TESTOSTERONE REGULATION OF THE SPERMATOGONIAL STEM CELL NICHE IN MICE

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of LIANG-YU CHEN find it satisfactory and recommend that it be accepted.

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Abstract

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Testosterone, a steroid hormone, is not only important for testes development but also regulates sperm production. Sperm differentiate from spermatogonial stem cells (SSCs) a cell population that continually differentiates into sperm and must proliferate to replenish the germ cells in the testes. The regulation of spermatogonial stem cell proliferation or differentiation is an important aspect of male fertility. This purpose of this project was to determine how testosterone regulates SSCs homeostasis. To determine how testosterone regulates SSC, a protocol to immunize mice against GnRH was used to stop pituitary gland secretion of LH and FSH to establish a depleted hormone environment in mice. Subsequent experiments determined the testosterone regulated cell mechanisms regulating SSC homeostasis.

We established that immunization of mice against GnRH suppressed SSC activity such that these cells did not have stem cell activity. However, treating immunized mice with testosterone propionate (TP) stimulated progressive SSC activity over 48 hr. The data show that testosterone functionally enhances SSC proliferation in immunized mice. Glial cell-derived neurotrophic factor (GDNF) is critical for the establishment and maintenance of SSCs. Therefore, it was hypothesized that testosterone regulates GDNF. Real-time RT-PCR showed that *gdnf* expression was induced in GnRH immunized mice testis after TP injection. In addition, the expression of *gdnf* and leukemia inhibitory factor (*lif*), another factor that regulates SSC activity, increased in

the testes of flutamide-treated mice when the flutamide block of androgen receptor was lost. To determine the specific testicular cell responding to testosterone to support SSCs, Sertoli and peritubular myoid cells were treated with testosterone. Testosterone inhibited *gdnf* expression in the Sertoli cells but significantly increased *gdnf* mRNA and protein expression in peritubular myoid cells. In contrast, pregnenolone, which is produced by Sertoli cells, suppresses *gdnf* expression in the PM cells. Based on these data, the SSC niche includes FSH regulation of GDNF expression by Sertoli cells while pregnenolone and testosterone regulate peritubular myoid cell expression of GDNF. Together, these factors regulate SSC proliferation and self-renewal in the testis. This regulatory loop provides a physiological and cellular model of SSC homeostasis in mammals.

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DEDICATION

When I looked back to the first time I landed in America, exciting and anxious feelings all come to mind. I was excited to have the chance to begin my next journey and also anxious trying to figure out how to immerse myself into this giant knowledge ocean. I want to dedicate this work to my parents who gave me this opportunity to pursue a PhD degree in America to learn different culture and attitude. I also want to thank Dr. McLean's research grant and Department of Animal Science fellowship and scholarship to support my PhD training and allow me to focus on my research.

CHAPTER 1

INTRODUCTION

In male, sperm and androgen are derived from the testis. Sperm not only fertilize the oocyte to form zygote, but also carry genetic information to the next generation. Leydig cells are stimulated by luteinizing hormone (LH) to produce testosterone, the most biologically important androgen for male reproductive fucnction. Moreover, testosterone also provides negative feedback to inhibit hypothalamic production of GnRH and pituitary secretion of LH. Testosterone is one of the most important factors for spermatogenesis by signaling the somatic Sertoli and peritubular myoid cells to support germ cell differentiation [1, 2].

Several mouse models demonstrate the testicular phenotype that may occur when testosterone signaling in the testis is disrupted. For example, Gendt *et. al.* [1] selectively eliminated androgen receptor expression from Sertoli cells in mice with the use of CRE-lox technology. Mice homozygous null for AR in Sertoli cells had significantly smaller testes, disruption of spermatogenesis, and germ cell apoptosis. Interestingly, analysis of the expression of several genes important for testis function in the Sertoli cell AR deficient mouse demonstrated the mRNA expression of anti-Mullerian hormone (AMH) increased, but androgen-binding protein (ABP), cyclin A1, and sperm-1 mRNA expression decreased [3]. AMH, secreted by Sertoli cells, inhibits Leydig cells to synthesize testosterone in adult mice. Therefore, ABP, cyclin A1, and sperm-1 are correlated with the decrease in spermatogenesis observed in these mice. ABP transports androgen to the epididymis to support sperm formation. Sperm-1 and cyclin A1 are

correlated with spermatogenesis during meiosis [4, 5]. The sperm-1 and cyclinA1 mRNA level decrease is likely associated with germ cell differentiation arresting in meiosis.

Follicle stimulating hormone (FSH), a trophic hormone, is secreted from the pituitary gland or adenohypophysis and regulated by hypothalamic produced gonadotropic releasing hormone (GnRH) and inhibin produced by the testis [1]. FSH receptor is a G protein coupled receptor that is only expressed by Sertoli cells in the male. FSH signaling regulates Sertoli cell proliferation in neonatal animals and regulates expression and sometimes secretion of factors important for spermatogonial differentiation. ABP expression by Sertoli cells is stimulated by FSH therefore allowing androgen to maintain a high concentration in the seminiferous fluid [6]. Inhibin acts through negative feedback to inhibit FSH release from the pituitary gland as a feedback mechanism for Sertoli cell regulation [7]. Further, FSH also is important for completion of meiosis during spermatogenesis [2]. When pachytene spermatocytes are cocultured with Sertoli cells without FSH treatment, spermatogenesis is arrested in the meiosis [2]. Similar results also have been reported by Kangasniemi et. al. [8] who demonstrated FSH receptor mRNA was expressed from stage VIII to II at higher levels compared to stages VII and VIII in the irradiated rat seminiferous tubules. These results suggest that FSH is important for supporting germ cell meiosis and possibly spermatid elongation. On the other hand, FSH also can stimulate Sertoli cells to secret growth factors, such as glial cell line-derived neurotrophic factor (GDNF), to regulate spermatogonial stem cells [9-11].

Spermatogonial stem cells (SSC) originate from primordial germ cells (PGCs) [12]. PGCs can be identified in the primitive streak at around 6.5-7.25-day post coitum (6.5-7.25 dpc) in the mouse embryo. After 7.5 dpc, PGCs migrate from the hindgut to the

urogenital ridge. During 10.75 to 11 dpc, sex-determining region of the Y chromosome (*Sry*) is expressed in the bipotential gonad and associated with *Dax1*, *Sox9*, and *Fgf9* expression leads to Sertoli cell differentiation from the coelomic epithelium [13-15]. At 11.25 dpc, Sertoli cells begin proliferation and *Sry* expression doubles to reach peak expression at 11.5 dpc. Simultaneously, anti-Müllerian hormone (AMH) is secreted by Sertoli cells to inhibit Müllerian duct formation [13]. After 12.5 dpc, the sex of the gonad can be identified which includes sex cord formation in the embryonic testis [12]. The number of PGCs increases through mitosis from 50 at the 7.5 dpc to 25791 at 13.5 dpc in each mouse gonad [16, 17]. At 13.5 dpc, PGCs differentiate into prospermatogonia and arrest in the G0/G1 phase until after birth [18, 19]. During postnatal day 1 to 6, prospermatogonia will immediately transform into gonocytes and migrate from central position of the seminiferous tubule to the periphery in the seminiferous tubules. When they arrive on the basement membrane, gonocytes will transform into spermatogonia or spermatogonial stem cells [20, 21]. However, not every gonocyte transforms into spermatogonial stem cell. Some of them proceed directly to differentiated spermatogonia eventually forming sperm [18, 22]. Recently, Yoshida *et. al*. showed that only gonocytes that express neurogenin 3 (Ngn3) transform into SSCs [21]. Ngn3 is a basic helix-loop-helix transcription factor and has been suggested to regulate the central nerve system, peripheral nerve system, and pancreatic cell differentiation in the mice embryo [23-26]. In the SSCs, Ngn3 expression is correlated with galectin 1 quantitative expression [21]. These researchers also suggested that the first wave of spermatogenesis originated from gonocytes without Ngn3 expression and these germ cells will go into meiosis directly and differentiate into sperm escaping speromatogonial mitosis stage [22]. In adult testes,

SSCs go through spermatogonial differentiation followed by meiosis, during which the chromosome number goes from diploid to haploid, differentiate into the spermatocytes and eventually spermatids to complete spermatogenesis [27].

In 1871, von Ebner first described "spermatogenesis wave" [28]. He worked with sections of fixed tissue from rats and measured the length of the spermatogenesis wave on longitudinal sections of isolated tubules 25-38mm in length [28-30]. Based on this information, Roosen-Runge *et. al.* determined the different stages of spermatocyte differentiation in rat testes [30]. Although they defined spermatogenesis into 8 stages in the 19th century, this result implies spermatogenesis is occurring not arbitrary or concurrent but through regulatory waves in the seminiferous tubules. The organization of spermatogenesis is complex requiring germ – somatic cell interaction. Germ cells appear to regulate the number of stages in individual species; however the regulation of the initiation of spermatogenesis is not clear. Therefore, the mechanisms regulating SSC homeostasis is an area of great interest.

Several factors have been shown to influence SSC homeostasis. During testes development, expression of kit ligand (*Steel* or stem cell factor) guides PGC migration to the embryonic gonad. If expression of either kit ligand or expression its receptor, kit, is lost, this results in the testes lacking germ cells and the seminiferous tubules have a Sertoli cell only phenotype [31]. Further, in adult testes, kit is expressed when SSCs differentiate into spermatogonia. Steroid hormones can influence spermatogonial survival, for example decreased androgen concentration in the testis can cause spermatogonia apoptosis. Until now, however, the SSC self-renewing mechanism was not well known, but the SSC niche, made by Sertoli cells and the basement membrane

surrounding SSCs, plays important roles in the SSC homeostasis [33, 34]. In the different types of stem cells, specific niche cells regulate stem cell proliferation and differentiation, such as intestinal and blood stem cells [35]. Therefore, in the testis microenvironment surrounding the SSCs, niche cells can be Sertoli cells and peritubular myoid cells, but the regulatory mechanism is still unclear. Therefore, the importance of endocrine factors, such as FSH and testosterone, to regulate spermatogonial differentiation or proliferation suggest that these factors may in addition regulate SSC activity [2, 9, 36-40]. FSH has been suggested to stimulate glial cell line-derived neurotrophic factor (GDNF) expression in the Sertoli cell. The specific function of GDNF will be described latter in the introduction but it is important for SSC proliferation and self-renewal. Testosterone is essential for meiosis completion and round spermtid formation.

Brinster *et. al.* [41] proposed that bFGF, GDNF, and GDNF family co-receptor α1 (GFRα1) could induce SSC proliferation in SSCs co-cultured with STO cells. Although these three factors regulate SSC proliferation in this system, testosterone and FSH both are important for spermatogonial differentiation and proliferation. Meistrich *et. al*. [42] irradiated rat testis with 6 Gy gamma-ray and, 18 weeks after irradiation, treated the rats with GnRH antagonist-Zoladex for 10 weeks. It turns out that spermatogenesis recovered about 14.5% compared with non-hormone treated irradiated rats. However, the sperm number didn't recover to pre-treatment levels. To determine the effect of FSH and testosterone on irradiated testis, Shetty *et. al.* [43] irradiated hypophysectomic rat testes by a single dose of 5 Gy and immediately treated the rats with testosterone. The result showed that the percentage of germ cell differentiation decreased to 56±4% compared with the hypophysectomy without testosterone treated group. These results suggest that

testosterone decreases spermatogenesis in the irradiated rat testes. Furthermore, gonadotropin hormones also play inhibition role for the spermatogenesis in the irradiated rat testes. Twenty-seven days after testes were irradiated, FSH antibody was injected everyday combined with GnRH antagonist and either testosterone or flutamide injection. Flutamide is an androgen antagonist by directly binding the androgen receptor and blocking its activation by testosterone or another androgen. The testosterone treatment group was intended to determine testosterone action without gonadotropin hormone effect. The results showed that FSH antibody combined with GnRH antagonist and flutamide treatment had a higher sperm head count compared with FSH antibody alone group. However, if testosterone was injected instead of flutamide, the sperm head count gradually decreased. In this experiment, they point out that FSH and testosterone could independently inhibit spermatogenic recovery in the radiated rat testes. Similar results have been reported in other mammals. Boekelheide *et. al.* [44] irradiated monkey's testes, *Stump-tailed macaques*, and treated with GnRH antagonist following the irradiation. The result was showed that 40-90% seminiferous tubules contain differentiated germ cells compared with non-hormone treatment group. These results indicate that FSH and testosterone not only prevent germ cell differentiation but also might enhance germ cell proliferation in the irradiated rodent testis.

Chapter 2

LITERATURE REVIEW

I. Physical structure of testes

In mammals, male gonads consist of two testes contained in the scrotum or pouch, which is suspended outside the abdominal cavity. The scrotum plays an important role in temperature regulation that is essential for normal testicular function. During male embryo development, the gonad translocates from the abdominal cavity into the scrotum. This process of testicular descent is an androgen dependent action. Failure of the testes to descend into the scrotum is called cryptochidism and results infertility.

The tunica albuginea is a thin tissue covering of the testis and branches to form septa that divides the seminiferous tubules into discrete lobules. The majority of the testis is composed of coiled seminiferous tubules along with cells in the interstitial space. Each seminiferous tuble starts and ends in the rete testis to form a loop of sperm producing tissue. The seminiferous tubule walls are composed of peritubular myoid cells and the germinal epithelium along with Sertoli cells. After spermatozoa formation in the seminiferous tubules, they are transported into a convoluted network of ducts known as the rete testis. The spermatozoa are carried by seminiferous fluid from the rete testis into efferent ducts that connect to the epididymis for transportation of sperm from the testis. The functions of the epididymis are to transport sperm to the ductus deferens during ejaculation, support sperm maturation so they become motile and sperm storage.

The walls of seminiferous tubules have a complex structure. There are three layers between interstitial and Sertoli cells including the peritubular myoid cells, fibroblasts, and the basement membrane. The specialized Sertoli cells contact with basement membrane forming a compartment, also known as niche, which surrounds the SSCs. The spermatogonial stem cell niche plays an important role in regulation of SSC differentiation and/or proliferation. In addition, there are specialized tight junctions made by Sertoli-Sertoli interaction that forms an exclusive environment known as the blood-testis barrier (BTB). The function of the BTB is to separate germ cell and the bloodstream to prevent back diffusion of seminiferous tubule interior fluid that contains germ cell specific antigens into the bloodstream. These proteins found on the developing sperm can induce an antibody response to destroy sperm production.

II. Hypothalamic-Pituitary-Testes axis

The hypothalamus is located below the third ventricle of the brain and above the median eminence. It can be divided into three major zones: the medial, lateral, and periventricular zones. The medial and periventricular zones contain the majority of structures or regions of the hypothalamus and the fiber system pertinent to central regulation of endocrine system. Each of three different zones of the hypothalamus can be subdivided into three groups of nuclei or areas: a rostral (anterior) group, a tuberal group, and posterior group [56]. One of these three areas has been considered the hypophysiotropic area that is tuberal group and also includes the paraventricular nucleus (located in anterior group). This area contains ventromedial nucleus, dorsalmedial nucleus, and arcuate nucleus. These three nuclei are located immediately above the

median eminence and adjacent to the third ventricle. The hypothalamus is connected to the pituitary gland both by a portal vascular system and by neural pathways. The neural system provides indirect brain regulation to the adenohypophysis through the portal vascular system. The preoptic area and the medial basal region of hypothalamus, especially the arcuate nucleus, contain the gonadotropin-controlling center that regulates gonadotropin-releasing hormone (GnRH) secretion. GnRH is secreted from peptidergic neurons which are regulated by dopaminergic, catecholaminergic, and endorphin-related neurons. These neurons regulate the frequency and amplitude of the release of gonadotropin releasing hormone (GnRH). GnRH is a decapeptide that is widely distributed in the central nervous system and other tissues. GnRH is released in hypothalamo-hypophyseal tract into the portal vascular system. The blood flow to the pituitary without dilution through the systemic blood system allows the maximal amount of GnRH to reach the adenohypophysis to stimulate gonadotropin release. The GnRH receptor is a seven transmembrane receptor [57] and GnRH binds to its receptor on the luteotrophic cells inducing receptor interaction with other membrane receptors. This receptor-receptor interaction stimulates opening of cell membrane calcium channels thereby increasing the calcium ion concentration in the cytoplasma of the target cells. This activation, in turn, results in the downstream activation of protein kinase C and calmodulin. This signaling increases LH gene expression and also stimulates LH release [58]. In addition, Oliver *et. al.* [59] identified a retrograde system between pituitary gland and hypothalamus. However, the regulatory mechanism of this system is still unclear.

