which is an early marker of the germ cell lineage that is fated to become PGCs. Co-receptors to vascular endothelial growth factor (VEGF) are found (NRP1; 4.7-fold and VE-Cadherin; 2.5-fold) as well as the tight junction protein Claudin5 (Cldn5; 4.0-fold) and the Notch pathway receptor Notch 4 (2.3-fold). To determine if these targets are specifically expressed on non-SSCs, clusters can be stained using fluorescently-labelled antibodies against each of these targets, and target-expressing and non-expressing cells can be separated using FACS. The SSC activity of each population can then be examined using spermatogonial transplantation. Subsequently, to confirm expression in differentiated cells, these markers can be screened using primary testes cells, from various postnatal ages. Currently, the most stringent marker to enrich for SSCs is Thy1, however as mentioned, it is expressed on other non-SSC spermatogonia [3]. Therefore, the expression of these newly identified markers can be compared to Thy1 expression in an attempt to further purify SSCs from committed cells in the $Thy1^{+}$ population.

Finally, in Chapter 4 it was observed that Wnt3a stimulated non-SSC differentiated cells in clusters and might artificially induce differentiation in vitro. Therefore, a comparison of the TCF/LEF-*lacZ* negative, dim and high populations described in Chapter 4 may also identify prospective markers that are gradually expressed as SSCs are fated to differentiation. To this end, TCF/LEF-*lacZ* clusters can be treated with Wnt3a and these populations can be separated by FACS. Because TCF/LEF-*lacZ* dim and high cells were mostly non-SSCs, a global gene expression analysis of these populations compared to the TCF/LEF-*lacZ* negative population can potentially reveal what cell-surface targets are gradually expressed as SSCs differentiate. Furthermore, this dataset can also be compared to that reported in my Supplemental Chapter and Appendix I to potentially unveil common cell-surface targets that might be useful to purify SSCs from non-SSC progenitor spermatogonia.

Clinical application of SSCs

The findings of this thesis are another step towards developing human SSC transplantation as a safe and viable fertility restoration technology. SSCs are expected to become an important resource to restore male fertility in cancer survivors. A clinical application of SSCs would involve the following paradigm: A testes biopsy from a cancer patient would be harvested prior to sterilizing chemotherapy and the SSCs could be transplanted back following clearance from the disease to restore fertility (Fig. 1).

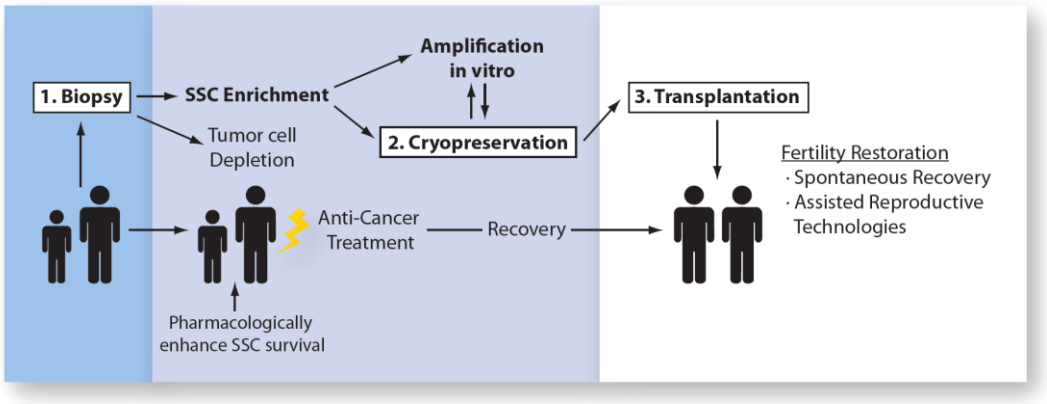

Figure 1: Proposed scheme of SSC-based fertility restoration for cancer patients.

One of the greatest weaknesses to this strategy is the sheer lack of SSCs to efficiently restore fertility following transplantation. As mentioned, the percentage of SSCs in the testis is small (0.01% of all germ cells in mouse testis) and homing efficiency of SSC is low (12%) [70]. Assuming that a recovered biopsy from a prepubertal testis will be small, it can be inferred that very few SSCs will be available for transplantation. Therefore, two strategies can be used to increase the effectiveness and safety of transplantation: 1) Enrichment using SSC markers to concentrate SSCs and eliminate any tumorigenic cells present in the cell preparation and 2) using ex vivo expansion of SSCs to increase their number.

