MECHANISMS OF SPERMATOCHORIAL STEM CELL DIFFERENTIATION

A Thesis in

Animal Science

by

Amy V Kaucher

©2010 Amy V Kaucher

Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science

December 2010
The thesis of Amy V Kaucher was reviewed and approved* by the following:

Jon M. Oatley
Assistant Professor of Reproductive Physiology
Thesis Advisor

Francisco J. Diaz
Assistant Professor of Reproductive Biology

Troy L. Ott
Associate Professor of Reproductive Physiology

Daniel Hagen
Professor of Animal Science
Chair of Animal Sciences Graduate Program Committee

*Signatures are on file in the Graduate School.
ABSTRACT

In mammalian testes, the actions of spermatogonial stem cells (SSC) provide a foundation for spermatogenesis during adult life. Like other tissue-specific stem cell populations, SSC initiate differentiation while also maintaining a cohort of undifferentiated stem cells via self-renewal. Decisions to self-renew or differentiate are controlled both extrinsically and intrinsically. These decisions must be tightly controlled, as dysregulation can lead to loss of tissue homeostasis, infertility or cancer. Currently, understanding of mechanisms regulating SSC fate decisions, particularly differentiation, is limited. Therefore, the goal of the studies herein was to identify and examine molecular factors required for fate decisions of SSC. Two proteins, signal transducer and activator of transcription 3 (Stat3) and Neurogenin 3 (Ngn3), were investigated. Stat3, a transcription factor, is part of the Jak/Stat signal transduction pathway. The Drosophila Stat3 homolog, Stat92E is necessary for self-renewal germline stem cells. Also, embryonic stem cells require Stat3 signaling to maintain pluripotency. Ngn3 is a transcription factor needed for the differentiation of neurons and β-cell progenitors. In the mammalian germline, expression of Ngn3 coincides with early germ cell differentiation. Furthermore, in rodents, the cytokine Glial cell line-derived neurotrophic factor (Gdnf) regulates SSC self-renewal and Ngn3 is known to be a Gdnf down-regulated gene. Despite this, to our knowledge, the functional roles of Stat3 or Ngn3 have yet to be examined in SSC fate decisions.

In Chapter II: Regulation of SSC differentiation by Stat3, the objective was to determine the effect of Stat3 mRNA reduction on SSC fate decisions. To address the objective, Stat3-specific shRNA was used to permanently reduce Stat3 expression in cultured Thy1+ germ cells. After functional germ cell transplantation, it was apparent that short hairpin RNA-mediated stable reduction of STAT3 expression in cultured SSCs abolished SSC ability to differentiate beyond the undifferentiated
spermatogonial stage following transplantation into recipient testes. Therefore, these results demonstrate that STAT3 promotes the differentiation of SSC.

The objective of Chapter III: Neurogenin 3 is a regulator of mouse spermatogonial stem cell differentiation was to determine if NGN3 plays a functional role in SSC differentiation. To address this, we studied the SSC-enriched Thy1+ germ cell population, which is also composed of non-SSC spermatogonia produced by differentiation. Withdrawal and replacement of Gdnf from cultured THY1+ germ cells resulted in an increase followed by down-regulation of Ngn3 gene expression, indicating that Ngn3 transcription is repressed by GDNF signaling. Within the THY1+ germ cell population, NGN3 expression was found in a sub-fraction of cells both in vivo and after long-term culture. Transient reduction of Ngn3 expression in cultured THY1+ germ cells by siRNA treatment increased SSC content after multiple self-renewal cycles without impacting spermatogonial proliferation overall, suggesting alteration of the balance between SSC fate decisions in favor of self-renewal. Lastly, it was found that the SSC differentiation factor STAT3 binds the Ngn3 promoter to regulate Ngn3 transcription in THY1+ germ cells. Collectively, these results indicate NGN3 plays a key role in regulating differentiation of SSCs via a STAT3-mediated mechanism.

Collectively, the data of these two studies indicate that Stat3 and Ngn3 work in conjunction to regulate SSC differentiation.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xi</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>xii</td>
</tr>
<tr>
<td>CHAPTER 1: REVIEW OF LITERATURE</td>
<td>1</td>
</tr>
<tr>
<td>STEM CELL BIOLOGY</td>
<td>1</td>
</tr>
<tr>
<td>Importance of studying stem cells</td>
<td>1</td>
</tr>
<tr>
<td>Classification of stem cells</td>
<td>2</td>
</tr>
<tr>
<td>SPERMATOGENESIS</td>
<td>3</td>
</tr>
<tr>
<td>Spermatogenesis in adults</td>
<td>3</td>
</tr>
<tr>
<td>Spermatocytogenesis</td>
<td>4</td>
</tr>
<tr>
<td>Spermatidogenesis</td>
<td>6</td>
</tr>
<tr>
<td>Spermiogenesis</td>
<td>7</td>
</tr>
<tr>
<td>First round of spermatogenesis</td>
<td>7</td>
</tr>
<tr>
<td>Inter-species differences</td>
<td>8</td>
</tr>
<tr>
<td>NICHE MICROENVIRONMENT</td>
<td>9</td>
</tr>
<tr>
<td>Sertoli cells</td>
<td>10</td>
</tr>
<tr>
<td>Interstitium</td>
<td>11</td>
</tr>
<tr>
<td>Leydig cells</td>
<td>12</td>
</tr>
<tr>
<td>Myoid cells</td>
<td>12</td>
</tr>
<tr>
<td>Vasculature</td>
<td>13</td>
</tr>
</tbody>
</table>
Production of Stat3 shRNA lentivirus ................................................................. 46
Stat3 reduction inhibits SSC differentiation in vivo ........................................... 47
DISCUSSION ........................................................................................................... 47
REFERENCES ........................................................................................................ 56
CHAPTER 3: NGN3 IS A REGULATOR OF MOUSE SSC DIFFERENTIATION IN VITRO .............................................................. 58
ABSTRACT ............................................................................................................. 58
INTRODUCTION ..................................................................................................... 59
METHODS ............................................................................................................. 61
Animals .................................................................................................................. 61
Isolation and culture of Thy1+ germ cells ............................................................. 62
Quantitative PCR analyses .................................................................................. 63
Western blot analyses .......................................................................................... 64
Fluorescent immunocytochemistry ...................................................................... 65
Transfection of cultured Thy1+ germ cells with siRNA oligonucleotides .............. 66
Germ cell transplantation and colonization analyses ............................................. 67
Chromatin immunoprecipitation .......................................................................... 68
Statistical analyses ............................................................................................... 69
RESULTS .............................................................................................................. 69
Ngn3 expression is down-regulation by Gdnf in cultured Thy1+ germ cells .......... 69
Ngn3 is expressed by a subpopulation of Thy1+ germ cells in vivo ..................... 70
Ngn3 is expressed by a subpopulation of Thy1+ germ cells in vitro ..................... 71
Ngn3 gene expression is regulated by the SSC differentiation factor Stat3 ........... 73
LIST OF FIGURES

Figure 1: shRNA action within a mammalian cell ................................................................. 51-52

Figure 2: Reduction of Stat3 by shRNA lentivirus ............................................................... 53

Figure 3: Permanent reduction of Stat3 reduces SSC capacity to differentiate in vivo .......... 54-55

Figure 4: Effects of Gdnf stimulation on Ngn3 mRNA abundance in cultured Thy1+ germ cells ........ 78

Figure 5: Relative Ngn3 mRNA abundance in the Thy1+ germ cell fraction of mouse testes .... 79-80

Figure 6: Effects of reducing Ngn3 mRNA abundance on SSC self-renewal in cultured Thy1+ germ cell populations ............................................................ 81

Figure 7: Reduction in Stat3 reduces Ngn3 mRNA abundance in Thy1+ germ cells ............. 82-83
I would like to acknowledge...

...my advisor, Dr. Jon Oatley, for his mentorship, for his patience, for challenging me and for giving me this opportunity. I learned much as a part of his lab group, none of which will soon be forgotten.

...my committee members, Drs. Francisco Diaz and Troy Ott, for their efforts and assistance.

...Drs. Jack Vanden Heuvel and Jerry Thompson for the use of their sonicator and assistance with ChIP assay optimization.

...the past and present members of the Oatley lab for supporting, brain-storming/trouble-shooting, laughing and commiserating with me.

...the faculty, staff and students of the Department of Animal Sciences for their support and kind words of encouragement along the way.
DEDICATION

This thesis is dedicated to my loved ones

– family and friends –

for their continuous support and unconditional love.
CHAPTER 1: REVIEW OF LITERATURE

STEM CELL BIOLOGY

Stem cells are defined by their ability to both maintain an undifferentiated stem cell population; as well as, give rise to specialized cells via differentiation (Morrison et al., 1997). The term stem cell was first used in 1868 by German biologist Ernst Haeckel in support of Darwin’s theory of evolution. In the late 19th century the term was applied to embryonic development (Haeckel, 1877) and hematopoiesis (Ehrlich, 1879). Since the inception of the term ‘stem cell’, effort has been dedicated to understanding these cells.

Importance of studying stem cells

The intrigue generated by the field of stem cell biology is not unsupported as these cells are required for development and maintenance of life; therefore, understanding the function of stem cells is of utmost importance. Treatments for diseases and infertility have been and remain to be found through stem cell biology. First, clinical treatments for diseases have been and remain to be discovered. For example, understanding the hematopoietic system, which is based on hematopoietic stem cells (HSC) has allowed for clinic success in developing bone marrow transplantation (Till and McCulloch, 1961; Till et al., 1964). Second, the field of regenerative medicine is grounded in stem cell research. Producing tissues, organs and limbs has been the goal of regenerative medicine. Through manipulation of stem cells biomimetic tissue for skin grafting, partial liver transplantation and heart valve transplants could one day be produced (Hodgkinson et al., 2009). Third, researchers have found that some forms of cancer, such as breast (Rudland and Barraclough, 1988) and prostate (van Leenders and
Schalken, 2001), are caused by the dysregulation of stem cells. Understanding these populations of cells would provide more effective treatments for some forms of cancer (Iwasaki and Suda, 2009). Additionally, studying germline stem cells could aid in treating infertility, preserving fertility in patients undergoing chemotherapy or providing a means for in vitro sperm production (Ogawa, 2008; Schlatt et al., 2009; Wyns et al., 2010). However, this technology can only be applied to males, because the female germline does not maintain stem cell identity into adulthood. Therefore, comprehending how stem cell populations function and why they function in a particular manner can be a means for correcting dysregulation.

Classifications of stem cells

Stem cells are broken into four classifications: omnipotent, pluripotent, multipotent and unipotent. Omnipotent stem cells are cells that are capable of forming a conceptus. A zygote is the classic example of an omnipotent stem cell. A zygote differentiates to become not only all three embryonic germ layers, ectoderm, endoderm, and mesoderm; but also forms the trophectoderm. The trophectoderm develops into the trophoblast, the peripheral cells of the conceptus that attach to the uterine wall. Pluripotent stem cells only differentiate to become cells of the three germ layers. Embryonic stem cells are a classic example of pluripotent stem cells. Multipotent stem cells are capable of becoming more than one cell type of a specific lineage but cannot differentiate into cell types outside of that lineage. For example, hematopoietic stem cells (HSC) have the capability to differentiate into erythrocytes, granulocytes, lymphocytes, monocytes and thrombocytes (Fifth report of the committee for clarification of the nomenclature of cells and diseases of the blood and blood-forming organs,
1950). However, HSC are incapable of becoming cells of different lineages such as neurons or epidermis. Finally, a unipotent stem cell has the capacity to become only one cell type.

Spermatogonial stem cells are an example of unipotent stem cells, because differentiation of SSC culminates in the production of spermatozoa (de Rooij and Russell, 2000). These four stem cell potencies are the foundation for embryonic development as well as adult tissue homeostasis.

Another classification of stem cells, tissue-specific stem cells, are undifferentiated cells that reside in tissues to regulate homeostasis of the tissue or to recover function of the tissue after injury. Organs and tissues supported by tissue-specific stem cell populations include hematopoietic (Alenzi et al., 2009), hepatic (Schwartz and Verfaillie, 2010), intestinal (van der Flier and Clevers, 2009), neuronal (Duan et al., 2008), pancreatic (Jun, 2008) retinal (Djojosubroto and Arsenijevic, 2008) and testicular (Phillips et al., 2010) tissue. Characteristically multi- or unipotent, tissue-specific stem cells differentiate to form a single cell type or multiple cell types of a single lineage. A well-characterized tissue-specific stem cell system based on multipotent stem cells is the hematopoietic system. HSC differentiate to become all cellular components of blood. An example of unipotent stem cell population is spermatogonial stem cells (SSC), which support spermatogenesis (de Rooij and Russell, 2000). Without SSC, fertility is lost due to the inability to produce germ cells. Therefore, tissue-specific stem cell populations are important for maintenance and survival of most tissues.
SPERMATOGENESIS

Spermatogenesis in adults

Conversion of a single diploid spermatogonium into mature haploid spermatozoa is referred to as spermatogenesis. Germ cell differentiation and specialization are the main objectives of spermatogenesis, where germ cells are transformed into an efficient vehicle for transmitting genetic material. During spermatogenesis one spermatogonium has the capacity to multiply and differentiate to form over 4,000 spermatozoa capable of fertilization (Russell et al., 1990). Undifferentiated germ cells reside on the basement membrane of the seminiferous tubule. As germ cells differentiate, they move away from the basement membrane, closer to the lumen of the seminiferous tubule (Russell et al., 1990). Once spermatogenesis is complete, the transformed germ cell is released into the lumen of the tubule and transported away from the testis for further maturation. Spermatogenesis can be divided into three portions: spermatocytogenesis, spermatidogenesis and spermiogenesis.