LH and follicle-stimulating hormone (FSH) are both secreted from folliculotrophic and luteotrophic cells that are basophilic cells in the pituitary. Both LH

and FSH are composed of α and β subunits. The α subunit of LH and FSH are identical so, function of each specific protein is determined by the β subunit. The receptors of LH and FSH are G protein-coupled receptors. In the testis, LH receptor is expressed on the Leydig cells and FSH receptor is expressed on the Sertoli cells. LH stimulates steroidogenesis in Leydig cells by activating cAMP production and downstream signaling to induce testosterone production. On the other hand, FSH receptor is only expressed by Sertoli cells and signals to support Sertoli cell function for sperm production. Moreover, FSH stimulates the cAMP pathway to trigger inhibin and androgen binding protein synthesis in Sertoli cells to regulate hormone synthesis, sperm production and sperm maturation in the epididymis.

The first description of a factor with the function of inhibin was reported by Mottram and Cramer in 1923 [60]. They found that seminiferous tubules were shrunken and disorganized while interstitial cells appeared normal in X-irradiated rats' testes. In addition, the histological appearance of cells in the anterior pituitary gland also changed with the appearance of "castration cells" [61, 62]. The results indicated that a negative feedback signal to the pituitary gland was lost and resulted in the formation of castration cells. In 1932, McCullagh used aqueous testicular extract to suppress castration cells in castrated rats and named this substance as inhibin [63]. Inhibin is a heterodimeric glycoprotein which contains one α subunit and either β_A or β_B forming inhibin A or B. Inhibins belong to the transforming growth factor β (TGF-β) superfamily. FSH is regulated not only by inhibin but also by activin. Activin has three subtypes: activin A ($\beta_A\beta_A$), activin B($\beta_A\beta_B$), and activin C($\beta_B\beta_B$). In cultured pituitary cells, activin can

stimulate FSH release. However, the physiological function of activin in male reproduction is still unclear.

III. Steriodogenesis

Testosterone regulates multiple aspects of sexual development and sexual differentiation in the male. It is also essential for the continual production of sperm by supporting multiple aspects of spermatogenesis. Loss of FSH signaling does not result in complete cessation of sperm production. However, loss of testosterone signaling for a short period of time results in a dramatic decrease in sperm released from the testis. Leydig cells through steroid synthesis produce testosterone.

A. Leydig cells

Leydig cells, also called interstitial cells, are located in the interstitial are of the testis between the seminiferous tubules. Leydig cells are the only cell in the testis that express LH receptors. The LH receptor is a G-protein coupled receptor (Gs) which activates adenylyl cyclase to convert ATP into cAMP. Protein kinase A (PKA) is activated by cAMP and phosphorylates CRBP to trigger steroidogenic acute regulatory protein (StAR) expression. The activity of StAR is important because it transports cholesterol into mitochondria. At the same time, cyctochrome P450 side chain cleavage enzyme cuts cholesterol side chain (removing 6 carbons at 21th position) to convert it into pregnenolone in the mitochondria. This step is the rate limiting step and followed by transport of progesterone into the cytoplasm. Progesterone is produced in the smooth endoplasmic reticular through the action of 3β hydroxyl steroid dehydrogenase. After

progesterone is synthesized, it is metabolized by 17α-hydroxylase into 17α-hydroxyprogesterone. Finally, testosterone is formed by the conversion of 17α-hydroxyprogesterone through 17,20-desmolase action. After testosterone is synthesized, it diffuses out of the Leydig cell and is transported in the blood associated with the liver produced steroid-binding protein. Testosterone acts on target tissues by dissociating from the steroid binding protein and binding to the androgen receptor. In the testis, testosterone diffuses across the interstitial space and into peritubular myoid and Sertoli cells that both express androgen receptor. In the seminiferous tubule, testosterone binds androgen-binding protein that is secreted from Sertoli cells and is directly transported to the epididymis to support its function.

B. Leydig cell populations during development

The Leydig cells have been characterized as having two populations in the rodent and three populations in human at different time points during development. During embryonic development, a population of cells positive for the orphan nuclear receptor steroidogenic factor 1 (*Sf1*) is found at the cranial end of the mesonephros. These cells are subsequently separated into two groups: one in the region of adrenal cortex and the other one in the gonad. During this time Sertoli cells secrete anti-Müllerian hormone (AMH) to inhibit Müllerian duct formation in male. The high concentration of AMH stimulates Leydig cell differentiation and intiates steroidogenesis in these cells. Interestingly, AMH directly inhibits testosterone production in the adult Leydig cells in vitro. Prior to fetal Leydig cell differentiation, the enzyme 17β-hydroxysteroid dehydrogenase type 3 is expressed in the developing seminiferous cords to convert

androstenedione to testosterone. After testicular development, 17β-hydroxysteroid dehydrogenase type 3 is only expressed in Leydig cells. In humans, based on testosterone concentration, Prince [118] suggests that human Leydig cells pass through three cell populations: 14-18 weeks of fetal life, 2 to 3 months after birth, and from puberty throughout adult life. However, there is no direct evidence to identify each individual Leydig cell population. These data reflect the complexity of Leydig cell development and testosterone production in the developing testis. Although testosterone production is independent of LH production during fetal development, the testis produces androgens that support testis and male reproductive tract development.

IV. Physiological function of testes

A. Spermatogenesis

The process of spermatogenesis starts with spermatogonial stem cell differentiation and includes all of the steps leading to the formation of spermatozoa. This process requires a different period of time depending on different species. For example, humans need 75-80 days for spermatogonia to differentiate into a sperm while rats need 54 days. Spermatogenesis also involves meiosis that results in the formation of haploid sperm due to chromosomal reduction from diploid to haploid. In addition, homologous recombination occurs during meiosis providing the mechanism leading to genetic diversity. Spermatogenesis includes spermotocytogenesis, meiosis and spermiogenesis. Spermatocytogenesis is the mitotic proliferation of spermatogonia that occurs prior to meiosis. In rodents, based on morphology, spermatogonia can be divided into several major groups. The first group of spermatogonia is the undifferentiated type A

spermatogonia including the: A*single*, A*paired*, and A*aligned* spermatogonia. Unidifferentiated spermatogonia represent a small percentage of the germ cell population in the testis. Following an unknown signal or loss of signal, spermatogonia convert from type A*aligned* to types A1~A4 spermatogonia. These spermatogonia are now considered differentiated spermatogonia and each conversion to a new cell type (i.e. A1, A2, A3 and A4) is associated with mitosis. The type A spermatogonia differentiate into intermediate spermatogonia and finally type B spermatogonia, the final germ cell type prior to the initiation of meiosis [64, 65]. At the first division of undifferentiated type of cells- A*paired* spermatogonial, are connected by an intercellular bridge that is maintained between all cells in the germ cell cohort as the germ cells undergo mitosis and expand in number. It is believed that the intercellular bridge allows cells to share their intracellular signal to synchronize their development [66].

Type B spermatogonium is the cell type that initiates the process of meiosis by becoming a primary spermatocye. Humans and monkeys also have type A and B spermatogonia. However, from morphological identification, type A spermatogonia, compared to the rodent, can be divided into two types: A*dark* and A*pale* [67]. After A*pale*, spermatogonia differentiate into type B spermatogonia. During meiosis, the morphology of the differentiating germ cells changes and each cell type was named based on the appearance of the chromosomes including preleptotene, leptotene, zygotene, pachytene and diplotene. In mice, as meiotic germ cells differentiate from preleptotene to leptotene cell types the cell shape changes from flattened to round shape. The chromatin appears as fine chromatin threads in leptotene primary spermatocytes. In the zygotene primary spermatocyte, the chromosomes become paired and form the synaptonemal

complex. In the pachytene primary spermatocyte, the cell and nuclear sizes increase signficantly. Furthermore, the chromosomal crossing-over and homologous recombination also occur in this stage. In the diplotene primary spermatocyte, the synaptonemal complex disappears and a dense round body is located in the central clear area. After first meiotic division, the germ cells are called secondary spermatocytes and then complete meiosis II to form spermatids.

The final phase of spermatogenesis is spermiogenesis which involves the differentiation and morphological reorganization of a round spermatid into an elongate spermatid with a condensed nuclei and flagella. Each progressive morphological and in many ways biochemical change in the spermatid is called a step. Depending on the species, there are 9-15 steps in spermiogenesis. The final product, spermatozoa is released from the germinal epithelium by Sertoli cells in a process called spermiation. This involves many proteases and the recovery and disposal of excess cytoplasm lost from the differentiating elongating spermatid. Once spermatids are released from the lumen of the seminiferous tubule, they are transported through the efferent ductules into the epididymis to begin the final stage of maturation. Although the round spermatid can fertilize egg through intracytoplasmic sperm injection (ICSI) [68], in the normal situation, spermatids are released from Sertoli cells lacking motility and the ability to fertilize an oocyte. Sperm obtain these characteristics during their transit in the epididymis. During the spermatid passage into the epididymis, the first step is that most of seminiferous fluid is reabsorbed by the efferent duct to concentrate the spermatids. The fluid pH value from efferent duct to the caput region of epididymus is drops from 7.3 to

the 6.5 [69]. However, it is not clear if the alteration of the ion content and pH change is important for spermatid maturation.

The epididymis releases factors, ions and other molecules that are important for sperm maturation and function. For example, beta-defensin 126 (DEFB126), formerly known as epididymal secretory protein 13.2 (ESP13.2) has been suggested to provide immune protection in the female reproductive tract [70]. Likewise epididymal secretions may be important for long-term metabolism in sperm for function. One example is the secretion and sperm uptake of free L-carnitine that is transported from the blood plasma through epididymal plasma and then into the spermatozoa, where it accumulates as both free and acetylated L-carnitine. It has been proposed that carnitine in the spermatozoa may serve as an intramitochondrial vehicle for an acyl group, which in the form of acyl CoA acts as a substrate for the oxidative process producing energy for sperm motility [71].

The epididymis is divided into three sections: caput, corpus and caudal. Each section has unique protein expression and different functions to support germ cell maturation. The proximal caput epididymis is primarily responsible for fluid uptake along with secreting factors that support sperm maturation. Sperm removed from the caput epididymis are not capable of naturally fertilizing an oocyte, are not motile and have a low degree of protein disulfide cross-linking. Sperm present in the corpus epididymis have increased disulfide cross-linking compared to the caput epididymis and a low percentage of sperm are capable of motility. Sperm in the corpus epididymis also have the capacity to specifically bind to an oocyte. The corpus epididymis is the primary extragonadal site of sperm storage. Sperm in the corpus epididymis are motile and

capable of fertilizing an oocyte under normal conditions. An important morphological change that occurs in sperm during the transit through the epididymis is the translocation of the proximal droplet, the final vestige of cytoplasm on the sperm, from the nuclear or head region of the sperm to the tail where it is eventually lost. Removal of the proximal droplet is likely important for efficient sperm motility.

B. Sertoli cells

Sertoli cells are columnar shape and extend from the basement membrane of seminiferous epithelium to the lumen. In a two-dimensional view, the cytoplasma of Sertoli cell is thin and forms arm-like structures toward the lumen. A three-dimensional view of Sertoli cells demonstrates that these cells are columnar and cylindrical in shape and surround the germ cells. However, based on the spermatogenesis stage, Sertoli cell shape can be divided into two different morphologies: type A and type B [72]. In type A Sertoli cells, mature spermatids reside within cytoplasmic crypts and are ready for release into the tubule lumen. However, in type B Sertoli cells, the cytoplasmic crypts are either barely observed or completely absent. The shape of Sertoli cells follows the change of the spermatogenic cycle [73]. In stages II, VII, VIII, IX-XI, and XIII-XIV, the shape changes dramatically. These changes also regulate Sertoli cell volume. In the rat morphometric analysis, Sertoli cell occupation of seminiferous tubules ranges from 20% to 29% of the total area in stages VI-VIII to XII-XIV [73]. These results suggest that Sertoli cells have high degree of plasticity and mobility to support spermatogenesis [74]. Tight junctions form and are present between Sertoli cells to prevent autoantigens going into the blood stream to induce immune system reaction. Gap junction also can be found between

Sertoli-Sertoli cells. Gap junctions are believed to be involved in the regulation of spermatogenesis cohort in the seminiferous tubules.

In the mature Sertoli cells, FSH helps supports spermatogenesis and stimulates the production of inhibin and aromatase for estrogen synthesis of Leydig cell produced testosterone. As mentioned before, FSH is a glycoprotein, binds to a specific G-protein coupled receptor and is a member of the superfamily of cysteine knot growth factors with two subunits, alpha and beta [75]. It has three cysteine disulfide bonds in each subunit. Both of them have a similar structure including two β-hairpin loops on one side of the protein and one cystiene knot on the other side. The protein structure appears curved because it is formed by the two subunit-inverted association.

The gene of FSH receptor is located in the chromosome 2p21 position in humans and on chromosome 17 in mice [76]. The size of gene is about 215 Kb which includes 10 exons and 9 intervening introns. The first 9 exons encode the extracellular domain and the last exon encodes transmembrane region, intracellular tail, and C-terminal end of hinge region. In addition, the N and C terminal regions are cysteine-rich and the extracellular domain includes nine leucine rich repeats [76]. It has been found that N terminal cysteine region is important for the receptor moving to the cell membrane [77]. The leucine rich regions participate in ligand binding [78].

FSH – FSHR binding induces a conformational change in the receptor that stimulates the Gsα subunit to exchange GDP to GTP and, concurrently, Gsα dissociates with Gβγ subunits. Therefore, Gsα activate adenylyl cyclase to produce cAMP, which, in turn, activates protein kinase A (PKA). PKA can directly transport into the nucleus to activate cAMP response element (CREB), which directly binds to DNA promoter region

allowing CREB-binding protein binding. This complex allows RNA polymerase to associate to the DNA and then drives transcription.

FSH regulates protein synthesis in Sertoli cells and some proteins, such as inhibin and stem cell factor (SCF), have been well studied. However, an oligonucleotide microarray analysis demonstrated that FSH regulated the expression of 100 to 300 genes and the mechanisms regulating many of these are still unknown [79].

The expression of inhibin, kit ligand, lactate dehydrogenase A, testibunin, and α_2-macroglobulin are regulated not only by FSH but also by spermatogenic cycle. The highest mRNA levels of inhibin are found in the stages XIII through IV and the lowest in stages VII through VIII. α_2-macroglobulin, a nonspecific protease inhibitor, is also differentially expressed during spermatogenesis with high activity in stages XIII through I. FSH stimulates SCF mRNA transcription in all stages of spermatogenesis, however, the highest expression is in stages II through VI and the weakest expression is in stages VII through VIII. Kit ligand, also known as SCF, is important for spermatogonial differentiation and has been shown to protect germ cells from apoptosis *in vitro* [80]. . Thus, FSH is important for maintaining Sertoli cell function in adult males and some of these functions are critical for germ cell differentiation.

C. Factors secreted by Sertoli cells

Sertoli cells secrete factors that can be categorized as growth factors and hormones. These factors influence cellular function, growth and differentiation at the molecular level through receptor-mediated signal transduction [81]. The following factors are a partial list of growth factors that are secreted bySertoli cells: insulin-like factors

(IGFs), transforming growth factor α (TGFα), transforming growth factor β (TGFβ), fibroblast growth factor (FGF), neurotropins, cytokines, kit ligand/stem cell factor, and glial cell-derived neurotrophic factor (GDNF). The general function of these factors and any specific function for testis function will be discussed below.

IGFs

The structure of IGFs is similar to insulin. These factors include IGF-I and IGF-II and influence Sertoli cells by stimulating DNA synthesis in proliferating Sertoli cells and stimulates adult Sertoli cells to produce lactate and transferrin. Also, IGF-I enhances FSH action on Sertoli cells. IGF-I is secreted from Sertoli cells and acts on Sertoli cells through autocrine mechanisms; however, IGF-I concentration in the interstitial space is high which so it is available to the basal surface of Sertoli cells without needing to cross the blood-testes barrier. This suggests that IGF-1 may be involved in Sertoli cell regulation of germ cell differentiation. On the other hand, IGF-I has been shown in vitro to regulate steroidogenesis in Leydig cells suggesting that a regulatory loop between Sertoli cells and Leydig cells involved IGF-1. However, functional evidence of this regulatory loop has not been established.