Human SSC enrichment

A leukemic cell-depletion strategy has been demonstrated before by selecting for MHC-I and CD45 in a mouse testis sample [88]. In order to enrich and concentrate human SSCs from a testis biopsy, surface markers for these cells have to be identified. The ideal strategy mimics that of rodents, that is, antibodies against the marker of interest and isolation of expressing and non-expressing populations using immunomagnetic separation or FACS. Finally, these populations would be assessed for SSC activity through the transplantation assay. Transplantation into human testes would present the ideal environment to regenerate spermatogenesis from human SSCs but is evidently not possible for experiments. As mentioned, human SSCs can colonize and survive in immune-compromised mouse testes [65]. Therefore, this presents a suitable alternative. To date, several markers expressed on mouse SSCs appear to be conserved on human SSCs, such as, α6-integrin, Thy1, GFRα1, and GPR125; although it should be noted that the expression of these markers on human SSCs is inferred by an ability to form in vitro clusters [277,278].

In vitro amplification of human SSCs

Recently, in vitro culture conditions were defined for human germ cells, in which they could be maintained for 15 – 20 weeks. Under these conditions, germ cells formed cluster-like accumulations [279]. This study used testicular somatic cells and human placenta-derived laminin as a feeder layer, however, fetal-calf serum was required in the culture media. An important consideration with the clinical application of SSCs is the use of animal-derived components which have the potential to transmit foreign pathogens in the patient. Studies with animal SSCs have used animal derived fibroblasts as feeder cells, which are unsuitable for the clinical application of human SSCs. In Chapters 3 and 4, I characterized a role for Wnt signalling in SSC regulation. These morphogens improved SSC survival and proliferation under feeder-free conditions and may be an important consideration for completely non-xenobiotic human SSC expansion. In particular, the addition of Wnt3a was observed to stimulate progenitor cells, which promoted

communication among germ cells in vitro and led to improved SSC maintenance. Thus, further study into human SSC culture should consider that survival of differentiated cells may be equally as important as directly pushing SSC maintenance.

Finally, besides autologous SSC transplantation following chemotherapy another strategy would be to pharmacologically stimulate the survival or proliferation of endogenous SSCs in the testis (Fig. 1). Wnt5a has potential in this regard since it can block cell death in vitro (Chapter 3) and the proposed in vivo studies with Wnt5a will help determine whether it can act as a survival factor in the testis. However, the discovery of other pathways that can push SSC proliferation will be important to stimulate endogenous human SSCs and for their amplification in vitro. The in vitro cluster-formation assay in Chapter 2 presents a rapid and easy assay for SSC activity. Recently, this assay has been expanded for use in high-throughput screening (HTS) to examine the effect of anti-cancer drugs on rat cluster cells [245]. In the same manner, clusters can screened against compounds listed in various chemical libraries containing a vast number of small molecules that can be individually added to SSC culture. Thus, in a similar manner to how anti-cancer drugs were assessed [245], the response of clusters to the small molecule library can be assessed using the HTS imaging system. Because cluster numbers indicate SSC activity, compounds that increase cluster numbers might promote SSC proliferation. Therefore, these particular compounds can be selected for further examination; compound-treated clusters can be transplanted to assess the extent of SSC proliferation. Furthermore, the effect of selected compounds on endogenous SSC proliferation and fertility recovery, following chemotherapeutic treatment, should also be examined. These studies would have a great impact into the discovery of factors stimulating SSC proliferation and the development of clinical applications for SSC-based male fertility restoration.

