Understanding the role of Glial cell line-derived neurotrophic factor (GDNF) in regulating stem and progenitor spermatogonia in adult mice and men

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ABSTRACT

Spermatogonial stem cells (SSCs), the foundational cells of spermatogenesis, are maintained through the delicate balance of self renewal or proliferation and differentiation. The growth factor glial cell line-derived neurotrophic factor (GDNF) has been shown to be critical in establishing this pool in the prepubertal animal, but its *in vivo* function in the normal adult testis has yet to be elucidated. We used a unique *in vivo* chemical-genetic approach to test the hypothesis that GDNF was essential in regulating stem and progenitor spermatogonia in the normal adult mouse. Using this experimental strategy, we were able to reversibly inhibit GDNF signaling for acute (2-3 days), intermediate (11 days) and prolonged (30 days) periods of time. Our results showed that this inhibition led to a progressive loss of cells expressing spermatogonial stem cell markers and transcripts. Taken together, we interpret these decreases as a loss of spermatogonial stem cells and/or progenitor spermatogonia. This interpretation is supported by the fact that all spermatogenic cells, including spermatogonia, were lost by maturation depletion after prolonged inhibition of GDNF signaling. These results are the first to provide direct evidence that GDNF regulates the numbers of spermatogonial stem cells *in vivo*, in the normal adult testis.

Based on our observations in the mouse and the striking similarity in spermatogenesis between rodents and humans, we hypothesized that this growth factor has a similar function in men. Specifically, we hypothesized that GDNF expression would be similar in the testes of normal men, when compared
to mice and rats, whose SSCs are GDNF-dependent. Our results show that GDNF mRNA levels in mice and men are almost identical. We also observed that the level of GDNF protein in rat total testicular fluid is identical to humans. These data suggest that GDNF could be essential in regulating human SSCs and progenitor spermatogonia. To further test this hypothesis, we compared GDNF mRNA levels in normal human testes with levels in testes of men who were diagnosed as being infertile due to the lack of spermatogenic cells, resulting in maturation arrest or a Sertoli cell-only (SCO) phenotype. Our results showed that there was an 80% decrease in GDNF mRNA expression in testes exhibiting the later phenotype, but not in testes with maturation arrest of spermatogenesis. To address whether this reduced expression in infertile individuals with the SCO phenotype was due to a complete state of hypo-function or a specific deficiency in their Sertoli cells, we measured transcripts encoding two other Sertoli cell products, Kit ligand and Clusterin. Expression of neither transcript was reduced in these SCO testes. Taken together, our data support the hypothesis that as in rodents, GDNF is essential for maintaining and regulating human SSCs and progenitor spermatogonia. These findings provide strong evidence that decreased GDNF expression may lead to the loss of SSCs, and consequently to human male infertility. Our results open up a new area of research geared towards clinical intervention using therapeutics to increase GDNF concentration in individuals with SCO testes with a few remaining SSCs. The use of such therapeutics might aid in rebuilding the numbers of these stem cells and increase
possibility that sufficient sperm are generated, either for natural or assisted reproduction.
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# TABLE OF CONTENTS

Abstract ii
Acknowledgements vi

Chapter 1: Literature Review 1
   Introduction 1
   Current state of Infertility 2
   Pathology of interest: Azoospermia 3
   Diagnosing Non-Obstructive Azoospermia (NOA) 6
   Treating Non-Obstructive Azoospermia 8
   A brief introduction to spermatogenesis 9
   Spermatogenesis in rodents and humans 10
   Morphological and molecular characteristics of undifferentiated spermatogonia in rodents and humans 11
   Sertoli cells and their role in spermatogenesis 14
   The role of Glial cell-line derived neurotrophic factor in spermatogenesis 15
   Post-transcriptional and post-translation regulation involving GDNF 17
   Summary 19
   References 21
   Figure legend 41
   Figures 42

CHAPTER 2: Understanding the role of GDNF in regulating stem and progenitor spermatogonia in normal adult mice 43
CHAPTER 3: Understanding the role of GDNF in regulating stem and progenitor spermatogonia in fertile and infertile men

Abstract 95
Introduction 97
Materials and Methods 101
Results 107
Discussion 113
Summary 120
References 121
Figure legend 126
Figures 129

CHAPTER 4: Conclusions 140
Introduction 140

The role of GDNF in regulating stem and progenitor
spermatogonia in vivo in the normal adult mouse testis 140

The role of GDNF in regulating spermatogenesis in fertile and infertile men 141

Conclusions and future directions 143

Figure legend 146

Figures 147

Curriculum Vitae 149
CHAPTER 1: LITERATURE REVIEW

Introduction:

The delicate nature of male fertility depends on the proper function of spermatogonial stem cells (SSCs). Extensive research using rodent models identifies glial cell-line derived neurotrophic factor (GDNF) as an essential regulator of the numbers of SSCs both in vivo and in vitro. From these studies, we know that in vivo GDNF over expression results in the formation of clusters of undifferentiated spermatogonia, which are eliminated by apoptosis, resulting in infertile mice [1]. Conversely, testes of GDNF null mice are devoid of most all germ cells within 7 days, and the few remaining germ cells do not replicate [2]. Mice haploinsufficient for GDNF also show a progressive loss of spermatogonia, spermatocytes and spermatids, resulting in a lack of germ cells in mature mice [1]. While informative, these studies demonstrate that GDNF is essential for establishing the stem spermatogonial pool in the context of an immature mouse testis. Prior to the studies described in this dissertation, the effects of the loss of GDNF signaling and the consequences of restoration of this signaling in the normal adult mouse testis have not been elucidated, and thus, proves to be a significant gap in our knowledge. In humans, the potential role of GDNF in the regulation of human SSCs is limited to the knowledge that Sertoli cells transcriptionally express GDNF [3]. Thus, defining the role of GDNF in the human testicular environment, and determining whether it is essential to the regulation of spermatogonial stem cells and progenitor spermatogonia is necessary to furthering our understanding of human male fertility. The studies outlined in this
thesis are an attempt to address these gaps in our knowledge about the role of GDNF in regulating numbers of SSCs in normal adult mice and men. Our results provide important evidence that GDNF concentration in the human testis is sufficient to regulate SSC and progenitor spermatogonia, as shown in the mouse. Furthermore, we demonstrate that GDNF expression is substantially reduced in a subset of men diagnosed with non-obstructive azoospermia. These results have important implications in understating the cause of infertility in a subset of infertile men. The long-term goal of these studies is to translate our findings into the clinic as a mode of growth hormone based therapy for a sub-population of infertile patients, where infertility stems from insufficient stimulation of spermatogonial stem cells by GDNF.

**Current state of Infertility:**

Infertility, as defined by the American College of Obstetricians and Gynecologists, the American Society for Reproductive Medicine (ASRM) and the World Health Organization (WHO), pertains to the inability of a couple to conceive after 12 months of actively trying [4, 5]. Currently, 15% of all couples will face challenges in conceiving, where infertility can be attributed to abnormalities in both, men and women [6]. However, when we take a closer look, we find that ~30-40% of these cases can be solely attributed to the male partner, giving good reason to investigate the causes of male infertility in an effort to alleviate some of the reproductive challenges these individuals face [7]. Apart from the financial and physical stress these couples or individuals deal with, infertility has been shown to directly correlate with a lower quality of life in these
individuals [8, 9]. Male infertility is initially determined based on sperm count, where normal men will have ~ 96 million sperm in their ejaculate; whereas infertile men will present with < 5 million sperm [10, 11]. Once diagnosed, treating these individuals with the aid of assisted reproductive technology (ART) is often challenging, and predicting outcomes of the treatment can be difficult [12-14]. To add to this, the possibility of successful outcomes is slimmer in individuals diagnosed with azoospermia, where patients lack sperm in their ejaculate. While sperm can be surgically retrieved from the testes of some of these men and used to fertilize an egg by ICSI [15], additional work in this field of clinical research still needs to be done to elucidate effective treatments which would enable more infertile men to conceive a child [16].

Pathology of interest: Azoospermia:

The most severe pathology of infertility is azoospermia, defined as the absence of sperm in the ejaculate, affecting ~ 1% of individuals and 10-15% of infertile population [17, 18]. Using diagnostic tools and genetic testing, clinicians can remarkably achieve upwards of a 90% accurate diagnostic rate in distinguishing between the two types of azoospermia; obstructive azoospermia (OA) and non-obstructive azoospermia (NOA) [19, 20]. Individuals with OA present with complete spermatogenesis, but have a blockage occurring in the vas deferens and/or epididymis. Surgeons can use reconstructive procedures to repair the blockage, potentially restoring reproductive function in individuals with obstructive azoospermia to a relatively normal state. In cases where the complete restoration of fertility is not feasible, these individuals are still able to
biologically conceive because surgical sperm retrieval occurs with relatively high efficiency [21, 22]. However, due to a primary defect in the testes of NOA patients, these individuals lack the ability to produce adequate numbers of sperm, making sperm retrieval (SR) more challenging [23]. In individuals diagnosed with the most severe form of NOA, the histologically-defined Sertoli cell only (SCO) phenotype, sperm retrieval is at best 20% successful [24]. Thus, these NOA individuals who wish to conceive often have to defer to multiple SR and ART procedures in order to have their own biological children. While most seminiferous tubules in these patients are without any germ cells, biopsies from these individuals do reveal foci of spermatogenesis and sperm in 30-60% of NOA men [18, 25-27]. Thus, even though NOA men have significantly reduced number of SSCs, therapies geared towards increasing the numbers of the remaining stem cells might increase the numbers of sperm in their testes, thereby increasing the possibility that they might conceive a child either naturally or with the aid of assisted reproductive technologies.

The etiology of NOA can be genetic (ex: Y chromosome microdeletions (YCMDs), congenital (ex: cryptorchidism), acquired (radiation, chemotherapy or trauma) or idiopathic (80% of the cases) [20, 28]. As previously mentioned, the etiology of NOA does not predict SR rates in NOA patients and as a consequence, clinicians cannot predict whether sperm retrieval will be successful. However, it is interesting to note an increase in the incidence of acquired cases. One such example can be attributed to the advancements in cancer therapies. While treatments consisting of exposure to radiation or high-
dose chemotherapy have contributed to a rise in individuals in remission, such treatments can be detrimental to an individual’s fertility [29-31]. For instance, exposure to chemotherapy has been linked to infertility stemming from a significant decrease in spermatogenic cells, including spermatogonial stem cells [29, 32-35]. Treatment has also been shown to induce DNA damage in sperm. Results from studies in rodents have shown a higher rate of DNA damage or chromosomal aneuploidy in germ cells of exposed animals [36-40]. With that said, it is important to keep in mind that the degree of severity in infertility is collectively dependent on factors such as age, dose of chemotherapy, and frequency of exposure [31, 41]. It is possible for individuals to experience a restoration in normal spermatogenesis, either immediately or after non-specific delays, which can last up to 4 years or more [42].

When it comes to preservation of fertility, cryopreservation of sperm prior to chemotherapy exposure is an option. However, only a minimal percentage of men pursue this option [43]. In addition, this is not a viable option for pre-pubertal individuals, who lack sperm. With the limited option of being treated with chemotherapeutic agents that are less toxic to their reproductive organs, these pre-pubertal individuals are faced with the dire reality that they could be infertile post-treatment [42]. As previously mentioned, in some cases, fertility can be restored many years later. However, there are also individuals who present with normal sperm counts post treatment, but experience a sequential loss of cells over time [42]. Thus, there is a growing need to provide an effective treatment plan for such individuals who later on in life express a strong desire to biologically
conceive. Research geared towards addressing restoration of fertility or preservation of SSCs in these individuals has been primarily based on studies performed in rodents. For instance, research in rodents exposed to chemotherapy has provided promising results showing that suppression of gonadotropins and testosterone, using gonadotropin-releasing hormone antagonists and anti-androgens, contributes to a recovery of surviving endogenous SSCs [30, 44-47] However, the results have yet to be recapitulated in clinical trials [48].

**Diagnosing Non-Obstructive Azoospermia (NOA):**

Non-obstructive azoospermia can be differentiated from obstructive azoospermia using family history, genetic testing, hormone analysis and physical examination. For instance, hormone analysis is used to identify individuals who are NOA due to hypogonadotropic hypogonadism (HH), an endocrine disorder, which leads to insufficient gonadotropin stimulation of spermatogenesis [49]. These individuals symptomatically present with low levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone due to improper function of the hypothalamus and/or pituitary gland [18, 49]. In this small subset of individuals, exogenous gonadotropins or gonadotropin-releasing hormone (GnRH) therapy aids in partial spermatogenic recovery and sperm retrieval [50, 51]. This recovery is due to increased stimulation of the testis by LH and FSH via their receptors on Leydig cells and Sertoli cells, respectively [49, 52].

In addition to hormone analysis, genetic testing is often used to identify the causes of NOA. One of the most commonly used diagnostic techniques
involves identifying individuals with microdeletions in the long arm of the Y chromosome referred to as AZF, accounting for ~10% of NOA cases [53]. AZF can be classified into four categories (AZFa, AZFb, AZFc, AZFb+c), each associated with a varying degree of severity in azoospermia [54, 55]. Majority of the cases can be attributed to an AZFc deletion (~80% of cases), followed by AZFb deletion (1%–5% of cases), AZFa deletion (0.5%–4% of cases) and AZFb+c deletions (1%–3% of cases) [54, 56, 57]. Individuals with the AZFa deletion are mostly diagnosed with SCO testicular pathology, implying a poor rate of sperm retrieval by biopsy or testicular sperm extraction [53, 58-61]. The pathology of individuals presenting with AZFb and AZF b+c deletions is similar to SCO individuals, with equally discouraging sperm retrieval (SR) success rates [53, 60, 61]. In men who present with complete AZFa, AZFb or AZF b+c microdeletions, SR is not recommended. Patients with AZFc deletions show signs of sporadic spermatogenesis, with promising SR rates (50%–70%) and a high probability of fatherhood by ICSI [53, 56, 62-65]. It is important to note that male children born to these individuals inherit the microdeletion and are infertile.

Even with the current innovations in diagnostic tools, histopathologic examination of testicular biopsies are still considered the gold-standard for diagnosing and confirming cases and severities of NOA. Visual examination has the unique perspective of distinguishing between cases of hypospermatogenesis, germ cell maturation arrest, Sertoli-cell-only, or a combination of pathologies [66]. The use of biopsies also aids in predicting the chances of successful sperm retrieval [67, 68].
Treating Non-Obstructive Azoospermia:

The current standard of care for NOA individuals interested in conceiving involves testicular sperm extraction: TESE and microdissection TESE (micro-TESE) [18, 26]. The main objective of these surgical procedures is to obtain a sufficient quantity of good quality sperm to be used with assisted reproductive technologies or for cryopreservation, while minimizing testicular damage and preserving vasculature. Typically, each seminiferous tubule of an NOA patient will only have 0–3 mature spermatids [69, 70]. In conventional TESE, randomly selected sections of seminiferous tubules are extracted and those biopsies are examined for active areas of spermatogenesis and sperm, which can be found in 30-60% of NOA men [18, 25-27]. However, this procedure requires the invasive acquisition of large testicular biopsies and can compromise androgen function in these individuals [71, 72]. Micro-TESE, developed by Dr. Peter Schlegel, provides a better alternative to TESE because active areas of spermatogenesis are identified with the aid of an operating microscope (Figure 1) [73]. This procedure begins with an incision in an avascular area of the testis, as in conventional TESE, and enlarged seminiferous tubules characteristic of active spermatogenesis are identified under ×15–25 magnification. As a result, 50-70 fold smaller biopsies are extracted and the chances of vascular injury or other complications are significantly decreased. In addition, this procedure leads to SR in one-third of the cases previously thought not possible [71, 73-75]. Patients needing additional surgeries are advised to wait at least 6 months between procedures to allow for adequate recovery. In turn, this recovery period
significantly improves the chances of sperm retrieval (25% vs. 80%) in a subsequent operation [71, 73]. When possible, cryopreservation of remaining testicular sperm is recommended, especially after successful sperm retrieval in NOA patients. However, the most successful strategy for conception in NOA individuals involves the use of fresh testicular sperm. It should be noted that successful sperm retrieval coupled with ART does not ensure successful live births. For instance, studies performed by Esteves and colleagues have reported significantly lower pregnancy rates and live births using sperm retrieved from NOA individuals [76-79]. In instances where live births were successful, the authors stated that additional follow up studies were required to elucidate the physical, neurological, and developmental outcomes of children conceived from sperm acquired from NOA individuals [80, 81].

As previously mentioned, recent findings are reporting focal areas of active spermatogenesis in NOA men. These focal areas must contain spermatogonial stem cells, which under the appropriate stimulus might expand in number and fill empty stem cell niches. This area of research is of particular interest to us because of the potential it holds towards creating fertility options for infertile patients who have a strong desire to have biological children.