TGFα

TGFα is a member of the epidermal growth factor (EGF) family. Sertoli cells secrete TGFα that can locally interact with germ cells and peritubular cells [40, 82]. EGF has also been shown to affect spermatogenesis. For example, decreased levels of serum EGF results in a significant decrease in sperm production. However, the concentration of EGF in the circulation is usually low so this suggests that circulation EGF might not have

physiological function in the testis and local production of EGF is important for sperm production.

TGFβ

TGFβ has three isoforms, including TGFβ$_1$, TGFβ$_2$, and TGFβ$_3$, which are all expressed by Sertoli cells. In the fetal rat testes, TGFβ$_1$ inhibits cord formation and also antagonizes EGF function [83]. Furthermore, cultured Leydig cells treated with TGFβ$_1$ results in inhibition of LH-induced testosterone production [84]. This result suggests that TGFβ$_1$ might reduce the activity steroidogenic enzymes' or may inhibit the production of mRNA of these emzymes. A similar result was demonstrated with TGFβ$_3$ treatment of Leydig cell *in vitro*. Another research group independently demonstrated that an antisense oligonucleotide of TGFβ$_1$ that inhibited TGFβ$_1$ synthesis in cultured pig Leydig cells resulted in an increase of testosterone production and steroidogenic enzyme levels. TGFβ$_1$ and TGFβ$_2$ expression is high in the prepubertal animal and then decrease to low levels in the adult. Interestingly, TGFβ$_3$ has a transient increase at the onset of puberty but the functional significance of this increase is not known [85].

FGF

bFGF is produced by Sertoli cells and its production is regulated by FSH. The FGF family of proteins has been shown to influence testes function. bFGF is one of the most studied FGF protein family members. The bFGF receptor is expressed by germ cells, Sertoli cells, Leydig cells, and peritubular myoid cells. It has been suggested that bFGF mediates the interaction between these testicular cells. In the mouse SSC, Brinster *et. al.* demonstrated that inclusion of bFGF in a serum free media is important for

proliferation of SSCs [41]. However, it is not known if bFGF acts directly on the SSCs or acts to stimulate the production of other factors by the STO feeder cells.

Kit ligand / stem cell factor (SCF)

SCF, also known as kit ligand, is important for primordial germ cell (PGC) viability and cell migration to the embryonic gonad. In the c-kit mutant mice, most PGC do not migrate to the urogental ridge due to a mutation of the kit receptor that prevents kit ligand activation of the kit [55, 86]. In addition, kit expression is also important for spermatogonial differentiation from undifferentiated spermatogonia to differentiated spermatogonia [87, 88]. Shinohara *et. al*. used kit ligand to culture with SSC in vitro and demonstrated that the number of SSCs in these cultures did not increase compared to controls [89]. The same results were confirmed by Yoshida *et. al.* using postnatal day 1 mouse testis cells [90]. In addition, they demonstrated that testes from day 1 mice did not stain for kit. However, after postnatal day 1, the c-kit expression gradually increases. The increase in kit expression may be associated with gonocytes that convert to type B spermatogonia without becoming SSCs first. These cells then continue to differentiate into sperm during the first wave of spermatogenesis that is accelerated compared to subsequent waves of spermatogenesis. However, in rats kit is expressed on undifferentiated spermatogonia before the postnatal stage but in neonatal rats it is only expressed on differentiated spermatocytes [91]. Thus, the expression of kit is varies between species and may play differential roles in the initiation of spermatogenesis

GDNF

GDNF is a member of TGFβ superfamily and neurotropin family. GDNF signals through a two-component receptor system to transfer signals: GPI-anchored co-receptor

called GFRα1 receptor and the Ret transmembrane receptor [92]. GDNF has been shown to be the most important regulator in the SSC self-renewal mechanism [93]. In mice that overexpress hGDNF in the testes, the SSC number dramatically increased [94]. Similarly, in the GDNF knockout mice, the number of SSCs is decreased compared to the wild type [93]. Interestingly, in the GDNF+/- mice [2], the number of SSCs is reduced and the apoptotic body can be identified in undifferentiated spermatogonia. The results suggest that GDNF concentration is very important for maintaining SSCs survival. Overall, these results illustrate that GDNF not only is essential for SSC proliferation but also can maintain the SSCs population.

V. BASEMENT MEMBRANE OF SEMINIFEROUS TUBULE

A. Morphological structure

The basement membrane of the seminiferous epithelium is located between seminiferous epithelium and the peritubular myoid cells [95]. It is composed of laminin, type IV collagen, heparan sulfate proteoglycan, and nidogen/entactin. In rodents, the basement membrane is the thin zone of extracellular matrix adjacent to the seminiferous epithelium. The clear zone around basement membrane is type I collagen fibrils in varying orientation. Preripheral to this collagen zone is a layer of flattened cells, known as peritubular myoid cells, followed by a layer of lymphatic endothelial cells. In large animals and primates, the seminiferous tubules are surrounded by a basement membrane adjacent to the type I collagen fibrils and multiple layers of myoid cells. Each myoid cell layer is surrounded by basement membrane material and is separated from one another by collagen fibrils, elastin fibrils, and microfibrils.

The basement membrane has been shown to maintain the columnar shape and polarity of Sertoli cells. In *in vitro* experiments, culture dishes coated with Matrigel, a basement membrane substrate, were used to culture Sertoli cells that formed columnar monolayers that were often 60 μm tall and similar is appear with in situ Sertoli cells [95]. In the Sertoli cell monoculture experiments, Sertoli cells produced laminin and collagen IV, but not fibronectin [96]. However, in myoid cell cultures, laminin, collagenIV, and fibroonectin are produced. These factors are important for basement membrane formation. In the mice, SSCs express α6-integrin that may anchor with laminin to allow SSC to attach and grow. On the other hand, the basement membrane might regulate SSC migration during the postnatal stage [97]. DNA microarry data shows that two genes, Col1a1 and Col1a2, were abundantly expressed in the seminiferous tubules of 6 days old mice compared with 60 days old mice. In the six-day-old mice, most germ cells are migrating from the lumen of the seminiferous tubule to the periphery, a process that may be regulated in part for the expression and presence of different substances such as Col1a1 and Col1a2. Pro-collagen I, a precursor of type I collagen, has consists of two α1 chains and one α2 chain. The α1 chain and α2 chain are encoded by two genes: Col1a1 and Col1a2. Immunohistochemical analysis demonstrated that procollagen I was detected on the cells that attached on the basement membrane, but was not expressed by the differentiated cells, such as leptotene spermatocytes, pachytene spermatocytes, round spermatids, and elongated spermatids. These data suggest that the basement membrane may play an important role in the germ cell mobility during spermatogenesis and SSC proliferation during self-renewal [97-99].

Peritubular myoid cells (PM cells), as described before, are embedded in the basement membrane. A limited amount of information is available about the mechanisms regulating the function of PM cells for spermatogenesis. Recently, in the PM cell androgen receptor specific knockout mice, testis size and sperm production were dramatically reduced. These data indicate that the loss of androgen signaling in the PM cells induces a decrease of sperm production. It also suggests that PM cells secrete essential factors to influence testes niche and this function may be regulated by testosterone.

Leukemia inhibitor factor (LIF) is a growth factor that is essential for the survival of different types of stem cells SSCs. LIF is a four-alpha-helix protein that contains three functional binding sites to interact with the receptor, gp130. In the mice testes, LIF is expressed in the PM cells and Sertoli cells. For SSC function, LIF may activate the JAK-STAT pathway to maintain SSCs population. It has been known that mice embryonic fibroblast cells (MEF) can secrete LIF to maintain embryonic stem cell survival. In experiments in which MEF are co-cultured with SSCs, the SSCs proliferated in the first seven days and then the cell number was maintained. These results suggest that LIF can influece SSC survival rather than self-renewal and proliferation. Overall, the PM-AR specific knockout mice model shows that losing testosterone leads to spermatogenesis aberration and LIF is essential for maintaining SSCs. It is not clear the relationship between testosterone and LIF expression.

VI. SPERMATOGONIAL STEM CELL (SSC)

A. Origin and development:

Primordial germ cells (PGCs) are the first germline cell in the embryo and therefore the SSC ancestor [12, 16, 100]. In mouse, PGCs are derived from proximal epiblast cells, adjacent to the extraembryonic ectoderm. It has been suggested that bone morphogenetic protein 4 (BMP4) and BMP8b are expressed in the extraembryonic ectoderm and targeted inactivation of either gene results in embryos devoid of both PGC and allantois [101]. Both BMP4 and BMP8b belong to the TGFβ superfamily. During gastrulation (about 6 days post coitum, 6dpc), PGCs move through the posterior primitive steak into the extraembryonic region [102]. At this time, *Fragilis* and *Smad1* are the first genes expressed in the PGC [103]. At about 7dpc, the newly formed extraembryonic mesoderm is moving to form the exocoelomic cavity. Some of the PGCs stop migration and form a cluster. At 7.2 dpc, *Stella* and tissue nonspecific alkaline phosphatase (TNAP) are expressed in the PGC cluster region. At 8.5 dpc, the endoderm is invaginating to form the hind-gut, and PGCs are carried along with the endoderm cells. At 9.5dpc, the majority of the PGCs are moving out of the hindgut and into the dorsal body wall and at 10.5dpc, PGCs are migrating into the gential ridges [104]. During PGC migration, targeted deletion of β2 integrin and *Fgf*8 along with natural mutations of *Steel* and *W* which code for kit ligand and kit, the receptor for kit ligand, result in improper PGC migration and few germ cells in the embryonic or adult testis. After PGCs have reached the gential ridges, sex determination and gene imprinting by either methylation or demethylation occur during this period of time.

The initiation of sex determination is regulated by the somatic cells in the embryonic gonad so the PGC differentiation into male germline cells is determined by environment. At 12.5 dpc, Sertoli cells have expressed sry and differentiated and testis

cords have formed. From 12.5 dpc, the germ cells are committed to male lineage and spermatogenesis; however, if PGCs are removed before 12.5 and mixed with 12.5 dpc female gential ridge cells, PGC enter the oogenesis pathway. Similarly, female PGCs are removed before 12.5 dpc or younger to the male genital-ridge; PGCs are male commitment. These experiments illustrate that the ultimate fate of a PGC may be determined by the somatic cells of the genital ridges. On the other hand, imprinted genes will be demethylated or methylated in the embryo a process known as epigenetic reprogramming. Imprinted genes are differentially expressed on the parental and maternal alleles before 12.5 dpc. An imprinted gene, such as insulin-like growth factor 2 (*Igf2*) and H19 fetal liver mRNA (*H19*), is differentially expressed on the single allele. *H19* is a paternally imprinted gene that is methylated in the sperm but unmethylated in oocytes. However, *Igf2* is a maternally imprinted gene that methylated in the oocytes but unmethylated in sperm [105].

In the male gential ridges, PGCs differentiate into prospermatogonia and enters mitotic arrest as G0/G1 phase. In contrast, in the female genital ridge, PGCs enter meiotic prophase as oocytes, and pass through leptotene, zygotene, and pachytene stages before arresting in diplotene at the time of birth [19, 106]. Oocytes resume meiosis prior to ovulation and only complete meiosis if fertilization occurs. Male germ cells remain in mitotic arrest until around the time of birth, depending upon the species.

During postnatal day 1 to 6, prospermatogonia will immediately transform into SSC and migrate from central position to the periphery in the seminiferous tubules. When they arrive on the basement membrane, gonocytes will transform into spermatogonia or SSCs [20, 21]. The steps in the formation of the SSC population are not known but, as

mentioned previously, the factor GDNF appears to be important. The SSC population continues to expand during prepubertal development and appears to be stable in adult animals. Thus, the establishment and maintenance of the SSC population requires a functioning stem cell niche in the seminiferous tubule.

B. Spermatogonial stem cell self-renewal

The ability of cells to self-renew and remain undifferentiated is a hallmark of stem cells, either embryonic or adult type. Two mechanisms have been proposed for SSC self-renewal. Clermont and Bustos-Obregon proposed the first self-renewal theory in 1968 [65]. It is also called A0/A1 theory or A0 theory. The scientists described a system that progenitor cells of the A1 spermatogonia are small in number and called A0 cells. These cells are normally quiescent and only divide when needed such as following insult or injury to the tissue. The A0 cells have an indefinite or very slow cell cycle. The cells that replenish the A1 spermatogonia are the A4 spermatogonia that can divide to form intermediate spermatogonia or return to the A1 spermatogonia cell type. This cell proliferation/self-renewal system would be maintained during the normal operation of the tissue when a small number of A4 spermatogonia can divide into A1 spermatogonia [22]. If the testes encounter irradiation, for example, A4 spermatogonia cannot go back to A1 spermatogonia and then A0 spermatogonia cell will divide into A1 cell.

Huckins and Oakberg proposed the second theory for spermatogonial self-renewal in 1971 [107, 108]. This theory is called A-single (As) theory because it is based on As spermatogonia as the stem cell. The As spermatogonia divide to either renew As spermatogonia or to become A-pr spermatogonia. Subsequently, Apr cells spermatogonia

divide to form A-al spermatogonia that reach 4, 8, or 16 cells clone. Most As, A-pr, and A-al spermatogonia divide during rat stage X-II and only occasionally in stages III-IX. At stages VII-IX, almost all Aal spermatogonia transform into A1 spermatogonia, a step that does not require mitosis and then the A1 spermatogonia resume the cell cycle. However, the regulatory mechanisms of A-*al* spermatogonia to divide into A1 spermatogonia is still unclear. Therefore, several models have been developed to investigate this process. For example, the vitamin A deficiency model, A-al spermatogonia arrest in the G0/G1 phase and do not progress to A1 spermatogonia. When vitamin A is administered, all spermatogonia in all tubules differentiate at the same time to A1 spermatogonia [109]. Thus, this model is beneficial to investigate the factors important in the transition of undifferentiated spermatogonia into differentiated spermatogonia.

C. Spermatogonial stem cell characteristics

The only assay to test for the presence of a SSC is the testicular transplantation technique. This approach requires SSCs to be injected into the testis of another animal and the establishment of donor-derived spermatogenesis is analyzed. Several proteins are enriched in SSCs, however to this date no specific marker for SSCs has been reported. The SSC markers that are enriched on SSCs compared to somatic or other germ cells of the testis provide the ability to select subsets of the cell populations from the testis. For example, Brinster and colleagues first identified the thymus 1 (Thy1.2, CD90) protein as being expressed by SSCs compared to other germ cells in the testis of mice [99]. Moreover, SSCs also express α6-integrin on the cell surface. The expression of some

markers is not consistent across species. For example, Ep-CAM is expressed on the surface of rat SSCs but not mouse [99].

D. Spermatogonial stem cell transplantation

The most important characteristic of SSCs is their ability to undego self-renewal [110]. Testis cell transplantation as a functional assay for spermatogonial stem cells was first reported in 1994 using mice [111-113]. The testes were enzymatically digested in order to create a single cell suspension of testicular cells that can be directly transplanted into recipient mice testis [113]. Only SSCs form colonies in the seminiferous tubules due to the stem cell characteristics and after several weeks, donor-derived spermatogenesis leads to sperm production from the donor cells. However, it is not easy to determine the effect of SSCs on the treatment because the SSCs population is low in the adult testis [107]. The marker proteins on the surface of SSCs can be used for cell isolation to enrich for SSCs for culture experiments [99]. In any case, SSC transplantation is best way to provide evidence that the cells in a suspension are SSCs, because specific protein markers have not been identified for SSCs.

E. Spermatogonial stem cell niche

The idea of niche came from Schofield who realized that the haematopoietic stem cells are regulated by specific region that is called niche [114]. This idea has been applied to all adult stem cells [33]. Moore and Lemischka define that niche cells can provide a sheltering environment where stem cells can sequester differentiation stimuli, apoptotic stimuli, and other stimuli that would challenge stem cell reserves [115]. However, in the

adult testis, the cells that compose niche is controversial. The physical compartment of SSC location suggests that the somatic cells of the seminiferous tubule participate as niche cells. Sertoli cells, peritubular myoid cells and basement membrane form this compartment. The interaction between Sertoli cell and SSC has been adressed more than basement membrane and SSC interaction.