Conclusion

SSCs are defined by their functional ability to self-renew and to differentiate to form sperm. In this thesis, I examine the mechanisms of this functional ability utilizing an in vitro culture system that recapitulates long-term SSC self-renewal and early differentiation. These traits manifest as distinct societies of SSCs and committed cells, termed clusters. In Chapter 2, I observed that cluster numbers in vitro correlated with SSC numbers and I defined cluster formation as a short-term assay for SSC activity. With the aid of this novel SSC assay, I implicate Wnt signalling in SSC regulation. In Chapter 3, I demonstrate that Wnt5a supports SSC renewal by promoting survival. This occurs through a non-canonical Wnt signalling mechanism. In Chapter 4, Wnt3a was shown to activate the β-catenin pathway selectively in differentiated cells. Wnt3a stimulated the cell cycle and promoted cluster formation, which resulted in improved SSC maintenance. Notably, these results not only characterize a role for Wnt signalling in both SSC self-renewal and differentiation but also speak of the importance for communication among the germ cell community to finely regulate proper SSC numbers. These results suggest that this communication may mediate the function of the cluster community to act as an in vitro niche. Prior to these results, the thought of committed germ cells contributing to the niche regulation of SSCs had not been effectively shown. The preliminary results of my Supplemental Chapter revealed potential targets of this cluster cell communication, such as Dlk1, which pushed SSC differentiation. Therefore, committed cells in the cluster community may show potential to express regulators of SSC self-renewal and differentiation. From my results it can be speculated that the cluster community may each independently regulate proper numbers of SSCs per cluster, which to come full circle, can explain perhaps why cluster numbers correlate faithfully to SSC numbers, as observed in Chapter 2.

In closing, this body of work defines an original technology to study SSCs, characterizes novel factors regulating SSC fate decision, and presents potential markers to definitively identify SSCs, thereby furthering understanding into the biology of SSCs.

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APPENDIX I: GENE EXPRESSION DATASET

Table 1: Gene expression comparison between TCF/LEF-*lacZ*$^{+}$ and TCF/LEF-*lacZ*^{neg} cluster cells (paired SAM, 2-fold and greater)

Probe ID	Gene Symbol	TCF/LEF-*lacZ*$^{+}$ cells		TCF/LEF-*lacZ*^{neg} cells		Fold change	P-value
		Sample mean	Sample variance	Sample mean	Sample variance		
830397	PTN	11.8548	2.9707	8.4066	0.0314	**10.9152**	0.0113