A brief introduction to spermatogenesis:

The development of new therapies for infertile men requires a fundamental understanding of the process by which sperm are formed, spermatogenesis. This process takes place in the testis, which is sectioned into two compartments. The majority of the testis is comprised of seminiferous
tubules, the site of spermatogenesis. The remaining ~10% of the testis is comprised of the interstitium, made up of Leydig cells, blood vessels, immune cells and vasculature [82, 83]. Spermatogenesis can be divided into 3 stages: mitotic proliferation of spermatogonia, meiosis, and spermiogenesis [84]. During the first phase of this process, some of the replicating spermatogonial stem cells begin to differentiate, thereby forming transit amplifying progenitor spermatogonia [85, 86]. These progenitors amplify through mitotic division. Stem cells differ from and progenitor cells in that they have the potential to replicate indefinitely, while progenitors proliferate a limited number of times [87]. Progenitor spermatogonia ultimately generate fully differentiated spermatogonia. In the mouse after 5 additional mitotic divisions, preleptotene spermatocytes are formed, and meiosis commences [87]. The finals steps of spermatogenesis begin with spermiogenesis, which refers to a cytological transformation in round spermatids, forming spermatozoa. These cells are then released into the lumen of the seminiferous tubule by a process called spermiation [84]. Spermatogenesis requires this sequential transition from one step to another. Disruption during any of these transitions or stages can lead to infertility. 

**Spermatogenesis in rodents and humans:**

In mammals, spermatogonial differentiation and germ cell development is a highly synchronized process, with spermatogonia, spermatocytes and spermatids at specific states of development always found together in what are the stages of the cycle of the seminiferous epithelium. There are 12 distinct stages in mice and 14 in rats. In almost all mammals, except great apes and
humans, one stage can be identified per cross-section of a seminiferous tubule and sequential movement through all the stages constitutes a complete cycle [88, 89]. One cycle lasts 8.62 and 13 days and spermatogenesis takes 34.5 and 51.6 days in mice and rats, respectively [90]. The organization of stages is different in humans. Clermont described six stages in the human, and 2-4 different stages can be found in the same seminiferous tubule cross-section, adding significantly to the challenge of identifying distinct germ cells and/or stages present in each cross-section [91]. In humans, spermatogenesis takes 64 days to complete, where one cycle takes 16 days [90]. Thus, when comparing histology of the seminiferous tubule in rodents and humans, we can conclude that rodents display a linear pattern with one stage present per cross section in rodents, while humans display a patchy pattern where a cross section contains germ cells present in multiple stages.

**Morphological and molecular characteristics of undifferentiated spermatogonia in rodents and humans:**

Collectively, spermatogonial stem cells and progenitor spermatogonia comprise a population of cells that are often called undifferentiated spermatogonia. Oakberg was the first to define these cells by their morphology, and classified them as $A_s$ single ($A_s$), $A_{pr}$ paired ($A_{pr}$) or $A_{al}$ aligned ($A_{al}$) spermatogonia. $A_s$ spermatogonia can be identified because they exist as single cells and lack intercellular bridges, which can be found between $A_{pr}$ or $A_{al}$ spermatogonia [92]. Two cells connected by an intracellular bridge are called $A_{pr}$ spermatogonia. Longer chains of connected undifferentiated spermatogonia are $A_{al}$
spermatogonia. All of these undifferentiated spermatogonia can be found in areas adjacent to the stem cell niche, are proposed to be associated with niche vasculature [93-95] and are present at all stages of the of the seminiferous epithelium. In the rodent testis, there are around 35,000 Aₖ spermatogonia, contributing to ~11% of the undifferentiated spermatogonial population and 0.03% of the germ cell population [90, 96]. In the mouse, spermatogonial stem cells (SSCs) represent a subpopulation of Aₖ spermatogonia that can asymmetrically divide to self-renew or give rise to Aᵥpr spermatogonia, depending on the appropriate environmental cues. It is estimated that each mouse testis contains 3,000 to 6,000 functional SSCs [95]. Once formed, Aᵥpr spermatogonia undergo clonal expansion to generate chains of Aₐₜ spermatogonia, which typically consist of 4, 8 or 16 cells. In the mouse, Aₐₜ spermatogonia differentiate to form A₁ spermatogonia, which sequentially undergo mitotic divisions to give rise to A₂, A₃, A₄, Intermediate (In) and Type B spermatogonia. Thus, through this clonal expansion, each SSC division has the capacity to expand as much as 4000-fold, giving rise to as many as 4096 spermatids [97]. Detailed long-term lineage tracing and transplantation studies have revealed two molecular markers whose expressions are restricted to Aₖ spermatogonia in the adult mouse testis, PAX7 and ID4 [98-101], and thus are currently the optimal markers for SSCs. These cells also express GFRα1, THY1, POU5F1, PLZF, NGN3, GRP125 and α6-Integrin, as do progenitor spermatogonia [91]. The transition from undifferentiated to differentiated spermatogonia is often marked by cKIT expression in Type A and B spermatogonia [102].
In humans, spermatogenesis begins with two distinct types of spermatogonia, \( \text{A}_{\text{dark}} (\text{A}_d) \) and \( \text{A}_{\text{pale}} (\text{A}_p) \) [82, 97, 103, 104]. \( \text{A}_d \) spermatogonia are referred to as the reserve stem cell population, which normally remain unproliferative unless significant numbers of spermatogenic cells are lost because of irradiation, chemotherapy or other injuries [97, 103, 105, 106]. Whereas, \( \text{A}_p \) spermatogonia proliferate regularly to self renew or undergo one division to form a type B spermatogonia. \( \text{A}_d \) and \( \text{A}_p \) spermatogonia are morphologically distinct; the nuclei of \( \text{A}_d \) spermatogonia contain a large vacuole-like cavity called a chromatin rarefaction zone [107]. In contrast, \( \text{A}_p \) nuclei do not have this zone and contain 1-3 nucleoli attached to the nuclear membrane. While there is no clear molecular definition of SSCs in human, von Kopylow and colleagues have shown evidence that FGFR3 is a reliable marker for \( \text{A}_d \) spermatogonia by demonstrating that FGFR3\(^+\) cells expressed the chromatin rarefaction zone [108]. Recently, our lab and other researchers have also observed a hybrid phenotype of dark and pale spermatogonia, where these cells share a few characteristics of both cell types [103]. While a true population of SSCs in humans remains to be identified, there is some evidence suggesting that a subset of \( \text{A}_p \) spermatogonia may also contribute to the SSC population. For instance, Schulze and colleagues have shown the presence of mostly \( \text{A}_p \) spermatogonia, among very rare \( \text{A}_d \) cells, in recipients of radiation therapy and in post pubertal individuals with cryptorchid testes [106]. Compared to rodents, each SSC division in humans only gives rise to 32 spermatids [97]. Like rodents, human undifferentiated spermatogonia express a broad range of molecular
markers comprising of UTF1, MAGE-A4, FGFR3, GFRα1, α6-INTERGRIN, PLZF, SALL4, UTF1, UCHL1 and THY1 [104, 105, 108, 109].

Sertoli cells and their role in spermatogenesis:

Spermatogenesis and male fertility relies on Sertoli cells to produce and respond to a complex spectrum of paracrine and endocrine factors influencing spermatogonia [110, 111]. SSC and/or progenitor maintenance and behavior is heavily influenced by the stem cell niche, created at least in part by the Sertoli cell [86]. Located at the basement membrane, these somatic multitaskers provide immunological, nutritional, and structural support as well as growth factors and cytokines that target different cells in the spermatogenic lineage. By forming tight junctions between neighboring Sertoli cells, they also create the blood-testis-barrier, which separates meiotic and haploid germ cells in the adluminal compartment from SSCs, progenitor spermatogonia and mature spermatogonia in the basal compartments of the seminiferous tubule [86, 112]. This barrier prevents molecules greater than 1000 daltons from moving into and out of the seminiferous tubule, protecting the germ cells from inflammatory interactions with leukocytes and antibodies [113-116]. Sertoli cells also have phagocytic activity and can degrade apoptotic cells or residual bodies, clearing the testicular environment of unwanted debris [117]. It is equally important to have adequate quantities of Sertoli cells, ensuring normal spermatogenesis by establishing proper testicular size, and germ cell and sperm yields [118]. Sertoli cells proliferate and mature during finite periods of development to form a stable population; where their maturation is marked by morphological changes in the
nucleolus, shedding of their proliferative activity, down regulation of anti-Mullerian hormone and aromatase, and up-regulation of GATA-1 expression [119]. Once mature and stable, Sertoli cells express Wilm’s tumor protein (WT1) and GATA-4 binding protein [120-123]. In addition, Sertoli cells express the transmembrane growth factor, Kit ligand (KIT1), which binds to the receptor ckit, to regulate the proliferation and/or differentiation of Type A spermatogonia [124-127]. Thus, due to their critical role, it is reasonable to assume that any abnormality in Sertoli cell number, maturation, and/or function can lead to a disruption in spermatogenesis and in some cases infertility.

The role of glial cell-line derived neurotrophic factor (GDNF) in spermatogenesis:

Sertoli cells aid in regulating SSC self-renewal or differentiation by secreting a variety of hormones, growth factors and cytokines into the SSC environment. Furthermore, they mediate the effects of follicle stimulating hormone (FSH) and testosterone on spermatogenesis, because of their expression of receptors for those hormones. [113]. Sertoli cells also secrete growth factors such as FGF2 and EGF, which have been shown to be necessary for in vitro SSC proliferation [128]. However, FGF2 and EGF are not solely enough to stimulate in vivo self-renewal of SSCs [128]. To date, GDNF, secreted by Sertoli cells, is the only paracrine factor in the testis shown to be absolutely required in vivo by SSCs [1, 129].

GDNF has been shown to be essential for neuronal, renal and testicular development [130]. For instance, GDNF knockout mice are embryonic lethal and die within the first day of birth due to severe abnormalities in renal and neuronal
development, such as absent ureteric buds, kidneys and enteric neurons [131-133]. Mechanistically, GDNF acts via a complex consisting of a glycosylphosphatidylinositol (GPI)-anchored cell surface ligand binding subunit (GFRα1) and a tyrosine kinase transmembrane protein (RET) [134, 135]. In the testis, the downstream effects of GDNF signaling lead to the phosphorylation and subsequent activation of multiple signaling pathways, such as the AKT and SRC, causing an increase in SSC proliferation [136-139]. Mice lacking the Ret or GFRα1 receptor subunits show the same phenotype of severe abnormalities in renal and neuronal development [140-143]. While Gdnf +/− mice do survive and are fertile, histological analysis of their seminiferous tubules reveal that spermatogenesis does not occur normally [1]. For instance, the histology of young Gdnf +/− mice reveal spermatids in ectopic positions or phagocytosed by Sertoli cells. In older Gdnf +/− mice, germ cells were absent in majority of the seminiferous tubules, resulting in a Sertoli cell only phenotype. In contrast, GDNF over expression under the testis-specific human translation elongation factor–1a (EF-1a) promoter in transgenic mice resulted in an accumulation of stem and undifferentiated spermatogonia [1, 144]. However, it is important to note that as these cells accumulated, they formed testicular tumors and led to a state of infertility because they failed to give rise to differentiated spermatogonia and eventually sperm [145]. Together, these elegant in vitro and in vivo findings provide strong evidence that altering GDNF signaling to SSCs throughout the life of the animal has a significant effect on their numbers.
Post-transcriptional and post-translation regulation involving GDNF:

MicroRNA’s (miRNA’s) have been widely studied and shown to be involved in regulating the stem cell niche at the post-transcriptional level. For instance, over-expression of miRNA-221 and miRNA-222 in the mouse has been shown to inhibit the effects of retinoic acid, halting the transformation of undifferentiated spermatogonia into differentiated spermatogonia, which are c-kit positive [146]. GDNF can actually enhance the expression of these miRNA’s, while retinoic acid dampens the effect [146]. Similarly, miRNA-146 has been implicated in affecting the expression of differentiation factors such as c-kit, Stra8 and Sohlh2 [147]. MicroRNA’s have also been shown to influence mouse SSC self-renewal. He and colleagues have shown that miRNA-20 and miRNA-106a influence self-renewal of spermatogonial stem cells by targeting Stat3 and Cyclin D1 [148]. On the other hand, there is limited information regarding the impact of post-translational modifications of GDNF on its biological activity and clinical use. In mammalian cells, GDNF exists as a preproprotein, which dimerizes during secretion and folds by forming a disulfide bond. Following this, it is modified via N-linked glycosylation and proteolysis [149]. Piccinini and colleagues have shown that GDNF is modified by N-linked glycosylation (Asn⁴⁹), which assists in the proteolytic processing of GDNF and makes it more stable, which may aid in its clinical use [149]. In regards to its therapeutic administration, it is recommend that GDNF, like other therapeutic proteins, be administered in a form inclusive of the protein structure and glycosylation pattern comparable to its native form [150]. When administered in this manner, studies in the pig have shown that pure
GDNF delivered intravitreally at a concentration of 100 ng has a half life of 1.5 day [151].
As previously mentioned, majority of our knowledge regarding the regulation of spermatogonial stem cells is based on studies in the mouse. Previous studies have shown that spermatogonial stem cells sustain spermatogenesis through a continual maintenance of the stem cell pool, either through self-renewal or differentiation [152]. In 2000, Meng and colleagues showed that glial cell line-derived neurotrophic factor (GDNF) was involved in SSC regulation, by influencing SSC self-renewal [1]. They specifically showed that transgenic mice overexpressing GDNF demonstrated an accumulation in undifferentiated spermatogonia and a termination in differentiating spermatogonia in their seminiferous tubules. On the other hand, GDNF heterozygous mice showed a progressive loss in spermatogonia [1]. In both scenarios, these mice were rendered infertile. Similarly, animals with deficiencies in Ret or GFRα1 exhibit similar phenotypes [2, 153, 154]. These data form the foundation of our current understanding in the field: GDNF influences SSC behavior, in vitro and in vivo. Specifically, we know that under high concentrations of GDNF, SSCs undergo self-renewal. In contrast, under low concentrations of GDNF, SSCs preferentially differentiate.

Despite the progress in our understanding of how SSCs are directly influenced by GDNF, there are still significant gaps in our knowledge. For instance, little is known about how SSCs are regulated in vivo in normal adult mice and humans. These gaps in knowledge primarily stem from the limitations we face in terms of the experimental methods available to specifically manipulate
SSC numbers or signaling to these cells in adult mice and men. We have been able to overcome these limitations in the mouse by using a unique chemical-genetic approach, allowing us to reversibly inhibit the downstream signaling effects of GDNF. Using this approach, we were able to prove that GDNF acutely regulates the numbers of spermatogonia stem cells in the normal adult mouse testis. Based on similarities in spermatogenesis between mice and men, we wanted to test the hypothesis that levels of this growth factor in fertile human testes are similar to those in rats and mice, thereby supporting the hypothesis that GDNF is essential for the \textit{in vivo} maintenance of human SSCs and progenitor spermatogonia. If true, then our hypothesis would predict that the testes of men with NOA (SCO) testes would express low levels of GDNF. Thus, it would follow that restoring GDNF levels in these men would aid in the partial recovery of spermatogenesis, by providing the appropriate stimulus needed for SSCs to self-renew and replenish the stem cell pool. As previously mentioned, with the exception of invasive surgeries with low success rates, there is no effective treatment for NOA patients. However, with the discovery of focal areas of spermatogenesis, access to SSCs can have direct implications in treating a subset of infertile individuals, opening up a new field of research and potential treatment for NOA [155-158].
REFERENCES


FIGURE LEGEND

Figure 1. Schematic showing the steps involved in microdissection- TESE. Figure adapted from Esteves (2015), reference # [20].
Figure 1.

Microdissection TESE requires use of an operating microscope.

Testicular suspensions are then immediately examined under the inverted microscope at 400x magnification to determine whether sperm is found.

Non-enlarged tubules are easily visualized (blue arrow) and usually contain no sperm.

Tubules with larger diameter (green arrow) are more likely to contain sperm, and can be identified under 15-25 magnification after opening the testis.

Microsurgically-guided excision of tiny volumes of testicular tissue containing the tubules with larger diameter is performed. Extracted specimens are placed in sperm culture medium and transferred to the IVF laboratory. Specimens are minced mechanically under the stereomicroscope to allow tubular break down and cellular content loss.
CHAPTER 2: Understanding the role of GDNF in regulating stem and progenitor spermatogonia in normal adult mice

This work cited in this chapter has been published:


Role in published work: I discussed experimental design, conducted the experiments testing the function of GDNF in the adult testis. Additionally, I contributed to the writing of the published manuscripts.
ABSTRACT

Male fertility is based on the delicate balance between re-newel of stem cells and their proliferation and differentiation. The growth factor glial cell line-derived neurotrophic factor (GDNF) has been shown to be critical in establishing this pool in the prepubertal animal, but its *in vivo* function in the normal adult testis has never been directly studied. Thus, the mechanisms regulating the size of this stem cell pool in the adult still remain unknown. Using a unique *in vivo* chemical-genetic approach, we were able to address this gap in our knowledge by investigating the effects of inhibition of the GDNF signaling complex in the normal adult testicular environment. The genetic aspect of this approach refers to the introduction of a single amino acid mutation (V805A) into the ATP binding site of Ret, the kinase subunit of the GDNF receptor. While this mutation does not affect normal GDNF signaling, it renders the receptor susceptible to inhibition by a bulky ATP competitive inhibitor, NA-PP1. Using this strategy, we were able to show that after the downstream effects of GDNF signaling was blocked in adult mice for 11 days, most spermatogonial stem cells were lost. The rare surviving cells expressed the stem spermatogonial markers, GFRα1 and Zbtb16. In addition, testicular spermatogonial stem cells (SSCs) message levels were reduced, with the most substantial reduction reported in Ret mRNA levels. Taken together, these decreases reflect the loss of spermatogonial stem cells and/or progenitor spermatogonia. Interestingly, when signaling was restored by cessation of NA-PP1 treatment, the remaining stem cells initiated the restoration of spermatogenesis. However, when the signaling complex was inhibited for a
prolonged period of 30 days, the process of spermatogenesis was permanently disrupted, indicating that the SSCs had been lost. These results are the first of its kind to provide direct evidence that GDNF regulates the numbers of spermatogonial stem cells \textit{in vivo}, in the normal adult testis.
INTRODUCTION

Spermatogonial stem cells are the foundational cells of spermatogenesis, and their preservation is essential for male fertility [1]. The mechanisms that control their preservation prevent the pathological accumulation of stem cells or, alternatively, the depletion of the stem cell pool. Sertoli cells have been shown to be an integral part of this regulation because they secrete GDNF, which has been shown to promote self-renewal over differentiation of replicating stem cells [1]. However, this has yet to be shown in vivo, in the context of a normal adult testicular environment.