Spermatocytogenesis

During spermatocytogenesis, the first phase of spermatogenesis, SSC differentiate into germ cells committed to differentiation and these lineage-committed cells proliferate to increase the spermatogenic pool. The most undifferentiated germ cells residing in adult seminiferous tubules lie on the basement membrane and are referred to as A-single (A_s) spermatogonia (Russell et al., 1990). The stem cell potential of germ cells is thought to be contained within this population of cells. However, recent studies have demonstrated that heterogeneity exists within the A-single spermatogonial population, possibly authenticating
existence of both stem and non-stem cell A_s spermatogonia (Nakagawa et al., 2010; Yoshida et al., 2007b). In 2000, de Rooij and Russell suggested that A_s spermatogonia, which undergo cytokinesis and presumably retain stem cell activity, be labeled as undifferentiated spermatogonia; whereas all other spermatogonia, which are connected by intercellular bridges, should to be considered differentiating. However, the dogma refers to A_s, A-paired (A_{pr}), A-aligned (A_{al}) spermatogonia as undifferentiated, A1-A4 are considered differentiating spermatogonia and Intermediate and type-B are regarded as differentiated spermatogonia (de Rooij and Russell, 2000). At some point, in response to either an intrinsic signal or extrinsic stimuli from the surrounding niche microenvironment, a SSC loses its “stemness” and differentiation is initiated. However, the factors required for initiation of SSC differentiation have not been elucidated. As differentiation progresses, A_s cells undergo mitosis but do not complete cytokinesis to form A-paired (A_{pr}) spermatogonia, which are connected via intracellular bridges, indicating synchrony between these germ cells (Dym and Fawcett, 1971). Therefore, A_{pr} spermatogonia (and all subsequent levels of differentiating/differentiated spermatogonia, which are formed by rounds of mitotic divisions) are connected by intracellular bridge(s), which are open channels 1-3 μm in diameter between germ cells (Weber and Russell, 1987). Intercellular bridges are formed by arrest of cytokinesis and allow for intercytoplasmic transport of regulatory substances and even organelles throughout interconnected germ cell clones (Morales et al., 2002; Vantela et al., 2003). As A_{al} germ cells transition into A-1 spermatogonia, c-Kit expression is once again upregulated, as it was during the gonocyte stage (Yoshinaga et al., 1991; Orth et al., 1996). At this point, each mitotic division leads to progression to next level of A type spermatogonia: A-1 become A-2, A-2 become A-3 and finally

5
A-3 become A-4 spermatogonia. Mitotic divisions also separate intermediate spermatogonia and B type spermatogonia. The next developmental change is the transition of type-B spermatogonia into primary spermatocytes during spermatidogenesis.

*Spermatidogenesis*

Meiosis is the hallmark of spermatidogenesis, the second phase of spermatogenesis. Primary spermatocytes undergo Meiosis I to form secondary spermatocytes, which then complete Meiosis II to form haploid round spermatids. The portion of the seminiferous epithelium that plays a crucial role in spermatidogenesis is the BTB (Dym and Clermont, 1970). Formed by tight and adherens junctions between Sertoli cells, the BTB separates basal and adluminal compartments of the seminiferous tubule and creates specific microenvironments within the compartments it creates (Cheng and Mruk, 2002; Mather et al., 1983). Primary spermatocytes, when differentiated from type-B spermatogonia, are located on the basal side of the BTB. Preleptotene spermatocytes cross the BTB, which corresponds to stage VIII of the cycle of seminiferous in both mice and rats and stage III of humans (Clermont, 1963; Leblond and Clermont, 1952; Oakberg, 1956). Progression of Meiosis I occurs within the adluminal compartment of seminiferous tubules (Russell et al., 1990). Disruption of the BTB is known to cause spermatogenic failure, and it is widely believed that the BTB is necessary to keep the adluminal compartment an immune-privileged site; however, the exact function of the BTB is unknown. Spermatidogenesis is completed when Meiosis concludes and germ cells are ready to undergo physical transformation into sperm.
Spermiogenesis

During spermiogenesis, the last phase of spermatogenesis, morphological changes convert round spermatids into spermatozoa. After meiosis is completed, germ cells are referred to as spermatids. They are spherical in shape, much like their preceding germ cells. No further mitotic divisions occur during spermiogenesis, indicating that the final steps are dedicated to physical metamorphosis of these cells. This process consists of four stages: Golgi, cap, acrosomal and maturation phases. During these phases, the nucleus elongates, a majority of the cytoplasm is shed and a motile tail piece is formed. A mature spermatozoon is comprised of two main portions - the head and tail. The head includes the nucleus and acrosome, which is an adaptation of the Golgi apparatus. Whereas, the tail is used for motility and includes the middle piece the principal piece. The final step of spermatogenesis is spermiation or release of spermatozoa from the Sertoli cell microenvironment into the lumen of the seminiferous tubule. Collectively, these events change spermatids into highly specialized spermatozoa, capable of transmitting genetic information.

First round of spermatogenesis

The first round of spermatogenesis is unique from all successive rounds of spermatogenesis. Germ cells that contribute to the first round of spermatogenesis lack a self-renewing stage. Using mice that expressed β-galactosidase only in Nuerogenin3 (Ngn3)-expressing cells, the first round of spermatogenesis was observed to be completed by prospermatogonia that bypass a self-renewing spermatogonial stage and pass directly into differentiation (Yoshida et al., 2006). This study provided evidence that spermatogonia that
contribute to later rounds of spermatogenesis undergo a self-renewal phase before differentiation, marked by expression of Ngn3. Prospermogonia not expressing Ngn3 that transition into spermatogonia pass directly to a differentiated state, contributing only to the first round of spermatogenesis. Successive rounds of spermatogenesis are supported by the self-renewal and differentiation of spermatogonia that undergo an Ngn3-expressing phase.

**Inter-species differences**

Though the outcome of spermatogenesis is universal between all mammals, there are distinct differences between species. One of the most common differences between species is the number of stages and length of the seminiferous epithelium cycle. In mice, the seminiferous epithelium has 12 stages and one cycle of the epithelium takes approximately 8.6 days to complete (Oakberg, 1956). However rats have a seminiferous cycle that contains 14 stages and lasts approximately 12 days (Clermont and Leblond, 1953). Full spermatogenesis takes 35 days to complete in mice (Clermont and Trott, 1969); whereas, this process requires 48 days to reach completion in rats (Clermont and Harvey, 1965). Most nonhuman primates have 12 stages in their epithelial cycle, which encompasses 9.5 - 11.5 days (Barr, 1973; de Rooij et al., 1986). Moreover, human seminiferous epithelial cycle consists of 6 stages, lasting 16 days (Clermont, 1963). Another spermatogenic dissimilarity found between species is the number of spermatogonial phases and divisions. In rodent species, A_{sr}, A_{pr} and A_{al} spermatogonia comprise the undifferentiated spermatogonia; whereas, A1-A4 are considered differentiating spermatogonia, and intermediate and type-B spermatogonia are referred to as differentiated. Therefore, in the rodent, the transformation of spermatogonia to spermatocyte includes 8-9
mitotic divisions (de Rooij and Russell, 2000). However, in humans, the only classifications of spermatogonia include A-pale ($A_p$), A-dark ($A_d$) and type-B spermatogonia (Clermont, 1966). $A_d$ are considered quiescent spermatogonia in humans because their chromatin is highly condensed within the nucleus, providing its dark appearance. Alternatively, $A_p$ are thought to be mitotically active type-A spermatogonia as their chromatin is not condensed, thereby appearing pale in color. Both $A_d$ and $A_p$ have slightly ovoid nuclear shaping. Type-B spermatogonia can be distinguished from other forms of spermatogonia as they display spherical nuclei that are slightly larger ($7.0 \, \mu m$) than that of $A_d$ ($6.0 \, \mu m$) and $A_p$ ($6.8 \, \mu m$) spermatogonia (Clermont, 1966). Additionally, in rodent species, a single longitudinal cross-section of a seminiferous tubule reveals a single stage of seminiferous epithelium. Conversely, in humans and some nonhuman primate species, a seminiferous tubule cross-section typically contains more than one stage (Schulze and Rehder, 1984). This phenomenon can be attributed to the helical orientation of spermatogenic progression in human and some nonhuman primate seminiferous tubules; unlike rodents, which progress in a wave-like pattern along the tubule. While these differences exist, they do not have a major impact on the outcome of spermatogenesis between species, which is the production of spermatozoa.

**NICHE MICROENVIRONMENT**

Spermatogenesis, like processes supported by other tissue-specific stem cell populations, is sustained by a niche microenvironment (Spradling et al., 2001). In Drosophila, the spermatogenic niche is comprised of hub, cyst and germline stem cells (Hardy et al., 1979). In mammals, the niche is comprised of cells both within and surrounding the seminiferous
tubules (de Rooij, 2009). Within the seminiferous tubules the niche is an assembly of somatic (Sertoli) cells that act to support the maintenance and differentiation of spermatogonial stem cells (Russell and Peterson, 1984). Other somatic cells such as Leydig and myoid cells contribute to the niche but do not have direct contact with the spermatogenic cells (de Rooij, 2009; Hermo et al., 2010). Sertoli cells of the mammalian testis function as both the hub and cyst cells of Drosophila, anchoring the stem cell niche to the basement membrane and also surrounding germ cells as they progress toward spermiation. Without functional niche microenvironments, production of sperm, and subsequent production of offspring, is impossible.

**Sertoli cells**

The most obvious and logical contributors to the SSC niche are Sertoli cells. Also referred to as nurse cells, Sertoli cells are the only somatic cells that reside within the seminiferous tubules and are supporters of germ cell development by providing structural support, secreting necessary cytokines and partitioning seminiferous tubules. Sertoli cells, first referred to as the branched cell (Sertoli, 1865), provide structural support for developing germ cells. As germ cells differentiate and move away from the basement membrane, Sertoli cells act as a scaffold to which germ cells attach. Sertoli cells comprise 15-20% of the seminiferous epithelium of rodent species; whereas, they occupy approximately 37% of the seminiferous epithelium of humans (Hess and Franca, 2005). It is estimated that ~40% of a Sertoli cell’s surface area is in direct contact with elongated spermatids, indicating the extent to which Sertoli cells contact germ cells (Franca et al., 1993). Sertoli cells interact with germ cells by
forming adherens and gap junctions to engulf the developing germ cells (Siu and Cheng, 2008). Another way Sertoli cells support germ cell development is through secretion of cytokines that serve as extrinsic cues to direct fate decisions of all germ cells, including SSC. Gdnf, which is necessary for SSC self-renewal, is thought to be secreted by Sertoli cells (Tadokoro et al., 2002). Other factors such as Bone morphogenic protein 4 (Bmp4; Pellegrini et al., 2003) and Activin A (Nagano et al., 2003) are secreted by Sertoli cells and may influence SSC fate decisions. Sertoli cells also support spermatogenesis by forming tight junctions with other Sertoli cells to form the blood-testis-barrier (BTB; Setchell, 2009). The BTB acts as a gateway between basal and adlumenal compartments of the seminiferous tubule (Wong and Cheng, 2005). Thought to maintain an immune-privileged environment within the lumen of seminiferous tubules, little is actually known about the role of the BTB. Though the BTB has not been shown to impact SSC fate decisions, it is necessary for successful completion of spermatogenesis (Fiorini et al., 2004). Without the function of Sertoli cells, germ cells are incapable of differentiating or surviving (Mruk et al., 2004, Siu et al., 2009). Mice are lacking functioning Sertoli cells are infertile, but fertility can be restored to these animals by transplanting donor Sertoli and germ cells in to the seminiferous tubules (Shinohara et al., 2003). Therefore, Sertoli cells are clearly a key component of the SSC niche.

**Interstitium**

Other components of the SSC niche are the cells which consist of interstitial tissue. Interstitial cells reside outside of the seminiferous tubules and include myoid and Leydig cells, as well as immune and vascular cells. When comparing portions the basement membrane of
the seminiferous tubule which border other seminiferous tubules or interstitial tissue; generally, undifferentiated spermatogonia favor orientations with closest proximity to interstitial space, suggesting that interstitial cells are an important component of the SSC niche (Chiarini-Garcia et al., 2001; Chiarini-Garcia et al., 2003). The interstitial tissue is comprised of Leydig cells, peritubular myoid cells as well as cells of the blood and lymph (de Rooij, 2009).

**Leydig cells**

A well-characterized function of Leydig cells is the production of testosterone, through stimulation by luteinizing hormone. Sertoli cells express receptors for testosterone. Administration of the compound leuprolide to recipient mice before germ cell transplantation decreases testosterone output by Leydig cells and increases SSC colonization efficiency, suggesting that testosterone may have a negative impact on SSC colonization (Ogawa et al., 1998). Leydig cells comprise approximately 9-16% of total testis volume in mammals and are thought to contribute to germ cell maintenance through the production of cytokines or testosterone (Kothari et al, 1978). The main factor produced by Leydig cells is testosterone which is known to promote maleness.

**Myoid cells**

Peritubular myoid cells form a monolayer surrounding the seminiferous tubules within the interstitial space in rodents. These cells have contractile properties that aid in spermiation and flow of spermatozoa through the seminiferous tubules before motility is gained (Skinner and Fritz, 1985). Contraction of myoid cells has been shown to be effected by factors such as
prostaglandins, oxytocin and transforming growth factor-β (Maekawa et al., 1996). Also, myoid cells may contribute to the SSC niche by influencing Sertoli cell function (Hoeben et al., 1999; Verhoeven et al., 2000).

**Vasculature**

Vascularization may also play an important role in developing, maintaining and contributing to the SSC niche as undifferentiated spermatogonia are preferentially located in areas of seminiferous tubules adjacent to blood vessels (Yoshida et al., 2007a). Factors delivered by the vasculature such as hormones, cytokines and nutrients may influence the distribution of undifferentiated spermatogonia within the seminiferous tubules. However, little is known about this phenomenon.