2100402	BGN	12.4804	2.5675	9.1361	0.0334	**10.1563**	0.0102
2850020	DLK1	11.8705	2.7679	8.6455	0.0079	**9.3502**	0.0109
4260241	DLK1	13.8536	1.4255	10.7927	0.0139	**8.3451**	0.0053
5810598	COL6A3	11.1113	7.1996	8.0921	0.0056	**8.1074**	0.0456
5390328	DCN	11.3606	2.9416	8.3930	0.0086	**7.8225**	0.0115
7210041	COL6A1	11.0610	6.0415	8.2254	0.0462	**7.1384**	0.0358
4060102	COL4A1	12.2840	0.2057	9.4505	0.3276	**7.1279**	0.0033
3610082	CXCL1	11.1823	4.5450	8.4217	0.0000	**6.7767**	0.0336
5360685	IGFBP7	13.2718	0.0429	10.5532	2.4345	**6.5825**	0.0087
3170280	COL6A1	10.6201	4.2856	7.9787	0.0005	**6.2395**	0.0345
2070152	TIMP2	11.2256	0.8159	8.6260	0.0531	**6.0613**	0.0060
7160167	EMP1	12.1163	0.1592	9.5328	0.4223	**5.9938**	0.0048
2850575	VCAM1	10.3966	5.3670	7.8924	0.0296	**5.6733**	0.0461
5130148	SPARC	12.0818	0.0090	9.6094	0.7004	**5.5496**	0.0038
2260551	COL4A2	11.2392	0.2685	8.7886	0.2164	**5.4661**	0.0047
1690187	LY6A	10.9672	0.0136	8.6310	0.2132	**5.0496**	0.0004
4230228	FBLN2	10.1346	2.3508	7.8800	0.0072	**4.7720**	0.0207
6940187	NRP1	10.4389	2.4433	8.2042	0.0826	**4.7068**	0.0429
1940370	TMSB4X	13.0678	0.0251	10.8403	0.4969	**4.6831**	0.0044
360743	NRP1	10.4862	1.9965	8.2737	0.0513	**4.6348**	0.0316
1690091	VIM	10.7321	0.9254	8.5592	0.0242	**4.5094**	0.0104
3400747	IGFBP4	10.5402	1.8719	8.3744	0.0007	**4.4871**	0.0189
7380524	PRELP	9.9193	2.6732	7.7555	0.0013	**4.4808**	0.0315
1820601	IGFBP3	11.1793	0.0179	9.0321	0.3006	**4.4297**	0.0006
4280139	DCN	9.8060	3.1657	7.6844	0.0204	**4.3520**	0.0356
4610072	CAV1	11.0282	0.0440	8.9817	0.5688	**4.1310**	0.0083
1260482	SPARC	10.7138	0.0447	8.7002	0.2665	**4.0380**	0.0038
3450180	TMEM176B	10.0513	1.0362	8.0472	0.0508	**4.0115**	0.0195
2690025	GSTM2	9.9535	1.6665	7.9588	0.0467	**3.9854**	0.0127
3840292	IFITM3	12.4244	1.7135	10.4309	0.4394	**3.9818**	0.0028
430386	CLDN5	10.5859	1.6052	8.6033	1.1197	**3.9519**	0.0002
7510072	GSTM2	9.9807	1.7905	7.9998	0.0333	**3.9474**	0.0162
5900452	CYR61	9.8319	0.5565	7.8616	0.0960	**3.9186**	0.0125
1820224	MARCKS	10.9796	0.8967	9.0226	0.1578	**3.8824**	0.0263
6550681	ESAM	10.7622	0.4551	8.8271	0.5844	**3.8241**	0.0001
870670	PCOLCE	9.6852	2.7503	7.7551	0.0140	**3.8109**	0.0404
4010082	CTGF	10.6700	0.5144	8.7881	0.5439	**3.6855**	0.0000
2480059	ANXA5	10.7194	1.2942	8.8677	0.0437	**3.6092**	0.0315
2450347	SERPINA3N	9.5626	2.2820	7.7578	0.0096	**3.4939**	0.0389
5080435	IGFBP4	9.8274	1.6176	8.0333	0.0965	**3.4679**	0.0132
2650019	6330406I15RIK	9.4640	2.3402	7.6787	0.0022	**3.4468**	0.0458
6420520	FOSB	10.0976	0.0414	8.3290	0.0034	**3.4073**	0.0004
870333	AHNAK	11.0047	1.5075	9.2380	0.0030	**3.4026**	0.0247
1400309	TIMP3	9.7647	1.2863	8.0176	0.0014	**3.3568**	0.0213