GDNF has been demonstrated to be critical in regulating the numbers of SSCs in in vitro and in vivo studies. For instance, long-term culture studies with GDNF have shown that it is required to maintain and expand SSCs, as measured by their ability to restore spermatogenesis when transplanted into a germ cell-deficient testis [2]. This suggests that GDNF preferentially promotes self-renewing replication of SSCs. If true, then inhibition of GDNF signaling would result in a rapid loss of some of these cells within one cell cycle, which is estimated to be 43-46 hours in duration [3-8]. Contradictorily, cultures enriched in mouse SSCs deprived of GDNF for 3 or 6 days result in a significant increase in the numbers of functional stem cells, as evaluated by transplantation, a result identical to what was observed when the cells were incubated with GDNF [9]. The numbers of spermatogonia that were non-functional stem cells substantially increased when GDNF was added to the cultures, suggesting that GDNF may not always preferentially promote self-renewal over differentiation. This
hypothesis was also challenged by evidence in rat stem spermatogonial cultures, where one stem cell would undergo self-renewing replication, while another in the same microenvironment would produce differentiating progeny [10]. Taken together, these results suggest that cell fate decisions may be regulated by mechanisms intrinsic to the stem cells themselves.

*In vivo* studies using transgenic overexpression or a gene knockout model of GDNF have tremendously contributed to our understanding of how GDNF regulates SSCs and progenitor spermatogonia by examining the consequences of altered GDNF expression from the time of birth or earlier. From these studies, we know that GDNF overexpression results in the formation of clusters of undifferentiated spermatogonia, where many of the cells in these clusters are eliminated via apoptosis, resulting in infertile mice with seminiferous tubules primarily lined only with a rim of spermatogonia or Sertoli cells [11]. However, the authors were unable to determine if these stem cells were functional because they expressed the transgene, and consequently, repeated their abnormal phenotype when transplanted into a germ cell-deficient testis [12, 13]. Similarly, testes of GDNF null mice are devoid of most germ cells within 7 days, and the few remaining germ cells did not replicate [13]. Mice haploinsufficient for GDNF also show a progressive loss of spermatogonia, spermatocytes and spermatids, resulting in a lack of germ cells in mature mice. While this may support the hypothesis that GDNF promotes self-renewal of stem spermatogonia, it should be noted that the first wave of spermatogenesis is initiated from gonocytes, and not stem spermatogonia [14]. This suggests that a complete deficiency in GDNF
at birth would result in the failure of the gonocytes to give rise to a stem spermatogonial pool of normal size and function. Taken together, previous in vivo studies have demonstrated that GDNF is essential for establishing the stem spermatogonial pool in the immature testis. However, direct analysis of the effects of the loss of GDNF signaling and the consequences of restoration of this signaling in the normal adult mouse testis have yet to be elucidated.

Based on the critical relationship between a functional stem cell pool and male fertility, we concluded that a new paradigm was necessary to allow one to study the downstream effects of GDNF signaling on spermatogonial stem cells within the physiological context of the normal adult testis. Therefore, we employed the use of a highly specific chemical-genetic approach, which allowed for the reversible inhibition of GDNF signaling [15-18]. This signaling normally occurs by first binding to the receptor, GFRα1. This dimeric complex then binds to the tyrosine kinase subunit of the receptor, Ret, which leads to activation of signaling by phosphorylation and autophosphorylation, and completes the signaling complex (Figure 1A) [19]. Our mice carry a single amino acid mutation (V805A) in Ret, which has no effect on baseline Ret kinase activity, but substantially increases its affinity for the ATP competitive inhibitor, NA-PP1 (Figure 1B) [20]. While Ret is also a subunit for receptors of other GDNF family members, knockouts of the ligand binding subunits for these other receptors have no direct effect on testis morphology or male fertility [21-23]. This unique chemical-genetic approach has allowed us to investigate the impact of the inhibition and restoration of the GDNF signaling complex on SSCs and progenitor
spermatogonia in the normal adult mouse testis. Using this approach, we tested the hypothesis that GDNF is essential for maintaining the spermatogonial stem cell pool in the normal adult testis. Our results show that the inhibition of GDNF signaling for acute, intermediate, and prolonged periods of time leads to the progressive loss of stem and progenitor spermatogonia. The data also show that restoration of GDNF signaling results in an amplification of the remaining stem cells, which fill empty stem cell niches in an effort to restore the stem cell pool and eventually, normalize spermatogenesis [24].

MATERIALS AND METHODS

Animals:

Mice carrying a mutation (V805A) in the ATP binding site of Ret were generated as previously described by Dr. Joseph Savitt, Department of Neurology, Johns Hopkins School of Medicine [25]. The frt-flanked neomycin resistance cassette in the targeting construct (Figure 2) was removed by crossing these mice with B6; SJL- Tg(ACTFLPe) 9205 DG M/J mice (Jackson Laboratories, Bar Harbor, ME). Homozygous Ret (V805A) mice were identified by PCR analysis of genomic DNA using primers that crossed the 5' LoxP site of the targeting construct:

Ret F (36580): CCTTGGGCCTGCTGAGCACGGG
RET R (36858): GGAGGCAGGAAGGCCTGTGC

PCR conditions were: 4 minutes at 95°C followed by 35 cycles of: 30 sec at 95°C, 45 sec for 57°C, 45 sec at 72°C, followed by a 7 min incubation at 72°C. Mice were 70-100 days of age at the start of the experiment and the Johns
Hopkins University Institutional Animal Care and Use Committee approved their use.

**Testing the efficacy of the different ATP competitive inhibitors of Ret (V805A):**

The abilities of different bulky ATP competitive inhibitors to block Ret (V805A) kinase activity were tested by Dr. Savitt. Full-length cDNAs for wild type Ret and Ret (V805A) were cloned into the pRK5 vector (BD Biosciences; San Diego, CA) and transfected into confluent HEK 293 cells using lipofectamine (Invitrogen, Carlsbad, CA). After three hours, medium with serum was added along with NA-PP1 or a related inhibitor. Cells were lysed 16 hours later, fractioned by SDS-PAGE, blotted onto nylon and incubated with Anti-phospho Ret (Y1062) (Santa Cruz Biotechnology, Santa Cruz, CA) and the ECL detection system (GE Healthcare, Piscataway, NJ). Some blots were re-probed for total Ret (Santa Cruz Biotechnology).

**Synthesis and administration of NA-PP1:**

NA-PP1 and related compounds were synthesized by the laboratory of Kevan Shokat (UCSF) as previously described [20]. To convert NA-PP1 into an HCl salt, 400 mg of NA-PP1 was dissolved in 40 ml of methanol plus 5 ml of 1.25M HCl in methanol. NA-PP1 was dissolved by heating and stirring, the salt was dried using a rotovap, dissolved in ethanol (62.5 mg/ml) and stored under nitrogen at -20°C. One part of NA-PP1 in ethanol was diluted into 9 parts in saline:cremophor EL (7:2) and 62.5 mg NA-PP1/kg body weight was injected subdermally between the scapulae. Vehicle-treated control mice were injected with 100 microliters of ethanol:saline:cremophor EL (7:2:1) per 100 grams body
weight. Additional studies showed that GDNF inhibition studies, a dose response study revealed that the minimal dose of NA-PP1 required for inhibition of GDNF signaling was 43.7 mg/Kg (Figure 3).

**Tissue Collection:**

Whole testes and seminiferous tubules were collected from mature male mice (Bl6SJL/J genetic background). Collection of tissues was approved by the Institutional Animal Care and Use Committee of Johns Hopkins University.

**Tissue Preparation and Fixation for Immunohistochemistry:**

Adult mouse testes were stripped of the tunica, releasing compacted seminiferous tubules. These tubules were manually separated in PBS, fixed for 2 hours in 4% paraformaldehyde at room temperature and washed in PBS (four times, 30minutes/wash).

**GFRα1 Immunohistochemistry in whole–mounts of seminiferous tubules of mice:**

4% PFA fixed tubules were blocked with PBS and 1% BSA (PBS-B) for 1 hr at RT in netwells in a 12-well plate. The tubules were washed with PBS once for 5 minutes and incubated overnight with primary antibody (1: 100, Goat anti-rat GFRα1, R&D systems, catalog # AF560) PBSB (1% BSA per 100ml PBS) at 4°C. The next day, the tubules were washed 6X, 15 minutes/wash in PBSBT (0.1% Triton X-100 in 1% BSA per 100ml PBS) and incubated overnight at 4°C with secondary antibody (200ul/well, 1:500 anti goat- Alexa 488) in PBSBT. Once the tubules came into contact with secondary antibody, the plate was wrapped in aluminum foil to prevent photobleaching of the fluorochrome. On the last day, the tubules were washed 6X for 15 minutes each with PBSBT and mounted as
described below.

**Zbtb16 (PLZF) Immunohistochemistry in whole-mounts of seminiferous tubules of mice:**

Gradual dehydration was performed on 4% PFA fixed tubules, followed by sequential washing for 10 minutes each in 25%, 50%, 75%, and 95% MeOH in PBS and twice in 100% MeOH. The tubules were then incubated in 3ml/net well of MEOH: DMSO: H2O2 (4:1:1) at RT for 2 hours and rehydrated for 10 minutes each in 3ml of 50% and 25% MeOH in PBS and twice in 3ml PBS for 15 minutes. Tubules were blocked in cold PBSMT (0.5% Triton X-100 in 2% nonfat dried milk powder per 100ml PBS) for 2 hours and incubated overnight in primary antibody (4 µg/ml AntiPLZF: goat anti-PLZF, R&D Research systems, AF2944) diluted in PBSMT at 4°C. The plate was placed in a tupperware container with water saturated paper towels to prevent the primary antibody from drying out. The following day, tubules were washed with cold PBSMT (2X 15minutes, 5X 1hr) and then transferred to a 24 well plate for an overnight incubation at 4°C with secondary antibody (4 µg/ml Alexa fluor-488 rabbit anti-goat IgG, Invitrogen, Carlsbad, CA) diluted in PBSMT. Once the tubules came into contact with secondary antibody, the plate was wrapped in aluminum foil. The following day, the tubules were washed in cold PBSMT (2x for 15 minutes and 4X for 1 hr) and PBS (2X for 10 minutes). Following washing, tubules were mounted as described below.
Cleaved caspase 3 Immunohistochemistry in whole-mounts of seminiferous tubules of mice:

4% PFA fixed tubules were blocked with 1% BSA in 1X PBS for 1 hour at RT and rinsed twice with PBS for 15 minutes. The tubules were then incubated with cleaved caspase 3 (1:500 Rabbit antibody, Cell Signaling Technologies, catalog # 96615) in 1% BSA per 100ml PBS overnight at 4°C on the shaker. The next day, the tubules were washed 6X, 15 minutes/wash in PBSBT (0.1% Triton X-100 in 1% BSA per 100ml PBS) and incubated in secondary antibody (1:1000 Donkey Anti-rabbit, Alexa fluor-555 donkey anti-rabbit IgG (H+L), Invitrogen, Carlsbad, CA, catalog # A31572) in PBSBT overnight at 4°C on a shaker. The next day, the tubules were washed 6X, 15 minutes/wash in PBSBT, followed by sequential washing for 10 minutes each in 25%, 50%, 75%, and 95% MeOH in PBS. The tubules were then rehydrated for 10 minutes each in 3ml of 50% and 25% MeOH in PBS and twice in 3ml PBS for 15 minutes. The tubules were then blocked with 1% BSA in 1X PBS for 1 hour at room temperature on a shaker and rinsed twice with 1X PBS for 15 minutes/each, and incubated overnight in primary antibody (1:1000 AntiPLZF: goat anti-PLZF, R&D Research systems, AF2944) diluted in PBSMT at 4°C. The next day, the tubules were washed 6X, 15 minutes/wash in PBSBT and incubated with secondary antibody (1:200 Alexa fluor-488 donkey anti-goat IgG, Invitrogen, Carlsbad, CA, catalog # A11055) diluted in PBSBT. Once the tubules came into contact with secondary antibody, the plate was wrapped in aluminum foil. On the last day, the tubules were
washed 6X, 15 minutes/wash in PBSBT, 2X with 1X PBS for 10 minutes/each and mounted as described below.

Mounting Seminiferous tubules:

The slides are prepared for mounting by placing four drops of clear nail polish on the slide to replicate the dimensions of the coverslip. The purpose of this is to create raised edges to prevent the cover slip from crushing the tubules. A few drops (2-3) of PBS are placed on the slide to which the tubules are added and separated using forceps. Any excess PBS was soaked up gently via a Kimwipe and a drop of Vectashield was placed to coat the tubules. The coverslip was placed and secured by sealing the edges with clear nail polish. The slides were allowed to dry for at least 20 minutes before observing.

Analysis of Zbtb16⁺, GFRα₁⁺ or cleaved caspase 3⁺ spermatogonia:

Digital images of Zbtb16⁺ and GFRα₁⁺ spermatogonia were captured with a Nikon Eclipse Microscope equipped with a cooled CCD camera (QImaging, Surrey, BC, CA) and imported into iVision (Biovision Technologies, Exton, PA). Zbtb16 or GFRα₁ marked cells were quantified based on their classification as A single (Aₛ) spermatogonia, a subpopulation of which are the functional stem cells, A paired (Aₚ) or as A aligned (Aₐ) spermatogonia, which form chains of 4-16 cells (Figure 4). A change in the ratio of Aₚ or Aₐ spermatogonia to Aₛ cells was indicative of increased differentiation of replicating stem cells. For cleaved caspase 3, whole mounts of seminiferous tubules were optically sectioned (2.3 µm) using a Zeiss LSM 710 Confocal Microscope and images of ZBTB16⁺ and / or cleaved caspase 3⁺ spermatogonia were captured and quantified. Same
settings were used for imaging negative controls (isotype control IgG).

**Whole testis fixation:**

To evaluate the duration in loss of functional stem cells due to inhibited GDNF signaling, testis were collected after treatment and fixed to observe the loss and depletion of mature spermatogenic cells. Testes were fixed in 5% glutaraldehyde in cacodylate buffer, postfixed in osmium tetroxide, embedded in Epon 812, and 1 micron thick sections stained with Toluidine blue. Four to six different cross sections per testis were evaluated and a minimum of 300 tubules per testis were examined for the presence or absence of spermatogonia, spermatocytes and/or spermatids. Tubule cross-sections that were devoid of all spermatogonia, spermatocytes and round spermatids were considered to lack spermatogonial stem cells. To assess any toxic or off-target effects, kidneys and livers were emersion fixed in Bouin’s fixative, embedded in paraffin and 5-micron sections stained with hematoxylin and eosin were examined.

**Measurement of transcript levels:**

RNA was isolated using RNAeasy kits (Quigen, Valencia CA), cDNA was synthesized using Superscript III (Invitrogen) and transcripts encoding Zbtb16, GFRα1, Ret and 18S rRNA were quantified using TaqMan primers (Life Technologies Corp, Carlsbad, CA). Standard curves for each assay were generated from cloned, sequence-verified cDNA standards and the amount of each transcript was normalized to the amount of 18S rRNA in each sample.

**Statistical analysis:**

Cell counts were analyzed using a nested ANOVA and other data were
analyzed by ANOVA. Statistical analysis used StatView (SAS Institute Inc, Cary, NC). Differences were defined as significant at $p \leq 0.05$.