**Effect of age on niche**

Function of the niche is highly important to SSC maintenance and fate decisions. Age is a factor which greatly impacts the function of the SSC niche (Ryu et al., 2006). It is natural that, with age, spermatogenesis becomes a less efficient process. This is due to break-down of the SSC niche microenvironment. In rodents, spermatogenic deterioration occurs between 12-24 months of age. However, when SSC are removed from niches of older males and transferred to niches of younger males, SSC were capable of maintaining spermatogenic integrity for more than 3 years, indicating that the niche greatly impacts SSC preservation and reproductive longevity.
EMBRYONIC DEVELOPMENT

Primordial germ cells

During embryonic development, the germline consists of primordial germ cells (PGC) in both male and female embryos. PGC arise from the epiblast, before differentiation of the three germ layers. By 7 dpc in mice, PGC lineage commitment is complete (Saitou, 2002). By 8.5 dpc, PGCs become transcriptionally inactive and undergo genomic reprogramming such as histone modifications and decreased DNA methylation (Seki et al., 2007). At this point in development, PGCs are located in the hindgut of the embryo. PGC must migrate to the genital ridge, an area of the embryo adjacent to the mesonephros, where gonadal development takes place. At 9 dpc in mice, the genital ridge is comprised solely of coelomic epithelium, and PGCs migration is initiated (Ikeda et al., 1994). Migration of PGCs from the embryonic hindgut is made possible by a chemoattractant gradient leading to the genital ridges (Molyneaux, 2003). Without expression of chemoattractants, germ cell migration is disorganized and most PGCs do not reach the genital ridge. After migration of PGCs to the genital ridge, the gonad still cannot be distinguished as a testis or ovary.

Importance of Sry gene

Proper testis development is dependent upon expression of Y-chromosome encoded sex determining region Y (Sry) gene during embryonic development. Around 11 dpc in mice, three phenotypic events in early development of maleness occur and include: aggregation of pre-Sertoli cells with PGCs, formation of seminiferous cords and promotion of Wolffian duct development. All three of these events are dependent upon Sry expression. Around 11dpc,
Sertoli cells differentiate from coelomic epithelium of the genital ridge. The hallmark of Sertoli cell differentiation is the expression of Sry (Schmahl et al., 2000). As they differentiate, Sertoli cells begin to aggregate with PGCs which by have completed their migration and are residing in the genital ridge. Next, Sertoli-PGC aggregates are encased by endothelial cells, which migrate from the mesonephros, causing the formation of testis cords (Buehr et al., 1993; Tilmann and Capel, 1999). At this same time, development of Wolffian ducts, predecessors to epididymides, vas deferens and secondary sex glands, is initiated by Sry. Without expression of the transcription factor Sry, antecedents of the female reproductive tract (oviduct, uterus, cervix and vagina) termed Mullerian ducts, develop. A protein called Mullerian inhibiting substance (Mis), a Sry-promoted factor, is secreted by Sertoli cells as they differentiate from their pre-Sertoli cell status at days 11-13 postcoitum (Martineau et al., 1997; Shen et al., 1994). After seminiferous chords are formed, encapsulating mesonephronic endothelial cells form a single layer of peritubular cells (Capel et al., 1999; Tilmann and Capel, 1999). Coelomic epithelial cells adjacent to seminiferous cords become immature Leydig cells. As Leydig cells differentiate, they produce 3β-hydroxysteroid dehydrogenase (3β-HSD), an enzyme specifically produced by Leydig cells, which is required for testosterone production. Testosterone synthesis supports maintenance and further development of the Wolffian duct system (Nordqvist and Tohonen, 1997). During this developmental period, no changes are occurring to germ cells within newly formed testis.
Prospermatogonia

Once made part of the developing gonad around dpc 14.5 in mice, PGCs are referred to as gonocytes (Culty, 2009) or prospermatogonia (de Felici, 2000). It is at this point that alkaline phosphatase activity is lost in the germ cells, a defining characteristic of prospermatogonia (Richards et al., 1999). Prospermatogonia are a heterogeneous cell population, displaying various gene expression patterns between fetal and neonatal periods, which include mitotically active and quiescent stages (Vergouven et al., 1991). As development progresses toward reproductive competency, some prospermatogonia transition into spermatogonial stem cells (SSC; Yoshida et al., 2006). Prospermatogonia are capable of persisting in seminiferous tubules of mice until approximately 5 days postpartum (dpp); however, transition from prospermatogonia to SSC begins around the time of birth (Huckins and Clermont, 1968). By 6 dpp, virtually all gonocytes have transitioned into SSC, differentiated directly into A1 spermatogonia that contribute to the first round of spermatogenesis or have undergone apoptosis, leaving SSC as the most primitive cells within the seminiferous tubules (Bellve et al., 1977; Culty, 2009; de Rooij and Russell, 2009; Yoshida et al., 2006). With proper regulation, SSC persist into adulthood and support continuous spermatogenesis throughout adult life.

SPERMATOGONIAL STEM CELL BIOLOGY

Spermatogonial stem cells are the basis for continuous production of spermatozoa throughout adult life. SSC are rare (~1 in 3,000 cells) in the testis (Tegelenbosch and de Rooij, 1993) and have two main functions (Oatley and Brinster, 2006). The first is to differentiate to produce the more advanced generations of germ cells that complete spermatogenesis and
ensure fertility. Second, SSC must continue to renew the stem cell pool. Without self-renewal of SSC, infertility is eminent because the foundation for spermatogenesis has been lost. As a result, a balance between differentiation and self-renewal must be maintained in order to preserve continual spermatogenic activity. Despite the knowledge of the existence of SSC for over 15 years (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994), knowledge of mechanisms controlling self-renewal and differentiation is limited.

**REGULATION OF SSC FATE DECISIONS**

Fate decisions of SSC are controlled both extrinsically and intrinsically. The niche microenvironment impacts SSC fates through extrinsic factors such as cytokines. Intrinsic control of SSC differentiation is controlled by cell signaling pathways and transcriptional regulators.

**Cytokines**

**Gdnf**

Gdnf is a cytokine produced by Sertoli cells (Trupp et al., 1995). Overexpression of Gdnf by Sertoli cells was shown to cause an accumulation undifferentiated spermatogonia and eventually formation of seminoma or germ cell tumors were observed within the seminiferous tubules of these mice (Meng et al., 2000; Yomogida et al., 2003). In contrast, mice carrying a single Gdnf-null allele displayed a loss of undifferentiated spermatogonia in vivo (Meng et al., 2000). In vitro, self-renewal of mouse and rat SSC require Gdnf supplementation to culture
media (Kubota et al., 2004a; Hamra et al., 2005). As expected, genes that are upregulated by Gdnf in cultured Thy1+ germ cells were found to promote self-renewal of SSC (Oatley et al., 2006). These data indicate that the cytokine Gdnf is required for the self-renewal of SSC.

_Csf1_

Another ligand, colony stimulating factor 1 (Csf1), is expressed by Leydig cells and peritubular myoid cells (Oatley et al., 2009). The Csf1 receptor (Csf1r) was found to be preferentially expressed by Thy1+ germ cells compared to Thy1-depleted testis cells. Addition of recombinant Csf1 to culture media increased SSC self-renewal by (Oatley et al., 2009), proving that Csf1 impacts SSC self-renewal.

_Signal transduction pathways_

_Akt/Sfk_

Gdnf signaling occurs through two pathways in SSC: Bruton agammaglobulinemia tyrosine kinase (Akt; Lee et al., 2007; Oatley et al., 2007) and Src family kinase (Sfk; Oatley et al., 2007). The Akt signaling pathway supports SSC survival and proliferation; whereas, Sfk signaling promotes transcription of genes important for self-renewal (Lee et al., 2007; Oatley et al., 2007).
Intrinsic factors

Gdnf up-regulated genes

Many other factors have been found to promote self-renewal of SSC by Gdnf signaling. Gdnf upregulated genes, such as Bcl6b, Etv5, Lhx1 and Oct6, are needed for self-renewal of SSC (Oatley et al., 2007; Wu et al., 2010).

Bcl6b

B-cell CLL/lymphoma 6, member B (Bcl6b) is a Gdnf up-regulated gene that has a role in promoting SSC self-renewal (Oatley et al., 2006). As a transcription factor, Bcl6b influences expression of other genes important for self-renewal. Bcl6b knock-out mice have reduced fertility and display decreased testis weights and seminiferous tubules devoid of germ cells. In studies where Bcl6b transcript abundance is reduced by siRNA, SSC had reduced ability to self-renew (Oatley et al., 2007), indicating Bcl6b is important for promoting SSC self-renewal.

Etv5

ETS variant 5 (Etv5) is another transcription factor that is upregulated by Gdnf signaling (Oatley et al., 2006). Disruption of Etv5 in the male mouse testis caused germ cell depletion (Chen et al., 2005). By 10 weeks of age, Etv5-null mice were infertile. Similar to studies conducted with Bcl6b, reducing transcript abundance of Etv5 by siRNA in cultured Thy1+ germ cells decreased SSC self-renewal (Oatley et al., 2007). Combined, these data indicate that Etv5 is needed for self-renewal of SSC.
**Lhx1**

Similarly, Lim homeobox protein 1 (Lhx1 or Lim1) was found to be regulated by Gdnf (Oatley et al., 2006). Lhx1 knockout mice display severe cranial abnormalities during embryonic development which causes neonatal lethality (Shawlot and Behringer, 1995). Interestingly, these Lhx1-null mice lack gonads and kidneys. When \( Lhx1 \) mRNA abundance is reduced by siRNA treatment in cultured Thy1+ germ cells, reduction of SSC survival is observed (Oatley et al., 2007). These data suggest that Lxh1 is not only important to embryonic organogenesis but also to the survival of SSC.

**Oct6/Oct4**

Expression of octamer-binding family member 6 (Oct6, also known as POU3F1) is regulated by Gdnf via the PI3k/Akt pathway (Wu et al., 2010). Oct6 is needed by cultured Thy1+ germ cells to protect against apoptosis and maintain SSC self-renewal (Wu et al., 2010). However, Oct4 (POU5F1), an important factor for maintenance of pluripotency of ES cell that has been implicated as a necessary factor for SSC self-renewal, is not regulated by Gdnf signaling and has recently been shown to not promote SSC self-renewal (Wu et al., 2010).

**Genes not up-regulated by Gdnf**

**Plzf**

A mutant mouse model carrying the luxoid (\( lu \)) mutation, a single nucleotide change in the \( Zfp145 \) gene that encodes for proteomyelocytic leukemia zinc finger protein (Plzf), causes the protein to be nonfunctional (Buaas et al., 2004). Mice carrying this mutation display
decreased spermatogenic efficiency with increasing age (Johnson and Hunt, 1971).

Homozygosity for the luxoid mutation disrupts spermatogenesis, eventually leading to a Sertoli-cell-only phenotype within the seminiferous tubules of these mice (Buaas et al., 2004). The testes of lu/lu mutants are capable of supporting donor germ cell derived spermatogenesis, proving that the luxoid mutation does not disrupt the niche microenvironment of the testis but that the mutation impairs stem cell activity. In the reciprocal experiment, lu/lu mutant testis cells are unable to colonize recipient seminiferous tubules and initiate donor derived spermatogenesis. However, it was never clearly addressed if the break-down in spermatogenesis and inability to reestablish spermatogenesis in recipient mice was due to the loss of self-renewing or differentiating capabilities in the lu/lu mutants. A similar study, published that same year using Plzf knockout mice, provided similar results; however, again there remained multiple interpretations of the results and no clear conclusions could be drawn about the involvement of Plzf in SSC self-renewal or differentiation (Costoya et al., 2004).

Taf4b

TATA-binding protein associated factor 4b (Taf4b) is a subunit of the complex required for transcription of RNA polymerase II (Liu et al., 2008). Disruption of Taf4b causes germ cell depletion over time (Falender et al, 2005a; Falender et al, 2005b). This progressive loss of germ cells indicates dysregulation of SSC fate decisions. However, Taf4b is expressed by multiple germ cell types, suggesting that the impact of Taf4b reduction in the male germline may not be SSC-specific.
CELL SURFACE PHENOTYPE OF SSC

Studying SSC biology is confounded by the inability to isolate pure populations of SSC because a unique cell surface phenotype to distinguish SSC from other spermatogonia has not been discovered (Oatley and Brinster, 2006). However, multiple phenotypic parameters have been established to enrich for SSC content, thereby bringing the scientific community closer to understanding the phenotype of SSC.

Thy1

Thymus cell antigen 1 (Thy1) is a glycosyl phosphatidylinositol (GPI) anchored glycoprotein molecule belonging to the IgG superfamily. Thy1 has proved to be an important cell surface molecule for enriching SSC in an experimental population (Oatley and Brinster et al., 2006; Oatley and Brinster, 2008). Selecting for Thy1 expressing germ cells cells increases the SSC content from cryptorchid adult testis 15-fold (Kubota et al., 2003). Thy1 selection increases SSC content in normal adult testes from ~1 in 3,000 cells (Tegelenbosch and de Rooij, 1993) to ~1 in 100 cells (Kubota et al., 2004a). This represents a 30-fold increase in SSC enrichment. Therefore, Thy1 is a cell-surface marker important for the study of SSC.

Gdnf receptor

Expression of Gdnf and its co-receptors Gfrα1 and c-Ret is important to maintenance of SSC and undifferentiated spermatogonia. Expression of the Gdnf co-receptor Gfrα1 was proven to be age dependent, being expressed higher in prepubertal pups versus during the neonatal or adult stages. Despite the importance of Gdnf signaling to SSC maintenance, Gfrα1-expressing
cells isolated from adult testes had a decrease in stem cell activity after transplantation analysis, compared to unselected control cells. In prepubertal pup testes, selection for Gfrα1 increased stem cell content by 2.5-fold (Ebata et al., 2005). However in neonatal testes, selection based on Gfrα1 expression decreased SSC content by 200-fold. Similarly, other Gdnf receptor components, c-Ret and Ncam, decreased SSC content in both prepubertal pup and adult testes. Collectively, these results indicate that, while they form the receptor for Gdnf, selection for Gfrα1, c-Ret or Ncam are not efficient means to enrich for SSC.