Probe ID	Gene Symbol	TCF/LEF-*lacZ*$^{+}$ cells		TCF/LEF-*lacZ*^{neg} cells		Fold change	P-value
		Sample mean	Sample variance	Sample mean	Sample variance		
840209	6330406I15RIK	9.3406	2.3772	7.5984	0.0409	**3.3454**	0.0374
3290195	PRKCDBP	9.9197	0.2170	8.1861	0.0365	**3.3256**	0.0048
1710193	GJA1	10.4291	0.0251	8.7141	0.4139	**3.2829**	0.0022
270324	TNC	9.5260	0.9166	7.8629	0.0005	**3.1669**	0.0181
5720646	S100A4	9.5252	2.6978	7.8708	0.0698	**3.1479**	0.0472
4760224	CTGF	9.9368	0.4554	8.2975	0.2446	**3.1151**	0.0003
6060050	CD93	10.1506	0.0000	8.5165	0.0806	**3.1038**	0.0007
5960497	TGFBR2	10.6910	0.1022	9.0722	0.1619	**3.0713**	0.0081
460594	HSPB8	9.6523	1.2031	8.0458	0.0045	**3.0451**	0.0230
1580519	ANXA3	9.8352	0.5456	8.2425	0.0234	**3.0162**	0.0157
2510390	IDH2	9.6461	1.0739	8.0927	0.0029	**2.9352**	0.0223
3990053	F2R	11.8303	0.0489	10.2847	0.1280	**2.9193**	0.0050
7400725	MGP	9.2161	1.4078	7.6850	0.0009	**2.8900**	0.0425
5420333	TNC	9.2940	0.7147	7.7810	0.0001	**2.8541**	0.0155
5720523	S100A11	12.9427	1.8852	11.4395	0.0157	**2.8348**	0.0480
6110736	B2M	13.4712	0.6797	11.9799	0.0008	**2.8114**	0.0173
510050	SPARC	9.6698	0.9036	8.2106	0.4401	**2.7495**	0.0010
5090184	ANXA3	9.4773	0.4388	8.0197	0.0094	**2.7464**	0.0134
1070224	EMP3	9.4722	1.6853	8.0179	0.0419	**2.7403**	0.0373
3520546	EMP3	10.4907	1.7035	9.0368	0.0136	**2.7395**	0.0466
630041	SCARA3	9.0311	1.3556	7.5941	0.0024	**2.7075**	0.0410
2370520	AHNAK	9.5974	1.1952	8.1615	0.0000	**2.7055**	0.0383
450397	9130005N14RIK	9.1436	1.4214	7.7194	0.0005	**2.6837**	0.0475
6860609	RBP1	10.2035	0.0056	8.7919	0.0271	**2.6602**	0.0002
3940242	SAMD9L	9.3990	0.3453	7.9909	0.0669	**2.6539**	0.0205
2070274	APP	12.2273	0.2191	10.8199	0.2645	**2.6525**	0.0310
2630333	ROBO4	9.7348	0.6087	8.3409	0.5004	**2.6278**	0.0002
5890255	CD93	10.0132	0.0658	8.6195	0.3455	**2.6276**	0.0017
3520066	MMP14	11.4521	0.7202	10.0676	0.0899	**2.6109**	0.0493
4730685	PMP22	9.1904	0.9673	7.8167	0.0009	**2.5912**	0.0361
5900544	GPIHBP1	9.4583	0.5704	8.0925	0.1906	**2.5773**	0.0015
1030273	CDH5	9.7130	0.4996	8.3647	0.2908	**2.5462**	0.0005
2140450	1110012O05RIK	11.9541	0.5119	10.6111	0.1324	**2.5368**	0.0457
1660376	1110032E23RIK	9.4827	0.0008	8.1406	0.1548	**2.5352**	0.0035
6110519	ELK3	9.7942	0.1378	8.4540	0.0543	**2.5318**	0.0091
5570253	B2M	13.4222	0.7423	12.0940	0.0070	**2.5109**	0.0333
7510154	ETS2	11.0462	0.0338	9.7245	0.0011	**2.4995**	0.0008
3420451	ESM1	9.6602	0.0316	8.3387	0.1072	**2.4994**	0.0004
4220280	GPIHBP1	9.4414	0.3054	8.1200	0.2924	**2.4990**	0.0000
3390243	CPE	8.9842	1.1248	7.6677	0.0055	**2.4905**	0.0385
7040201	THY1	9.6410	1.5180	8.3247	0.1662	**2.4903**	0.0235
1470605	RNF213	9.5497	0.7868	8.2416	0.1420	**2.4762**	0.0066
5080326	S100A6	11.0694	1.0343	9.7627	0.0044	**2.4737**	0.0355
270681	E030026I10RIK	9.7137	0.9540	8.4171	0.0070	**2.4565**	0.0486
6940278	BMP1	9.4720	0.1723	8.1792	0.0002	**2.4501**	0.0040
4040037	TPM4	11.0495	0.4141	9.7616	0.0168	**2.4417**	0.0071
1410543	LOX	8.9429	0.3234	7.6553	0.0042	**2.4412**	0.0122
3890059	CYR61	8.8403	0.3454	7.5648	0.0022	**2.4208**	0.0127
1500102	ACSL5	9.5957	0.6715	8.3366	0.0288	**2.3933**	0.0142
620647	2210013O21RIK	10.4587	0.2053	9.2065	0.3188	**2.3821**	0.0483
160402	OGN	8.8799	0.0032	7.6354	0.0305	**2.3694**	0.0011