RESULTS

Characterization of Ret (V805A) mice and their response to NA-PP1:

To permit for the reversible inhibition of GDNF signaling, the Ret kinase domain was engineered to allow for the substitution of an inert small molecule, which acted as a high affinity inhibitor of Ret's kinase activity. This V805A mutation was selected by comparing the sequence of RET to other kinases that have been similarly targeted (Figure 5). Transient transfection analysis confirmed that this mutation did not affect baseline kinase activity; but rather, it made RET susceptible to inhibition by NA-PP1 (Figure 6). Mice homozygous for this mutation were generated and tested for the ability of NA-PP1 to inhibit Ret by injecting it into pregnant mice from embryonic day 9 until birth. Homozygous pups born to these mothers died by post-natal day 2 due to hypoplastic kidneys, reiterating the Ret knockout phenotype [26]. However, treating adult male Ret (V805A) mice or wild-type mice for 30 days with NA-PP1 did not affect body weight or kidney & liver histology (Figure 7 & 8). Additionally, there was no difference in testes weights of wild-type and Ret (V805A) mice treated with NA-PP1 for 20 days, respectively (Figure 7). Thus, NA-PP1 has no evident toxicity or off-target effects in adult mice.
Inhibition of the GDNF signaling complex in the adult leads to the loss in expression of cells expressing the stem spermatogonial markers, GFRα1, Zbtb16 and Ret:

To test the hypothesis that GDNF is required by spermatogonial stem cells in the normal adult testis, adult male Ret (V805A) mice were treated with NA-PP1 for 5, 11 or 20 days and examined for GFRα1+ and Zbtb16+ spermatogonia. Keeping in mind that it takes ~35 days for one full cycle of spermatogenesis to take place in the mouse, these time-points were chosen because they represent acute (5 days), intermediate (11 days), and prolonged (20 days) periods of inhibition [27]. In mice where GDNF signaling was inhibited for 5 days, we observed a more rapid loss in GFRα1+ spermatogonia when compared to Zbtb16+ spermatogonia and control mice (Figure 9B). After 11 days of inhibition, very few GFRα1+ cells remained, and after 20 days of inhibition, there were no surviving GFRα1+ cells in the seminiferous tubules of these mice (Figure 9A). In contrast, there was no change in numbers of Zbtb16+ spermatogonia 5 days of treatment, when compared to mice treated with vehicle (Figure 9A). However, after 11 days of treatment, there was a significant loss in the numbers of these spermatogonia, and after 20 days of treatment, there was a complete loss of Zbtb16+ spermatogonia (Figure 9A). To account for any off target effects of NA-PP1, wild-type mice were also treated with NA-PP1 for 11 days (Figure 9A). No abnormalities in spermatogenesis were detected.

To quantify these results, we determined the numbers of GFRα1+ or
Zbtb16\(^+\) A\(_s\), A\(_{pr}\) and A\(_{al}\) spermatogonia per mm\(^2\) of tubule surface. Ret (V805A) mice were treated for 5 or 11 days with NA-PP1 or with vehicle. After 5 days of inhibition, densities of GFR\(\alpha_1\)^+ A\(_s\), A\(_{pr}\) and A\(_{al}\) spermatogonia were significantly reduced to 32%, 15% and 7% of vehicle-treated controls, respectively (Figure 9B). After 11 days of inhibition, the densities of Zbtb16\(^+\) A\(_s\), A\(_{pr}\) and A\(_{al}\) spermatogonia were significantly reduced to 12%, 21% and 6% of control, respectively (Figure 9B). In addition to quantification of cells, we also measured the levels of transcripts encoding Ret, GFR\(\alpha_1\) and Zbtb16 (Figure 10). Ret mRNA and GFR\(\alpha_1\) mRNA testicular levels were similar in control mice. After GDNF signaling was inhibited for 20 days, the most pronounced decline was observed in Ret mRNA expression (9%), followed by GFR\(\alpha_1\) (34%), when compared to controls. The expression of Zbtb16 mRNA decreased steadily and in a linear pattern to 33% of controls. Taken together, the data demonstrate that in the normal adult testis, inhibiting the GDNF signaling complex results in the rapid loss of spermatogonial stem cell and/or progenitor spermatogonia, as defined by message levels and SSC markers. These data also provide in vivo evidence that the RET gene is a direct target of the signal transduction cascade associated with GDNF in the testis.

Inhibition of the GDNF signaling complex for 30 days leads to a loss of all functional spermatogonial stem cells in the normal adult testis:

While figure 10 shows a reduction in message levels of Ret, GFR\(\alpha_1\) and Zbtb16 after 20 days, these transcripts were still detectable, raising the issue of
whether inhibition led to a complete loss of stem and other undifferentiated spermatogonia or whether this inhibition only reduced the expression of the stem cell markers to levels undetectable by immunocytochemistry. To test the first possibility, we injected adult Ret (V805A) and wild-type mice with NA-PP1 for 30 days and then collected testicular tissue immediately afterwards or after an additional 35 days (Figure 11). Ret (V805A) mice were treated for 30 days based on data in figure 10, which predicted that this time point was required for the complete disappearance of transcripts encoding Ret, GFRα1 and Zbtb16. Thus, mice treated for such a prolonged period of time would be lacking in stem and progenitor spermatogonia, and therefore, deficient in spermatogenic cells 35 days later (Figure 12). Our results were consistent with this prediction; the testes of NA-PP1-treated Ret (V805A) mice were smaller (55.4 + 4.4 mg) than the testes of vehicle-treated mice (217 + 9.4 mg (mean + SEM)). In addition, examination of ~300 tubules from each of 5 treated wild type mice demonstrated that 97± 2.5% (mean + SEM) of the tubules displayed normal spermatogenesis (Figure 11 A&B). By contrast, not a single spermatogenic cell was found in the seminiferous tubules of the five Ret (V805A) treated mice, leading us to conclude that the spermatogonial stem cells were lost during the 30 days of treatment with NA-PP1 (Figure 11 C&D).

A few stem spermatogonia survive after inhibition of the GDNF signaling complex for 11 days:

To elucidate how rapidly the loss of GDNF signaling leads to the loss of stem spermatogonia, we treated Ret (V805A) mice and wild-type mice for 11
days with NA-PP1 and analyzed their testes 35 days post-treatment. Thirty-five days post-treatment, testes of all wild-type mice were morphologically normal, as expected (Figure 13 A&B). Seminiferous tubule whole-mounts of Ret (V805A) collected 24 hours after the last injection with NA-PP1 revealed the presence of very few GFRα1+ cells, all of which were As spermatogonia (Figure 13 C&D). Overall, these mice had morphologically normal testes. In contrast, testes of Ret (V805A) mice collected 35 days later showed that 97% of the seminiferous tubules contained either no spermatogenic cells or only elongate spermatids (Figure 14). However, the remaining 3% of the tubules exhibited dense clusters of GFRα1+ spermatogonia located in concentrated areas of the seminiferous tubule, correlating with areas of active spermatogenesis (Figure 13 E&F). These clusters were similar in number (0.24 ± 0.14 clusters/mm²; mean ±SEM) to the number of the individual GFRα1+ As spermatogonia on tubules that were collected 24 hours after the last of the 11 injections of NA-PP1 (0.46±0.14 cells/mm²). Taken together, the data suggest that inhibition of the GDNF signaling complex for 11 days leads to the loss of approximately 97% of the stem spermatogonia. Conversely, restoring GDNF signaling allows the remaining stem and undifferentiated spermatogonia to proliferate in an effort to rebuild the tissue. Some spermatogonial stem cells or progenitor spermatogonia are lost after inhibition of the GDNF signaling complex for 2 days:

Based on the hypothesis that GDNF promotes self-renewal over differentiation of replicating spermatogonial stem cells, we predicted that Ret
(V805A) mice treated with NA-PP1 for two days, the approximate duration of the one cell cycle, would result in the significant loss of spermatogonial stem cells and/or progenitor spermatogonia. To test this hypothesis, Ret (V805A) mice were injected with NA-PP1 or vehicle for 2 days and their testes were examined 44 days later (Figure 15). As expected, the testes of all control mice were normal. However, we noted considerable heterogeneity in the histology of tubules of NA-PP1 treated mice: 25.6% of the tubules were morphologically normal, 9% contained Sertoli cells and only a few elongate spermatids, and the remaining tubules were missing a generation or two of germ cells, but contained spermatogonia and/or preleptotene spermatocytes (Figure 15). These results demonstrate that inhibition of the GDNF signaling complex for as few as 2 days causes loss of spermatogonial stem cells and/or progenitor spermatogonia. To further understand what was contributing to the loss of A$_s$$^+$, A$_{pr}^+$ and A$_{al}$ spermatogonia, we investigated whether the inhibition of the GDNF signaling complex for acute periods of time (2 and 3 days) increased the incidence of apoptosis by co-staining and quantifying apoptotic undifferentiated spermatogonia. However, in both control and treated mice, less than 3% of the Zbtb16$^+$ cells expressed the marker of apoptosis, cleaved caspase 3. Overall, based on the similar numbers of apoptotic cells in treated and control mice, we concluded that apoptosis is not a significant regulator of numbers of GFR$\alpha_1$ spermatogonia (Figure 16 & 17). Additional studies in the lab have shown that this loss of SSCs and progenitor spermatogonia due to inhibition of the GDNF signaling complex can be attributed to preferential differentiation of GFR$\alpha_1$$^+$
DISCUSSION

A unique *in vivo* approach to investigating the behavior of adult spermatogonial stem cells in the mouse:

Current approaches to inhibit receptor signaling in the adult, such as inducible Cre-mediated recombination, are lengthy, inefficient and irreversible. We were the first to use a simple and unique chemical-genetic approach to investigate the direct regulation of any adult stem cell population by a single growth factor. This approach insures efficient inhibition by coupling a mutated ATP binding site of the kinase subunit of the receptor with an ATP competitive inhibitor. There are three advantages to this experimental strategy that make it particularly relevant to stem cell biology: 1) it involves a completely normal pool of stem cells, 2) we can analyze the *in vivo* response of stem cells to acute and prolonged changes in signaling from a specific growth factor, and 3) the inhibition is reversible. Using this approach, we were able to investigate the *in vivo* effects of inhibition of the GDNF signaling complex on stem and progenitor spermatogonia in the normal adult mouse testes.

GDNF has previously been shown to be required for the establishment of the stem cell pool *in vivo* in the immature testes and during maturation. However, to date, investigators have not addressed how this growth factor regulates SSCs and progenitor spermatogonia, and thus, spermatogenesis in the normal adult mouse. Our data are the first to prove that this growth factor is required for the maintenance of these stem cells in the normal adult testicular environment. We
showed that *in vivo* inhibition of the GDNF signaling complex for only two days leads to the loss of stem cells and/or progenitor spermatogonia. This result is in contrast to the report that *in vitro* stem spermatogonial numbers increase when cultured in the absence of GDNF for 3 or 6 days [9]. When Ret signaling was inhibited for 30 days, we observed a loss in almost all germ cells. In addition, our observation that many stem spermatogonia remain after 11 days of inhibition of the GDNF signaling complex suggests that GNDF is not solely responsible for SSC regulation and that other intrinsic or extrinsic factors may affect the cells response to GDNF.

The current hypothesis in the field states that GDNF promotes self-renewal over differentiation of replicating stem and other $A_s$ spermatogonia [1]. In support of this hypothesis, we showed that inhibition of GDNF signaling led to more of a rapid decline in GFR$_{\alpha1}^+$ spermatogonia when compared to Zbtb16$^+$ spermatogonia. Since almost all GFR$_{\alpha1}^+$ spermatogonia co-express Zbtb16, the sequential loss of GFR$_{\alpha1}$ expression, followed by Zbtb16 expression suggests that GDNF suppresses differentiation [29]. However, we did not observe an increase in the ratio of $A_{pr}$ or $A_{al}$ spermatogonia to $A_s$ spermatogonia, suggesting that this regulation may be more complex than originally thought. **Acute inhibition of the GDNF signaling complex leads to a heterogeneous testicular environment:**

Our data show that *in vivo* inhibition of the GDNF signaling complex for acute periods of time, 2 to 3 days, results in the loss of some spermatogonial stem cells and progenitor spermatogonia, contributing to tubules (8.7%) that are
devoid of all but a few elongate spermatids forty-four days post-treatment. Additionally, in all but 26% of the remaining seminiferous tubules, we observed tubules that lack one or two generations of spermatogenic cells. Based on work by Oakberg and Clermont, where they defined the amount of time required to complete each phase of spermatogenesis, we estimated that the tubules lacking preleptotene spermatocytes, pachytene spermatocytes or round spermatids in these animals had lost stem and other undifferentiated spermatogonia around 26 days, 17 days or 9.2 days after the start of inhibition, respectively [27, 30]. This explains why we continued to report the loss of cells after the treatment period had ended. Taken together, we interpret this heterogeneous loss of spermatogenic cells as evidence of a transient loss of SSCs or progenitor spermatogonia, which leads subsequently to the loss of at least one generation of more mature spermatogenic cells, showing that inhibition for even 2 days can disrupt spermatogenesis.
SUMMARY

Using a unique chemical-genetic approach, we have been able to study the consequences of the reversible inhibition of the downstream effects of GDNF signaling on stem spermatogonia in a normal adult testis. The experiments outlined in this chapter demonstrate that inhibiting the downstream effects of GDNF signaling cascade causes a sequential loss of stem cells as identified by the SSC markers, GFRα1 and Zbtb16, respectively. In addition, the loss of GDNF signaling leads to a loss in SSC message levels, with the most pronounced decrease occurring in Ret mRNA levels. We have also shown that SSCs and progenitor spermatogonia respond to this loss differentially; where some stem cells are lost when the GDNF signaling complex is inhibited for only 2 days, while others persist for up to 11 days. Due to the reversible nature of our model, we were able to demonstrate that once treatment with NA-PP1 had ceased and GDNF signaling was restored, the remaining stem cells amplified to restore the stem cell pool. In conclusion, these data provide evidence in support of the following hypothesis: GDNF is essential in regulating the self-renewal and differentiation of SSC and progenitor spermatogonia in a normal mature testis.
REFERENCES


FIGURE LEGEND

Figure 1. The Chemical-Genetic approach used to reversibly inhibit the GDNF signaling complex. Figure 1A. Normal GDNF signaling: Dimeric GDNF is cross-linked to two GFRα1 subunits. Together, they bind to two subunits of the receptor tyrosine kinase, Ret. Upon phosphorylation of Ret (red star), an intracellular signaling cascade is initiated. Figure 1B. Inhibited GDNF signaling: Treatment with a competitive ATP inhibitor, NA-PP1, blocks the phosphorylation of mutated Ret (blue star), which leads to the inhibition of the GDNF signaling cascade.

Figure 2. Targeting vector used to introduce the Ret (V805A) mutation into ES cells. The targeting vector was constructed using a BAC clone isolated from a 129J mouse genomic library (RPCI-22). The target sequence was composed of a 1.4 kb Age I-Hind III fragment containing exons 14 and 15, where a valine to alanine missense mutation was introduced at the 805 position of Ret, and placed upstream of an FRT-Neo-FRT selection cassette, flanked by loxP sites.

Figure 3. Determining the minimal dose of NA-PP1 required for inhibition of GDNF signaling. In order to determine the minimal dose of NA-PP1 necessary to inhibit signaling, Ret (V805A) mice (n=2-3/dose group) were injected for 5 continuous days with four different doses of NA-PP1. A NA-PP1 dose of 43.7 mg/Kg was determined to be the minimal dose required for inhibition.

Figure 4. Example of quantitation of Zbtb16⁺ and classification as Aₖ, Aₚr or Aₐl spermatogonia. Images captured with a Nikon Eclipse Microscope equipped with a cooled CCD camera (QImaging, Surrey, BC, CA) at 20-25X were imported into
iVision (Biovision Technologies, Exton, PA) and quantified using their morphological differences.

Yellow: Single cells
Orange: Paired cells held together by cytoplasmic bridges
Green: Chains of cells connected by cytoplasmic bridges

Figure 5. A comparison of the sequence alignments of RET to other kinases that have been targeted using a high affinity inhibitor of kinase activity. The shaded residues correspond to v-Src residue 338 in the ATP binding pocket of protein kinase subdomain V.

Figure 6. Kinase activity of Ret (V805A) is inhibited by ATP competitive inhibitors. HeK293 cells transfected with pRK5 plasmids encoding wild-type RET or RET V805A were used to test kinase activity. Phosphorylation of RET was examined with the use of immunoblots. Figure 6A: Immunoblot of phospho-RET in cells transfected with wild-type Ret and incubated with or without NA-PP1 or 1NM-PP1 show that there was no effect of the inhibitor on kinase activity of wild-type Ret. Figure 6B: Cells expressing Ret (V805A) incubated with or without 100 nM of four different ATP competitive inhibitors show that 100 nM of NA-PP1 inhibits Ret kinase activity without affecting total Ret. Figure 6C: Dose-dependent effect of NA-PP1 on kinase activity of Ret (V805A). Figure 6D: Structures of the ATP competitive inhibitors used.

Figure 7. Body and testis weights of wild type mice (A) and Ret (V805A) mice (B) treated for 30 days (left) or 20 Days (right) with vehicle or NA-PP1.

Data (n=4) are presented as means + SEM. These mice were sacrificed 24 hours
post-treatment. The results confirm that there were no off-target effects of drug
treatment on these mice.

Figure 8. The effect of NA-PP1 treatment on the kidneys and livers of Ret
(V805A) mice. Ret (V805A) mice treated with vehicle for 30 days were used as
controls. NA-PP1 treatment for 30 days revealed no effect on kidney or liver
histology. Tissues were stained with hematoxylin and eosin (n=5/group), and
bars are equal to 20 microns (kidney) or 80 microns (liver). Arrows point to
glomeruli in the kidney.