**c-Kit**

Kit ligand receptor c-Kit is a part of the cell-surface phenotype of HSC, ES cells and PGCs (Snodgrass et al., 1992; Yoshinaga et al., 1991). Selection by allowing germ cells to adhere to collagen type IV or fibronectin was found to enrich for cells expressing c-Kit (Shinohara et al., 1999). Adherence of germ cells to collagen type IV did not impact SSC content. However, fibronectin-selected germ cells decreased SSC content by 3-fold (Shinohara et al., 1999). Similarly, selection for c-Kit expression by fluorescent activated cell sorting (FACS) did not enrich for SSC (Shinohara et al., 1999). Additionally, cryptorchid testes, which have been shown to be enriched for SSC in their total germ cell population (Shinohara et al., 2000a), contain very few c-Kit expressing cells (Shinohara et al., 2000c). These data indicate that c-Kit is not expressed by SSC and thus not necessary for their function.
α6-integrin/β1-integrin

The heterodimer of α6- and β1-integrin is important for binding to the extracellular matrix laminin. Laminin-selected germ cells were enriched for cells expressing both α6-integrin and β1-integrin (Shinohara et al., 1999). Adhesion of germ cells to laminin increased recipient SSC content 3-fold compared to unselected control cells (Shinohara et al., 1999). Selection for α6-integrin by FACS increased colonization of the recipient testis 8.4-fold compared to unselected control cells (Shinohara et al., 1999). Transplantation analysis of testis cells selected for β1-integrin by FACS showed a 3.8-fold increase in colonization efficiency compared to unselected testis cells and a 14.6-fold increase in colonization compared to the β1-integrin depleted cell fraction (Shinohara et al., 1999), indicating enrichment for SSC. Also, homing of SSC to their niche is influenced by β1-integrin. SSC lacking β1-integrin have reduced homing efficiency and lowered ability to colonize the seminiferous epithelium (Kanatsu-Shinohara et al., 2008). Similarly, disruption of β1-integrin expression by Sertoli cells also decreases SSC homing (Kanatsu-Shinohara et al., 2008). Collectively, these data indicate that α6- and β1-integrin are a functional part of the cell surface phenotype of SSC.

MHC

Major histocompatibility complex class I (MHC-I) molecules had been thought to not be expressed by spermatogonia, yet that theory had not been proven or refuted by sound evidence. Therefore, the existence of MHC-I molecules on the cell surface of the cryptorchid SSC was explored (Kubota et al., 2003). Using FACS analysis and functional transplantation, SSC of the cryptorchid testis were found to be MHC-1-negative.
**Similarities to other tissue-specific stem cell populations**

Exploration of possible shared cell surface phenotypes between SSC other tissue-specific stem cell populations has uncovered similarities and distinct differences between these cell types (Kubota et al., 2003). Because work has been conducted to discover the cell surface phenotypes unique to other tissue-specific stem cell populations, translation of this knowledge to SSC was attempted. Hematopoietic stem cells (HSC) are known to express CD24, CD34, c-Kit, Thy1 and Sca1 (Goodell et al., 1996); therefore, these proteins were examined in SSC (Kubota et al., 2003). SSC of adult testes were found to be c-Kit-negative and Thy1-positive. Further investigation of SSC phenotype revealed α6-integrin and CD24 is expressed by SSC; whereas, SSC do not express αv-integrin, CD34 or Sca1. The MHC-I-negative, c-Kit-negative, Thy1-positive cell fraction of the adult testis is enriched for SSC. Additionally, the Hoechst dye efflux assay identified a testis side population that, unlike other adult stem cell populations such as HSC (Goodell et al., 1996), contains no stem cell activity (Kubota et al., 2003). Therefore, SSC share some, but not all, characteristics of other tissue-specific stem cell populations.

**STUDY OF SSC**

**Transplantation**

Exploration of the kinetics of donor cell colonization of recipient seminiferous tubules indicated that donor germ cell colonization of the recipient testes could be categorized in three phases (Parreira et al., 1998; Nagano et al., 1999). During the first week after transplantation, germ cells are spread at random throughout seminiferous tubules and some germ cells are able to translocate to the basement membrane. From 0-4 hours after germ cells transplantation,
most of the cells were free-floating in the lumen of seminiferous tubules. By 24 hours post-transplantation, germ cells appear to interact with Sertoli cells and few germ cells are freely floating in the seminiferous lumen; although, it is clear that most of the germ cells have not yet reached the basement membrane. One week after transplantation, some germ cells have reached the basal membrane, and the rest have presumably been phagocytosed. Second, donor germ cells that reach the basement membrane proliferated during the 1 week to 1 month time period. Mitotic proliferation of transplanted germ cells was observed during this time frame. Third, from 1 month to 4 months, germ cells continue to proliferate and differentiate to form colonies of donor-derived spermatogenesis in the recipient testis. Colony number per testis did not increase between 1 and 4 months after transplantation; however, individual colony size increased significantly during this period.

Both enrichment for SSC in the injected donor cell population and the status of the recipient seminiferous tubule influence the efficiency with which spermatogenesis was established (Shinohara et al., 2002). First, germ cells isolated from cryptorchid testes are known to be enriched ~24-fold for SSC, due to the lack of differentiated cells caused by a block in spermatogenesis that is observed with increased intra-testicular temperature (Shinohara et al, 2000a). Cryptorchid-derived germ cells had 21-fold greater colonization of busulfan-treated recipient testes than non-enriched, wild-type germ cells. Therefore, enriching a population of germ cells for SSC content increases the efficiency with which these cells colonize the seminiferous tubule (Shinohara et al., 2002). Also, transplantation of donor SSC could compete with endogenous SSC and establish spermatogenesis within non-busulfan-treated wild-type
testes. While no competition was observed between donor and endogenous germ cells in non-busulfan-treated testes, SSC-enriched germ cell populations were capable of competing for niches within seminiferous tubules of non-busulfan-treated mice to successfully establish spermatogenesis.

To date, transplantation is the only irrefutable means to determine the content of SSC in a population of testis cells, because there is no known specific cell surface phenotype for SSC (Oatley and Brinster, 2006; Oatley and Brinster, 2008; Phillips et al., 2010). The effectiveness of tissue-specific stem cell transplantation as a mode to determine stem cell content of a population is dependent on the ability to mark or track donor stem cells once in the recipient. A method to identify and trace donor SSC in recipient testes is through the use of transgenic animals. Without this system, the existence of mammalian germline stem cells would remain speculation. Also, germ cell transplantation has opened an entire avenue of research pertaining to SSC, the coordination of the early stages of spermatogenesis and fertility. Additionally, development this method has allowed for clinical exploration of SSC transplantation to treat infertility in humans. Though germ cell transplantation was an innovative and effective mechanism, transplantation alone was limited in its scope as a research tool because extensive manipulation of testis cells before transplantation was not possible.
Culture

The process of developing a serum-free culture condition required discovering a base media, feeder cells and growth factors necessary for SSC self-renewal. The optimal combination of base media and feeder cells was found to be Minimal Essential Media-alpha (MEMα) with mitotically inactivated SIM mouse embryonic thioguanine and ouabain resistant fibroblast (STO) feeders (Kubota et al., 2004a). The first reported growth factors to support SSC maintenance and expansion in vitro were stem cell factor (Scf) and glial cell line-derived neurotrophic factor (Gdnf; Kubota et al., 2004a). Of six growth factors (Insulin-like growth factor-1 (Igf1), Leukemia inhibiting factor (Lif), Fibroblast growth factor-2 (Fgf2), Epidermal growth factor (Egf), Scf, and Gdnf) selected for examination, only Scf and Gdnf increased SSC content of cultures over a 7 day period. Gdnf was shown to be important for maintenance of undifferentiated spermatogonia in vivo (Meng et al., 2000), so its necessity for culture was logical; however, the result that Scf maintained SSC in culture was interesting because the receptor for Scf, c-Kit, is not expressed by SSC (Kubota et al., 2003). Further experimentation revealed that 40 ng/mL of recombinant human Gdnf supported self-renewing expansion of SSC in serum-free culture conditions, and the final formulation of growth factors that supported the greatest amount of SSC expansion in vitro included: 40 ng/mL Gdnf, 300 ng/mL rat Gfrα1 fusion protein and 1 ng/mL recombinant human Fgf2 (Kubota et al., 2004b). The current culture conditions used by the Oatley laboratory includes culture of germ cells isolated on the basis of expression of Thymus cell antigen 1 (Thy1) by MACS isolation, maintained on mitotically inactivated STO feeders with serum-free media supplemented with Gdnf (40 ng/mL) and Fgf2 (1 ng/mL; Oatley et al., 2009).
Importance

Most significantly, as germline stem cells, SSC are important because they are the only stem cell that contributes genetically to the next generation. Therefore, manipulation of the male germline could be accomplished through SSC. This would be important to altering the phenotype of offspring such as correcting mutations that cause disease such as autism, diabetes, Down’s syndrome or heart disease. Also, SSC contribute directly to fertility. Dysregulation of SSC fate decisions impact spermatogenesis and cause subfertility or infertility. Furthermore, the isolation, culture and cryopreservation of SSC in humans could produce options for preserving fertility in men undergoing cancer treatment, which has the risk of ablating endogenous spermatogenesis. Finally, SSC are integral to the idea in vitro spermatogenesis, where SSC could be influenced to undergo spermatogenesis in culture to produce sperm without the testis. This process would be important for men who need to undergo radical orchiectomy but still wish to father offspring. In conclusion, as the only stem cells of the germline, studying SSC has the capacity to greatly impact human medicine.

Despite the knowledge of molecular mechanisms regulating SSC self-renewal, little is understood about the factors contributing to SSC differentiation. In the future, studying mechanisms underlying differentiation of SSC should be an experimental focus.
REFERENCES


derOoij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. J Androl 2000: 21:776-98.


CHAPTER 2: REGULATION OF SSC DIFFERENTIATION BY STAT3

ABSTRACT

Signal transducer and activator of transcription 3 (Stat3) is an important transcription factor involved in fate decision determination of various stem cell populations. Pluripotency of mouse embryonic stem cells is maintained through Stat3 signaling activated by Leukemia inhibiting factor (Lif). Also, Stat signaling promotes male germ line stem cell self-renewal in Drosophila. Without proper regulation of SSC, disruption in spermatogenesis can occur, leading to subfertility or infertility. Due to the complexity of the spermatogenic system, studying fate decisions of SSC is a difficult task, limiting the knowledge of these processes. The objective of this study was to determine the role of Stat3 in regulating self-renewal or differentiation of SSC. To achieve this, Stat3 was reduced in cultured mouse SSC via lentiviral mediated shRNA transduction resulting in 74.7% reduction of gene expression. Upon transplantation into recipient seminiferous tubules, SSC with reduced Stat3 expression were unable to reestablish spermatogenesis; whereas, spermatogenesis was regenerated from control SSC with normal Stat3 expression. SSC with reduced Stat3 expression produced colonies of undifferentiated spermatogonia present as only single cells or short chains of spermatogonia within recipient seminiferous tubules. These results indicated that lack of Stat3 expression blocked differentiation of SSC beyond the undifferentiated spermatogonial stage of development.
INTRODUCTION

The continual process of producing spermatozoa throughout adulthood is known as spermatogenesis. The foundation of this process is spermatogonial stem cells (SSC), which have the capacity to self-renew to maintain a population of stem cells, as well as, to differentiate into A-paired spermatogonia that, through a series of phenotypic and morphological changes, become spermatozoa. Since the discovery of SSC, studies have been conducted to investigate molecular mechanisms influencing differentiation and self-renewal. Much of this work focuses on mechanisms underlying self-renewal. Glial cell line derived neurotrophic factor (Gdnf) was shown to regulate SSC self-renewal both in vivo and in vitro (Kubota et al., 2004b; Meng et al., 2000). Additionally, proteins which are up-regulated by Gdnf, such as B-cell CLL/lymphoma 6 member B (Bcl6b) and ezrin-radixin-moesin (Erm) proteins are important to self-renewal of SSC (Oatley et al., 2007). However, to date, little is known about factors promoting SSC differentiation.

To study SSC differentiation, long-term cultures of Thy1+ germ cells isolated from seminiferous tubules serve as a model of SSC proliferation and differentiation. When in culture, these cells are capable of both differentiation and self-renewal, yielding a heterogeneous population comprised of SSC as well as non-stem cell spermatogonia. Upon transplantation, only SSC are capable of reinitiating spermatogenesis in recipient testes; therefore, germ cell transplantation assays exclusively examine SSC content of experimental cell populations.
used in conjunction, SSC culture and transplantation provide a means to explore the effects of individual molecules on SSC fate decisions.

One means to accomplish this goal is through RNA interference (RNAi).  Two forms of RNAi are small interfering RNA (siRNA) and short hairpin RNA (shRNA).  siRNA is double stranded RNA 19-25 nucleotides in length that create transient reduction of a specific mRNA transcript by incorporation into the RNA induced silencing complex (RISC) machinery, which facilitates degradation of mRNA transcripts corresponding to siRNA sequence.  Typically, siRNA are directly introduced into a cell by means of lipofection or electroporation.  Short hairpin RNA works in a similar fashion, but mRNA transcript reduction is continuous.  Lentivirus delivery of shRNA plasmid DNA allows for infection of a cell and incorporation of plasmid DNA into the host genome.  Under control of a constitutively active promoter, the shRNA is constantly transcribed.  To function, shRNA transcripts form a hairpin loop, which is cleaved from the transcript to create double stranded siRNA that functions as previously described to reduce the abundance of a specific transcript.  These methods of regulating transcript abundance are effective and convenient means to explore the effect of molecules on proliferation and differentiation of SSC.

Stat3 has been implicated in controlling fate decisions in other stem cell populations.  In *Drosophila* male germ-line cells, somatic hub cells produce the ligand Unpaired to promote JAK/STAT signaling in germline stem cells to maintain self-renewal (Kiger et al., 2001; Tulina and
Moreover, the Jak2/Stat3 signaling pathway promotes maintenance of pluripotency in mouse ES cells by initiating transcription of *Octomer 4 (Oct4)* and *Nanog*, via activation by Lif (Smith et al., 1988; Hao et al., 2006). A similar role was found for Fibroblast growth factor 2 (Fgf2) in human ES cells (Wang et al., 2005; Xu et al., 2005). However, the role of Stat3 has not yet been determined in SSC. Therefore, this study was conducted to examine the effect of reduction in Stat3 on differentiation and proliferation of SSC using short hairpin RNA (shRNA).