The number of SSCs likely determines the number of niches during the neonatal stage. Shinohara *et. al.* [116] used 5-9 days old W/W^v mice as recipient to receive different ages of donor testicular cells. The proportion of SSCs in the cell population from day 5-12 old donors was larger than from14-20 weeks donor testicular cells. These data imply that the niche number can be altered during neonatal development of the testes. Moreover, it also suggests that the Sertoli cells support niche to regulate SSC because the testicular cells they used contain Sertoli cells which help SSC to build up extra niche leading to the testis size increase [117]. In the infertile Steel (Sl) mutant mice, Sertoli cells lacking steel factor prevents SSC differentiation into spermatogonia resulting in azoospermia [3]. The same result also happens in the kit mutant mice (W/W^v) and when the kit receptor on the SSC is mutated. Therefore, when SSCs isolated from Sl mice were transplant into W mice testis, spermatogenesis was recovered. These results suggest Sertoli cells play an important role in the testis SSC niche to regulate SSCs differentiation.

Sertoli cells secrete GDNF to induce SSCs proliferation and possibly self-renewal. Meng *et. al.* [11] demonstrated by using GDNF knock-out mice that if GDNF expression is lost the testes will lose SSCs. However, if niche cells overexpress GDNF, SSC number will dramatically increase without sperm production. Thus GDNF

concentration is a key regulator for the SSC homeostasis. Sperm loss, tumor formation and SSC apoptosis are all phenomena that occur in mice with GDNF overexpression. Therefore, lacking GDNF expression not only regulates neuron development but also SSC maintenance in the testis. Interestingly, $GDNF^{+/-}$ mice have sperm production but the spermatogenesis is disturbed. Furthermore, the Sertoli cell only seminiferous tubular is often observed in the aged $GDNF^{+/-}$ mice. Similar results are observed in the FSHR and AR knock-out mice [4].

The function of peritubular tubular myoid cells (PM cells) in the testis niches has been illustrated recently. Zhang *et. al.* used the Cre/loxP method to knock-out AR in PM cells [119]. The testes in these mice were smaller than controls and sperm production was significantly decreased compared to the normal mice. Most researches demonstrate that testosterone is important for spermatogenesis in the Sertoli cells but Zhang's results suggest testosterone can also influence spermatogenesis through PM cells. Spermatogenesis needs a precise system to regulate sperm production. From SSC proliferation and differentiation, if one of those cells does not function correctly, it might lead to SSC overproliferation or apoptosis. In this project, we ask many questions to investigate what the testosterone role is for the SSC niche in the testis. In addition we asked what the regulatory mechanism of GDNF is and what is the function of other members in the testes niche. Multiple experiments were designed to investigate these questions to help us understand the overall the function of niche.

F. SSC activity associated with aging

The age related decline in testosterone is due to testicular aging and at least 30% of men greater than 60-70 years of age have low testosterone concentrations [120]. Testicular aging may be due to decreased Leydig cell function or changes in the pulsatility of LH leading to a decreased production of testosterone. Similar changes occur in mice with declines in the serum testosterone at 15 months of age and severe depletion of germ cells and the presence of vacuoles in the seminiferous epithelium [121]. Indeed, up to 70% of the seminiferous tubules in 15-month-old mice show degeneration. Ryu *et al*. [122] showed that SSCs from 12-24 month old mice maintain their activity when transplanted into the testes of young mice suggesting that SSCs are functional past the lifespan of the male. These authors concluded that infertility in old males results from deterioration of the SSC niche and a failure of the niche to support the appropriate balance between stem cell self-renewal and differentiation [122]. These results, taken with the report that the lack of AR expression in peritubular myoid cells leads to oligospermia [119], indicate that testosterone is needed to support the SSC niche and sperm production throughout the lifespan of the male. Likewise, the decrease in serum testosterone levels during aging in males leads to a decrease in the number of SSCs resulting in decreased sperm production.

G. Testosterone signaling in the testis

The classical mechanism in which a steroid hormone stimulates gene expression occurs when the hormone diffuses through the plasma membrane, binds to a specific protein receptor that is then activated leading to binding of the hormone-receptor complex to specific DNA elements. The steroid-receptor complex acts to recruit co-activator

proteins to stimulate or in some cases repress transcription. Androgen receptor (AR) is expressed in Sertoli, Leydig and peritubular myoid cells in the testis [123]. Multiple genes regulated by testosterone *in vivo* have been identified [124] however; the cells in the testis that express these genes have not been determined. Furthermore, the molecular mechanisms in which testosterone regulates cell function in the testis is not clear. To date, the only gene expressed by Sertoli cells in the testis known to be induced by AR-DNA interactions in Rhox5 [125]. In addition, there is evidence that AR binds to two separate DNA motifs: classical ARE and AR-selective ARE. The difference between these elements is that other steroid hormone receptors can bind to the classical ARE while only AR binds to the AR-selective ARE [126].

Interestingly, not all testosterone-regulated genes contain an ARE in their promoters. Eacker et al. [127] used two transgenic mouse models and gene microarrays to investigate androgen regulation in the testis and Sertoli cells. Their results show that 35% of T-regulated genes (out of 60) did not contain an ARE in the gene promoter. One conclusion from this work is that testosterone-AR complex can bind to previously unidentified response elements. Another possibility is that testosterone is acting to stimulate a rapid signaling cascade that results in downstream transcription. The AR has been associated with caveolin in lipid rafts in a ligand dependent manner within minutes of DHT treatment in a prostate cancer cell line [128]. This activation has been reported in Sertoli cells from 15-day old rats. In this study, testosterone stimulates AR localization to the plasma membrane, Src kinase activation resulting in CREB stimulation [129].

Several cells in the testis express AR in the testis including Sertoli cells, Leydig cells and peritubular myoid cells. Reports of AR expression in differentiating germ cells

has been controversial because of conflicting data on which germ cells express AR. The data indicates that AR expression is present in some differentiating germ cells in certain species. Elongated spermatids have been shown to express AR and the AR-specific co-regulator, SNURF/RNF4. In addition, we have unpublished data that bovine gonocytes express AR during the first weeks of life in bull calves. However, AR expression in differentiating germ cells is not required for spermatogenesis because transplantation of germ cells from the naturally occurring AR mutants called *tfm* (testicular feminized) mice into the testes of wild type mice resulted in complete germ cell differentiation [130]. These results mean that germ cells do not need to express AR in order to differentiate into sperm. However, numerous reports have demonstrated that AR expression in Sertoli cells is essential for germ cell differentiation into sperm. Likewise, AR expression in Leydig cells is necessary for complete germ cell differentiation. Interestingly, elimination of AR expression in peritubular myoid cells does not eliminate complete germ cell differentiation but results in decreased sperm production in adult mice [119]. However, the specific mechanisms and function genes regulated by testosterone in these cells have been difficult to characterize.

H. Testosterone regulation of spermatogonia

The classical mechanism in which a steroid hormone stimulates gene expression occurs when the hormone diffuses through the plasma membrane, binds to a specific protein receptor that is then activated leading to binding of the hormone-receptor complex to specific DNA elements. The steroid-receptor complex acts to recruit co-activator proteins to stimulate or in some cases repress transcription. Androgen receptor

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Testosterone is essential for testis development and sustained spermatogenesis. However, it is interesting that following cytotoxic damage to the testis in rats from multiple toxicants including radiation recovery of spermatogonial proliferation is blocked

by testosterone [138]. In addition, FSH also blocks spermatogonial proliferation independently of testosterone following irradiation [139]. The negative action of FSH and testosterone on spermatogonial proliferation in this model is independent of SSCs and is due to the somatic environment. Radiation does not alter the functional ability of rat SSCs to colonize the seminiferous tubules of recipient immunodeficient nude mice. The block on spermatogonial differentiation in irradiated rats is actually due to damage to the somatic cells in the testis. This was demonstrated with the use of SSC transplantation by the transplantation of germ cells from irradiated rats into the testes of irradiated or untreated recipient rats [140]. Suppression of testosterone was necessary for colonized rat SSCs to differentiate in the testes of irradiated rat recipients but testosterone suppression was not required if the rat SSCs were injected into the seminiferous tubules of control recipient rats. Interestingly, transplantation of rat SSCs into the seminiferous tubules of bulsulfan-treated or irradiated mouse testes demonstrated that the colonization of the rat SSCs and subsequent spermatogonial differentiation were not inhibited by the high intratesticular testosterone concentrations in irradiated mice. Thus, the block of spermatogonial differentiation due to high concentrations of FSH and testosterone following irradiation appears to be specific to rats.

I. Regulation of GDNF expression

Cellular and physiological actions of RET activation by GDNF binding have been demonstrated in the kidney, neural tissue and the testis [141]. However, regulatory signals that induce GDNF production and release have not yet been clarified in any of these tissues. The greatest amount of work investigating induction of GDNF expression

and release has been conducted in astrocytes or cell lines derived from neural support cells. This work has demonstrated several compounds, cellular mechanisms and signaling pathways that lead to transcription of the GDNF gene.

The potential importance of GDNF as a therapeutic agent against neurodegenerative disease and addiction has resulted in investigation of how several drugs stimulate GDNF production. The treatment of C6 glioblastoma cells with FK960, an anti-dementia drug, resulted in increased mRNA and protein levels of GDNF. The effect of FK960 on GDNF mRNA expression was due to phosphorylation of mitogen-activated protein/extracellular signal-regulated kinase (Mapk3, also referred to as ERK1) that subsequently leads to activation of CREB and increased c-Fos expression [142]. Similarly, the herb *Rehmennia glutinnosa* (RG), a treatment for dementia, induces GDNF expression in C6 glioblastoma cells. The cell mechanism leading to GDNF expression was phosphorylation of MapK3 and the activation of protein kinase C (PKC). In this study, the PKC induction of GDNF gene expression was independent of Mapk3 [143]. In another study with C6 glioblastoma cells, thapsigargin, a compound that stimulates Ca^{+2} discharge from the endoplasmic reticulum, induced GDNF mRNA expression through both MAPK-dependent and possibly MAPK–independent pathways. Although Ca^{+2} was involved in the GDNF induction, the Ca^{+2} dependent PKC was responsible for the increase in GDNF expression; instead the PKCδ pathway was linked to GDNF expression [144]. The authors could not rule out the possibility that PKCδ did not induce GDNF mRNA expression independently of MapK3. Therefore, the details of the Ca^{+2} induced induction of GDNF expression have not been specifically determined. Another study focusing on the mechanism of how the antidepressant amitriptyline induces

expression of GDNF mRNA demonstrated it was through rapid activation of MapK3 [145]. Generally in neural cells the induction of GDNF mRNA production and protein release is due to activation of the MEK/MAPK signaling pathway.

During embryonic kidney development the GDNF gene is expressed in the matnephrogenic mesenchyme (MM) before ureteric bud induction. One factor, GDF11, has been suggested to induce GDNF expression in the MM because GDF11 knockout mice do not form a ureteric bud, a process dependent on GDNF expression [146]. However, a direct association between GDF11 and GDNF has not been established and subsequent work suggests GDF11 may not be required for kidney development [147]. To our knowledge no other extra-cellular signal has been identified in the embryonic kidney that stimulates a cell to produce GDNF. Regulation of GDNF mRNA expression during early kidney development has focused on the activation of the GDNF gene resulting from the interaction of gene products in a network of transcription factors and regulators of these factors. However, phenotypic analysis of the developing kidney in mouse knockouts has been used to identify several transcription factors important in GDNF expression. In vivo and in vitro studies have demonstrated that the transcription factor Pax2 plays a role in GDNF expression. Deletion of the Hoxa11/Hoxc11/Hoxd11 gene cluster also results in loss of GDNF expression in the embryonic kidney although these mice have normal expression of Pax2 [148]. It is not known if these factors act downstream of Pax2 expression or are necessary to interact with Pax2 to stimulate GDNF expression [149]. Six2, a member of the *Six* family of homeobox containing transcription factors, also activates GDNF gene expression [150]. The last factor shown to be involved in GDNF expression in the embryonic kidney is Eya1 (mammalian ortholog of eyes

absent in Drosophila). Eya1 does not directly induce expression of genes, but is a co-factor that binds other factors to induce transcription [149].

While is it known that GDNF is very important in SSC biological activity in the testis, little is known about what regulates its expression in this tissue. GDNF mRNA and protein have been localized in Sertoli cells [151], spermatogonia and spermatids [152] in the mouse. Similar results have been reported for rats and humans [31]. In addition to regulating SSCs, GDNF has been reported to, in combination with FSH, increase the number of Sertoli cells in testicular fragments in culture from 6-day-old rats [154]. However, this is the only report that shows GDNF has any action on Sertoli cells.

FSH increases the expression of GDNF 3 hr after treatment in cultures of primary Sertoli cells from 14-day old mice [155]. In fact, these researchers suggest that FSH stimulation via homeostatic control is a major regulator of GDNF concentration in the testis. However, this conclusion is based on data in which a gonadotropin-releasing hormone antagonist (Nal-Glu) is used to suppress FSH. This approach would also suppress testosterone production so the role of testosterone in this homeostatic regulation is not known [155]. FSHR is a G-protein coupled receptor (GPCR), which upon activation leads to G_{α} stimulation of adenylate cyclase (AC) to produce cAMP. The increase of the second messenger cAMP can have multiple effects in the cytoplasm of Sertoli cells. An increase in cAMP is usually associated with activation of cAMP dependent protein kinase A (PKA) that activates other proteins through phosphorylation. In pre-pubertal rat Sertoli cells, FSH stimulation leads to PKA phosphorylation of the nuclear transcription factor cAMP regulatory element-binding protein (CREB), [156]. In prepubertal rats, induction of gene expression through this pathway in Sertoli cells has

been described [157]. Activation of FSHR will also lead to signaling through other pathways and this response differs depending upon the level of differentiation of the cell and the presence of other factors. For example, FSH stimulated increase of cAMP in Sertoli cells from rats 5 and 11 days after birth resulted in a PKA dependent activation of Mapk3 leading to induction of cyclin D1 [158]. The increase of intracellular cAMP leading to cytoplasmic changes in Sertoli cells does not always involve PKA. In Sertoli cells from 10-day-old rats, FSH, through the action of cAMP but independent of PKA, enhances IGF-I dependent AKT1 phosphorylation [159]. In another study, FSH stimulated activation of phosphatidylinositol 3-kinase (PI3K) resulted in phosphorylation of AKT1 in Sertoli cells from 10 day old rats [160]. Activation of AKT1 was shown to be involved in the expression of *cyp19a1* (aromatase) and estradiol production. From these data, it is apparent that FSH mediates responses in Sertoli cells through multiple signaling cascades. In addition, multiple signaling pathways active in Sertoli cells result in the activation of CREB indicating this is an important transcription factor regulating Sertoli cell function.

In another study, the mouse Sertoli cell line TM4 was used to investigate regulation of GDNF expression. FGF2 at 25 ng/ml stimulated GDNF expression 5-fold in TM4 cells 48 hr after treatment while FSH stimulated GDNF expression 2-fold in TM4 cells 3-24 hr after treatment [161]. Stimulation of GDNF expression by FGF2 in TM4 cells was suppressed by pre-treatment of the TM4 cells with the phosphatidyl inositol 3-kinase (PI3K) inhibitor wortmannin and the mitogen-activated protein kinase (MAPK) signaling inhibitor PD98059. However, neither inhibitor alone or when added together completely abolished the FGF2 stimulated increase in GDNF expression. This indicates

other cellular mechanisms in addition to the MAPK and PI3K pathways are involved in FGF2 induction of GDNF expression. How wortmannin or PD98059 affected FSH induction of GDNF in TM4 cells was not investigated [161]. These authors reported that when primary mouse Sertoli cells were treated with FGF2 and FSH the expression of GDNF mRNA increased. Specifically, FSH increased GDNF expression 2.1 fold 6 hr after treatment in primary Sertoli cells from 10-day old mice. Similarly, FGF2 treated resulted in a 1.92 fold increase in GDNF mRNA levels 48 hr after treatment [161]. However, experiments with wortmannin and PD98059 were not performed with FGF2 or FSH treated primary mouse Sertoli cells. How FGF2 regulates GDNF expression *in vivo* is difficult to understand based on the fact that FGF2 has been shown to play a role in SSC survival *in vitro* (see above sections) but SSCs do not express GDNF [162]. Similarly, the role of FGF2 in the testis is complicated by the fact that the FGF2 knockout mouse is fertile [163]. In summary, while several transcription factors involved in GDNF expression in the embryonic kidney have been described, the signal or signals that induce this expression is not clear. Likewise, the complex transcriptional network is just being described. Thus, information regarding the cellular and molecular mechanisms regulating GDNF expression in the testis is lacking and represents a gap in the field. Similarly, information about GDNF expression by Sertoli cells is based on experimentation using prepubertal rodents as the source of Sertoli cells. Sertoli cell response to FSH declines in sexually mature animals, however it is still biological important, especially for spermatogonial differentiation. Therefore, details about how FSH may contribute to maintenance of SSCs by regulating the expression of GDNF or other factors in sexually mature animals have not been determined.