Figure 9. The \textit{in vivo} effect of inhibition of the GDNF signaling complex on the
testicular environment of Ret (V805A) mice. Figure 9A. Zbtb16\textsuperscript{+} and GFR\textalpha\textsubscript{1}\textsuperscript{+}
spermatogonia were identified on whole mounts of seminiferous tubules of Ret
(V805A) mice injected daily with NA-PP1 for 5, 11 or 20 days (N=4-6 mice/group).
Controls included Ret (V805A) mice treated for 20 days with vehicle and wild-
type mice treated for 11 days with NA-PP1 (N=4-6 mice/group). Only a few faintly
stained GFR\textalpha\textsubscript{1}\textsuperscript{+} cells were present on the tubules of Ret (V805A) mice treated
for 5 or 11 days (see arrows), while the density of Zbtb16\textsuperscript{+} spermatogonia only
decreased after 11 days. Both GFR\textalpha\textsubscript{1}\textsuperscript{+} and Zbtb16\textsuperscript{+} cells were completely
absent in animals treated with NA-PP1 for 20 days. Figure 9B. Quantitation of
GFR\textalpha\textsubscript{1}\textsuperscript{+} (n=3) and Zbtb16\textsuperscript{+} (n=4) A\textsubscript{s}, A\textsubscript{pr} and A\textsubscript{al} spermatogonia in Ret (V805A)
mice treated with NA-PP1 (white bars) or vehicle (black bars). Data are
expressed as mean \pm SEM.

Figure 10. The \textit{in vivo} effect of inhibition of the GDNF signaling complex on SSC
message levels in Ret (V805A) mice. Ret, Zbtb16 and GFR\textalpha\textsubscript{1} message levels
were measured in Ret (V805A) mice that were injected daily with NA-PP1 for 5, 11 or 20 days. (N=4-6 mice/group). Message levels in treated mice were compared to Ret (V805A) mice were injected with vehicle for 5 or 11 days. Data (mean + SEM; n=5-6/group) are expressed as the numbers of molecules of each transcript divided by numbers molecules of 18S rRNA in the same sample. The inhibition of GDNF signaling resulted in the progressive loss of Ret, GFRα1 and Zbtb16 message levels, with the most pronounced decrease noticed in Ret message levels.

Figure 11. The in vivo effect of inhibiting the GDNF signaling complex for 30 days on the testicular environment of Ret (V805A) mice. Figure 11 A&B. The testicular histology of wild-type treated for 30 days with NA-PP1 and testes collected 35 days thereafter, (N=5 mice). Figure 11 C&D. The testicular histology of Ret (V805A) treated for 30 days with NA-PP1 and testes collected 35 days thereafter, (N=5 mice). The white arrowheads in panels B and D point to Sertoli cell nuclei, bar = 20 microns.

In panel B: white arrows = spermatogonia
Black & white arrowhead = the nuclei of a pachytene spermatocyte
Black & white arrow = a round spermatid
White & black arrowhead = a nucleus of an elongate spermatid

Figure 12. Differential sensitivities of SSC resulting from inhibition of GDNF signaling. Top panel: This figure depicts maturation depletion and repletion after the loss of many spermatogonial stem cells, which eventually lead to the loss of mature spermatogenic cells over time. The yellow boxes indicate the types of
spermatogenic cells lost with increasing time, where each series of boxes represent ~8.6 days. Bottom panel: This figure depicts maturation depletion after the loss of all spermatogonial stem cells, which eventually leads to the loss of all germ cells over time. The pink boxes indicate the types of spermatogenic cells lost with increasing time.

Figure 13. The \textit{in vivo} effect of inhibiting the GDNF signaling complex for 11 days on the testicular environment of Ret (V805A) mice. After inhibiting the GDNF signaling complex for 11 days, the remaining stem spermatogonia begin to rebuild the stem cell pool. The histology is representative of testes of Ret (V805A) and wild type mice treated for 11 days, and collected either 1 or 35 days post treatment (n=4 mice/group), bar = 20 microns.

Panels A, C & E: one micron thick cross sections of seminiferous tubules
Panels B, D & F: whole mounts of tubules immunostained for GFR$\alpha_1^+$ spermatogonia Arrowheads = rare and weakly stained GFR$\alpha_1^+$ A$_s$ spermatogonia
Black & white arrowhead= spermatocyte
White arrow= 10 GFR$\alpha_1^+$ A$_{al}$ spermatogonia cells in a chain, indicative of dense patches of spermatogonia

Figure 14. The predominant phenotype associated with the \textit{in vivo} inhibition of the GDNF signaling complex for 11 days on the testicular environment of Ret (V805A) mice.

Majority to the tubules in mice treated with NA-PP1 and testes examined 35 days thereafter had lost almost all spermatogonial stem cells, lacking spermatogenic
cells and elongate spermatids, bar = 20 microns (N=4). Panel A: low power image of a NA-PP1 treated Ret (V805A) mouse of a testis cross section. Panel B & C: testicular cross-sections representing the 97% rate of maturation depletion in these animals, where only Sertoli cells (arrow head, panel B) and elongate spermatids (arrow head, panel C) were remaining.

Figure 15. The acute in vivo effect of inhibiting the GDNF signaling complex for 2 days on the testicular environment of Ret (V805A) mice. A high degree of diversity was observed in Ret (V805A) mice in which GDNF signaling was inhibited for only 2 days and testes collected 44 days later. Bar = 20 microns (N=4). Panel A: shows normal spermatogenesis containing all spermatogenic cell types (White arrows = spermatogonia or preleptotene spermatocytes). Panel B: shows a cross-section lacking pachytene spermatocytes. Panel C: shows a cross-section lacking round spermatids. Panel D: shows a cross-section lacking elongate spermatids (White & black arrowheads). Panel E: shows a cross-section containing only Sertoli cells and elongated spermatids. Panel F: shows a cross-section lacking both round spermatids (Black & white arrows) and pachytene spermatocytes (Black & white arrowheads).

Figure 16. Zbtb16+ and cleaved caspase 3+ spermatogonia in Ret (V805A) mice treated with Na-PP1. The inhibition of the GDNF signaling complex does not lead to an increase in apoptosis in Ret (V805A) mice. Majority of cells expressing cleaved caspase 3 did not co-express Zbtb16, making these co-stained detectable, but rare. Figure 16 A. The white arrow points to the sole apoptotic cell (red) at the end of a chain of Zbtb16+ cells (green). Image show cells in a 2.3
micron section of a whole mount imaged by confocal microscopy using a 25X objective. Figure 16 B. The left panel shows a chain of 4 Aal spermatogonia co-stained for Zbtb16 (green) and cleaved caspase 3 (red). Middle panel show just the cleaved caspase 3+ cells (red). The right panel shows just the Zbtb16+ cells (green). Image show cells in a 2.3 micron section of a whole mount imaged by confocal microscopy using a 40X objective.

Figure 17. The in vivo effect of inhibiting the GDNF signaling complex on apoptosis. Treating Ret (V805A) mice for 2 and 3 days with NA-PP1 does not lead to an increase incidence of apoptosis. Because cells co-stained for Zbtb16 and cleaved caspase 3 were rare in Ret (V805A) mice injected with Na-PP1 or vehicle, cells expressing only cleaved casapse 3 were quantified. Data are means + SEM, n=3/group.
Figure 1.
Figure 3.

Dose-Response of Testis Ret mRNA Concentration to 5 Days of Treatment with NA-PP1
Figure 4.
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Figure 6.

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P-Ret (Tyr 1062)

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P-Ret (Tyr 1062)

Ret

C.

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P-Ret (Tyr 1062)

D.

- PP1
- NA-PP1 (NA)
- 1NM-PP1 (1NM)
- 2NM-PP1 (2NM)
Figure 7.
Figure 8.
Figure 9.
Figure 10.
Figure 11.
Figure 12.

Germ cell maturation depletion and repletion after loss of many but not all stem spermatogonia:

Increasing Time

Germ cell maturation depletion after loss of all stem spermatogonia:

Increasing Time
Figure 13.
Figure 14.
Figure 16.
Figure 17.
CHAPTER 3: Understanding the role of GDNF in regulating stem and progenitor spermatogonia in fertile and infertile men.
ABSTRACT

Chapter 2 proves that GDNF is essential in maintaining spermatogonial stem cells (SSCs) and progenitor spermatogonia in the adult mouse testis. If this growth factor has a similar function in men, we hypothesize that GDNF expression and the distribution of its receptor, GFRα1, would be similar in the testes of normal men, when compared to mice and rats, whose SSCs are GDNF-dependent. Our results show that GDNF mRNA levels in mice and men are similar, while GDNF protein concentrations in the total testicular fluid (TTF) of men (1800 pg/ml) is 36% of that of mice. Because SSCs are situated on the basal side of the blood-testis barrier, the fluid that surrounds these stem cells is continuous with the testicular interstitial fluid (TIF). Thus, in an effort to be thorough, we compared GDNF protein concentrations in TIF, TTF, and seminiferous tubule fluid (STF). Since the collection of TIF is difficult in mice and men, we collected this fluid from rats using a previously validated protocol and compared GDNF levels in TIF, STF and TTF, collected from the same animals. Results show that GDNF protein levels in rat TTF is identical to humans, but is substantially lower in rat TIF. This suggested that GDNF could be essential in regulating human SSCs and progenitor spermatogonia. To further test this hypothesis, we compared GDNF mRNA levels in normal human testes with levels in testes of men who were diagnosed as being infertile due to the lack of spermatogenic cells. Our results showed that infertility was associated with an 80% decrease in expression of this transcript. To address whether reduced GDNF expression in individuals with the Sertoli-cell-only (SCO) phenotype was
due to a complete state of hypo-function or a specific deficiency in their Sertoli cells, we measured transcripts encoding two Sertoli cell products, kit ligand and clusterin. Expression of neither transcript was reduced in these SCO testes. As GDNF regulates the numbers of spermatogonial stem cells and progenitor spermatogonia in the adult mouse testes, we next tested the hypothesis that numbers of GDNF responsive spermatogonial stem cells and progenitor spermatogonia, as identified by expression of GFRα1, were higher in mice and similar in humans and rats. Results showed that numbers of these cells were highest in humans, intermediate in rats and low in mice. However, in all three species, areas of tubules with relatively high numbers of GFRα1+ cells were interspersed with areas with none. To verify the localization of GFRα1 to human SSCs and progenitor spermatogonia, we co-stained these seminiferous tubules with UCHL1, an established marker for A<sub>d</sub>, A<sub>p</sub> and Type B spermatogonia. Our results verified the localization of GFRα1 to a portion of the UCHL1<sup>+</sup> cells, indicating their ability to bind to GDNF. Taken together, our data support the hypothesis that as in rodents, GDNF is essential for maintaining the population of human SSCs and progenitor spermatogonia. These data are the first indication that depressed GDNF expression may lead to loss of SSCs, and consequently human male infertility. This finding opens up the possibility that clinical procedures to increase GDNF concentration in individuals with SCO testes with a few remaining SSCs might aid in rebuilding the numbers of these stem cells, and increase the possibility that sufficient sperm are generated either for natural or assisted reproduction.
INTRODUCTION

Understanding the regulation of SSCs and progenitor spermatogonia in humans is central to understanding male fertility. This regulation is dependent on the balance between SSC self-renewal and their production of transit-amplifying progenitor spermatogonia, which replicate and give rise to differentiated spermatogonia [1]. Most of our knowledge regarding the regulation of mammalian SSCs and progenitor spermatogonia comes from studies of rodents. In the mouse, SSCs exist as a subpopulation of A_s spermatogonia, which are sparsely distributed along the length of the seminiferous tubule. These SSCs asymmetrically divide to self-renew or give rise to A_pr spermatogonia [2, 3], which then undergo clonal expansion through mitotic divisions to generate chains of A_al spermatogonia. These cells give rise to type A and B spermatogonia, which are irreversibly committed to the ultimate formation of spermatids [2]. In contrast to the mouse, humans and other primates have two functional populations of SSCs (A_dark and A_pale). A_dark spermatogonia represent the reserve stem cells, and A_pale spermatogonia represent the mitotically active stem cells that sustain spermatogenesis [4-7]. Unlike the mouse, progenitor spermatogonia in humans replicate only once before giving rise to B spermatogonia [8]. This suggests that for humans to produce the numbers of sperm required for fertility, the numbers of sperm produced per gram of testis are not substantially different in men and mice.

In the mouse, GDNF has been shown to be required for maintaining and expanding SSCs in vitro. For instance, work in our lab has shown that increasing
the concentration of GDNF around seminiferous tubules in vitro stimulated the proliferation of SSCs and progenitor spermatogonia [9]. Additional in vivo studies have shown that GDNF is essential for establishing the stem cell pool in the immature testis, and for maintaining these cells in the normal adult testis [9-11]. Using a specific mouse model, we were able to confirm this in the context of a normal adult testicular environment and demonstrate that GDNF is a primary regulator of both the replication and differentiation of SSCs and progenitor spermatogonia [9, 12]. When we inhibited GDNF signaling for 2 or 3 days, a significant decrease in replicating A₆ spermatogonia was observed, followed by differentiation, generating Type A1 spermatogonia [12]. This loss of SSCs and progenitor spermatogonia continued with increasing periods of inhibition of GDNF signaling, until the testes were devoid of stem and progenitor spermatogonia [9].

As previously mentioned, most of our understanding of the regulation of SSCs is based on studies of the mouse. There is limited information on how GDNF is involved in regulating stem and progenitor spermatogonia in humans. Studies have shown that as in the mouse, Sertoli cells in humans transcriptionally express GDNF [13, 14]. However, it remains to be shown whether GDNF levels in the normal human testes are adequate to stimulate replication or suppress differentiation of SSCs or progenitor spermatogonia. In addition, no one has directly compared the expression of GDNF in situ in the testicular environment of fertile vs. infertile men. Neither has there been a determination of whether altered expression of GDNF is a characteristic of
infertility *per se*, or whether it is only occurs when testes exhibit the Sertoli cell-only (SCO) phenotype and have significantly reduced numbers of spermatogonial stem cells. This chapter aims to resolve these gaps in our knowledge. We began by comparing fertile humans to fertile mice and rats, in an effort to understand the level of similarity in GDNF regulation between these mammalian species. Secondly, in an effort to understand the role of GDNF in supporting human fertility, we compared GDNF message levels in fertile and infertile men. We also address the distribution of GFRα1, the ligand-binding domain of the GDNF receptor in the human testis, and compare it to the distribution in rodents. In the mouse testis, GFRα1 is expressed exclusively by spermatogonial stem cells and progenitor spermatogonia [12]. While there are molecular similarities between these cell types in mice and men, varying reports indicate that GFRα1 localizes to different cell types in the human testis [15]. Davidoff and colleagues report that this receptor subunit is expressed by human Leydig and Sertoli cells, while others report that GFRα1 localizes to a subset of A_d and A_p spermatogonia [13, 16]. The studies outlined in this chapter also address this discrepancy.

Based on the similarities between human and mouse spermatogenesis, we have used comparisons to the rodent models to further our understanding of human fertility [7]. Specifically, we hypothesize that the concentrations of GDNF in the human, mouse and rat testes are similar and thus, the level in the human testis is sufficient to be an important regulator of SSCs and/or progenitor spermatogonia. If this is true, then we hypothesize that GDNF mRNA would be
substantially reduced in infertile men, whose testes were lacking in stem cells. Lastly, we hypothesize that the same cell types in mice, rats and men express GFRα1, the ligand binding subunit of the GDNF receptor. To test our hypotheses, GDNF message and protein levels were measured and compared in fertile mice, men and rats. In addition, we compared GDNF mRNA levels in human testis with normal spermatogenesis, to individuals who presented with SSCs but failed to produce sperm due to spermatogenic arrest, and individuals who presented with rare SSCs, resulting in a Sertoli cell-only phenotype. Although reduced GDNF expression is not noted in germ cell-deficient mice, we chose to measure GDNF mRNA levels in individuals with SSCs but reduced numbers of spermatogenic cells due to maturation arrest for a thorough comparison [17]. Lastly, distributions and relative numbers of GFRα1+ cells on seminiferous tubules were compared between species. To further define the population of human spermatogenic cells GFRα1 was localized to, we co-stained with another marker for human stem and progenitor spermatogonia, UCHL1. The findings outlined in this chapter are unique because they represent the first direct comparison of GDNF message levels in testes of infertile men to normal fertile men. Taken together, our data further support the hypothesis that GDNF is an important regulator of human spermatogonial stem cells and progenitor spermatogonia and that reduced GDNF expression reflects a specific deficit in the function of Sertoli cells.
MATERIALS AND METHODS

Tissue Collection:

Whole human testes were collected from beating-heart organ donors by the organ transplant center of the University of Pittsburgh Medical Center. Testis biopsies were collected by Dr. Peter Schlegel as part of standard clinical care during microdissection testicular sperm extraction at Weill Medical College of Cornell University. Collection and analysis of human testes was approved by the Institutional Review Boards of Weill Medical College of Cornell University, The University of Pittsburgh Medical School and the Johns Hopkins Bloomberg School of Public Health.