**METHODS**

*Isolation and culture of Thy1+ germ cells*

Cultures of Thy1-expressing germ cells were established as previously described (Oatley and Brinster, 2006). Briefly, Thy1-expressing germ cells were isolated from seminiferous tubules of prepubertal (6-8 dpp) mice using magnetic activated cell sorting. Mice used for culture were B6.129S7-Gtrosa26 (designated Rosa; The Jackson Laboratory, Bar Harbor, ME). Isolated cells were maintained on a monolayer of Lif-secreting STO cells (American Type Culture Collection; Manassas, VA), that were mitotically inactivated by mitomycin-C treatment (Sigma-Aldrich). Cultures were maintained in serum free conditions with supplementation of Glial cell line-derived neurotrophic factor (Gdnf; 20 ng/mL of media; R&D Systems; Minneapolis, MN) and Fibroblast growth factor 2 (Fgf2; 1 ng/mL of media; BD Biosciences; San Jose, CA). Germ cells were passaged to fresh STO cells every 7 days. Seven days encompasses one in vitro self-renewal cycle of ~6 days for SSC (Kubota et al., 2004b).
All procedures using animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee (Protocol # 25358 & 25714).

**Production and purification of shRNA lentivirus**

HEK 293 cells (American Type Culture Collection; Manassas, VA) were grown to ~70% confluence in HEK growth media (Dulbecco’s Modified Eagle Media (Invitrogen; Carlsbad, CA) with 10% fetal bovine serum (HyClone; Logan, UT). At approximately 70% confluence, growth media was removed and replaced with Opti- Minimal Essential Media-α (Invitrogen; Carlsbad, CA). Lipofectamine 2000 (60 µL; Invitrogen; Carlsbad, CA) was mixed with 5 µg pMD2.G envelope plasmid DNA (Addgene plasmid 12259; Cambridge, MA), 10 µg psPAX2 lentiviral packaging plasmid DNA (Addgene plasmid 12260) and 9 µg Mission Stat3 shRNA plasmid DNA (clone ID NM_011486.3-1238s1c1; shRNA sequence: CCGGCCTGAGTTGAATTATCAGCTTCTCGAGAAGCTGATAATTCAACTCAGGTTTTTG; Sigma-Aldrich; St. Louis, MO) or Mission shRNA lentiviral non-target control plasmid (Sigma-Aldrich; St. Louis, MO). Twenty-four hours after transfection, medium was changed back to HEK growth media. After 48 hours of culture with growth medium, supernatant was collected, from which virus was purified. Medium was collected and virus purified for 3 consecutive days. Lentiviral particles were collected by ultracentrifugation (at 50,000 x g for 1.5 hours at 4°C on a Sorvall Evolution RC Superspeed centrifuge; Kendro Laboratory Products; Thermo Scientific;
Wilmington, DE) through a 20% sucrose-PBS gradient. Concentration of virus was determined by RetroTek HIV-1 p24 Antigen ELISA kit (ZepToMetrix; Buffalo, NY) and is expressed as pg of virus/mL.

**Quantitative PCR for Stat3**

Total cellular RNA was extracted from cells using TRIZol reagent (Invitrogen; Carlsbad, CA). Only RNA samples with an absorbance 260/280 nm reading ratio of greater than 1.80 and less than 2.01 were used. Possible genomic DNA contamination was removed by degradation by the enzyme DNase I. RNA (1 µg/reaction) was reverse transcribed using the Super Script III reverse transcriptase kit (Invitrogen). Complementary DNA (cDNA) was examined for relative abundance of either Stat3 or Ngn3 by qPCR with the SYBR Green assay kit (Platinum SYBR Green qPCR SuperMix-UDG w/ROX; Invitrogen; Carlsbad, CA). Primers specific for Stat3 were designed using Primer Express III software (Applied Biosystems; Forward: GACCTGCAGCAATACCATTGAC; Reverse: CCGTTATTCCAAACTGCATCA). Ribosomal protein S2 (Rps2) mRNA was used for standardization and primers were designed with Primer Express III software. Assays were conducted (cycle settings: 50°C for 2 minutes, 95°C for 5 minutes and 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds followed by a dissociation stage) and data were collected on the 7500 Fast Real-Time PCR System (Applied Biosystems). Comparisons were made by normalizing Stat3 expression to that of Rps2. Calculation of relative transcript abundance was accomplished using the formula: relative transcript abundance = $0.5^{(CT_{Stat3} - CT_{S2})}$ (Oatley et al., 2007).
Transplantation of shRNA lentivirus transduced cultured Thy1+ germ cells

Cultured Thy1+ germ cells were collected by trypsinization and suspended in serum-free medium at a concentration of 2x10^6 cells/mL. Transplantation of donor cells was conducted as previously described (Oatley and Brinster, 2006). Busulfan-treated F1 recipient mice were anesthetized before transplantation by intraperitoneal injections of averting (240 mg/kg; Sigma-Aldrich) and ketoprofin (5 mg/kg; MP Biomedicals; Solon, OH), and virus-treated germ cells were injected into the efferent ducts of recipient mice at a concentration of 2x10^6/mL. Experimental cell suspensions were allowed to fill recipient seminiferous tubules. Recipient mice were F1 progeny of C57BL/6 crossed with 129SvCP mice treated with busulfan (60 mg/kg of body weight; Sigma-Aldrich; St. Louis, MO) to ablate endogenous spermatogenesis.

Analyses of donor SSC colonization of recipient testes

Six weeks after transplantation, recipient testes were analyzed for donor cell colonization of seminiferous tubules. Only SSC are capable of reestablishing spermatogenesis, and because Rose donor germ cells were used for all transplantations, recipient testes were collected and exposed to X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; G-Biosciences; Maryland Heights, MO) to detect β-galactosidase protein which appears blue within seminiferous tubules of recipient testes after exposure to X-gal. Colonies of donor-derived spermatogenesis were observed and quantified using an SZ51 microscope (Olympus; Center Valley, PA) and images of recipient testes were taken using an SZX10 microscope.
(Olympus) and an Axioskop 2 plus microscope (Carl Zeiss, Inc.; Dublin, CA) with a DP71 camera (Olympus) with DP Controller imaging software (Olympus).

RESULTS

Production of Stat3 shRNA lentivirus

To examine the role of Stat3 in SSC proliferation and differentiation in vivo, lentivirus encoding a Stat3 targeting shRNA under control of the constitutively active human U6 promoter was produced to permanently reduce levels of Stat3 mRNA in cultured Thy1+ germ cells. Cultured cells transduced with the shRNA lentivirus constitutively produce Stat3-specific shRNA, which is cleaved by Drosha in the nucleus, transported to the cytoplasm, transformed into small interfering RNA (siRNA) by Dicer-mediated removal of the hairpin and incorporated into RNA-induced silencing complex (RISC) machinery. This complex then functions to bind and cleave endogenous Stat3 transcripts homologous to the siRNA (Figure 1). Additionally, lentiviral transduction results in stable genomic incorporation of the retrovirus; thus shRNA transgene is retained following mitosis, ensuring that daughter cells will possess this form of RNAi. Stat3 gene expression was reduced by 74.7% in cells transduced with Stat3-specific shRNA lentivirus compared to testis cells transfected with the non-target control shRNA lentivirus (Figure 2).
**Stat3 reduction inhibits SSC differentiation in vivo**

To test the role of Stat3 in SSC function in vivo, cultured Rosa Thy1+ germ cells transduced with Stat3-specific or non-target control shRNA were transplanted into recipient testes. One recipient testis from each mouse received Stat3 shRNA lentivirus treated germ cells, while the other testis received germ cells treated with control shRNA lentivirus; therefore, each mouse served as its own control. Recipient testes were evaluated for reestablishment of spermatogenesis 6 weeks later, which encompasses ~1.2 rounds of spermatogenesis in mice (Clermont and Trott, 1969). Colonies arising from cells transduced with Stat3-specific shRNA lentivirus consisted of single cells and short cohorts of LacZ-expressing germ cells (Figure 3A and B). In comparison, cells treated with control shRNA lentivirus produced colonies of full spermatogenesis throughout seminiferous tubules of recipient animals (Figure 3C). These colonies were densely packed with germ cells, indicating that presumably all stages of spermatogenesis were present (Figure 3D).

**DISCUSSION**

Spermatogonial stem cells serve as a foundation for continuous production of spermatozoa throughout adulthood (Oatley and Brinster, 2006; Phillips et al., 2010). Studying mechanisms that lead to self-renewal or differentiation of SSC is important to understanding factors leading to fertility or infertility. With better understand of these mechanisms, comes a greater ability to diagnose and correct male infertility. Also, SSC can serve as a model for other tissue-specific stem cell populations. Because both long-term culture and functional
transplantation of SSC are possible, dynamics of SSC self-renewal and differentiation can be explored more easily than other tissue-specific stem cell populations. To date, a greater understanding of factors affecting self-renewal exists compared to knowledge of differentiation of SSC. The purpose of this study was to investigate the role of the transcription factor Stat3 as a regulator of SSC differentiation. Lentiviral shRNA was used to reduce Stat3 transcript in cultured Thy1+ germ cells and this treatment impaired the ability of SSC to reestablish spermatogenesis in recipient testes.

The SSC culture system used in the present study supports both self-renewal and differentiation of SSC. Therefore, both stem cells and non-stem cell spermatogonia are present within these cultures. Upon germ cell transplantation, only SSC are capable of colonizing a recipient’s seminiferous epithelium and reestablishing spermatogenesis. Because of this, effects observed in donor-derived germ cell colonies originate from transplanted SSC. Another technique used within this study is gene expression modification by means of shRNA, which causes a reduction in transcript abundance of a specific gene – such as Stat3 – using a cell’s endogenous RNAi machinery (Figure 1B).

When differentiation of SSC or other undifferentiated spermatogonia (A-paired and A-aligned) is impaired, spermatogenesis is unable to progress beyond early stages. In this situation, seminiferous tubules would only contain single cells or short chains of spermatogonia but no further differentiated cells. This type of germ cell failure is a cause of infertility as no
spermatozoa would be produced. Over time, such dysfunction may lead to accumulation of SSC or primitive spermatogonia within the testis, indicating increased frequency of self-renewal at the expense of differentiation. Conversely, when SSC are only capable of differentiation at the expense of self-renewal, the stem cells pool would become depleted. In this case, the first few rounds of spermatogenesis would appear normal; however, without self-renewal to replenish SSC, spermatogenesis would cease. Impaired development beyond the A-single and A-aligned spermatogonial stage in Stat3 shRNA transducer cells indicates that Stat3 plays a major role in regulating the differentiation of SSC and likely other undifferentiated spermatogonia.

To our knowledge, this study is the first to reveal an unequivocal determinator of SSC differentiation. This study shows that continuous reduction of Stat3 by shRNA causes a block in SSC and early germ cell differentiation, indicating that Stat3 functions to promote SSC differentiation in vivo. This was evident as only single or short chains of donor derived spermatogonia were observable after transplantation. Hence, this study is the first to report a direct connection between Stat3, and SSC differentiation.

In mouse embryonic stem cells, Stat3 regulates maintenance of pluripotency through increased transcription of Nanog and Oct4 (Hao et al., 2006; Smith et al., 1988). Additionally, in the Drosophila male germ line, Stat92E, the single Drosophila Stat isoform, promotes germ line stem cell self-renewal (Kiger et al., 2001; Tulina and Matunis, 2001). This study indicates a different role for Stat3 was adopted by mouse SSC. Further work should be conducted to
elucidate the activator of Stat3 in SSC and to identify the specific downstream targets of Stat3 in SSC which facilitate differentiation.
Figure 1: shRNA action within a mammalian cell. After transduction with shRNA lentivirus, shRNA DNA is incorporated into host genome under transcriptional control of the U6 promoter (1). Once transcribed, mRNA transcripts form hairpin conformation (2). The Drosha/Posha complex transforms hairpin into pre-shRNA (3); which is transported from the nucleus by Exportins (4). Once in the cytoplasm, the hairpin loop is cleaved by Dicer to form double stranded siRNA (5); which is incorporated into RISC/Argonaut complex (6) to cleave and inactivate mRNA transcripts with sequences that are bound by siRNA (7), causing decreased transcript and protein abundance.
Figure 2: *Reduction of Stat3 by shRNA lentivirus*. Stat3 mRNA abundance after shRNA lentivirus transduction with 5.5 MOI of Stat3-specific or non-target control shRNA virus and 3 days of puromycin selection. Data are represented as fold-difference of relative Stat3 transcript abundance from control treated cells (n=1).
Figure 3: Permanent reduction of Stat3 reduces SSC capacity to differentiate in vivo. Cultured Rosa Thy1+ germ cells were transduced with either non-target control or Stat3-specific shRNA lentivirus. Cultures were selected for successful transduction with puromycin for 3 days before being transplanted into busulfan-treated recipient mouse testes at a standard concentration of 2x10^4 cells per µL of injection fluid. Each mouse had one testis receive control shRNA lentivirus treated germ cells, whereas the other testis received germ cells treated with Stat3-specific shRNA lentivirus. Six weeks (~1.2 rounds of spermatogenesis) later, recipient testes were collected and examined for Rosa donor SSC derived spermatogenesis. Each colony of spermatogenesis is clonally derived from a single SSC. (A) A single SSC capable of colonizing the seminiferous epithelium and surviving 6 weeks after transplantation, but unable to differentiate to A-paired spermatogonia, demonstrating a loss of differentiation capacity at the stem cell stage (bar = 25 µm). (B) Early germ cell differentiation failure as observable by short chains of germ cells without ability to further differentiate to complete spermatogenesis (bar = 25 µm). (C) Recipient testis that received control shRNA lentivirus treated SSCs, displaying typical reestablishment of spermatogenesis (bar = 2.5 mm). (D) A colony of donor derived spermatogenesis originating from a single control shRNA lentivirus treated SSC (bar = 2 mm). Note the densely packed LacZ expressing germ cells, indicative of completion of spermatogenesis.
REFERENCES