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CHAPTER THREE

Active immunization against GnRH results in loss of spermatogonial stem cell biological activity in mice

Abbreviated title: Hormone regulation of germline stem cells

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Abstract

Spermatogonial stem cells (SSCs) play an integral role in sustained sperm production through self-renewing proliferation and differentiation. Proper function of the hypophyseal-pituitary-gonadal (HPG) axis to supply gonadotropins to somatic cells of the testis is essential for spermatogenesis in mammals. Direct influence of the HPG axis on SSC function is poorly understood. We evaluated SSC biological activity in mice with a disrupted HPG axis using active immunization against GnRH and SSC transplantation. Transgenic donor mice expressing β-galactosidase in germ cells were actively immunized against GnRH and one month after the final booster, testicular cells were collected and transplanted into busulfan treated mice. Colonies of donor-derived spermatogenesis in recipient testes were evaluated after transplantation to assess biological activity and number of SSCs in the injected cell suspensions. GnRH immunization resulted in a significant decrease in testis weight, and reduced serum and intratesticular testosterone concentrations to 8.2 % and 4 % of non-immunized controls, respectively. Serum FSH concentrations in GnRH immunized mice decreased 2.4-fold compared to controls. Colonization of SSCs from GnRH immunized donors could not be detected in the seminiferous tubules of recipient mice. In contrast, colonization of SSCs from control donors was observed. Importantly, treatment of GnRH immunized mice with testosterone propionate for 0-72 hours followed by transplantation of testis cells resulted in colonization of SSCs and the initiation of spermatogenesis in recipient testes. Collectively, these results support the conclusion that normal function of the HPG axis, and thus testosterone is essential for SSC biological activity in the adult mouse testis.

Introduction

Spermatogenesis is the complex process by which a diploid spermatogonial stem cell (SSC) produces differentiating spermatogonia that have the ability to undergo proliferation and differentiation to eventually become mature haploid spermatozoa (1). The key element to continuation of this process throughout the lifetime of the male is the dual actions of SSCs to undergo both self-renewing mitosis and differentiation to produce daughter progeny that are committed to terminal differentiation into spermatozoa (2). It is essential that SSCs maintain these actions for fertility to be sustained in males. Despite their critical importance, very little is known about the endocrine and paracrine regulation of SSCs. In addition, little information is available about how SSCs regulate their own cell fate decision of self-renewal or differentiation, or how the testicular stem cell niche microenvironment influences cell fate.

Identification of factors that regulate SSCs and contribute to the stem cell niche in the testis is an active area of research. One niche factor identified, as an essential to regulator of SSC activity is glial cell line derived neurotrophic factor (GDNF). Mice null for GDNF die right after birth and testis morphology at this point of development is normal. However, mice that had decreased expression of GDNF due to targeted gene disruption (GDNF +/- mice) had progressive loss of differentiating germ cells and spermatogonia (3). Transgenic mice that over-express GDNF accumulate undifferentiated spermatogonia suggesting that this factor acts in the testis to suppress spermatogonial differentiation (3, 4). The systemic or local factors that regulate GDNF and other factors influencing SSCs in the testis are unknown. However, the pituitary produced protein

follicle-stimulating hormone (FSH) has been shown to induce GDNF expression in prepubertal mouse Sertoli cells *in vitro* (5).

A major contributor to the spermatogonial stem cell niche is the Sertoli cell, the somatic cell of the seminiferous epithelium (6-8). Sertoli cell function is regulated by pituitary released FSH and Leydig cell produced testosterone that is regulated by pituitary released luteinizing hormone (LH). The production and release of FSH and LH from the anterior pituitary is regulated by hypothalamic gonadotropin releasing hormone (GnRH). Together, these hormones form the hypophyseal-pituitary-gonadal (HPG) axis and are essential for testis function and production of spermatozoa (9). However, the influence of the HPG axis on SSC activity is unknown.

SSC-enriched cell fractions have been obtained from a variety of animals, transplanted into the testes of recipients and in many cases colonize the recipient testis and initiate donor-derived spermatogenesis (10, 11). Additionally, SSCs can be maintained in culture and retain the ability to colonize the testes of recipient animals (12-14). The mouse has been used primarily to investigate the regulation of SSCs both *in vivo* and *in vitro*. Transplantation into the testes of a recipient male is the only assay to definitively identify the presence of SSCs in a cell suspension and study their biological activity. Using this assay, SSC colonization is enhanced in a low gonadotropin recipient environment (15, 16), indicating that gonadotropins or testosterone may have an impact on the SSC niche microenvironment. One method for disruption of the HPG axis is through active immunization against GnRH (17, 18), thus blocking GnRH from reaching the pituitary and eliciting a release of FSH and LH. Hypogonadotropic hypogonadism is a condition represented by dysfunction of the HPG axis resulting in low to undetectable

concentrations of gonadotropins and testosterone causing severe to moderate oligospermia in men (19, 20). Depending on when during development and how severely this condition represents to an individual, hormone replacement therapy may not remedy the disorder. Understanding the effects of a disrupted HPG axis on spermatogonial stem cell biological activity is important for designing treatment regimens for individuals with hypogonadotropic hypogonadism. Similarly, the identification of systemic factors that regulate SSCs and contribute to the testicular stem cell niche is important in deciphering the molecular mechanisms involved in SSC homeostasis. The objective of this study was to evaluate the biological activity of SSCs in adult mice with a disrupted HPG axis using active immunization against GnRH.

Materials and Methods

Donor Mice, active immunization against GnRH, and SSC transplantation

Adult ROSA26 mice on a C57/B6 background (B6-Gtrosa26, Jackson Laboratory, Bar Harbor, ME) which express *LacZ* in all postnatal germ cell types were used as donors. All animal procedures were approved by the Washington State University Animal Care and Use Committee. Donor ROSA26 (n = 9 in 3 groups) mice were actively immunized against GnRH with a fusion protein cocktail (15). The immunization regimen consisted of a primary immunization in modified Freund's complete adjuvant followed by two booster injections in incomplete Freund's adjuvant one month apart each. Adult donor ROSA26 (n = 6) mice not receiving any immunization were used as controls. One month after the final booster donor mice were killed by CO_2 inhalation and blood collected by cardiac puncture. With all donors, both testes were removed, weighed, and one testis fixed in Bouin's solution for subsequent histological evaluation. Total germ cell populations were collected from the other testis by two-step enzymatic digestion as previously described (21) and suspended at a concentration of $1X10^6$ cells/ml in injection medium (DMEM with 0.004% trypan blue). Approximately 10^6 of donor germ cell suspension was then microinjected into the seminiferous tubules of recipient C57 adult male mice (n=3 mice/donor) treated with busulfan (40 mg/kg) 4wk prior to transplantation to deplete endogenous germ cells as previously described (21). With all recipients one testis was injected with donor germ cells while the contralateral testis was left as a non-injected control.

Mice treated with testosterone propionate (TP) were immunized against GnRH as described above. Testosterone propionate was dissolved in corn oil and mice were treated with 0.4mg/kg body weight. Based on the half-life of testosterone propionate, mice were injected at time = 0 and time = 48 hr and sacrificed at time = 0, 24, 48 and 72 hr after treatment. At sacrifice the testes were removed, digested as described and germ cells injected into the testes of recipient mice (n=3 mice/donor). Treatments were repeated with 3 groups of GnRH immunized mice for each TP treatment, equaling 12 total donors.

For all transplant experiments, recipient mice were evaluates for donor SSC colonization approximately six weeks after transplantation. Briefly, testes were fixed in 4% paraformaldehyde for 1hr at 4^{o}C, followed by washing in LacZ rinse buffer (0.2 M sodium phosphate, pH 7.3; 2 mM MgCl2; 0.02% NP40; 0.01% sodium deoxycholate) and incubation with X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) overnight at 37^{o}C. The next day, stained testes were washed twice with LacZ rinse buffer followed by rinses in 50% and 70% ethanol and finally stored in 70% ethanol at room temperature. Testes were then evaluated for donor colonization using a dissecting microscope at 10-40X magnification. The number of blue stained donor colonies of spermatogenesis was manually counted and digital images were captured (Leica DFC 280 digital camera).

GnRH donor mouse analysis

Donor testes fixed in Bouin's solution were processed for microscopic evaluation of spermatogenesis within seminiferous tubules using light microscopy. The average percentage of total regressed seminiferous tubules without active spermatogenesis was

determined by dividing the number of regressed tubules in three microscopic fields by the total number of seminiferous tubules within the same three fields. Efficacy of the GnRH immunization was determined based on serum testosterone and FSH concentrations. Blood was collected from donor mice used for transplantation at sacrifice by cardiac puncture. Additionally, intratesticular testosterone concentration was assayed in a separate group of GnRH immunized mice (n=8) by snap freezing testes in liquid nitrogen. Testicular cytosols were subsequently prepared from frozen testes by homogenization of the whole testes in PBS (pH 7.4). Serum and intratesticular testosterone concentration was then measured by RIA (DSL-400; Diagnostic Systems Laboratory Inc., Webster, TX).

Serum FSH concentration was determined using FSH assay kit purchased from the National Hormone and Peptide program (Dr. Parlow). Briefly, serum (1:25 dilution) was mixed with the rabbit anti-mouse FSH antibody and I^{125}-FSH and incubated at room temperature overnight. Secondary antibody and polyethylene glycol were added to precipitate the resulting insoluble antibody-protein complex and incubated at room temperature for 15 min. followed by centrifugation. The supernatant was discarded and FSH concentration was determined. The interassay coefficient of variation was 17%.

Statistical analysis

All data were analyzed using the SAS system software with the Proc GLM function. Differences between means for testis weight and percentage of seminiferous tubule cross sections with germ cells, mouse serum and intratesticular testosterone concentrations, mouse serum FSH concentration and colonization of recipient testes were

determined using the Duncan test for significance. Differences between treatments were considered significant at $p < 0.05$. Data are presented as the mean ± SEM.

Results

Effects of GnRH immunization on testicular weight and spermatogenesis

Mice were immunized against GnRH to investigate the impact of testosterone and FSH on SSC function. The antigen was a recombinant fusion protein generated from cDNA containing seven GnRH cDNA sequences inserted within the chicken ovalbumin gene (11). Mice were immunized at sexual maturity (6 wk of age) followed by two booster injections 4 and 8 wk after the initial immunization. The first series of experiments were conducted to evaluate the biological effect of GnRH immunization. As can be seen in Figure 1a, testis weight was significantly lower in mice immunized against GnRH when compared to controls. A 63% ± 7.4 reduction in testicular weight was observed in GnRH immunized mice compared to controls.

To investigate the effects of active immunization against GnRH on spermatogenesis donor testes were processed for microscopic evaluation. The seminiferous tubules in GnRH immunized mice were regressed and contained disrupted spermatogenesis (Figure 1b). In contrast, control donor testes contained virtually no regressed seminiferous tubules (Figure 1c). In addition, as can be seen in Figure 1b, the interstitial space between seminiferous tubules in testes of GnRH immunized males was disrupted resulting in altered seminiferous tubule architecture.

Effects of GnRH immunization on hormone concentrations

Although the observed block in germ cell differentiation is a strong indicator that GnRH immunization suppressed testosterone production, serum from GnRH immunized

and control mice was collected to evaluate pituitary and testicular hormone production. Both serum and intratesticular testosterone concentration was measured to evaluate the degree of reduced gonadotropin production by the pituitary in response to immunization against GnRH. In serum of GnRH immunized mice testosterone concentration was significantly ($p < 0.05$) reduced to 8%$\pm$0.3 of controls (Figure 2a). Because low serum testosterone concentrations may not reflect the concentration of testosterone in the testis, intratesticular testosterone concentration was also measured in GnRH immunized and control mice. Similar to serum testosterone concentrations, intratesticular testosterone in GnRH immunized mice significantly ($p< 0.01$) reduced to 4% ± 0.2 of controls and less than 2 ng/ml (Figure 2b).

The effect of GnRH immunization on pituitary FSH secretion was determined by measuring FSH concentration in the serum of treated and control males (Figure 2c). The FSH concentration in GnRH immunized mice (10.5 + 0.74 ng/ml) was significantly ($P<0.05$) reduced to 42% of that in control mice (24.8 + 1.47 ng/ml).

Effects of GnRH immunization on SSC biological activity

To examine the biological activity of SSCs in testes of GnRH immunized mice, testis cells were transplanted into seminiferous tubules of busulfan-treated recipient mice. Six weeks after transplantation, colonies of donor spermatogenesis derived from SSCs of control donors (2.6 $\pm$ 0.8 colonies/recipient testis) could be detected. However, colonies of donor-derived spermatogenesis could not be detected in any of the 10 recipient testes transplanted with cells from the testes of GnRH immunized donors (n=6).

Effects of TP treatment on re-initiation of SSC biological activity in testes of GnRH immunized mice

Based on the GnRH-immunized mice SSC transplantation experimental data, we hypothesized that testosterone regulates SSC activity *in vivo*. To test this hypothesis, ROSA26 GnRH immunized mice were treated with TP and controls were ROSA26 GnRH immunized mice injected with vehicle. Mice were sacrificed 0, 24, 48 and 72 hr after treatment and a single cell suspension of testis cells from each mouse was transplanted into the testes of recipient wild-type busulfan treated mice. Beginning 24 hr after TP treatment SSC colonization from GnRH immunized donors could be detected (Figure 3a-d). Consistent with previous experiments, SSCs from GnRH immunized non-TP treated controls and time 0 TP treatment did not colonize the testes of recipient mice. Interestingly, as the time after the testosterone injection into the GnRH-immunized mice progressed (i.e. 24-48-72 hr after TP injection) increased SSC colonization occurred, suggesting that TP treatment stimulated SSC activity in testes of GnRH immunized mice previously containing quiescent SSC populations (Figure 3e).

Discussion

Regulation of SSC homeostasis is complex and appears to vary between neonatal and adult mice. We investigated SSC homeostasis in adult mice with the use of a potent GnRH immunization protocol. A normal functioning HPG axis is essential for male fertility to provide gonadotropins to Leydig and Sertoli cells which supports their functions in the sperm production process. Disruption of the HPG axis leads to a condition of hypogonadotropic hypogonadism with associated infertility. In many instances this condition can be treated with administration of exogenous hormones resulting in regained fertility; however, this treatment is not always 100% effective (20). In this study we evaluated the effects of disrupting the HPG axis in the adult animal on SSC biology. Disruption of the HPG axis was achieved with active immunization against GnRH and testis cell transplantation was used as an assay to evaluate SSC activity. Immunization of mice against GnRH resulted in suppression of testosterone production, testicular regression and a loss of the ability of SSCs to colonize the testes of recipient mice. Thus, these data lead to the conclusion that loss of a functional HPG axis in adult mice results in suppression of stem cell activity by possibly forcing SSCs into a quiescent or inactive state. Furthermore, treating GnRH-immunized mice with testosterone propionate stimulated SSCs such that the cells colonized the testes of recipient mice following transplantation. These results suggest that testosterone is a regulator of SSC activity in adult mice, possibly through a pathway involving the stimulation of SSCs from a quiescent into an active state. This hypothesis is supported by the observation of varying active states of SSCs in mouse testes (21).