Collection of Testicular Fluids:

Human testis fluids were collected by percutaneous aspiration from patients who were undergoing vasectomy reversals and who had given informed consent for the procedure [18]. Human blood was collected by venipuncture of the same patients. All patient identifiers had been extracted from the samples prior to our use. Collection of human testis fluid and blood was conducted by Jon Jarrow, M.D. and this collection and our analyses were approved by the Institutional Review Boards of the Johns Hopkins University School of Medicine and the Bloomberg School of Public Health. Seminiferous tubules and testis fluids were collected from mature male mice (Bl6SJL/J genetic background) and from Sprague Dawley rats. Collections of tissues and fluids from mice and rats were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University. Seminiferous tubule and interstitial fluid was collected from
60-day old Sprague Dawley rats as previously described by Turner and colleagues [19]. Briefly, two small holes were made on the basal end of mature rat testis and interstitial fluid expressed from the testis by centrifugation at 54XG for 15 minutes. The testes were then decapsulated and washed in three separate baths of PBS. After blotting of the tubules with a gauze pad, the tubules were macerated by expressing them through a 3 cc syringe into a polycarbonate centrifuge tube. Seminiferous tubule fluid was expressed from the tubules by centrifugation at 10,000 x g at 4°C for 30 minutes. Total testicular fluid was recovered from mice and rats by centrifugation of macerated tubules at 10,000 rpm at 4°C for 30 minutes, to separate the fluid from the tissue. To remove debris, all fluids were collected and re-spun for an additional 10 minutes at 10,000 rpm at 4°C. Serum was separated from trunk blood by allowing the blood to clot for 30 minutes-1hr, and then centrifuged at 3,000 rpm at 4°C for 15 minutes.

**Tissue Preparation and Fixation for Immunohistochemistry:**

Normal adult mouse and rat testes were stripped of the tunica, releasing compacted seminiferous tubules. These tubules were manually separated in PBS, fixed for 2 hours in 4% paraformaldehyde at room temperature and washed in PBS (four times, 30 minutes/wash). Human testicular tissue from beating heart donors was obtained from the University of Pittsburgh and transported on ice in Belzer UW cold storage solution (Bridge to Life, Columbia, SC) within ~24-36 hours of being harvested. Human tubules were processed identically to rat and mouse tubules, with the exception of being manually separated in pre-warmed
DMEM/F-12 (Life Technologies). Results with these tubules were confirmed using human seminiferous tubules that were immediately fixed in Pittsburgh and then shipped to Baltimore, in Belzer UW cold storage solution and on ice.

**Message Level Detection:**

Due to an increased level of sensitivity, digital PCR was used to detect GDNF message levels in the human and mouse. RNA was isolated from tissue using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) and cDNA was synthesized using the SuperScript III First Synthesis kit (ThermoFisher Scientific, Waltham, MA), using random hexamers as primers (InVitrogen, Carlsbad, CA). Digital PCR was conducted using the Life Technologies QuantStudio 3D System and Taqman real time PCR assays (InVitrogen). 18S rRNA, DDX4, kit ligand and clusterin mRNA were assayed using Taqman assays and the StepOnePlus Real-Time PCR System (ThermoFisher Scientific, Waltham, MA). A standard curve of cloned plasmids encoding 18S rRNA, DDX4, kit ligand or clusterin mRNA was run with each assay of these transcripts. All data were normalized to the amount of 18S rRNA in each sample. Negative controls included cDNA samples synthesized without reverse transcriptase and PCR reactions run with water instead of cDNA.

**Protein Level Detection:**

GDNF protein was measured in human, mouse and rat testicular fluid and rat seminiferous tubule and interstitial fluids using the GDNF Emax ImmunoAssay System (Promega, Madison, WI). Testicular fluids in humans and mice and all fluids in the rat were diluted to at a 5:95 (fluid: buffer) dilution with
sample 1X buffer (provided in the kit). OD was measured using a fluorescent plate reader (DT 800 Multimode detector (Beckman Coulter, Sykesville, MD).

Serum in humans, mice and rats were measured at a 10:90 dilution (fluid: sample 1X buffer). A standard curve (15.625 pg/ml to 1 ng/ml of GDNF) was run in duplicate in with each assay. Analysis of standard curves from four independent assays demonstrated that the lowest standard that produced a response in the assay different from no GDNF was a concentration of 31.25 pg/ml GDNF, as determined by the means, standard deviation of the means and SEM.

GFRα1 Immunohistochemistry in whole –mounts of seminiferous tubules of fertile humans, mice and rats:

4% PFA-fixed tubules were blocked with PBS and 1% BSA (PBS-B) for 1 hr at RT in netwells in a 12-well plate. The tubules were washed with PBS once for 5 minutes and incubated overnight with primary antibody (1: 100, Goat anti-rat GFRα1, R&D systems, catalog # AF560) PBSB (1% BSA per 100ml PBS) at 4°C. The next day, the tubules were washed 6X, 15 minutes/wash in PBSBT (0.1% Triton X-100 in 1% BSA per 100ml PBS) and incubated overnight at 4°C with secondary antibody (200ul/well, 1:500 Alexa 488-anti goat IgG) in PBSBT. On the last day, the tubules were washed 6X for 15 minutes each with PBSBT and mounted as described below. Whole mounts of seminiferous tubules were optically sectioned (2.3 µm) using a Zeiss LSM 710 Confocal Microscope and GFRα1+ cells examined. Same settings were used for imaging negative controls (isotype control IgG or without primary antibody). Brightness and contrast of images were adjusted so that GFRα1+ cells in all species and GFRα1+ cells co-
stained with UCHL1+ in humans had the same brightness.

Human UCHL1 immunohistochemistry in whole-mounts of seminiferous tubules of fertile men:

4% PFA fixed seminiferous tubules were washed with PBS once for 5 minutes and incubated overnight with primary antibody (1:1000, Biogenesis, catalog # 7863-0507) in PBSB at 4°C. The next day, the tubules were washed 6X for 15 minutes each in PBSBT and incubated overnight at 4°C with Donkey anti-Rabbit IgG (H+L) Alexa Fluor 488 conjugate (1:200, Invitrogen Molecular Probes, catalog # A-21206) in PBSBT. On the last day, the tubules were washed 6X for 15 minutes each with PBSBT and mounted in VectaShield with DAPI (Vector Laboratories, catalog # H-1200). Tubules were examined by confocal microscopy as described above.

Human UCHL1 and GFRα1 immunohistochemistry in whole-mounts of seminiferous tubules of fertile men:

4% PFA fixed human seminiferous tubules were washed with PBS once for 5 minutes and incubated overnight with UCHL1 primary antibody (1:1000; Biogenesis, catalog # 7863-0507) and GFRα1 primary antibody (1:500, R & D systems, catalog # AF560) in PBSB at 4°C. Controls were incubated with non-immune IgG. The next day, tubules were washed 6X for 15 minutes each in PBSBT and incubated overnight at 4°C with Donkey anti-Rabbit Alexa Fluor 488 (1:200, IgG (H+L), Invitrogen Molecular Probes, catalog # A-21206) and Donkey anti-goat IgG Alexa Fluor 546 (H+L) conjugate (1:500, IgG (H+L), Invitrogen Molecular Probes, catalog # A-11056) in PBSBT. On the last day, the tubules
were washed 6X for 15 minutes each with PBSBT and mounted in VectaShield with DAPI (Vector Laboratories, catalog # H-1200). Tubules were examined by confocal microscopy as described above.

**Mounting Seminiferous tubules:**

Seminiferous tubules were mounted on slides by placing four drops of clear nail polish on the slide. The purpose of this is to create raised edges to prevent the cover slip from crushing the tubules. A few drops (2-3) of PBS are placed on the slide to which the tubules are added and separated using forceps. Any excess PBS was soaked up gently via a Kimwipe and a drop of Vectashield was placed to coat the tubules. The coverslip was placed and secured by sealing the edges with clear nail polish. The slides were allowed to dry for at least 20 minutes before observing.

**Quantitation of GFRα1⁺ spermatogonia in fertile humans and mice:**

Human, rat and mouse seminiferous tubules were imaged on a Zeiss 710 confocal microscope, under 25X magnification. Tubules were analyzed by scanning along the length of the tubules and 2.3 μM (1 Airy unit) thick optical sections captured with a 46.5 pinhole. The image within the parameter was categorized as LOC (lots of cells), FC (few cells) or NC (no cells) as described below. Then the tubule was moved to the next section to be analyzed and quantified. This was done till the entire tubule was analyzed and the process was repeated for the next tubule to be quantified. Cells that were faintly and intensely stained were counted, as long as the continuous outline of the cell was stained.
<table>
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<tr>
<th>GFRα1⁺ counts:</th>
<th>Lots of Cells (LOC):</th>
<th>Few Cells (FC):</th>
<th>No Cells (NC)</th>
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<tr>
<td>Mouse</td>
<td>&gt; 4 GFRα1⁺ cells</td>
<td>1-4 GFRα1⁺ cells</td>
<td>No GFRα1⁺ cells observed in the microscopic field</td>
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<tr>
<td>Human</td>
<td>&gt; 16 GFRα1⁺ cells</td>
<td>1-6 GFRα1⁺ cells</td>
<td>No GFRα1⁺ cells observed in the microscopic field</td>
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</table>

**Statistical Analysis:**

Statistical analyses on message levels, protein levels, and GFRα1 quantification was performed using GraphPad Prism Version 6.0 (GraphPad Software, La Jolla, CA). Data obtained for message and protein levels were analyzed by ANOVA or an unpaired t-test using Welch’s correction. Statistically significant differences were defined as P< 0.05.

**RESULTS**

**GDNF Message levels in testes of normal men and mice:**

The concentration of GDNF mRNA levels in normal human testes has not been evaluated. However, we predict that if GDNF plays a similar role in regulating SSC numbers in human and mouse testes, levels of GDNF mRNA would be similar. Our initial attempts to measure GDNF message levels relied on quantitative RT-PCR. However, we were unable to detect transcript levels in our human samples because of the relative insensitivity of the human GDNF assay. We were able to overcome this challenge by using the novel and more sensitive technology of digital PCR to measure the expression of GDNF mRNA in testes of
fertile men and mice. Our comparison revealed that GDNF mRNA levels are similar in human and mouse testes (Figure 1). Controls included replacement of the sample with water or with testis RNA that was subjected to conditions for cDNA synthesis without the addition of reverse transcriptase. The negative controls were identical to background in the assay.

**GDNF protein levels in total testes fluid of normal humans and mice:**

While GDNF mRNA levels in mice and humans did not differ, it was possible that there were significant differences between the translational regulation of GDNF synthesis or in the turnover of GDNF protein in these two species. Therefore, we used a two-antibody enzyme-linked immunoassay to measure GDNF in mouse and human total testicular fluid. As a control, we also measured GDNF protein levels in human and mouse serum (Figure 2). Results revealed that GDNF protein levels in total testis fluid in human testes (~1800 pg/ml) were 64% of those of the mouse (~5200 pg/ml) (Figure 3), which was a statistically significant difference. In contrast, GDNF levels in human and mouse serum were below the sensitivity of the assay.

**GDNF protein levels in total testicular fluid, interstitial fluid and seminiferous tubule fluid of rats:**

Growth factors in seminiferous tubule fluid are separated from contact with the basal compartment of the tubule by the blood-testis-barrier, which isolates the SSCs and progenitor spermatogonia from GDNF and other growth factors present in the adluminal compartment, which contains seminiferous tubule fluid (STF). Thus, the SSCs are directly exposed to the fluid in testicular interstitial
compartment (TIF). To understand the subtleties in GDNF concentration in these three distinct types of testicular fluid environments, we isolated these fluids and individually measured GDNF protein concentrations. To overcome the challenge of collecting interstitial fluid in mice and men, we deferred to the use of Sprague Dawley rats. It has previously been shown that GDNF mRNA and protein are positively correlated in the rat, and that GDNF regulates A\textsubscript{s} replication and differentiation in vitro [20-22]. TIF was collected from the testes of 4 mature male rats using an established protocol [19], and GDNF protein levels in this fluid were compared to levels in seminiferous tubule fluid (STF) and total testicular fluid (TTF), collected from the same animals. Results show that the concentration in rat STF (~4200 pg/ml) is 5-fold higher than in TIF (~800 pg/ml) (Figure 4). However, the concentration of GDNF in total testis fluid of rats (~2600 pg/ml) is similar to the concentration in humans (~1800 pg/ml) (Figure 3). However, when compared to mouse concentrations (~5200 pg/ml), the GDNF concentration in TTF of rats is ~50% lower (Figure 3). Taken together, these data show that GDNF concentrations in the testicular interstitial fluid are significantly lower than in total testicular fluid, but still sufficient to support the SSC population in rats. Secondly, based on similar TTF protein concentrations in rats and men, we hypothesize that the GDNF concentrations in TIF of men are rats are similar, and, thus, the concentration in human TIF is adequate for sustaining the SSC pool.

The comparison of GDNF, DDX4, kit ligand and clusterin mRNA expression in the testes of fertile and infertile men:
The long-term goal of this research is to test the hypothesis that GDNF is required for the \textit{in vivo} maintenance of human SSCs. If true, the testicular GDNF mRNA concentration would be substantially lower in a subset of infertile men diagnosed with the Sertoli cell-only phenotype, when compared to fertile men or infertile men whose testes contained SSCs, but who were diagnosed with azoospermia due to maturation arrest of spermatogenesis. We therefore compared GDNF mRNA levels in fertile human testes, testes of men who were infertile due to maturation arrest and men whose testes exhibited an SCO phenotype. A complete description of the samples analyzed and the diagnosis provided by Brian Robinson, MD, Department of Pathology, Weill Cornell Medical College, can be found in Table 1. To test this hypothesis, we used digital PCR to compare the expression of GDNF mRNA in these three distinct groups of men. GDNF mRNA levels were similar in fertile men and in the testes of men who were infertile due to maturation arrest of spermatogenesis. However, measurement of GDNF message levels in SCO individuals were significantly, 80\% and 87\%, lower when compared to men with normal testes or NOA men with maturation arrest, respectively (Figure 5A). To address the question of whether reduced GDNF expression in individuals with the SCO phenotype was due to a complete state of hypo-function or a specific deficiency in their Sertoli cells, we measured transcripts encoding two other Sertoli cell products, kit ligand and clusterin. Expression of neither transcript was reduced in these SCO testes (Figure 5 C&D). Instead, the expression of both transcripts were significantly increased in SCO testes, possibly reflecting a higher percentage of RNA of
Sertoli cell origin due to the lack of germ cells in these samples. This lack of
germ cells in the SCO testes was confirmed by the almost complete absence of
DDX4 mRNA, a marker of germ cells. (Figure 5B). Thus, the data suggest that
decreased GDNF expression observed in individuals with the SCO phenotype
might be attributed to a specific dysfunction in their Sertoli cells, rather than a
complete state of hypo-function.

Expression of GFRα1 in the adult testis of normal humans, mice and rats:

GFRα1, a well-established marker for mouse SSCs and progenitor
spermatogonia is expressed in the adult human testis [15, 16, 23]. While its
expression has previously been localized to A_d, A_p, B spermatogonia in cross-
sections of human seminiferous tubules, neither the relative numbers of these
cells nor their distribution along the length of human seminiferous tubule have
been evaluated. However, if GDNF is the primary regulator of the numbers of
GFRα1+ spermatogonia, based on the trend observed in GDNF protein levels,
the numbers of these cells in mice should be higher than in men, but numbers in
men and rats should be similar. We used whole mount immunocytochemistry of
mature human, rat and mouse tubules to assess this prediction. Post-pubertal
human organ donors with normal spermatogenesis and mature male mice and
rats were used for immunohistochemical analysis; two of the human donors were
between 25-49 years of age. Results show that the density of GFRα 1+
spermatogonia was substantially higher in the seminiferous tubules of all four
men, when compared to mice and rats (Figure 6). However, scanning across
tubules revealed a similar pattern of areas of high and low densities of GFRα1+
cells in human and mouse tubules (Figure 6 A-F). Our results show that ~65% of each mouse and human tubule was covered with high density patches of GFR\(\alpha_1^+\) spermatogonia, and the rest of the tubule contained low densities or the complete lack of these cells (Figure 7). In the rat, we observed a more consistent pattern of chains of cells distributed along the span of the tubule (Figure 6 G-I). Human seminiferous tubules fixed immediately after acquisition, but processed for immunocytochemistry at a later date displayed identical pattern of staining (data not shown).