CHAPTER 3: NEUROGENIN 3 IS A REGULATOR OF MOUSE SPERMATOГОNIAL STEM CELL DIFFERENTIATION

ABSTRACT

Like other tissue-specific stem cell populations, spermatogonial stem cells (SSC) undergo differentiation while also maintaining a pool of stem cells through self-renewal. These two fate decisions must be tightly controlled, as dysregulation can lead to sub-fertility and eventually infertility. Currently, understanding of the mechanisms regulating SSC fate decisions is limited, particularly factors involved in differentiation. In rodents, the cytokine Glial cell line derived neurotrophic factor (Gdnf) is a regulator of SSC self-renewal. In mice, expression of the basic helix-loop-helix transcription factor Neurogenin 3 (Ngn3) coincides with differentiation of early spermatogonia, but a functional role is not known. Therefore, the objective of this study was to determine whether Ngn3 plays a role in SSC differentiation. To address this, the SSC-enriched Thy1+ germ cell population was studied. Withdrawal and replacement of Gdnf from cultured Thy1+ germ cells resulted in an increase followed by a decrease in Ngn3 mRNA abundance, indicating that Gdnf may be a suppressor of Ngn3 transcription. Within the Thy1+ germ cell population, Ngn3 expression was found in a sub-fraction of cells both in vivo and after long-term culture. Transient reduction of Ngn3 by siRNA treatment in cultured Thy1+ germ cells increased SSC content after multiple self-renewal cycles without affecting spermatogonial proliferation overall, suggesting alteration of the balance between SSC fate decisions in favor of self-renewal. Lastly, it was found that the SSC differentiation factor Signal transducer and activator of transcription 3 (Stat3) binds the Ngn3 promoter to regulate Ngn3 transcription in Thy1+ germ cells. Collectively, these results indicate Ngn3 plays a key role in regulating SSC differentiation through a Stat3 mediated mechanism.
INTRODUCTION

Spermatogenesis is the process of spermatozoa production in adult males, relying on the self-renewal and differentiation of a tissue-specific stem cell population referred to as spermatogonial stem cells (SSC). These activities are important for continual spermatogenesis, as self-renewal ensures maintenance of the stem cell pool; whereas, differentiation is the first step in germ cell progression to A-paired spermatogonia, eventually leading to mature spermatozoa (de Rooij and Russell, 2000). Disruption of either self-renewal or differentiation will lead to infertility (Oatley and Brinster, 2006). Currently, a specific cell surface phenotype for SSC has not been described; consequently, isolating a pure population cannot be accomplished. Therefore, deciphering mechanisms regulating SSC functions has relied on studying SSC-enriched cell fractions. Isolation of the Thymus cell antigen 1 (Thy1)-expressing cell population from adult mouse testes results in ~30-fold enrichment for SSC (Kubota et al., 2004a). While SSC are enriched in this fraction, it is heterogeneous, consisting of a mixture of stem cells and non-stem cell spermatogonia (Oatley et al., 2010). Without the ability to isolate a pure stem cell population, studying SSC specifically in heterogeneous cell fractions, including Thy1+ germ cells, requires functional transplantation to prove a stem cell phenotype, which for SSC is reestablishment of spermatogenesis (Brinster and Avarbock, 1994). The technique for transplanting donor germ cells into recipient testes not only demonstrates existence of SSC but provides a means to assay for stem cell content of an experimental germ cell population (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). In addition, development of a serum free system for long-term culture of Thy1+ germ cells has facilitated a variety of mechanistic studies on the regulation of SSC fate decisions (Kubota et al., 2004a; Kubota et al.,
Together germ cell transplantation and long-term SSC culture have provided a means to study SSC self-renewal and differentiation, but knowledge of mechanisms regulating the dynamics of SSC fate decisions is sparse.

Currently molecular mechanisms underlying self-renewal are better understood compared to that of differentiation. However, the first steps in deciphering the mechanisms regulating SSC differentiation have been undertaken in studies by Oatley et al. (2010), that identified Signal transducer and activator of transcription 3 (Stat3) as a key molecule regulating this SSC fate decision. Also, expression of Neurognin 3 (Ngn3), a basic helix-loop-helix (bHLH) transcription factor, was reported to coincide with early stages of spermatogonial differentiation and was used as a marker for SSC differentiation (Nakagawa et al., 2010; Yoshida et al., 2004). Ngn3 was initially discovered in neural crest cells of mouse embryos (Sommer et al., 1996) and works in conjunction with other bHLH transcription factors to control gene expression. Basic HLH transcription factors have a protein structure containing a single loop of amino acids connecting two $\alpha$-helices, adjacent to a DNA-binding domain (Murre et al., 1994). These proteins form heterodimers with other bHLH proteins and bind E-box sequences of DNA, promoting transcription (Chaudhary and Skinner, 1999; and Murre et al., 1989). Functioning in this manner, Ngn3 has been linked to differentiation of multiple types of progenitor cells, including those of gastrointestinal, hepatic, neuronal and pancreatic lineages, as well as embryonic stem cells (Adewumi et al., 2007; Gradwohl et al., 2000; Lee et al., 2003; Schonhoff et al., 2004; and Wang et al., 2007). In cultured Thy1+ germ cells, large-scale microarray analysis indicated that Ngn3 gene expression is down-regulated by Glial cell line-derived
neurotrophic factor (Gdnf; Oatley et al., 2006), a growth factor necessary for self-renewal of SSC (Kubota et al., 2004b; Meng et al., 2000). Hence, previous studies suggest that Ngn3 could be involved in SSC differentiation but direct evidence of this role has not been reported. Therefore the objective of this study was to determine whether Ngn3 controls the differentiation of SSC.

METHODS

Animals

Cultures of Thy1+ germ cells were established from B6.129S7-GtROSA26Sor/J mice (designated Rosa; The Jackson Laboratory, Bar Harbor, ME), which are C57BL/6 mice that express the E. coli LacZ transgene in all germ cells and most other cell types, allowing for identification of reestablished, donor-derived spermatogenesis after germ cell transplantation. Recipient mice for germ cell transplantation assays were the F1 progeny of 129SvCP x C57BL/6 matings, which are immunologically compatible with Rosa donors. At 6 weeks old, recipient mice were treated with the chemotoxic agent busulfan (60 mg/kg of body weight; Sigma Aldrich; St. Louis, MO) to deplete endogenous spermatogenesis and mice were utilized for transplantation ~2 months later. All experiments not involving cultured Thy1+ germ cells or germ cell transplantation were conducted with germ cells or tissues from inbred C57BL/6 mice (The Jackson Laboratory). All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee (Protocol # 25358 & 25714).
Isolation and culture of Thy1+ germ cells

Thy1+ germ cells were collected from either 6-8 days postpartum (dpp) pups or 3 month old (adult) mice using magnetic activated cell sorting (MACS) as described previously (Oatley and Brinster, 2006). Briefly, testes were collected from donor mice and the tunicae albugineae were removed. Pup seminiferous tubules were digested into single cell suspensions by incubation with 0.25% trypsin/EDTA (Cellgro, Mediatech, Inc.; Manassas, VA) and DNase (7 mg/mL; Sigma-Aldrich) at 37°C for 10 minutes; whereas, adult testes were first subjected to collagenase (1mg/mL; Worthington; Lakewood, NJ) digestion (37°C for 15 minutes) and extensive washing to separate seminiferous tubules and remove interstitial cells. After digestion, clumps of cells and debris were removed by passing the cell suspension through a 40 μm cell filter. Cells were incubated with rat, anti-mouse, Thy1 antibody conjugated to magnetic microbeads (50 μL/mL; Miltenyi Biotech; Auburn, CA) for 30 minutes and washed to remove residual antibody, followed by fractionation using MACS separation columns (Miltenyi Biotech). Cells that bound to magnetic microbeads and were unable to pass through the magnetized column were designated as Thy1+ germ cells; whereas, cells of the flow-through fraction were classified as Thy1-depleted cells. Both Thy1+ and Thy1-depleted cell fractions were collected for protein and RNA isolation. For immunocytochemistry and culture of germ cells, only Thy1+ cell fractions were used.

For primary cultures, Thy1+ germ cells were suspended in mouse serum-free media (mSFM; Kubota et al., 2004a; and Kubota et al., 2004b) supplemented with recombinant human GDNF (20 ng/mL of media; R&D Systems; Minneapolis, MN) and recombinant human Fibroblast
growth factor 2 (Fgf2; 1 ng/mL of media; BD Biosciences; San Jose, CA). Cells were seeded onto mitotically inactivated LIF-secreting STO feeder cell monolayers (American Type Culture Collect; Manassas, VA) and maintained in humidified incubators at 37°C with an atmosphere of 5% CO₂ in air. Medium with growth factors was changed every second day. Every 7 days, which encompasses one in vitro SSC self-renewal cycle of 6 days (Kubota et al, 2004b), germ cells were diluted 1:2 or 1:3 and plated onto fresh STO feeder cell monolayers.

**Quantitative PCR (qPCR) analyses**

RNA was extracted from cell and tissue lysates using Trizol reagent (Invitrogen; Carlsbad, CA) or the AllPrep DNA/RNA/Protein Mini kit (Qiagen; Valencia, CA), according to manufacturer’s instructions. RNA concentration and purity (as determined by 260/280 nm absorbance reading ratios) were determined using the NanoDrop 2000 spectrophotometer (Thermo Scientific; Wilmington, DE). Only samples with a 260/280 nm absorbance reading ratio of greater than 1.75 were used for qPCR analysis. Possible genomic DNA contamination was removed by digestion with DNase I. For each sample, 1µg of RNA was reverse transcribed with oligo(d)T priming using the Super Script III reverse transcriptase kit (Invitrogen). Complementary DNA (cDNA) samples were assayed for *Ngn3* gene expression using validated *Ngn3* TaqMan Gene Expression Assay kit (Applied Biosystems, Foster City, CA). To make quantitative comparisons between samples, *Ngn3* expression was normalized to expression of the constitutively expressed gene *Ribosomal protein S2* (*Rps2*). qPCR was conducted and data were collected on the 7500 Fast Real-Time PCR System (Applied Biosystems). Quantitative comparisons were made by normalizing *Stat3* expression to that of *Rps2*. Calculation of relative
transcript abundance was accomplished using the formula: relative transcript abundance = \(0.5^{(CT_{of\ Stat3} - CT\ of\ S2)}\) (Oatley et al., 2007).

**Western blot analyses**

Protein lysates were isolated from cells or whole tissue using RIPA lysis buffer (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) and from Thy1+ or Thy1-depleted germ cell fractions using AllPrep DNA/RNA/Protein Mini kit (Qiagen). Protein concentration of each RIPA lysate sample was determined using the BCA protein assay (Pierce; Rockford, IL). For isolated Thy1+ or Thy1-depleted cell fractions, low yield of cells necessitated that a standard number of cells were lysed (5-6x10^5 cells total per fraction) and protein concentrations were not determined. For each RIPA lysate, 25 μg of protein was loaded per well in Novex 4-12% Tris-Glycine gels (Invitrogen; Carlsbad, CA), and the entire protein lysate of each isolated Thy1+ and Thy1-depleted germ cell fraction was loaded per well. For standardization, equal numbers of cells were lysed in both Thy1+ and Thy1-depleted germ cell fractions. Gel electrophoresis was conducted using XCell SureLock Mini-Cell electrophoresis system (Invitrogen) at 125 V for 90 minutes. Proteins were transferred to nitrocellulose membrane (Bio-Rad; Hercules, CA) using the XCell II Blot Module (Invitrogen) at 25 V for 120 minutes. Blots were then blocked against non-specific antibody binding by incubation with 5% BSA in TBS-T (tris-buffered saline with 0.1% Tween-20; Sigma Aldrich) for 2 hours at room temperature with gentle shaking. Blots were probed with sheep, anti-human, Ngn3 primary antibody (AF3444; 1 μg/mL; R&D Systems) overnight at 4°C with gentle shaking. On the next day, blots were incubated with secondary donkey, anti-sheep, IgG antibody conjugated to horseradish peroxidase (HRP; sc-2473; 80
ng/mL; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at room temperature with gentle
shaking. HRP activity was then detected using PicoWest Chemilumnescent Detection kit
(Pierce). The signal was detected and digital images were captured after 30 seconds of
exposure using the ChemiDoc XRS Molecular Imaging System (Bio-Rad; Hercules, CA).
Afterward, blots were stripped of antibodies using Restore Western Blot Stripping Buffer (Fisher
Scientific) for 15 minutes. Blots were again blocked with 5% BSA in TBS-T for 2 hours at room
temperature. For normalization, blots were incubated with primary rabbit, anti-human,
Tubulin-β antibody (NB600-936SS; 1.2 μg/mL; Novus Biologicals; Littleton, CO) overnight at 4°C
and with secondary goat, anti-rabbit, IgG antibody conjugated to HRP (sc-2004; 80 ng/mL; Santa
Cruz) for 2 hours at room temperature. HRP activity was detected and digital images were
taken after 15 seconds of exposure. Densitometric analysis was conducted using the ChemiDoc
XRS Molecular Imaging System and Quantity One software (Bio-Rad). Expression of Ngn3
protein was normalized in each sample by dividing signal intensities of Ngn3 by that of Tubulin-
β.