Several approaches to neutralize or suppress GnRH function including active immunization administration of GnRH antagonists have been utilized to investigate hormone regulation of spermatogenesis (22). In rats, GnRH antagonist treatment significantly reduces serum and testicular testosterone leading to decreased numbers of germ cells in seminiferous tubules (23). Similarly, active immunization against GnRH in rats resulted in testicular and epididymal regression (24-26). Meachem et al. (26) reported that intratesticular testosterone was 3.9% of controls following GnRH immunization in rats. Results of the current study were similar in which immunization of mice against GnRH resulted in reduction of intratesticular testosterone concentrations to 4% of wild-type controls. In normal rats, the concentration of testosterone is in excess of what is needed to support sperm production (27). Significant loss of elongating spermatids occurs in rat testes when intratesticular testosterone is below 10 ng/ml (27). We report that intratesticular testosterone concentration of GnRH immunized mice was less than 2 ng/ml resulting in the loss of function of testosterone-supported cells and a decrease in the number of differentiating germ cells.

In contrast to testosterone, GnRH immunization did not result in complete suppression of FSH production in mice. Serum FSH concentration was reduced to 40% of wild-type controls, a similar result to what has been reported in rats. The concentration of FSH in GnRH immunized rats was reported to be reduced to 34% of controls in one study (26) and 40% of controls in another (25). The production and regulation of FSH is complex in animals in which GnRH is suppressed artificially. Treating GnRH immunized rats with testosterone leads to an increase in serum FSH concentrations (25, 26). The mechanism responsible for this increase is testosterone induction of FSHβ subunit

expression in the pituitary (28). This response results in difficulty to distinguish the effect of testosterone treatment versus FSH action on the initiation of germ cell differentiation in GnRH immunized rats. Therefore, passive immunization against FSH has been used in conjunction with GnRH immunization to investigate the restoration of germ cell differentiation after testosterone treatment using this model (24). These studies have demonstrated that FSH is important for the initiation of spermatogonial differentiation and testosterone supports spermatocyte and spermatid differentiation (26). Although GnRH immunization in mice in this study resulted in a 60% decrease in FSH serum concentration, the remaining serum FSH likely exerts a biological effect on Sertoli cell function. Therefore, to investigate why SSCs in GnRH immunized mice were incapable of colonizing the testis of recipient mice, we focused on testosterone regulation.

While testosterone is essential for normal sperm production it can have a negative effect on certain aspects of germ cell differentiation. For example, administration of a low dose of testosterone in combination with a low dose of estradiol as implants will suppress LH secretion resulting in low physiological concentrations of testosterone (24). The reduced endogenous testosterone production does not support germ cell differentiation and the testes regress. Irradiation of rodents and humans can cause temporary loss of differentiating germ cells. The endocrinology associated with the recovery of spermatogenesis after this treatment has been extensively investigated in rats (29). In the rat, low doses of radiation cause azoospermia for up to 60 wk by blocking the differentiation of type A spermatogonia (30). Following irradiation, the concentration of intratesticular testosterone and serum FSH are elevated in rats compared to non-irradiated controls (30). Treatment of irradiated rats with GnRH analogs stimulates germ cell

differentiation in the testis. However, when irradiated rats are treated with GnRH analogs and testosterone the resumption of germ cell differentiation is blocked (30). FSH can also block germ cell differentiation in the rat testis after irradiation although testosterone treatment causes a more severe phenotype (31). In *jsd* mice, which have a mutation in the retrogene *mUtp14b,* spermatogenesis ceases after the initial wave (32). Interestingly, testosterone inhibits spermatogonia differentiation in *jsd* mice suggesting that similar mechanisms regulate spermatogonia differentiation in rats and mice (33). Taken together, these data suggest that somatic cells of the testis are important in the initiation of spermatogenesis following irradiation because FSH and androgen receptors are present in Sertoli cells while androgen receptors are also present in peritubular myoid and Leydig cells. Indeed, it has been demonstrated that spermatogonia differentiation is blocked following irradiation due to damage of the somatic cell environment in the seminiferous tubule (34).

Regulation of SSCs in the testis is limited and difficult to investigate since self-renewal and production of differentiated daughter progeny occurs at the same time. Studies in invertebrates suggest the stem cell fate decision is strictly regulated by autonomous self-renewal by the stem cell and influence by its microenvironment or niche (20, 35). In mammals, the Sertoli cell has been suggested as a key factor in the establishment and maintenance of the stem cell niche in the testis. Both FSH and testosterone regulate Sertoli cell action in the testis and loss of the production of either molecule could have profound effects on the ability of Sertoli cells to maintain SSC niches. Additionally, other testosterone responsive somatic cells in the testis may also contribute to the spermatogonial stem cell niche. Testosterone can signal through

multiple cells that express the androgen receptor such as the peritubular myoid and Leydig cells in addition to Sertoli cells. Future research will focus on determining the cells, factors and mechanisms that define the endocrine support of the mouse SSC niche.

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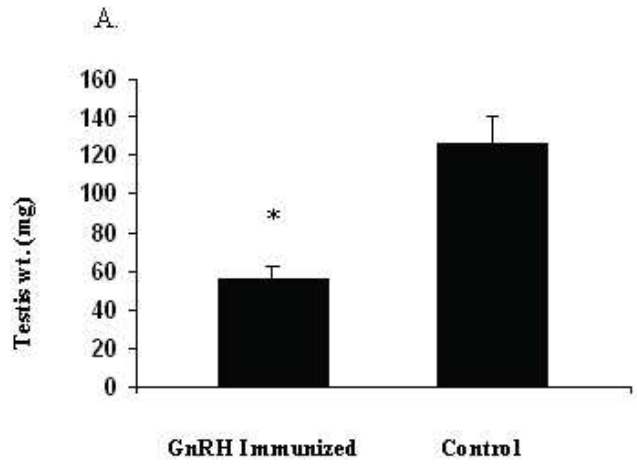


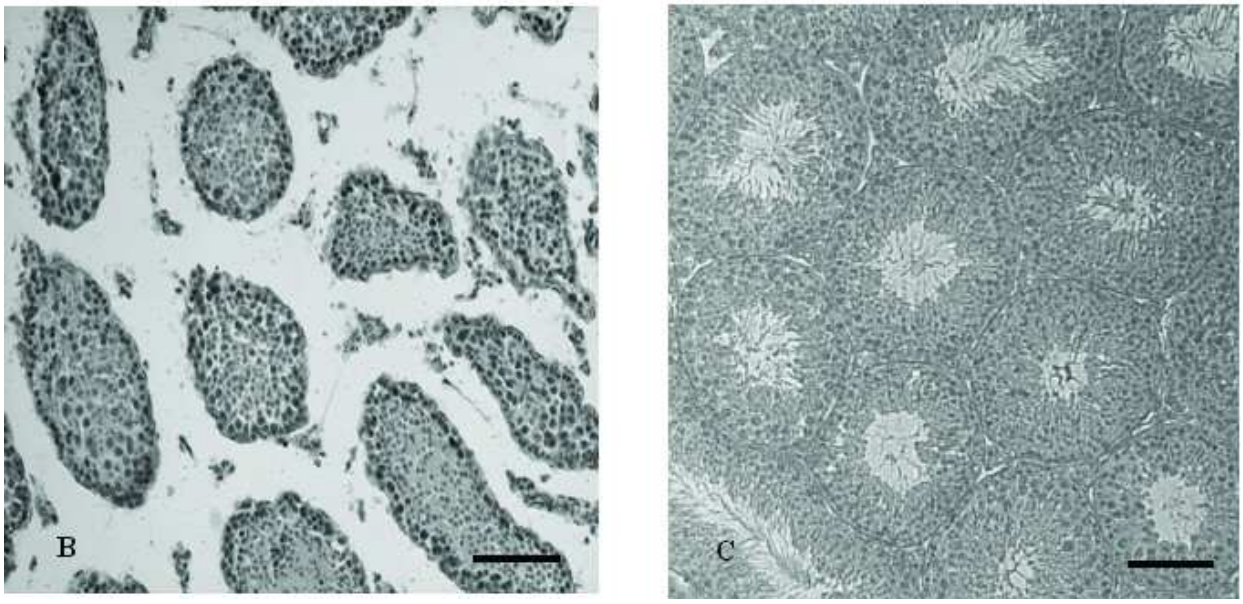


Figure 1. Effects of GnRH immunization on testis size and spermatogenesis in adult mice. A: Average testis weight (mg) of GnRH immunized and non-treated control mice. Data are presented as the mean ± SEM and * denotes significantly different at $P<0.05$. B: Testis cross-sections of GnRH immunized and control mice. The seminiferous epithelium is disorganized in the testis of the GnRH immunized mouse. In addition, few meiotic germ cells are present and the seminiferous tubule architecture is compromised. In contrast, the control testis (C) has seminiferous tubules containing all differentiating germ cells at different stages of spermatogenesis. Bars = 100 µm.

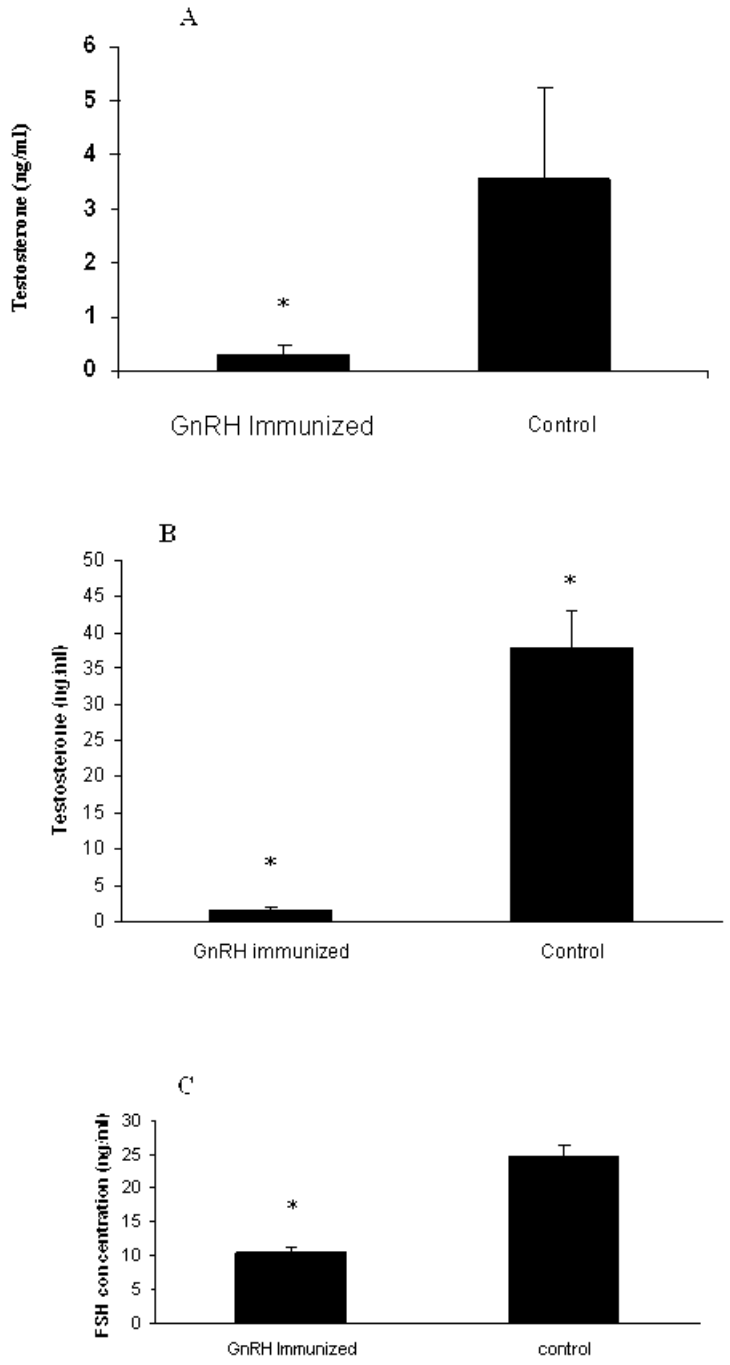
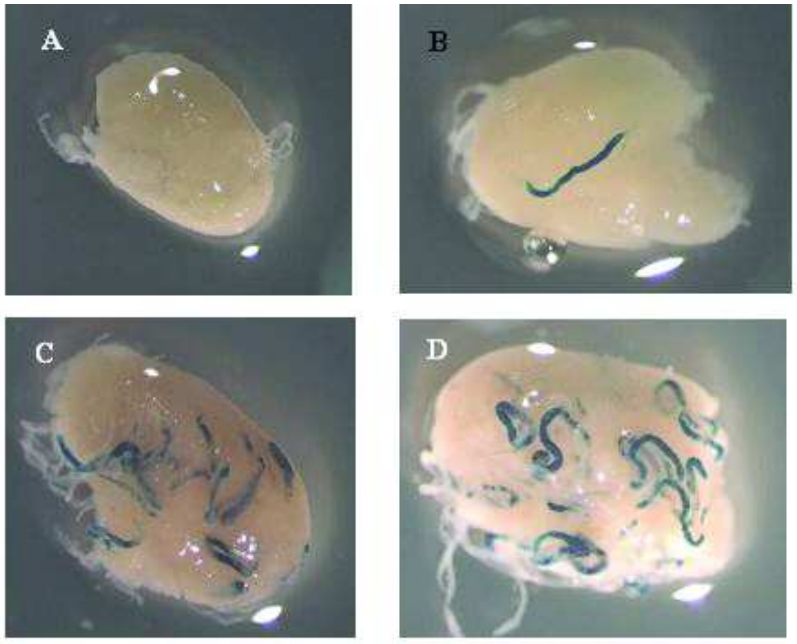


Figure 2. Effects of GnRH immunization on testosterone and FSH concentration in adult mice. Average serum testosterone (A), intratesticular testosterone (B) and FSH (C) concentrations in GnRH immunized and control mice. Data are presented, as the mean ± SEM and * denotes significantly different at $P < 0.05$


A
B
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D

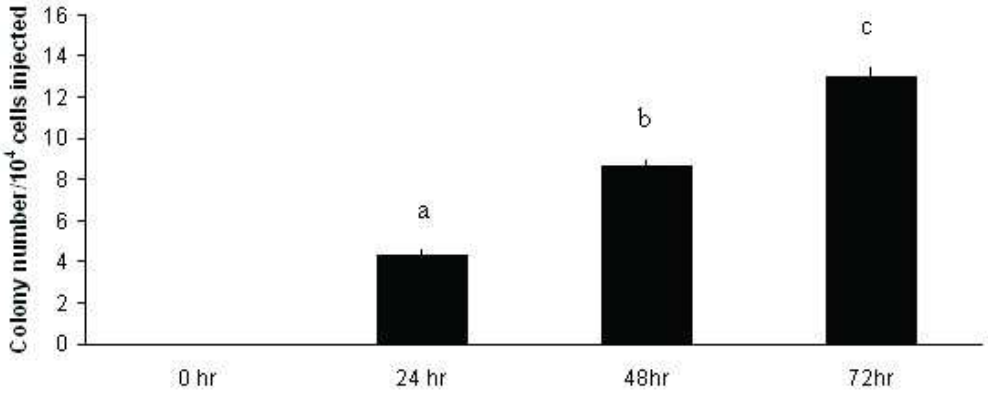
Colony number/10^4 cells injected
16
14
12
10
8
6
4
2
0
a
b
c
0 hr
24 hr
48hr
72hr

Figure 3. Evaluation of SSC biological activity in GnRH immunized, non TP treated control, and GnRH immunized mice treated with TP for 24, 48, or 72 hr. Rosa26 transgenic mice that express β-galactosidase in all cells were used as donors, thus donor spermatogenesis derived from colonized SSCs are visible as blue stained areas within recipient seminiferous tubules. A: Representative testis from recipient mice transplanted with testis cells from GnRH immunized mice treated with TP for 0 (A), 24 (B), 48 (C), and 72 hr (D). Bars = 2 mm. E: Average number of SSC derived colonies of donor spermatogenesis in recipient testes transplanted with testis cells from GnRH immunized mice treated with TP for 0, 24, 48, or 72 hr. Data are presented as mean+SEM and bars with different letters are significantly different at $P<0.05$.