**Expression of GFR\(\alpha_1^+\) & UCHL1\(^+\) spermatogonia in the seminiferous tubules of normal fertile men:**

To understand whether this patchy distribution of spermatogonia was reproducible when a different marker was used to identify the cells, we stained seminiferous tubules from a fertile individual for UCHL1, a spermatogonial marker [15]. Results revealed a similar pattern of high and low densities of UCHL1\(^+\) spermatogonia (Figure 8). We next co-stained human seminiferous tubules with GFR\(\alpha_1\) and UCHL1 to verify that GFR\(\alpha_1\) was indeed localizing to SSCs and progenitor spermatogonia. Immunohistochemical analysis of tubules processed from one individual show co-stained cells (Figure 9). To make it easier to visualize the density of GFR\(\alpha_1^+\), a black and white version is also provided in the figure. From this, we can conclude that a population of human spermatogonial stem cells and/or progenitor spermatogonia express the ligand binding subunit of the receptor needed for a response to GDNF.
DISCUSSION

Expression of GDNF mRNA and protein in the testes of normal humans, mice and rats:

These studies were grounded in the hypothesis that if GDNF regulates the numbers of human SSCs and progenitor spermatogonia, as it does in the mouse and rat, then the human testis expression of GDNF mRNA and concentration of GDNF protein in testes fluids would be similar to that of rodents and sufficient to trigger the GDNF signaling cascade. Digital PCR, a highly precise method, revealed that GDNF mRNA levels are almost identical in human and mouse testes. This motivated us to compare GDNF protein concentrations in the testicular fluids of humans, mice and rats, and in the interstitial and seminiferous tubule fluids of rats. Our results showed that GDNF proteins levels are 2.7-fold higher in the mouse vs. man. This could be due to possibility that translation of GDNF mRNA is more efficient in the mouse or perhaps, that the protein is cleared more rapidly from the human testis. Nonetheless, the fact remains that the concentration of GDNF in human total testicular fluid is almost identical to its concentration in rat total testicular fluid. This suggests that as in rodents, the concentration of GDNF surrounding human SSCs is sufficient to act as a mitogen and cell survival factor for these stem cells.

GDNF stimulates target cells through a single high-affinity binding site on GFRα1, the ligand binding subunit of the GDNF receptor. The affinity of this binding reaction as estimated by Kd is 11 pM [24, 25]. Thus, when viewed in the context of the kinetics of binding of GDNF to its receptor, the concentration of
GDNF in the human testis fluid (60 pM) is sufficient to drive the occupancy of a substantial percentage of GDNF receptors on SSCs and progenitor spermatogonia, thereby initiating the downstream effects of GDNF signaling in these cells. However, we acknowledge that Sertoli cells secrete many proteins vectorially, resulting in varying protein concentrations in different fluids of the testis, like the seminiferous tubule fluid and interstitial fluid [26-31]. Therefore, it is possible that the GDNF concentration in human interstitial fluid, which is continuous with the fluid that bathes SSCs and progenitor spermatogonia, differs from its concentration in the total testicular fluid, which is a mixture of the two testis fluids. Because collecting TIF and STF was not feasible in mice and men, we examined these fluids collected from rat testes, for which there is a well-established protocol. For a complete comparison across species, we also measured GDNF concentration in total testicular fluid from the rat. The results of this experiment revealed: (1) GDNF concentration in total testis fluid of rats is nearly identical to that of humans, and (2) the concentration of GDNF in testicular interstitial fluid in the rat (27pM) is 40% lower than in the STF. Due to the striking similarity in GDNF concentrations in the total testicular fluid of men and rats, we extrapolated relationships between fluids in the rat as a model to understand the consequences of GDNF protein levels on SSCs and progenitor spermatogonia in humans. Inherent in this thought are two underlying assumptions: 1) the concentrations of GDNF mRNA and protein in the human testes are positively correlated as they are in the rat [22], and 2) as shown in the rat, we estimate the
concentration of GDNF in TIF of a fertile man is 31% of its concentration in TTF, or 19pM.

**Evidence that GDNF is necessary for the maintenance of human spermatogonial stem cells and progenitor spermatogonia:**

The estimates of GDNF concentration in TIF of the fertile human testis have important implications in understanding the role GDNF has in contributing to infertility. We predict that the 80% decrease in expression of GDNF mRNA in the human SCO testis translates to a GDNF concentration in the TIF of these infertile men of approximately 2pM, a concentration, which is significantly lower than the Kd of GDNF for its receptor. This would leave most GDNF receptors unoccupied, depriving SSCs and progenitor spermatogonia of the necessarily stimulus.

We recognize that a reduction in GDNF expression in SCO testes, when viewed by itself, might not be viewed as proof of Sertoli cell dysfunction, but rather as a consequence of the loss of stimulus of Sertoli cells from spermatogenic cells. However, GDNF mRNA levels were not reduced in testes that had significantly reduced numbers of germ cells due to maturation arrest of spermatogenesis. We also recognized that decreased GDNF expression in the SCO testis might reflect an overall hypofunction of Sertoli cells and not a specific dysfunction. Thus, to distinguish between these two possibilities, we also quantified transcripts encoding two Sertoli cell products, kit ligand and clusterin. Expression of neither kit ligand mRNA nor clusterin were reduced in SCO testes, indicating that the SCO phenotype may be associated with a specific deficit in the
Sertoli cells. Taken together, our data suggest that a decrease in GDNF concentration in the fluid that bathes SSCs or progenitor spermatogonia is caused by a specific deficit in Sertoli cell function and is one probable cause for the diminished numbers and function of human SSCs associated with the clinical SCO phenotype.

We also acknowledge that GDNF may not be the sole regulator of the numbers of these cells in humans, leading us to propose that other intrinsic or extrinsic factors act in concert with GDNF to regulate the numbers of SSCs and progenitor spermatogonia, and that some of these other growth factors may not be expressed in the SCO testis. Thus, a requirement for investigating growth factors as treatments of human male infertility must begin with the identification of a full repertoire of growth factors implicated in regulating the numbers, replication and differentiation of these cells.

A comparison of GFRα1+ spermatogonia in fertile men, mice and rats:

Using a mouse model, we have previously shown that GDNF regulates the numbers and replication of SSCs and progenitor spermatogonia, stimulates their expression of GFRα1 and suppresses their differentiation [9, 12]. If GDNF is the primary regulator of the numbers of SSCs and progenitor spermatogonia, then mice should have higher densities of GFRα1+ spermatogonia along the surface of their seminiferous tubules than rats and humans. However, this is not what we observed. Our data show that densities of GFRα1+ cells were highest in humans, intermediate in rat tubules and lowest in mouse tubules. The observation of dense GFRα1+ spermatogonia in human tubules is consistent with
the conclusion that humans have a much higher ratio of SSCs and progenitor spermatogonia vs. differentiated spermatogonia, than do rodents [7, 32]. However, we were surprised by the observation of long chains of GFRα1+ cells, which appeared to be connected, in both rats and humans. We consider these cells to function primarily as progenitor spermatogonia and analogous to Aal spermatogonia. Such long chains of GFRα1+ Aal spermatogonia are rarely observed in the mouse and do not normally exceed more than 4 cells in a chain [9, 12]. However, in mice, longer chains of Aal spermatogonia, comprising of 8 or 16 cells, can be identified with the use of other markers. We have interpreted the lack of expression of GFRα1 in these longer chains as indicating that these cells have already initiated differentiation. Thus, longer chains of GFRα1 spermatogonia in rats and humans may indicate that differentiation of SSCs and/or progenitor spermatogonia is suppressed for a longer period of time; even through GDNF concentration is higher in the mouse, when compared to the rat or the human.

These differences also suggest the involvement of other intrinsic or extrinsic factors contributing to the regulation of the numbers or function of these SSCs in humans. This hypothesis is consistent with the fact that there are two different populations of SSCs in humans, a reserve and rarely dividing pool and an active population of SSCs that sustain normal spermatogenesis. The hypothesis that multiple growth factors regulate SSCs in vivo is also consistent with what is known about stem cells in many other tissues [33-35]. For instance, in bone marrow hematopoietic stem cells (HSCs), Wnt3a and throbopoietin are
required for maintenance, while BMP and hedgehog signaling pathways promote self-renewal and long-term function, respectively [36, 37]. In addition, Notch signaling regulates formation of progenitors from HSCs, in specific blood cell lineages [32]. The regulation of hair follicles is even more complex because they contain two types of stem cells, which are regulated by different cytokines, and undergo mitosis at different times in the hair follicle cycle [6]. Given the complex biology of spermatogonial stem cells and progenitor spermatogonia, and the differences we have noted between humans and rodents, it is reasonable to conclude that multiple growth factors regulate the numbers, replication and differentiation of these cells. However, taken together, the results outlined in this chapter lead us to propose that GDNF does play a significant role in sustaining SSCs and progenitor spermatogonia in humans, as it does in mice.

Evidence that GFRα1 identifies human spermatogonial stem cells and progenitor spermatogonia:

Lastly, we were interested in confirming that the cells expressing GFRα1 were SSCs and progenitor spermatogonia. One way to do this was to co-stain seminiferous tubules from fertile individuals with GFRα1 and UCHL1. Valli and colleagues have previously demonstrated that 92% of UCHL1+ human spermatogonia do not express the differentiation marker, Kit [6, 15, 38]. Based on their findings, we concluded that UCHL1 broadly identifies human SSCs and progenitor spermatogonia. Our results showed the same pattern of high and low density areas of UCHL1+ spermatogonia, as previously noted with GFRα1. In addition, we were able to visualize co-localization of spermatogonia in whole
mounts of seminiferous tubules, suggesting that some human spermatogonial stem cells and/or progenitor spermatogonia express the ligand binding subunit of the receptor needed to respond to GDNF. However, we acknowledge that the tubules analyzed come from one fertile individual, and that additional samples need to be analyzed quantitatively to account for the degree of overlap between these markers.
SUMMARY

Glial cell line-derived neurotrophic factor (GDNF) has been shown to be expressed in the human testis, bringing to light the question of whether it is required for human male fertility and, whether it targets the same cell-types in humans, as it does in mice [13]. Our data strongly support the hypothesis that GDNF is an important regulator of human spermatogonial stem cells and progenitor spermatogonia and that an inadequate concentration of GDNF can lead to the loss of numbers and/or function of these cells. When viewed from a therapeutic perspective, our data suggest that increasing intratesticular levels of GDNF in these infertile men might increase SSC numbers and function. In turn, this could increase the possibility that sufficient sperm are generated for either natural or assisted conception.
REFERENCES


FIGURE LEGEND

Figure 1. A comparison of GDNF mRNA concentration in testes of normal men and mice. Transcripts were quantified by digital PCR, and data (means + SEM; n=3 for mice and n=6 for humans) were normalized to 18S rRNA.

Figure 2. GDNF protein levels in human and mouse serum. Overall, transcript levels in humans (n= 5) and mice (n=4) were undetectable because they were below the level of sensitivity of the assay. Data are presented as pg/ml concentrations.

Figure 3. GDNF protein levels in the total testicular fluid of fertile men, rats and mice. Data (means +SEM; n=4 for humans and n=5 for rats and mice) are presented as pg/ml and pM of GDNF protein. For rats and mice, total testicular fluid was extracted from one testis per animal. In humans, total testicular fluid was collected from individuals undergoing vasectomy reversals. Statistical significance is identified by the different letters over the bars.

Figure 4. GDNF protein levels in fertile rat seminiferous tubule fluid (STF), testicular interstitial fluid testis fluid (TIF), and total testis fluid (TTF). Data (means + SEM; n=4) are presented as pg/ml and pM concentrations. Statistical significance is identified by the different letters over the bars.

Table 1. Characterization of fertile and infertile men.

Figure 5. A comparison of GDNF, DDX4, kit ligand (KL) and clusterin mRNA concentration in testes of fertile and infertile men. Numbers of transcripts encoding GDNF (means + SEM, N=6/group) (Fig. 7A), DDX4 (means + SEM, N=6-7/group) (Fig. 7B), KL (means + SEM, N=6-7/group) (Fig. 7C) and clusterin
(means + SEM; n=6/group) (Fig. 7D) were normalized to 18S rRNA. Bars with different letters represent statistical differences for each gene.

Figure 6. Localization of GFRα1 spermatogonia in seminiferous tubule whole mounts of fertile men, mice and rats. Confocal images of human (A-C), mouse (D- F) and rat (G-I) whole mounts were taken at a 25X magnification. Figures A, D and G show a high density of GFRα1+ cells juxtaposed to images (B, E and H) of whole mounts with a lower density of GFRα1+ cells. Additional areas of these tubules were devoid of GFRα1+ spermatogonia, and these areas appeared no different from negative control tubules. Negative controls included the replacement of primary antibody with isotype control IgG (data not shown) or the exclusion of primary antibody (C, F and I). Edges of the seminiferous tubules are outlined in white lines.

Figure 7. Quantification of the distribution of GFRα1+ spermatogonia in fertile mice and men. Percentage of the surface of human and mouse seminiferous tubules with a high, or no GFRα1+ cells were quantified. Data from tubules from three separate human and mouse testes are expressed as the mean + SEM of the area of the tubule.

Figure 8. Localization of UCHL1+ spermatogonia in seminiferous tubule whole mounts of fertile men. Panel A shows an area of the tubule with lots of UCHL1+ spermatogonia (>25 cells per image), identified as an area of high density. Panel B shows an area of the tubule with a few UCHL1+ spermatogonia (1-25 cells per image), identified as an area of low density. Negative controls included the
replacement of primary antibody with isotype control IgG (panel C) or the exclusion of primary antibody (data not shown), and were identical to areas of the tubule where no UCHL1+ spermatogonia were identified. All images are 2.3 micron thick optical sections of seminiferous tubule whole mounts, imaged using confocal microscopy.

Figure 9. Co-localization of spermatogonia GFRα1+ and UCHL1+ spermatogonia in seminiferous tubule whole mounts of fertile men. Tubules were immunostained for both proteins and 2.3 micron thick optical sections were captured by confocal microscopy. Green (UCHL1) and red (GFRα1) channels were captured separately. Top panel: Results for expression of UCHL1 (A, F, J) alone, GFRα1 (B, E, H, I) alone, and both proteins together (C, D, G) are shown. These images reveal considerable heterogeneity in the intensity of cellular expression of GFRα1 and co-localization. While many cells express both proteins (See box in 5C as well as D, E and F), some cells only express UCHL1 (See white arrow in C, and G & H), while others only express GFRα1 (See yellow arrow in C, and I & J). Bottom panel: The same image displayed in black and white. Brightness and contrast of the entire image was adjusted to aid in the visualization of GFRα1+ spermatogonia.
Figure 1.
Figure 2.

\[ y = 0.0014x + 0.2925 \]
\[ R^2 = 0.99843 \]

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Figure 3.
Figure 4.
Table 1.

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<td>Testis, right (biopsy): Spermatogenic arrest at secondary spermatocyte stage, with focal germ cell degeneration. Mild peritubular fibrosis. Leydig cells unremarkable. In most tubules, the spermatogenic arrest is present at the primary spermatocyte stage, although a few secondary spermatocytes are present. Definitive spermatids were not identified.</td>
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<td>Right testis biopsy: Late maturation arrest: Seminiferous tubules contain fairly normal numbers of spermatogonia that mature to the level of secondary spermatocytes. However, only occasional mature sperms are noted. Basement membranes are not thickened and normal numbers of Leydig cells are present in the interstitium.</td>
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<td>48</td>
<td>Late MA</td>
<td>Testis, right, biopsy: Spermatogenic arrest at secondary spermatocyte stage, see note. Leydig cells are unremarkable. Note: Degenerative germ cells are seen in some tubular lumina. There are structures which could possibly represent spermatids, but they are most likely to be degenerating pyknotic germ cell nuclei. Minimal peritubular fibrosis is seen.</td>
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<td>Testis, right (biopsy): Germ cell aplasia (Sertoli only pattern) is present in 100% of tubules. Leydig cells are present, unremarkable.</td>
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<td>27</td>
<td>SCO</td>
<td>Testis, right (biopsy): Germ cell aplasia (Sertoli cell only pattern). Sertoli cells only: 100% of tubules Leydig cells: Present, unremarkable.</td>
<td>No Y chromosome microdeletions</td>
</tr>
<tr>
<td>40</td>
<td>SCO</td>
<td>Testis, left (biopsy): Sertoli-only testis, with mild peritubular fibrosis. Leydig cells, unremarkable.</td>
<td>Not tested</td>
</tr>
<tr>
<td>33</td>
<td>SCO</td>
<td>Testis, left (biopsy): Germ cell aplasia (Sertoli cell only pattern). Active spermatogenesis (complete maturation): 0% of tubules. Sertoli cell only pattern: 100% of tubules. Tubular atrophy: 10% of tubules Leydig cells: Present, unremarkable.</td>
<td>No Y chromosome microdeletions</td>
</tr>
<tr>
<td>49</td>
<td>SCO</td>
<td>Testis, right (biopsy): Germ cell aplasia (Sertoli only pattern) is present in 100% of tubules. Leydig cells are present.</td>
<td>No Y chromosome microdeletions</td>
</tr>
<tr>
<td>32</td>
<td>SCO</td>
<td>Testis, right, biopsy: Sertoli-only testis with moderate peritubular fibrosis. Leydig cells, unremarkable.</td>
<td>No Y chromosome microdeletions</td>
</tr>
<tr>
<td>29</td>
<td>MA</td>
<td>Spermatogenic arrest at primary spermatocyte stage, with rare secondary spermatocytes. Peritubular fibrosis, moderate. Leydig cells, unremarkable.</td>
<td>No Y chromosome microdeletions</td>
</tr>
<tr>
<td>37</td>
<td>MA</td>
<td>Testis, right (biopsy): Maturation arrest. Maturation arrest (primary spermatocyte): 100% of tubules. Leydig cells: Present, unremarkable. Intratubular germ cell neoplasia: Absent. Peritubular fibrosis: Absent. Inflammation: Absent. Note: Although the biopsy shows uniform arrest at the primary spermatocyte, increased numbers of giant spermatogonia (without atypia) are noted the presence of which has been associated with a &quot;mixed atrophy&quot; picture [Sigg C and Hedinger C. Virchows Arch. (1983)].</td>
<td>No Y chromosome microdeletions</td>
</tr>
<tr>
<td>40</td>
<td>Cadaveric (Right testis)</td>
<td>Complete spermatogenesis</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td>Cadaveric (Left testis)</td>
<td>Complete spermatogenesis</td>
<td>Not tested</td>
</tr>
<tr>
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</tr>
<tr>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Cadaveric (Left testis)</td>
<td>Complete spermatogenesis</td>
<td>Not tested</td>
</tr>
<tr>
<td>31</td>
<td>SCO</td>
<td>Germ cell aplasia (Sertoli cell only pattern), Sertoli cells only: 100% of tubules. Leydig cells: Present, unremarkable. Intraductal germ cell neoplasia: Absent. Peritubular fibrosis/hyalinization: Focal, mild. Inflammation: Absent.</td>
<td>No Y chromosome microdeletions</td>
</tr>
<tr>
<td>27</td>
<td>SCO</td>
<td>Germ cell aplasia (Sertoli cell only pattern) present in 100% of tubules. Leydig cells: Present, unremarkable. Intraductal germ cell neoplasia: Absent. Peritubular fibrosis: Present. Inflammation: Absent.</td>
<td>No Y chromosome microdeletions</td>
</tr>
<tr>
<td>33</td>
<td>SCO</td>
<td>Germ cell aplasia (Sertoli cell only pattern) in 100% of tubules. Leydig cells: Present, increased. Intraductal germ cell neoplasia: Absent. Peritubular fibrosis: Absent. Inflammation: Absent.</td>
<td>AZFb+c terminal</td>
</tr>
<tr>
<td>34</td>
<td>MA93%/SCO2%</td>
<td>Maturation arrest (primary spermatocyte): 93% of tubules. Sertoli cells only: 2% of tubules. Tubular atrophy: 5% of tubules. Leydig cells: Present, unremarkable. Intraductal germ cell neoplasia: Absent (OCT-4 immunostain is negative). Peritubular fibrosis: Present, moderate. Inflammation: Absent.</td>
<td>No Y chromosome microdeletions</td>
</tr>
<tr>
<td>40</td>
<td>Cadaveric</td>
<td>Complete spermatogenesis</td>
<td>Not tested</td>
</tr>
<tr>
<td>40</td>
<td>Cadaveric</td>
<td>Complete spermatogenesis</td>
<td>Not tested</td>
</tr>
<tr>
<td>40</td>
<td>Cadaveric</td>
<td>Complete spermatogenesis</td>
<td>Not tested</td>
</tr>
<tr>
<td>25</td>
<td>Cadaveric</td>
<td>Complete spermatogenesis</td>
<td>Not tested</td>
</tr>
</tbody>
</table>
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Figure 9.
CHAPTER 4: CONCLUSIONS