Fluorescent immunocytochemistry

Cultured and freshly isolated Thy1+ germ cells adhered to poly-L-lysine coated cover
slips were fixed with 4% paraformaldehyde and incubated in PBS-T (phosphate buffered saline
with 0.1% Triton X-100; Sigma Aldrich) to permeabilize membranes. Non-specific antibody
binding was blocked by incubating cells in 10% normal goat serum in PBS-T for 1 hour at room
temperature. Rabbit, anti-mouse, Ngn3 primary antibody (sc-25655; 1.33 μg/mL; Santa Cruz)
was added to the cells and incubated overnight at 4°C. Cells incubated with normal rabbit IgG
(1.33 μg/mL; Santa Cruz) in place of primary antibody served as a negative control. Secondary goat, anti-rabbit, IgG antibody conjugated to AlexaFluor 488 (A11008; Invitrogen; Eugene, OR) was added to the cells for 1 hour at 4°C followed by mounting onto glass slides with Vectashield mounting medium containing DAPI (Vector Laboratories, Inc.; Burlingame, CA). The cells were examined for Ngn3-expression using an Axioskop 2 plus microscope (Carl Zeiss, Inc.; Dublin, CA) and a DP71 camera (Olympus) with DP Controller imaging software (Olympus). Five different fields of view (20x magnification) were examined per coverslip for total cell number (determined by DAPI staining) and Ngn3-expressing cells (determined by nuclear FITC staining). Percent of total cells expressing Ngn3 was determined by dividing the number Ngn3-expressing cells by the total number cells (i.e. DAPI stained nuclei).

**Transfection of cultured Thy1+ germ cells with siRNA oligonucleotides**

Cultured Thy1+ germ cells from Rosa donors were transfected with 75 pmol of validated ON-Target plus siRNA oligonucleotides (non-target control or Ngn3-specific; Dharmacon; Lafayette, CO) for 24 hours. DNA was transfected into cells with Lipofectamine 2000 reagent (60 μL; Invitrogen) in Opti-Minimal Essential Media (Opti-MEM; Invitrogen). Twenty-four hours after transfection, Opti-MEM media was replaced with mSFM supplemented with Glial cell line-derived neurotrophic factor (Gdnf; 20 ng/mL of media; R&D Systems; Minneapolis, MN) and Fibroblast growth factor 2 (Fgf2; 1 ng/mL of media; BD Biosciences; San Jose, CA). To determine the reduction of gene expression by siRNA, RNA was isolated using Trizol reagent (Invitrogen) from siRNA transfected cells 24 hours and 7 days after transfection. Relative Ngn3 transcript abundance was evaluated by TaqMan qPCR.
In order to assay for stem cell content of experimental germ cell populations, transplantation of cultured Thy1+ germ cells was conducted as described previously (Oatley and Brinster, 2006). On day 7 of culture, and for 3 consecutive weeks thereafter, an aliquot of Ngn3-specific and non-target control siRNA treated cells were transplanted into recipient testes to evaluate stem cell content. Remaining cells that were not transplanted were subcultured and subjected to another siRNA treatment. Cells were treated with siRNA and collected for transplantation on day 0, 7 and 14, and a final transplantation without a subsequent siRNA treatment was conducted on day 21. Because the self-renewal cycle of SSC in vitro is ~6 days (Kubota et al., 2004b), this strategy examined SSC activity over greater than 3 self-renewal cycles.

**Germ cell transplantation and colonization analyses**

Transplantation of cultured Thy1+ germ cells was conducted as described previously (Oatley and Brinster, 2006). Briefly, cultured Thy1+ germ cells were collected by trypsin/EDTA digestion and suspended in mSFM at a concentration of 2x10^6 cells/mL. Busulfan-treated F1 recipient mice were anesthetized before transplantation by intraperitoneal injections of avertin (240 mg/kg; Sigma-Aldrich) and ketoprofin (5 mg/kg; MP Biomedicals; Solon, OH). A ventral incision was made into the abdomen of the recipient, exposing the testes. For each recipient testis 7 µL of cell suspension was infused into the rete testis filling 80-90% of surface seminiferous tubules. Six weeks after transplantation, recipient testes were collected, detunicated, fixed in 4% PFA and incubated with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; G-Biosciences; Maryland Heights, MO) to label β-galactosidase-expressing
Rosa donor germ cells within seminiferous tubules of recipient testes. Donor-derived colonies of spermatogenesis were examined and counted using an SZ51 microscope (Olympus; Center Valley, PA). Images of colonies were taken using an SZX10 microscope (Olympus) equipped with a DP71 camera (Olympus) and DP Controller imaging software (Olympus).

**Chromatin Immunoprecipitation**

Freshly isolated adult Thy1+ germ cells were examined for physical interaction of Stat3 protein and the Ngn3 promoter DNA using the ExactaChIP Human/Mouse Stat3 Chromatin Immunoprecipitation kit (R&D Systems), following manufacturer instructions. Briefly, after isolation from adult testes, 5x10^6 Thy1+ germ cells were fixed with 1% formaldehyde in PBS to cross-link protein-DNA complexes, and then lysed with 500 µL of Lysis Buffer (R&D Systems). Germ cells were sonicated using a Sonifier 250 Cell Disruptor (Branson Ultrasonics Corporation; Danbury, CT) to a fragment genomic DNA to a size of ~1 kilobase. Lysates were collected via centrifugation, diluted with 1 mL of Dilution Buffer (R&D Systems), and incubated with sheep, anti-human/mouse, Stat3 antibody conjugated to biotin (Part 965817; 5 µL per 5x10^6 cells lysed; R&D Systems) overnight at 4°C with rotation. Negative controls were cells incubated with normal sheep IgG as the primary antibody (R&D Systems). Lysates were incubated with streptavidin conjugated agarose beads (Sigma-Aldrich) to precipitate protein-DNA complexes. Chelating resin solution (R&D Systems) was added to the collected lysate to detach DNA from the isolated DNA-protein complexes. Precipitated DNA fragments were purified using DNeasy DNA purification kit (Qiagen). PCR was then conducted with primers (Forward: GTTGGTGAGCCCTGGAGACCATAT; Reverse: CTGGCCCTGGCCCTGGGCAC) designed to
recognize a 200 bp fragment of the distal Ngn3 promoter region (3548-3748 bp upstream of the transcriptional start site). PCR products were separated on a 0.9% agarose gel and imaged using the ChemiDoc XRS Molecular Imager System (Bio-Rad). Positive controls consisted of PCR with validated primers for the v-myc myelocytomatosis viral oncogene homolog (c-Myc) promoter region, a known target of Stat3 (Forward: AAAAATAGAGAGTGGAAG; Reverse: TGGAATTACTACAGCGAGTCAGAA).

**Statistical analyses**

All numerical data are presented as Mean ± SEM for three replicates of each experiment. Means of treatment groups were compared using the independent t-test function of SPSS version 17.0 statistical software package (Chicago, IL), and effect of treatment overall was made using the general linear model with either the univariate ANOVA (with Tukey’s post hoc test) or repeated measures function. P-values less than 0.05 were considered significant.

**RESULTS**

*Ngn3 expression is down-regulated by Gdnf in cultured Thy1+ germ cells*

Gdnf stimulation is essential for self-renewal of rodent SSC (Kubota et al., 2004a; Meng et al., 2000; Yomogida et al., 2003). Mining of gene expression microarray data releaved multiple genes down-regulated by Gdnf in cultured Thy1+ germ cells containing SSC (Oatley et al., 2006). One gene that fit this profile was *Ngn3*. To confirm the microarray result, qPCR was conducted on cultured Thy1+ germ cells subjected to Gdnf withdrawal and replacement as described previously (Oatley et al., 2006). Withdrawal of Gdnf for 18 hours from cultured
Thy1+ germ cells resulted in an increase ($P = 0.03$) in $Ngn3$ transcript abundance by 4.8-fold. Upon replacement of Gdnf for 4 hours, $Ngn3$ transcript abundance was reduced ($P = 0.05$) to near the levels prior to Gdnf withdrawal (Figure 4). These results show that $Ngn3$ is a Gdnf down-regulated gene implicating a role in regulating the differentiation of SSC.

**$Ngn3$ is expressed by a subpopulation of Thy1+ germ cells in vivo**

Despite previous reports showing expression of $Ngn3$ transcript by Thy1+ germ cells, characterization of Ngn3 expression within this heterogeneous cell population had not been reported. Quantitative PCR analyses revealed that $Ngn3$ transcript abundance is 1.7-fold ($P = 0.05$) and 2.3-fold ($P = 0.003$) greater in the Thy1+ germ cell fraction of pups compared to the Thy1-depleted germ cell fraction and whole testis cell population (Figure 5A). In adult testes, expression of $Ngn3$ was also 1.8-fold ($P = 0.05$) and 3.2-fold ($P = 0.01$) greater in the Thy1+ cells compared to Thy1-depleted cells and whole testis cells, respectively (Figure 5A). Differences in Ngn3 protein expression were determined by Western blotting. Using a commercially available antibody validated to detect Ngn3 in other tissues, a distinct band at ~15 kDa was detected in lysates of whole testis and Thy1+ germ cells from mice. The predicted size of Ngn3 is 23.5 kDa. Unfortunately, previous studies examining Ngn3 expression in the testis have not reported Western blot analyses; thus, the 15 kDa band may represent the testis isoform of Ngn3. In agreement with qPCR results, both pup and adult Thy1+ cells had greater expression of Ngn3 protein compared to Thy1-depleted germ or whole testis cells of pups (Figure 5B and D) and adult mice (Figure 5C and D). Expression of Ngn3 protein in the Thy1+ germ cell fraction of pups was 2.3-fold ($P < 0.001$) and 8.9-fold ($P < 0.001$) greater compared to the corresponding
Thy1-depleted germ cell fraction and whole testis cell population, respectively (Figure 5D). Similarly, expression of Ngn3 protein in the adult Thy1+ germ cell fraction was 1.3-fold (P = 0.05) and 19.1-fold (P < 0.001) greater compared to the Thy1-depleted germ cell fraction and adult whole testis cell population, respectively (Figure 5D). Immunocytochemical analyses revealed that Ngn3 was expressed by 30.5 ± 2.9% and 28.2 ± 1.6% of cells within the germ cell fractions of pup (Figure 2E) and adult (Figure 2F) testes, respectively. These results indicate that Ngn3 is expressed by a subpopulation within the Thy1+ germ cell fraction consistent with heterogeneity of this germ cell population containing SSC and other primitive spermatogonia produced by SSC differentiation. Given that SSC represent a small percentage of the Thy1+ cell fraction; these results indicate Ngn3 expression may be restricted to SSC and daughter cells produced by initial differentiation of SSC.

**Transient reduction of Ngn3 expression enhances SSC self-renewal in vitro**

As a model for in vivo SSC, cultured Thy1+ germ cells were examined for Ngn3 expression. Immunocytochemical analyses showed that 25.1 ± 3.4% of cultured Thy1+ germ cells express Ngn3 similar to the expression profile in vivo (Figure 6A). To examine the role of Ngn3 in regulation of SSC fate decisions, expression of Ngn3 was transiently impaired in cultured Thy1+ germ cells and assayed for changes in SSC content with functional transplantation. Cultured Thy1+ germ cells from Rosa donor mice were transfected Ngn3-specific siRNA for 24 hours resulting in a reduction (P = 0.002) of Ngn3 expression by ~58% compared to cells transfected with non-target control siRNA (Figure 6B). This response was transient, and 7 days after treatment, Ngn3 mRNA abundance was not different (P = 0.965)
from controls. To assay for effects of transient reduction of Ngn3 mRNA abundance on SSC fate decisions, cultured Thy1+ germ cells were treated with siRNA every 7 days for 21 days, which encompasses greater than 3 in vitro self-renewal cycles of 6 days (Kubota et al., 2004b). On days 7, 14 and 21, cultured germ cells were collected, and a portion of the cells were transplanted into recipient seminiferous tubules to determine SSC content, and the remaining cells were treated with siRNA and cultured for another 7 days. Based on transplantation analyses, SSC content was found to be increased by Ngn3 siRNA treatment over the entire 21 day period but was not statistically significant (P = 0.06; Figure 6C). At 7 days of siRNA treatment, SSC content in Ngn3 siRNA treated cultures 1.1-fold of the controls which was not different (P = 0.32). However, after 14 days of siRNA treatment (i.e. 2 siRNA treatments and self-renewal cycles) SSC content was 2.2-fold greater (p = 0.04) in Ngn3 siRNA treated cultures compared to control siRNA treated. At 21 days (i.e. 3 siRNA treatments and self-renewal cycles), SSC content in Ngn3 siRNA treated was 1.7-fold of that in the non-target control siRNA treated cultured which was not different (P = 0.119). While differences in SSC content were observed, the total numbers of germ cells in Ngn3 siRNA treated cultures compared to controls was not different (P = 1.0, 0.884, 0.478, and 0.966 for Day 0, 7, 14 and 21, respectively) at any time point (Figure 6D). Division of SSC results in either self-renewal or differentiation; thus, during steady-state conditions, the balance between these fates would be equal. Therefore, a change in SSC content without associated difference of total germ cell number indicates alteration in the balance of fate decisions. In this respect, increase of SSC content following reduction in Ngn3 mRNA abundance indicates a tip in the balance to favor self-renewal at the expense of differentiation.
**Ngn3 gene expression is regulated by the SSC differentiation factor Stat3**

Previous studies showed Stat3 is a regulator of SSC differentiation (Oatley et al., 2010). Reduction of Stat3 expression caused an increase of SSC content without a change in spermatogonial proliferation overall, identical to results of the current study with transient impairment of Ngn3. Therefore, the impact of Stat3 on Ngn3 expression was investigated. Reduction of Stat3 expression by siRNA treatment in cultured Thy1+ germ cells resulted in a reduction (P = 0.002) in Ngn3 mRNA abundance by ~25% compared to cells treated with control siRNA (Figure 7A). Next, it was tested if Stat3 physically interacts with the Ngn3 promoter domain. Using a bioinformatics approach, a canonical Stat3 binding sequence, TTGGGCCAA, was identified in the distal promoter region of the Ngn3 locus (Figure 7B). Sonication was used to fragment chromatin and Stat3-DNA complexes following cross-linking and ChIP assay was used to extract these complexes. Two-hundred bp PCR primers were designed to amplify the region of the Ngn3 promoter containing the Stat3 binding sequence, and PCR was conducted with Stat3 ChIP-extracted DNA. This assays showed that Stat3 bound the region of the Ngn3 promoter containing the Stat3 consensus element (Figure 7C). These results indicate that the regulator of SSC differentiation within mouse SSC is controlled, in part, by Stat3 promoting transcription of Ngn3.