CHAPTER FOUR

Testosterone regulates factors to influence spermatogonial stem cell homeostasis in mice testis niche

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Abstract

Testosterone, secreted Leydig cells in the testis, is involved in the regulation of spermatogenesis. Lacking testosterone or an androgen receptor (AR) mutation results in the arrest of germ cell differentiation at the round spermatid step. Testis size in animals lacking testosterone signaling also decreases compared to the testis in animals with physiological testosterone serum concentrations and wild type androgen receptor. Loss of testosterone signaling in the testis suppresses spermatogonial stem cell activity in adult mice. However, it is still unclear how testosterone affects SSCs and the testis stem cell niche. Here, we demonstrate that testosterone can enhance GDNF expression in GnRH-immunized mice after testosterone agonist treatment. Similar results were observed in the flutamide-treated mice testes. Specifically, the loss of flutamide suppressed testosterone signaling resulted in increased *gdnf* expression. Cultured peritubular myoid cells (PM cells) express gdnf and testosterone increases *gdnf* expression in a dose and time dependent manner. In contrast, testosterone suppressed gdnf expression in cultured Sertoli cells. The expression of GDNF protein was demonstrated by Western blot and, similar to the gene expression results, testosterone treated increased GDNF expression. Furthermore, leukemia inhibitory factor (*lif*) expression is increased by the T treatment in the PM cells. Finally, to rule out estrogen effect in the PM cells in culture, we assayed for the present of aromatase mRNA. The PM cells were negative for aromatase gene expression supporting our conclusion that testosterone regulates GDNF and *lif* synthesis in the PM cells and is an important component of the SSC niche in the adult testis.

Introduction

Spermatogonial stem cells (SSC) are the source of differentiating germ cells for sperm formation in mammalian testes. In mice, SSC are surrounded by Sertoli cells and peritubular myoid cells in a microenvironment called the SSC niche [1]. Although the SSC niche is not clearly defined, Sertoli cells have been described to be important in regulating SSC homeostasis by secreting such factors askit ligand, also called stem cell factor and glial cell line-derived neurotrophic factor (GDNF) (1, 2). During SSC differentiation into sperm, c-kit has been described as a differentiation marker (3-6). The ligand for c-kit, kit ligand is secreted from Sertoli cell and potentially directs SSC differentiation into spermatogonia leading to the eventual production of sperm. GDNF has been reported to trigger SSC self-renewal mechanisms to maintain the SSC population in the testes (7-9). GDNF, a member of the TGF-beta family, is a dimerized protein which binds to the rearranged during transformation (RET) receptor and glial cell line-derived neurotrophic factor receptor alpha 1 (GFR alpha 1) activating the Akt pathway potentially inducing SSC proliferation (8; 10-16). In GDNF $^{+/-}$ mice SSC number decreased due to apoptosis. These results suggest that GDNF concentration is important for maintaining SSC survival and proliferation in the mice testis (7). Furthermore, it has been suggested that GDNF secretion from Sertoli cells is regulated by follicle stimulating factor (FSH) (11; 17). However, the mechanism that regulates GDNF expression is unclear.

FSH, secreted from the anterior pituitary gland under the regulation of gonadotropin releasing hormone (GnRH), is important the spermatogenesis in male. In the testis, FSH receptor (FSHR) is only expressed on the Sertoli cells (18; 19). In the FSH-beta subunit knockout mice, sperm production decreases to around 40% compared to the wild type mice (20). The relationship between FSH and SSC regulation is not known, however, FSH is known to regulate

spermatogonial proliferation. For example, FSHR mRNA expression increases at around XII ~ I stages and decreases from stages II to VII in the rat seminiferous epithelium (21). Spermatogonia proliferation and differentiation occurs during stages XII to I and is supported by FSH (19; 22). Data support the hypothesis that FSH influences the initiation of germ cell differentiation during spermatogenesis more than latter differentiation. Moreover, Sertoli cell in culture shows that FSH can induce GDNF expression in the immature and adult mice testis (23-25). These data indicate that FSH acts through Sertoli cells to regulate GDNF secretion that directly influences SSC homeostasis. However, it is unclear what signal pathway FSH activates to regulate GDNF expression. Despite the FSH and GDNF regulation mechanism, other factors and cells are likely involved in this regulation. This hypothesis is supported by the FSH-beta knockout mouse model (20). In this model, sperm are still continually produced although the number of sperm produced is decreased. Therefore, it suggests that other SSC regulatory mechanisms are maintained. Further, we previously demonstrated that testosterone could enhance SSC biological function from GnRH-immunized-mice testis by stimulating the ability of SSCs to form colonies in the recipient mice testes following transplantation. This demonstrates that testosterone can regulate SSCs.

Androgen receptor (AR) is expressed in both Sertoli cells and peritubular myoid cells (PM) (26). The ligand of AR, testosterone, is secreted from Leydig cells through regulation by luteinizing hormone (LH), and is the major steroid hormone in the testis. During the sperm formation, androgen receptor (AR) expression is maximum in the Sertoli cells at around stage VII~VIII which is when spermatocytes are transforming into round spermatids (27). The Sertoli cell specific AR knockout mouse model shows that germ cell differentiation is arrests before round spermatids are formed (28-29). A new role of PM cells to support spermatogenesis in the

mouse testis has recently been revealed (30). PM cells are located next to the basement membrane and are the major source of leukemia inhibitory factor (LIF) in the mouse testis (31). LIF has been reported to activate SSC proliferation by signaling through gp130 and gp190 receptor in the *in vitro* culture (31; 32). These data indicates that PM cells play an important regulator for the SSC homeostasis in the testis niche. However, it is not known what other factors are also secreted form PM cells. Recently, an additional function of PM cells was reported in the PM cell-AR specific knockout mouse model (30). In the PM cell-AR specific knockout mice, testis size is smaller which is most likely due to decreased sperm production. Previously, our lab demonstrated that GnRH immunized mice induces testosterone deficiency resulting in a loss of SSC activity. Treating these mice with testosterone resulted in the increase of SSC colonization in the testis of recipient mice suggesting that testosterone replenishment activates SSC activity. These data raise three questions - what is the role of androgen in the SSC niche? Next, are Sertoli cells the only cells that produce GDNF in the testis? Then, is FSH is the only contributor in the regulation of GDNF expression? Our data indicates that testosterone not only regulates spermatogenesis through Sertoli cells but also maintains SSC homeostasis through PM cells. Furthermore, GDNF regulation mechanism not only involves FSH but also testosterone in the mice testes.

Materials and Methods

Mice:

Six to 10 week-old C57Bl/6NCrj (C57), GFP^+ transgenic mice and androgen receptor-deficient testicular feminization mice (Tfm) were used for all experiments. C57 and GFP^+ mice were purchased form Jackson Laboratory. C57 mice were used for testosterone and flutamide treatment experiment, PM cells and Sertoli cells *in vitro* culture experiments. GFP^+ mice were used as SSC donors for transplantation experiments. Animal maintenance and surgery protocols are following NIH guidelines and approved by Washington State University institutional animal care and use committee.

Cell culture:

All reagents were purchased from Sigma unless otherwise noted. Sertoli cells and PM cells were isolated from C57Bl/6NCrJ mice testes and cultured in DMEM/F12 medium (1:1). Sertoli cells PM cells were cultured in DMEM/F12 (1:1) with 10% fetal bovine serum. The PM isolation protocol was modified from Palombi *et al.* (33). Testes were digested with 0.5% collagenase type IV in HBSS at 37^oC for 30 minutes and washed three times with HBSS to remove interstitial cells. Tissue was further digested with 0.5% collagenase type IV and 1% hyaluronidase in HBSS digestion buffer at 37^oC for 20minutes to release PM cells. The supernatant was collected and applied on the top of discontinuous Percol gradient (20% ~ 50%) to increase PM cells purity. The purity was assessed by staining cells with alpha-actin antibody and showed that approximately 95% of the cells were PM cells consistently across cell preparations. PM cells were cultured in the DMEM/F12 with 10% FBS for one week, and then

treated with 10^{-5}M testosterone (Sigma) in serum free DMEM/F12 medium for assaying GDNF gene and protein expression.

The Sertoli cell isolation protocol was based on Wu *et. al*. (34) protocol. Briefly, testes were digested in HBSS containing 2.5g/L trypsin, 1g/L collagnase type IA, 1g/L hyaluronidase and 10mg/ml DNaseI (Sigma) at 37^{o}C for 10 minutes. The tube was placed on ice and Sertoli cells were collected by centrifugation at 150 x *g* for 5 minutes. A total of 10^{6} cells were cultured per well in DMEM/F12 with 10µg/ml insulin and 5µg/ml transferrin medium at 34 ^{o}C for 2 days to allow cells to attach to the culture plate. After two days of culture, cells were treated with 20mM Tris-HCl (Invitrogen) at room temperature for 2.5 minutes to release germ cells. To recover Sertoli cells before treatment, cells were cultured in DMEM/F12 medium supplement with 0.1% bovine serum albumin (DMEM/F12-BSA) at 34 ^{o}C for 24 hours. Sertoli cells were treated with either 10^{-5}M testosterone or 20ng/ml FSH in the DMEM/F12-BSA medium for detecting GDNF mRNA expression.

GnRH vaccinnation and flutamide treatment

Six to ten week-old male mice were used for GnRH vaccination (35) and flutamide treatment (100mg/kg of body weight) to decrease testosterone concentration in the testes. The antigen was an ovalbumin-GnRH fusion protein and mixed with Fruend's complete adjuvant for the primary immunization. Booster immunizations were ovalbumin-GnRH fusion proteins in Freund's incomplete adjuvant given four and eight weeks after the primary immunization. Following the immunization protocol, mice were treated with testosterone propionate dissolved in corn oil (0.4 mg/Kg of body weight) and used for experiments. Flutamide was dissolved in corn oil and injected daily (100mg/kg of body weight) into mice (n=5) while controls were injected with corn

oil only (n=5) for a total 45 days. At the end of the treatment regime, mice were killed daily for five days and testes removed for RNA purification which is used to determine gene expression by quantitative RT-PCR.

Immunohistochemistry for the SSC transplantation:

SSCs-GFP$^+$, Thy1$^+$ magnetic column isolated, were co-cultured with PM cells in the 12 wells culture plate and treated DMEM/F12 medium with or without 10^{-5}M testosterone (SSCs:PM cell = 10^6:10^6). After 48, 72 and 168 hours, cells were trypsinized and transplanted into W/W^v mice testis for 10 weeks. After 10 weeks, testes were fixed in 4% paraformaldehyde and embedded in the wax. 7μm sections were fixed on the Rite-On (Gold Seal) micro slide. Slides were gone through histoclear and a serious of different concentration alcohol to dewax and rehydrate. The procedure of immunohistochemistry was following Schmidt *et. al.* (36). The slide was placed in hot boiling 10mM sodium citrate (PH 6.0) for antigen retrieval. 3% Hydrogen peroxidase (in methanol) were used to quench endogenous peroxides followed by PBS washes and 10% goat serum was applied on the slide to block non-specific binding. The slide was incubated with anti-GFP (1:200) in 4 ^{o}C overnight and followed by several washes in PBS. The HRP conjugated secondary antibody (1:1000) was applied and incubated at room temperature for one hour. After rinsing in PBS, the sections were incubated in streptavidin/peroxidase substrate (Vector) for 30 miuntes followed by rinsing in PBS. 3,3'-diaminobenzidine (Vector) was applied to the section and allowed to develop for four to ten minutes followed by light Hematoxylin counter stain.

RNA purification, quantitative RT-PCR (qRT-PCR)

Total RNA was purified with the use of Trizol reagent (Invitrogen, Carlsbad, CA). The isolation procedure was described previously (36) and cDNA was synthesized from 5µg total RNA and by M-MLV reverse transcriptase (Progema) using an oligo(dt)12-18 primer (Invitrogen) in total 20µl reaction solution. The cDNA (2 µl) was used as template for qRT-PCR. Taqman primers (Applied Biosystems) for *gdnf* (Mm00599849_m1), *gfra1* (Mm00833897_m1), *ret* (Mm00438304_m1) and mitochondrial ribosomal protein S2 (Mm00475529_m1) were used to assess gene expression following the manufacture's protocol. The gene expression was determined by using the Qgene method (36). The primer for the aromatase and beta-actin was designed with the use of Primer 3 (Biological workbench 3.2, San Diego Supercomputer Center). The sequences of primers are *aromatase* F: *ccttcagccttttggctttg*, R: *atttccacaaggtgcctgtc* and *beta-actin* F: *gaagcatttgcggtggacgat*, R: *tcctgtggcatccacgaaact*. The PCR was performed in 30 sec for 94 ^{o}C, 30sec for 58 ^{o}C and 30sec for 72 ^{o}C with 30 cycles amplification.

In situ hybridization:

The primer of GDNF for the in situ probe was designed by Primer 3 and the primer was F: *ttcgcactgtagcaggaatg* and R: *tgggctatgaaccaaggag*. The amplified product, also the probe for in situ, was 300 base pairs and inserted into pGEM-T vector (Promega) for sequencing. A digoxigenin (DIG) RNA labeling kit (Roche) was used for DIG labeling of the probe. The protocol of in situ was modified from Jansen *et. al.* (37) and described briefly. The Tfm and normal mice testes were embedded in the paraffin and sectioned at eight µm and fixed on In Situ PCR Glass slides (Perkin Elmer). After fixation in 4% paraformaldehyde, slides were washed three times in TBS buffer (50mM Tris-HCL and 150mM NaCl, PH 7.5) and treated with

200µg/ml proteinase K at 37 ^{o}C for 20 minutes. After three more TBS washes, slides were treated with100mM Tris (PH 8.0) and 0.5% acetic acid to reduce background. Slide were dehydrated rinsed with chloroform and incubated with probe at 51.2 ^{o}C overnight. Parafilm was removed from the slide and rinsed with SSC buffer for 30 minutes followed by washing. Next, sections were blocked in the 2% normal sheep serum for one hour. After blocking step, anti-digoxigenin-alkaline phosphatase Fab fragment antibody (1:1000, Roche) was applied to the slides and incubated at 37 ^{o}C for 3 hours. The slide was washed with 150mM NaCl three times and then rinsed with alkaline phosphatase buffer (50mM Tris-HCl and 100mM $MgCl_2$) for 10 minutes. The chromagen solution (1 NBT/BCIP pellet dissolved in the distilled water, Roche) was applied on the slide and then incubated in TE buffer (10mM Tris-HCl and 1mM EDTA) until the color developed.

Protein assay and Western blotting:

Cells were rinsed with TSE buffer (10mM Tris, 250 mM sucrose and 0.1mM EDTA; 4°C) and collected into 20ml Pyrex grinder for fifty grind at 4 ^{o}C to disperse cell membrane. The protein pellet was collected by 10,000 G centrifugation and the concentration was determined by Lowry method (Bio-Red). GDNF protein expression was determined by Western blot. A total of 5µg of protein/well was loaded into 12.5% bis-acrylamine (Bio-Red) gel. After electrophoresis, the protein was transferred into PVDF membrane (Invitrogen) for protein detection. The membrane was incubated with 10% Casein-PBS buffer (Sigma) for blocking nonspecific protein at 4^{o}C overnight. The primary antibody of GDNF (1:200, Santa Cruz Biotechnology) or actin-HRP (1:10,000, Santa Cruz Biotechnology) was applied and the blot incubated at the room temperature for one hour. The membrane was washed with a 0.05% PBS-Tween followed by

incubation with the secondary anti-rabbit-HRP antibody for GDNF antibody (1:10,000) for one hour at room temperature. Following three washes with PBS-Tween buffer, luminal reagent (Santa Cruz Biotechnology) was applied and the antibody-protein complex was visualized by Kodak image station 2000MM system.

Statistical analysis:

The Qgene analytical method was used to normalize gene expression (36). The statistical difference was determined by SPSS software with Student t test analysis and the P-value less than 0.05 was considered significant.

Results

gdnf expression in GnRH-immunized mice and flutamide-treated mice.

Suppression of testosterone production in mice results in loss of SSC biological activity based on spermatogonial stem cell transplantation. Treating mice immunized against GnRH with testosterone restores colonization potential of SSCs when transplanted into the testes bulsulfan treated mice. To determine how testosterone stimulates SSC activity, mice were immunized against GnRH, treated with testosterone, the testis removed for RNA isolation and the expression of genes associated with SSCs analyzed. First, the *gdnf*, *gfr-alpha1* and *c-ret* genes were evaluated with the use of Taqman RT-PCR. The results indicate that testosterone induces the expression of *gdnf* and *c-ret* in a time dependent manner in the testis (Figure 1) however; the expression *gfr-alpha 1* did not change (data not shown). The expression of *gdnf* increased at 48 and 72 hours after testosterone treatment when compared to the time 0 and RNA from the testes untreated, wild type mice. Moreover, *c-ret* expression was significantly increased 72 hours after testosterone treatment compared to time 0.