4280402	EPHX1	9.1112	0.3144	7.8674	0.0000	**2.3682**	0.0095
Probe ID	**Gene Symbol**	**TCF/LEF-*lacZ*$^{+}$ cells**		**TCF/LEF-*lacZ*^{neg} cells**		**Fold change**	**P-value**
		Sample mean	**Sample variance**	**Sample mean**	**Sample variance**		
5270021	EMCN	9.2739	0.3201	8.0340	0.2826	**2.3619**	0.0001
6450372	FGF7	8.7794	1.0870	7.5423	0.0018	**2.3573**	0.0479
4250059	NOTCH4	10.3374	0.0540	9.1098	0.1872	**2.3419**	0.0009
3800215	0610031J06RIK	9.1732	0.0945	7.9507	0.1244	**2.3336**	0.0164
6380379	PON2	10.1510	0.0656	8.9353	0.9012	**2.3225**	0.0192
4540372	LOC100046802	9.4795	0.1600	8.2662	0.1367	**2.3186**	0.0001
4900519	SGK1	10.9056	0.0060	9.6930	0.1189	**2.3176**	0.0016
5550671	LY6C1	9.4044	0.0982	8.2003	0.0549	**2.3039**	0.0003
1450491	SERPINA3H	8.8361	0.6531	7.6357	0.0004	**2.2981**	0.0281
5670634	SYNPO	10.4734	0.2551	9.2766	0.0492	**2.2924**	0.0020
7050446	LOC100046883	9.9806	0.1168	8.7886	0.0482	**2.2847**	0.0113
3370279	E030026I10RIK	9.6812	0.5153	8.5033	0.0016	**2.2625**	0.0269
4480333	PPIC	12.1253	0.2217	10.9512	0.0385	**2.2566**	0.0195
6590167	FCGRT	9.5111	0.1097	8.3382	0.0007	**2.2548**	0.0036
4210538	PEA15	9.5818	0.0000	8.4116	0.0098	**2.2504**	0.0003
610019	ANXA6	10.2916	0.1985	9.1291	0.0046	**2.2385**	0.0042
6370020	SCL0001849.1_2273	8.7589	0.7585	7.6025	0.0000	**2.2291**	0.0408
6840180	S100A10	10.0615	0.5562	8.9090	0.0160	**2.2231**	0.0417
6770356	MEST	10.5867	0.6820	9.4370	0.0195	**2.2187**	0.0222
7040243	D0H4S114	9.9574	0.0000	8.8111	0.0512	**2.2135**	0.0014
10167	EG630499	11.4615	0.9218	10.3588	0.0190	**2.1475**	0.0410
4060075	NFIB	9.6648	0.6649	8.5700	0.0004	**2.1359**	0.0434
6350086	SLC6A8	8.7611	0.4497	7.6709	0.0102	**2.1291**	0.0161
3870072	MMP2	9.7794	0.6290	8.6945	0.2594	**2.1212**	0.0028
4480364	AEBP1	10.7166	0.2533	9.6345	0.0013	**2.1171**	0.0097
4610451	GHR	8.9915	0.5192	7.9121	0.0129	**2.1133**	0.0450
5090156	MBNL1	10.1638	0.3264	9.0881	0.0012	**2.1078**	0.0201
4920288	IGFBP3	9.0096	0.5557	7.9348	0.0122	**2.1065**	0.0488
4150678	0610007C21RIK	11.2382	0.2980	10.1657	0.0427	**2.1030**	0.0353
7550360	TCF4	11.2260	0.0220	10.1538	0.0762	**2.1027**	0.0079
2900592	SFXN1	9.4930	0.4558	8.4259	0.0579	**2.0952**	0.0084
6940577	TSPAN6	9.1129	0.0235	8.0530	0.0005	**2.0847**	0.0006
430132	LGALS9	8.8215	0.0520	7.7621	0.0261	**2.0842**	0.0067
2340181	LOC100048710	8.9896	0.0337	7.9375	0.1020	**2.0736**	0.0008
770347	MAML2	8.6850	0.3809	7.6358	0.0200	**2.0693**	0.0387
5700446	B230343A10RIK	8.6784	0.6187	7.6473	0.0001	**2.0435**	0.0435
7510452	ICAM2	8.9822	0.2118	7.9521	0.2379	**2.0421**	0.0002
5810398	RNASE4	8.7445	0.1538	7.7246	0.0105	**2.0278**	0.0139
1300747	LOC100046120	9.2133	0.6336	8.1986	0.0036	**2.0205**	0.0396
430451	MMRN2	9.3333	0.0502	8.3196	0.0520	**2.0190**	0.0001
2710446	0610007C21RIK	11.6129	0.1827	10.6005	0.0220	**2.0172**	0.0214
4180577	SFXN1	9.3259	0.7610	8.3157	0.1081	**2.0141**	0.0185
6200189	RNPEPL1	9.1395	0.0139	8.1323	0.0436	**2.0099**	0.0050
1820148	SYDE1	8.5909	0.3150	7.5858	0.0005	**2.0071**	0.0222