**DISCUSSION**

Previous studies showed the importance of Gdnf signaling for self-renewal of SSC (Kubota et al., 2004a, Meng et al., 2000; Oatley et al., 2006; Oatley et al., 2007). Decreased Gdnf expression in vivo leads to spermatogenic failure with increasing age, indicating a lack of
self-renewal capability by undifferentiated spermatogonia (Meng et al., 2000). Additionally, overexpression of Gdnf in vivo causes aberrant self-renewal of undifferentiated spermatogonia (Meng et al., 2000; Yomogida et al., 2003). Also, exposure to Gdnf is essential for SSC self-renewal in vitro (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004b; Ryu et al., 2005). Previous studies showed that Gdnf up-regulated genes play major roles in SSC self-renewal (Oatley et al., 2006; Oatley et al., 2007). Thus, genes down-regulated by Gdnf may be regulators of SSC differentiation. The current study shows that Gdnf down-regulated Ngn3, indicating a major role in SSC differentiation.

In tissues other than the testis, Ngn3 expression has been linked directly to differentiation. Expression of Ngn3 by progenitor cells of the embryonic pancreas causes commitment to β-cell lineage (Gradwohl et al., 2000). In addition, expression of Ngn3 controls differentiation of neural progenitors in the hippocampus (Salama-Cohen et al., 2006), retina (Ma et al., 2009) and spinal cord (Lee et al., 2003), as well as endocrine cells of the gastrointestinal tract (Lee et al., 2002). Furthermore, conditional expression of Ngn3 by embryonic stem cells induces differentiation and emboid body formation (Treff et al., 2006). Therefore, Ngn3 appears to have a conserved function in several tissue-specific stem cell populations.

Studies of the male germ line indicate that Ngn3 is marker of early spermatogonial differentiation and is expressed by a subset of cells within this heterogeneous population (Nakagawa et al., 2007; Nakagawa et al., 2010; Yoshida et al., 2006). The current study shows
that Ngn3 is expressed by a sub-population of the Thy1+ germ cell fraction, which contains SSC and undifferentiated spermatogonia (Oatley et al., 2010). The function of Ngn3 in SSC was investigated in vitro using functional germ cell transplantation to quantify stem cell content of Ngn3 siRNA treated cultured Thy1+ germ cells. Over time, an increase in SSC content was measured in cultures of SSC when Ngn3 mRNA abundance was reduced. By the second siRNA treatment, SSC content had increased 2.2-fold in Ngn3 siRNA-treated cultures versus non-target control treated cultures, without affecting total germ cell number in culture suggesting increased SSC self-renewal at the expense of differentiation. These results indicate that Ngn3 regulates SSC differentiation. These results are similar to the effects of reducing Stat3 mRNA abundance in cultures of SSC (Oatley et al., 2010). A molecular connection between Stat3 and Ngn3 would explain the similarities in their function. Previous studies showed that Stat3 promotes Ngn3 transcription in β-cells in vitro (Baeyens et al., 2006), thus we examined whether a similar mechanism exists in SSC. In cultured Thy1+ germ cells, transient reduction of Stat3 expression by siRNA treatment decreased Ngn3 gene expression, signifying a link between these molecules. A possible mechanism by which Stat3 increases Ngn3 gene expression is regulation of promoter activity. Stat3 binds to DNA through palindromic binding sequences, the most common of which is TTNNNNNAA (Seidel et al., 1995). This Stat3 binding sequence was located within the distal promoter region of Ngn3. Using ChIP assay, it was found that Stat3 interacts with the Ngn3 promoter at this canonical Stat3 binding site. These results indicate that Ngn3 is a downstream target of Stat3 signaling in mouse SSC to regulate differentiation (Figure 8).
To date, activators of the Jak2/Stat3 pathway in SSC have not been discovered. One possible cytokine is Leukemia inhibiting factor (Lif), which is enhances germ cell proliferation in vitro (Kanatsu-Shinohara et al., 2007; Kubota et al., 2004b). Also, Lif activates Jak2/Stat3 signaling in embryonic stem cells to promote pluripotency (Matsuda et al., 1999). While Lif enhances germ cell culture, specific function of this growth factor is not known. If Lif acts to activate Jak/Stat pathway in SSC, perhaps its function is not promotion of self-renewal but maintenance of differentiation, to ensure both stem and non-stem cell spermatogonia are present, which may be needed for SSC survival in vitro. Also, the binding partner of Ngn3 protein in SSC has not been identified. One of many helix-loop-helix proteins expressed by germ cells and SSC is Inhibitor of DNA-binding (Id) proteins; which lack a DNA binding domain, therefore binds to other helix-loop-helix proteins to function (Benezra et al., 1990; Norton et al., 1998). Id proteins are also referred to as Inhibitor of differentiation proteins, alluding to their possible role in SSC fate decisions. Id proteins may bind to Ngn3 in SSC, sequestering its function, promoting continued self-renewal and maintenance of stem cell function. However, once Id protein expression decreases or Ngn3 expression becomes more abundant than that of the Id protein, the balance is tipped in favor of differentiation. While Ngn3 is not expressed or is masked by the Id protein, stem cell identity may be maintained. Furthermore, since Ngn3 is suppressed by Gdnf, a Gdnf upregulated protein, such as B-cell CLL/lymphoma 6, member B (Bcl6b), may act as a suppressor of Ngn3 transcription or translation, decreasing Ngn3 expression as a result of Gdnf signaling. Also, the heterodimeric component of Ngn3-DNA binding complex is unknown in SSC. Various bHLH proteins bind to E-box sequences which may dimerize with Ngn3, causing transcription of genes important for differentiation. Additionally,
the target of Ngn3 transcriptional activation has yet to be elucidated. In other tissues, Ngn3 has been shown to activate the transcription of Isl1, Hlx9, NeuroD, Nkx2.2, Pax4, Pax6 and Pdx1 (Gradwohl et al., 2000), but the function of these genes has not been reported in SSC or other germ cells.

In agreement with Ngn3 function in other tissues, this study illustrates that SSC differentiation is regulated by Ngn3 likely through a Stat3-mediated mechanism. To date, reports indicate that Ngn3 may be a conserved marker for tissue-specific stem or progenitor cell differentiation (Gradwohl et al., 2000; Lee et al., 2003; Sommer et al., 1996; Treff et al., 2006). Therefore, SSC may serve as a valuable model for other, less easily studied stem cell populations. Additionally, this report increases understanding of the molecular mechanisms governing SSC differentiation, dysregulation of which is a possible cause of male infertility. In the United States, 1 in 6 couples are infertile and 40% of these are the result of a male factor (Chandra et al., 2005). Therefore, understanding the mechanisms controlling SSC differentiation is of great importance to treating male infertility.
Figure 4: Effects of Gdnf stimulation on Ngn3 mRNA abundance in cultured Thy1+ germ cells.

Relative Ngn3 mRNA abundance normalized to that of Rps2 in cultured Thy1+ germ cells continually exposed to Gdnf (20 ng/mL), subjected to Gdnf withdrawal for 18 hours, or following 4 hours of Gdnf replacement after 18 hours withdraw. N = 3 different cultures; data are Mean ± SEM. * denotes significant difference (P < 0.05). Data are expressed as relative fold change compared to cells cultured without withdrawal of Gdnf.
Figure 5: *Relative Ngn3 mRNA abundance in the Thy1+ germ cell fraction of mouse testes.*

(A) Abundance of *Ngn3* mRNA in Thy1+ and Thy1-depleted germ cell fractions and the whole testis cell population from prepubertal pup and adult mouse testes. *Ngn3* gene expression was normalized to that of *Rps2* (*n* = 3 different cell preparations). Data are Mean ± SEM. Bars with different letters are different (*P* < 0.05). (B) Densitometric analysis of Western blot images (*n* = 3 experiments with different cell preparations). Data are Mean ± SEM. Bars with different letters are different (*P* < 0.05). (C) Representative image of Western blot analysis for expression of *Ngn3* in Thy1+ and Thy1-depleted cell fractions and whole testis cell populations from prepubertal pups. Top panel is *Ngn3* expression and bottom panel is the same blot reprobed for expression of Tubulin-β. (D) Representative image of Western blot analysis for expression of *Ngn3* in Thy1+ and Thy1-depleted cell fractions and whole testis cell populations from adult mice. Top panel is *Ngn3* expression and bottom panel is the same blot reprobed for expression of Tubulin-β. (E) Representative immunocytochemical analysis of *Ngn3* expression in freshly isolated Thy1+ germ cells from pup testes. Total nuclei were determined by DAPI staining and *Ngn3*-specific staining was identified by FITC fluorescence (inset = negative control image). (F) Representative immunocytochemical analysis of *Ngn3* expression in freshly isolated Thy1+ germ cells from adult testes. Total nuclei were determined by DAPI staining and *Ngn3*-specific staining was identified by FITC fluorescence (inlet = negative control). Bars in E and F are 50 μm.
**A**

Relative Ngn3 transcript abundance

- **Thy1+**
- **Thy1-depleted**

<table>
<thead>
<tr>
<th></th>
<th>Pup</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thy1+</strong></td>
<td><img src="Image" alt="Bar Graph" /></td>
<td><img src="Image" alt="Bar Graph" /></td>
</tr>
<tr>
<td><strong>Thy1-depleted</strong></td>
<td><img src="Image" alt="Bar Graph" /></td>
<td><img src="Image" alt="Bar Graph" /></td>
</tr>
</tbody>
</table>

**B**

NGN3/Tubulin-β

- **Thy1+**
- **Thy1-depleted**

<table>
<thead>
<tr>
<th></th>
<th>Pup</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thy1+</strong></td>
<td><img src="Image" alt="Bar Graph" /></td>
<td><img src="Image" alt="Bar Graph" /></td>
</tr>
<tr>
<td><strong>Thy1-depleted</strong></td>
<td><img src="Image" alt="Bar Graph" /></td>
<td><img src="Image" alt="Bar Graph" /></td>
</tr>
</tbody>
</table>

**C**

- **Ngn3** 15 kDa
- **Tubulin-β** 50 kDa

**D**

- **DAPI**
- **FITC**
- **Overlay**

**Pup**

**Adult**
**Figure 6: Effects of reducing Ngn3 mRNA abundance on SSC self-renewal in cultured Thy1+ germ cell populations.** (A) Immunocytochemical examination of Ngn3 protein abundance by cultured Thy1+ germ cells. Representative images from immunocytochemistry conducted on cultured Thy1+ germ cells. Images show staining for nuclei (DAPI) and Ngn3-specific fluorescence (FITC; inset = negative control image). Bars are 50 μm. (B) Relative Ngn3 mRNA abundance in cultured Thy1+ germ cells 24 hours and 7 days after treatment with 75 pmol of Ngn3-specific siRNA. Ngn3 mRNA abundance was normalized to Rsp2. * denotes significant difference from control (P < 0.05). (C) SSC content of cultured Thy1+ germ cells after control or Ngn3-specific siRNA treatment. Cultures were treated with siRNA on Day 0, Day 7 and Day 14 and then transplanted 7 days later. Values are presented as fold-difference of control siRNA SSC content. N = 3 experiments with different cultures. Data are Mean ± SEM. * denotes difference (P < 0.05) from control at each time point. (D) Total numbers of cultured Thy1+ germ cells at each time point after Ngn3-specific or control siRNA treatment. n=3 experiments with different cultures. Data are Mean ± SEM).
Figure 7: Reduction in Stat3 reduces Ngn3 mRNA abundance in Thy1+ germ cells. (A) Relative Ngn3 mRNA abundance in Thy1+ germ cells treated with non-target control or Stat3-specific siRNA. Data are presented as fold-difference compared to siRNA-treated germ cells. n=3 experiments with different cultures. Data are Mean ± SEM. * denotes statistically significant difference from control. (B) Partial sequence of the Ngn3 promoter region containing a Stat3 binding sequence (in red) that was amplified by primers (in grey) designed to recognize this region of the promoter. (C) Representative image of PCR analysis of ChIP assay showing amplification of a 200 bp region of the Ngn3 promoter within DNA fragments bound to Stat3 protein isolated from adult Thy1+ germ cells. Lane: 1) Molecular weight marker (100 bp); 2) Stat3 ChIP of Thy1+ germ cell protein probed for Ngn3 promoter region by PCR; 3) IgG control ChIP of Thy1+ germ cell protein probed for Ngn3 promoter region; 4) Stat3 ChIP of whole testis protein probed for Ngn3 promoter region; 5) Stat3 ChIP of Thy1+ germ cell protein probed for c-Myc promoter region; 6) IgG control ChIP of Thy1+ germ cell protein probed for Ngn3 promoter region; 7) Stat3 ChIP of whole testis protein probed for Ngn3 promoter region. IgG antibody served as a negative control for the Stat3 ChIP assay, and the c-Myc promoter region served as a positive control. N = 3 experiments with different cell preparations.
A

![Graph showing relative Ngn3 transcript abundance with two bars: Control siRNA and Stat3-specific siRNA. The bar for Stat3-specific siRNA is significantly lower than the control.](image)

B

TTGGGCCAA...  
TTGGGCCAA...

C

![Image of a gel with bands numbered 1 to 7.](image)
REFERENCES


deRooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. J Androl 2000: 21:776-98.


Murre C, McCaw PS, Vaessen H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB, Weintraub H, Baltimore D. Interactions between heterologous helix-loop-helix
proteins generate complexes that bind specifically to a common DNA sequence. Cell 1989: 58:537-44.


Sommer L, Ma Q, Anderson DJ. Neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. Mol Cell Neurosci 1996: 8:221-41.