Introduction:

Male fertility hinges on the delicate balance between renewal of stem cells and their proliferation and differentiation, a balance necessary for maintaining the stem cell pool and supporting spermatogenesis. The growth factor glial cell line-derived neurotrophic factor (GDNF) has been shown to be critical in regulating this balance in the prepubertal mouse, but its in vivo function in the normal adult mouse testis has never been directly studied. Filling this gap in our knowledge becomes particularly important when we consider the fact that the mouse is a representative model for investigating spermatogenesis in humans. However, in spite of significant overlap in similarities between species, we acknowledge the presences of subtle differences. In order to overcome these differences, we used testicular tissue biopsies to directly study the effects of GDNF in fertile and infertile men. The findings from our results provide significant support for the hypothesis that in humans, GDNF is essential for maintaining the pool of spermatogonial stem cells, as it is in mice.

The role of GDNF in regulating stem and progenitor spermatogonia in vivo in the normal adult mouse testis:

Using a unique in vivo chemical-genetic approach, we were able to investigate the effects of inhibition of the GDNF signaling complex on stem and progenitor spermatogonia in the normal adult mouse testes. We were the first to use this approach to investigate the direct regulation of any adult stem cell population by a single growth factor. There were three advantages to this
Experimental strategy: 1) it involved a completely normal pool of stem cells, 2) we could analyze the in vivo response of stem cells to acute and prolonged changes in signaling from a specific growth factor, and 3) the inhibition was reversible. Our results demonstrate that inhibiting the GDNF signaling complex for varying periods of time causes a sequential loss of stem cells as identified by the SSC markers: Ret, GFRα1 and Zbtb16, respectively. We also show that SSCs and progenitor spermatogonia respond to this loss differently; where some of these cells are lost when the GDNF signaling complex is inhibited for only 2 days, while others persist for up to 11 days. We thought that this loss of cells would be marked by an increase in apoptosis. Surprisingly, we observed no such increase. Thus, we propose that this loss occurs because the SSCs fail to re-new, and instead preferentially differentiate into type A1 spermatogonia; a hypothesis consistent with the current model in the field (Figure 1). Taken together, these data provide evidence in support of the hypothesis that GDNF is essential in regulating the self-renewal and differentiation of SSC and progenitor spermatogonia in the normal mature mouse testis.

The role of GDNF in regulating spermatogenesis in fertile and infertile men:

Glial cell line-derived neurotrophic factor (GDNF) has been shown to be expressed in the human testis. However, the question remains as to whether it is required for human male fertility and, whether it targets the same cell-types in humans, as it does in mice. Our data strongly support the hypothesis that GDNF is an important regulator of human spermatogonial stem cells and progenitor spermatogonia, and that an inadequate concentration GDNF can lead to the loss
of numbers and/or function of these cells. Our motivation to pursue translational studies stemmed from the surprising similarity between the histology of Ret (V805A) mice injected with NA-PP1 for 30 days and what clinicians were reporting in their Sertoli cell-only patients. In both cases, the seminiferous tubules were devoid of almost all germ cells, while primarily retaining Sertoli cells lining the rim of the tubule. The long-term goal of our research is to be able to alleviate some of the fertility challenges these individuals face, and in turn, improving their quality of life.

Our translational work began with the comparison and observation that GDNF mRNA levels were almost identical in human and mouse testes. This motivated us to compare GDNF protein concentrations in the testicular fluids of humans, mice and rats. Results showed that the concentration of GDNF in human total testicular fluid is almost identical to its concentration in rat total testicular fluid; suggesting that as in rodents, the concentration of GDNF surrounding human SSCs is sufficient to act as a mitogen and cell survival factor for these stem cells. Based on this similarity, we extrapolated relationships between fluids in the rat as a model to understand the consequences of GDNF protein levels on SSCs and progenitor spermatogonia in humans. These assumptions allowed us to estimate that the concentration of GDNF in the testicular interstitial fluid (TIF) of a fertile man is 31% of its concentration in the total testicular fluid. This becomes particularly important because it predicts that the 80% decrease in expression of GDNF mRNA in the human SCO testis translates to a GDNF concentration of ~2pM in the TIF of these infertile men, a
concentration much lower than the Kd of GDNF for its receptor, GFRα1. Such a low concentration would leave most GDNF receptors unoccupied, depriving SSCs and progenitor spermatogonia of the stimulus necessary to maintain the stem cell pool and sustain spermatogenesis. To distinguish between the possibility of whether decreased GDNF expression in the Sertoli cell-only (SCO) testis was due to an overall hypo-function or a specific dysfunction of Sertoli cells, we quantified transcripts encoding two Sertoli cell products, kit ligand and clusterin. Expression of neither kit ligand mRNA nor clusterin were reduced in SCO testes, indicating that the SCO phenotype may be associated with a specific deficit in Sertoli cell function. Taken together, these data provide a potential explanation for the diminished numbers and function of human SSCs associated with the clinical SCO phenotype.

Conclusions and future directions:

The impact of this research really resonates when viewed from a clinical and therapeutic perspective. Our data suggest that increasing intratesticular levels of GDNF in these SCO men might increase SSC numbers and function, and increase that possibility that sufficient sperm are generated for either natural or assisted conception. Focal areas of spermatogenesis, which can be found in SCO individuals, would be the main targets of such an approach. Figure 2 shows such a scenario, where a seminiferous tubule with normal spermatogenesis (lower right) is surround by tubules that are otherwise Sertoli cell-only. The objective would be to stimulate the few remaining SSCs and progenitor spermatogonia to re-new and differentiate into sperm, by exposing these cells to
elevated GDNF concentrations. This, in turn, could increase the success rates and efficiency of sperm retrieval through micro-TESE surgeries. However, we acknowledge that stem cell regulation is a complex process, requiring several growth factors to work in concert. Thus, the first prerequisite to such a therapeutic approach would require a thorough understanding of all other factors involved. Then, it would be worth measuring the message and protein levels of the top candidate growth factors that are common in fertile and infertile men using digital PCR/RT-PCR and ELISA, respectively. These data would help to get a sense of which growth factors are up or down regulated in infertile individuals. To test if these growth factors regulate the behavior and numbers of SSCs and progenitor spermatogonia, we can culture alginate encapsulated seminiferous tubules from fertile men, in the presence of individual growth factors at varying concentrations. Previous in vitro studies in the lab have shown that when isolated seminiferous tubules from fertile mice were cultured in the presence of a physiologically relevant concentration of recombinant GDNF (250 pg/ml or 21.6 pM), we observed an increase in replication of $A_s$, $A_{pr}$, and $A_{al}$ spermatogonia. These in vitro results mimic our in vivo results, and are in agreement with the hypothesis stating that GDNF regulates SSCs and progenitor spermatogonia. To test whether these growth factors work in a cooperative fashion, we can culture these tubules in the presence of different combinations and concentrations of candidate growth factors. Based on which combinations yield replication of SSC and progenitor spermatogonia, we will know which growths factors should be considered for therapy and get a sense of their
effective concentrations. Moving forward, we acknowledge that tittering concentrations is extremely important. We have previously observed the formation of clusters of stem and progenitor spermatogonia in the mouse, when the seminiferous tubules were exposed to higher concentrations of GDNF. Thus, to prevent the potential formation of tumorigenic clusters, we must be cautious of the concentrations. From here, we will have to test the selected growth factors at their respective concentrations \textit{in vivo}. One approach would involve using a mouse model, where we could surgically attach a slow release device containing these growth factors to the testes. The ideal mouse model would mimic the SCO phenotype, where we could also find the presence of focal areas with active spermatogenesis. If a specific mouse model is not available, we could also induce a SCO like state in our Ret (V805A) mice using NA-PP1. The goal would be to observe an improvement or restoration in spermatogenesis. If we could achieve this \textit{in vivo}, then we would have the preliminary data to start entertaining the idea of moving towards clinical trials.
FIGURE LEGEND

Figure 1. The current model in the field suggests that under low concentration of GDNF, SSCs preferentially self-renew. Conversely, when exposed to high concentrations of GDNF, these cells move towards differentiation.

Figure 2. Testicular cross-section (x400) showing a seminiferous tubule with normal spermatogenesis (lower right) surrounded by tubules that are otherwise Sertoli cell-only. Adapted from Silber, S.J., et al., Round spermatid injection. Fertil Steril, 2000. 73(5): p. 897-900.
Figure 1.

GDNF in the mouse:

Spermatogonial Stem Cells & Progenitor Spermatogonia

A_{single}

GDNF

A_{paired}

Progenitor Spermatogonia

A_{aligned}

Type A_1
Dolly Singh

EDUCATION
June 2016  **Doctor of Philosophy (Ph.D.),** Department of Biochemistry and Molecular Biology, Division of Reproductive Biology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD.

**Dissertation:** Glial cell line-derived neurotrophic factor (GDNF) is required in vivo for the maintenance of human spermatogonial stem cells and progenitor spermatogonia (Dr. William Wright).

May 2007  **Bachelor of Science in Biology (BS),** Florida State University, Tallahassee, FL.

RESEARCH EXPERIENCE
Aug. 2010-  **Graduate Student,** Department of Biochemistry and Molecular Biology, Division of Reproductive Biology, Johns Hopkins Bloomberg School of Public Health, MD.

My doctoral dissertation is focused on understanding the role of GDNF (Glial cell-line derived neurotrophic factor) in regulating human spermatogonial stem cells and spermatogenesis, and compare it to the mouse. The overall goal is to translate these findings into the clinic as a mode of therapy for a sub-population of infertile patients.

- Experienced in working with cadaveric human testes derived from hospitals or organ donation centers
- Developed and performed molecular, biochemical and immunological assays on human testicular tissue (fertile and infertile patients).
- Analyzed tissue using confocal microscopy, Digital PCR, ELISA, Western blots
- Presented research findings at the Society for the Study of Reproduction and the American Society of Andrology
- Mentored masters’ and undergraduate students with their research projects
- Co-founded the “Lab Student Scientist Group”
- Organized the Biochemistry and Molecular Biology Journal Club

July 2009- Aug. 2010  **Research Assistant,** Department of Biochemistry and Molecular Biology, Division of Reproductive Biology, Johns Hopkins Bloomberg School of Public Health, MD.

This independent research project focused on investigating the effect of inhibition of GDNF (Glial cell-line derived neurotrophic factor) on mouse spermatogonial stem cells and progenitor spermatogonia, using a novel chemical- genetic approach. The results show the acute effect lack of GDNF signaling has on
maintaining the stem cell pool and supporting spermatogenesis in the mouse (Savitt et al., 2012).
- Developed and performed Immunohistochemical assays on mouse testicular tissue
- Analyzed tissue using fluorescence microscopy
- Quantified spermatogonial cells using iVision software
- Collected mouse testicular tissue and generated RNA and cDNA from tissue
- Performed QRT-PCR and analyzed data for multiple genes to show a novel trend of the effect of inhibition of GDNF signaling on stem cell in the mouse testis

Oct. 2007- Aug. 2008 **Research Assistant**, Department of Oncology, Division of the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University, Baltimore, MD.
- Synthesized peptides using Fmoc chemistry
- Purified peptides by reverse-phase HPLC
- Verified molecular weights by Maldi-TOF

January - Dec. 2006 **Undergraduate Researcher**, Department of Biological Science, Program in Neuroscience, Florida State University, Tallahassee, FL (Dr. Debra Fadool).
- Subjected super-smeller mice to various odor stimulants and vehicle at different time-points
- Collected and interpreted trends in these data

**TEACHING EXPERIENCE**

Oct.- Dec. 2015 **Teaching Assistant**, Department of Biochemistry and Molecular Biology, Division of Reproductive Biology, Johns Hopkins Bloomberg School of Public Health, MD.
**Course title:** Fundamentals of Reproductive Biology
- Graded Midterm and Final Exams (Dr. Janice Evans and Dr. Barry Zirkin)

Aug. – Sept. 2007 **Science Teacher**, Sea-to-See, Department of Biological Science, Office of Science Teaching Activities Program (OSTA), Florida State University, Tallahassee, FL.
- Set-up interactive exhibits with live marine animals
- Taught Elementary School children about marine habitats and the importance of environmental preservation using visual aids
- Facilitated interactions between the children and live marine animals
- Aided in the maintenance of these animals at the FSU marine laboratory

June- **English Teacher**, Uceda School of English, Orlando, FL.
Aug. 2007  Taught conversational English and elementary grammar as a second language to adults from a variety of cultural backgrounds.
- Developed and implemented interactive activities, lesson plans, quizzes and exams
- Promoted an inclusive and comfortable learning environment in the classroom

- Mentored students interested in careers in science, engineering, mathematics, health, or medicine
- Tutored high school students in all subjects
- Guided them through the college application process
- Motivated students to achieve their long-term career goals

**SELECTED PUBLICATIONS:**
Published:


**SELECTED PRESENTATIONS:**
Talks:


Singh, D. Direct Evidence that GDNF regulates number of Undifferentiated spermatogonia in normal, mature testes. *43rd Annual Meeting Society for the Study of Reproduction: “The Intersection Between Genetics, Genomics, and
Reproductive Biology”, 2010.

Posters:
Singh, D., Parker, N., Falk, H., Smith, B., Wright, WW. Changes in stimulation by Glial cell line-derived neurotrophic factor (GDNF) have rapid effects on the replication of stem and other undifferentiated spermatogonia within a mature seminiferous epithelium and on expression by these cells on Gfra1. American Society of Andrology- XXII North American Testis workshop, 2013.

PROFESSIONAL MEMBERSHIPS:
2015- Present ASBMB: American Society for Biochemistry and Molecular Biology
2015- Present AMWA: American Medical Writers Association
2014-2015 LSSG: Lab Scientist Student Group, Vice President
2014-2015 Biochemistry and Molecular Biology Journal Club Co-organizer
2013-2014 AAAS: American Association for the Advancement of Science

PROFESSIONAL TRAINING:
2015 Human Subjects Research Training
Johns Hopkins School of Public Health
2013 Hazard Communication Training
Johns Hopkins School of Public Health
2010 Radiation Safety Training
Johns Hopkins School of Public Health
2009 Animal Care and Use Training
Johns Hopkins School of Public Health
May 2009 Vaccine Science and Policy Certificate, Department of Biochemistry and Molecular Biology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD.
2007 HIPPA and Research Training Certification
Johns Hopkins University