A second approach was used to suppress testosterone signaling to investigate the expression of these genes in the testis. Suppression of testosterone production followed by testosterone treatment will result in an increase of FSH production by the pituitary. FSH can induce the expression of *gdnf* in cultured Sertoli cells; therefore we used flutamide to specifically suppress testosterone signaling in the testis of mice. Mice were treated with flutamide for 45 days, a time period selected based on the 32-35 day length of time required for complete germ cell differentiation in mice. Flutamide, an androgen receptor antagonist, was used to block testosterone signaling and then the treatment was stopped so endogenous testosterone could signal through the androgen receptor. The data demonstrate that *gdnf* expression in the

flutamide-treated mice testes increases at day 45 of flutamide treatment compared to controls (corn oil only, open bar) and continues to stay elevated on days 1, 2 and 3 after flutamide injection was stopped (Fig. 2A). The expression of *lif* also increased at day 2 and day 5 after flutamide treatment comparing to the control (Fig. 2B). These results suggest that testosterone regulates *gdnf* and *lif* expression in the mice testis.

Tesoterone regulation of *gdnf* expression in cultured Sertoli and PM cells

Testosterone and flutamide treatment indicates testosterone induces the expression of several genes important for SSC activity. To determine the specific cells expressing these factors, Sertoli and PM cells were isolated and individually cultured. Cell preparations were treated with or without testosterone to analyze *gdnf* and *lif* expression. It has been shown that FSH induces *gdnf* expression in immature rat Sertoli cells. Thus, we used FSH treatment as a positive control to measure *gdnf* expression (Fig. 3A). In contrast to FSH treatment, testosterone does not induce *gdnf* expression in Sertoli cells (Fig. 3A). Androgen receptor is expression in Sertoli cells and the PM cells of the testis. Therefore, PM cells were isolated and treated with testosterone to determine *gdnf* expression. The result demonstrates that testosterone significantly increasesd *gdnf* expression in three and six hours after treatment compared to the controls (Fig. 3B). Next, we analyzed *lif* expression in PM cells after testosterone treatment. Testosterone significantly increased *lif* expression one, three and six hours after treatment compared to control (Fig. 3C). To determine long-term effect of testosterone treatment in culture, Sertoli and PM cells were treated with testosterone for 24 hours (Fig. 4). In 24 hours culture, testosterone significantly decreases *gdnf* expression in the Sertoli cell while significantly increasing *gdnf* and *lif* expression

in PM cells compared to controls (Fig. 4A-C). Interestingly, FSH treatment did not affect *gdnf* expression in Sertoli cells at 24 hours culture.

PM cells not only express androgen receptor but also express estrogen-receptor beta. Aromatase can convert testosterone into estrogen. So to confirm that testosterone is the steroid hormone inducing *gdnf* expression in PM cells, the expression of aromatase was assayed in cultured PM cells. The results show that aromatase is not expressed in the cultured PM cells (P). Male and female brain (B), epididymis (E) and Sertoli cells (S) were positive for aromatase expression (Fig. 4D).

Gdnf mRNA in situ hybridization.

To support testosterone directly effect in the PM cells, the *gdnf* expression in the Tfm testes was used to compare wild type mice. Tfm is a dominant spontaneous mutation on the X chromosome leading to androgen receptor defeat. The data show that *gdnf* expresses in both Sertoli cells and PM cells in the wild type mice testis (Fig. 5A and C). In the mice, Sertoli cells express *gdnf* indicating that testosterone signaling is not required for expression (Fig. 5D, arrow head). However, *gdnf* expression (arrow) in the PM cells in Tfm mice is weak and not expressed in every PM cells. This result suggests that the role of testosterone in the PM cells is to enhance *gdnf* expression rather than activated in the mice testes.

GDNF protein expression in Sertoli and PM cells.

The GDNF protein expression in Sertoli and PM cells was determined by Western blot. As can be seen in Figure 6, testosterone increases GDNF expression compared to the control (Fig. 7).

Therefore, GDNF protein is expressed by cultured PM cells and can be induced by testosterone. Overall, these results suggest that testosterone enhances GDNF expression in the PM cells.

Transplantation of SSCs co-cultured with PM cells into W/W^v mice testis

Thy-1^+ SSCs were isolated from adult GFP^+ mice (6~10 weeks) and cultured with PM cells in the DMEM/F12 medium with or without 10^{-5}M testosterone for three and eight days. Cells were transplanted into W/W^v mice for ten weeks after in vitro cultured treatment. Results show that the percentage of colonization in testosterone treatment group was significantly increased than the non-treatment group in the 3 days culture (Fig. A, B and E). Therefore, there is no significant different between treatment and non-treatment groups in the 8 days culture (Fig. C, D and E). Overall, results indicate that testosterone could enhance GDNF and LIF expression in the PM cells and thus support SSCs proliferation.

DISCUSSION

Testosterone has many well-documented roles in supporting germ cell differentiation in the testis by primarily signaling through Sertoli cells (28; 38-42). The androgen receptor has been detected in multiple cell types of the testis including Sertoli, Leydig, PM and germ cells, however testosterone signaling is not needed for germ cell differentiation (43). In contrast, we have shown that testosterone is important for SSC biological activity in mice and the decrease of intratesticular testosterone concentration below 10 μm results in a loss of the ability of SSCs to colonize the testes of recipient mice. Treatment of mice with intratesticular testosterone concentrations below 10 μM with testosterone propionate restores the ability of SSCs from these mice to colonize the testes of recipient mice. The goal of the research presented here was to determine the cells and mechanisms that stimulate the activation of the SSCs. We demonstrated that testosterone stimulates the expression of *gdnf* and *lif in vivo* through androgen receptor specific signaling. Similarly, we report a significant increase in *ret* expression *in vivo* following testosterone treatment. Based on the in vivo data, we refined our approach to determine the cell or cells responding to the testosterone by isolating specific cells in the testis that are closely associated with SSCs. Two cell types, Sertoli and PM cells were investigated based on their proximity to SSCs and potential contribution to the SSC niche and the fact that both cell types express androgen receptor. Although Sertoli cells have been reported to express *gdnf* in culture, the testosterone-regulated induction of *gdnf* is specific to the peritubular myoid cells but not Sertoli cells. In fact, testosterone suppressed *gdnf* expression in cultured Sertoli cells. The induction of *gdnf* is not limited to mRNA in PM cells because cultured PM cells produce GDNF and testosterone treatment results in a significant increase in the GDNF protein expression. We demonstrated that PM cells express *gdnf* mRNA in vivo with the use of in situ hybridization

supporting our hypothesis that this cell type contributes to the SSC niche and regulates SSC activity in adult mice. Finally, due to the fact that several steroid hormone receptors are expressed in PM cells, we demonstrated that testosterone was indeed inducing the expression of *gdnf* with the use of the androgen receptor specific antagonist flutamide and by showing that aromatase is not expressed in cultured PM cells. These data are significant because they demonstrate a novel mechanism for testosterone regulation of spermatogenesis by contributing to the spermatogonial stem cell niche. Similarly, these data suggest that PM cells are intimately involved in SSC homeostasis in adult mice.

GDNF has been demonstrated to play crucial roles in the kidney, neuron and gonad development. GDNF research in the gonads has focused on neonatal development and less PM cell addressing its role in the adult testis (44-46). In the GnRH-immunized mice model, *gdnf* expression increases after testosterone propionate injection. However, several researchers have demonstrated that supra-physiological testosterone concentrations can block spermatogenesis in rodents. Continuously high levels of testosterone suppress the release of GnRH from the hypothalamus thereby limiting the amount of LH secreted by the pituitary. In addition, sperm production is interrupted due to suppressed Sertoli cell function. In contrast, testosterone in adult mice appears to contribute to some aspect of maintaining SSC homeostasis. Although poorly defined in the testis, it is thought the main somatic contributor to the SSC niche is the Sertoli cell. SSCs are likely not just regulated by Sertoli cells but also controlled by the whole microenvironment-niche.

PM cells also appear to contribute to the SSC niche. The main function of PM cells is to regulate seminiferous tubular contraction to move elongated-spermatids to the rete testis and eventually the epididymis. Androgen regulation of PM cell function have been investigated by

several groups due to the fact that PM cells express androgen receptor and are in close proximity to Leydig cells. Piquet-Pellorce *et. al.* (47) treated PM cells, isolated from 20 day-old rat pups, with10 picomolar testosterone *in vitro* and showed that testosterone did not affect LIF expression. However, we wanted to indentify testosterone effect on the adult PM cells because the SSC data was generated with adult mice immunized against GnRH. The formation of the SSC population and gene expression in the testis is quite different in the neonatal and adult testis. Therefore, these factors may result in *lif* expression to be differentially regulated in adult PM cells. LIF, as well as GDNF, have been shown to induce SSC proliferation in culture and also is a very important growth factor for the embryonic stem cell proliferation. Kanatsu-Shinohara *et. al.* (31) showed that LIF can only induce SSC proliferation in the first week of culture. After the initiation period, LIF does not enhance SSC proliferation but maintains SSC population. This result suggests that the initiation of SSC proliferation needs LIF and GDNF. Interestingly, our data indicates testosterone is involved in *lif* expression in adult PM cells. In the flutamide-treated mice testis, *lif* mRNA expression increases 2-5 days after flutamide suppression of testosterone signaling is lost. In addition, testosterone treatment can induce *lif* expression in one hour.

SSC culture shows that GDNF prolongs SSC proliferation. It is likely that multiple factors are expressed in the testis niche to regulate SSC differentiation and proliferation. These factors are necessary to prevent unregulated proliferation or differentiation leading to SSC depletion. This model suggests that the initiation of SSC proliferation might need LIF and GDNF in combination in the testis niche. Potentially GDNF and LIF act synergistically to regulate SSCs. For example LIF may maintain SSC population or initiate SSC proliferation when GDNF expression is low. This hypothesis is suggested from our flutamide-treated mouse model. Two days after 45 days flutamide treatment, *lif* expression increased and also increased five days after flutamide

treatment was stopped. In the *gdnf* expression profile, five days after flutamide-treatment was stopped there was not a significant increase in *gdnf* expression. This suggests that *lif* expression might maintain SSC population or induce SSC proliferation.

Cultured PM cells respond to testosterone treatment by increasing the expression of GDNF mRNA and protein. This result suggests that PM cells contribute to the SSC niche and regulate spermatogenesis but supporting SSC proliferation and self-renewal. Interestingly, Zhang *et. al.* showed that testis size and sperm number are both decreased in the PM cell-AR specific knockout mice PM-ARKO mice (30). These data indicate that loss of androgen regulated PM cell function results in decreased sperm production. The decrease in sperm production in the PM-ARKO mice may be due to a loss of SSCs due to decreased SSC proliferation and self-renewal. This data supports our hypothesis that androgen induced PM cell function is critical to maintain the SSC population in adult mice. Indeed, take together these results suggest that the development of PM cells is important for spermatogenesis as well as Sertoli cells. Our data also points out that PM cells also contribute SSC homeostasis through GDNF and LIF production in the SSC niceh. Similarly, PM cells secrete laminin that has been used to enrich for SSCs because SSCs express alpha-6 and beta-2 integrins (48).

Co-culture of SSC with PM cells demonstrated that PM cells could enhance SSCs proliferation in the first three days of culture. Therefore, after eight days culture does not show any significantly different between treatment groups. Similar results also showed in the Kanatsu-Shinohara *et. al*. (31) work. SSCs co-culture with MEF cells that secrete LIF can initiate SSCs proliferation in the first seven days culture but maintenance SSCs number afterward. Based on our data, T can enhance GDNF and LIF expression to promote SSCs proliferation in the first three days co-culture, but the eight days culture does not show any different in the

transplantation results. Transplantation is a good way to measure T stimulated GDNF expression efficiency in PM cells. Furthermore, T effect on the kinetic of SSCs also can be determined by this method. Future experiments will focus on the kinetic effect of T on the PM cells cocultured with SSCs. We are also interested to determine if long-term culture with PM cells might change SSC normal response to culture conditions.

The theory that SSCs are dormant in the seminiferous tubules has been proposed based on data from Yoshida S. *et. al.*(49). These researchers used neurogenin 3 (NGN 3) conjugated with GFP transgenic mice to shows that type A spermatogonia can migrate from vascular-associated niche to the non-vascular-associated location in the seminiferous tubule. It is not clear if NGN3 is expressed in SSCs, however, the data suggests that not every SSC is active and only a small portion of SSC enter into spermatogenesis. It also suggests that SSCs are mobile and the vascular-associated area has a higher concentration of factors such as FSH and testosterone than non vascular-associated areas. Similarly it has been suggested that other niches are present in the seminiferous tubules microenvironment (50). Thus, the necessary location and environment for the SSC niche may include proximity to a vascular area such that somatic cells are exposed to the necessary systemic factors to induce the expression of signals that regulate SSC homeostasis.

The regulation of SSC homeostasis is complex and may be based on cell location, proximity to vascular niches and association with more than one somatic cell. It appears SSCs need GDNF to induce SSC proliferation and this is regulated by endocrine factors including FSH stimulating Sertoli cells and testosterone signaling in PM cells. Although this model explains aspects of SSC homeostasis in adult mice, it is likely that other mechanisms regulate SSC proliferation in prepubertal mice because testosterone production does not increase until three weeks after birth.

However, in the adult based on our data and we conclude that two cells secrete GDNF to regulate SSC activity.

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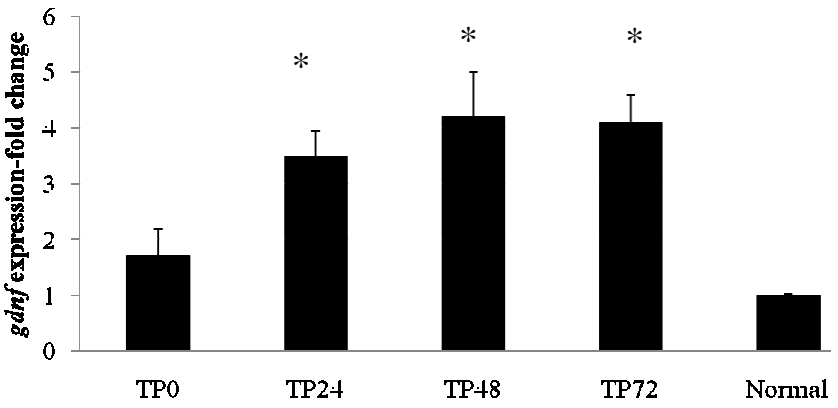
A.



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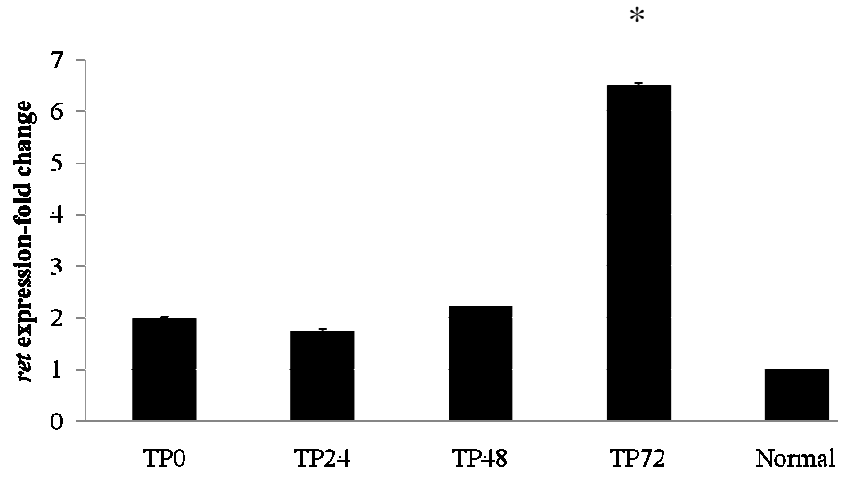


Figure 1. Testis gene expression following testosterone treatment in GnRH-immunized mice. Testosterone propionate (0.4 mg/kg) was injected into GnRH-immunized mice and mice were sacrificed at 24, 48 and 72 hr after treatment for testis RNA purification. Taqman primers were used to assay *gdnf* and *c-ret* expression in samples. (A) The expression of *gdnf* following testosterone propionate (TP) treatment. (B) The expression pattern of *c-ret* following TP treatment. Bars are mean + SEM, * $p<0.05$ comparing to the normal mice.

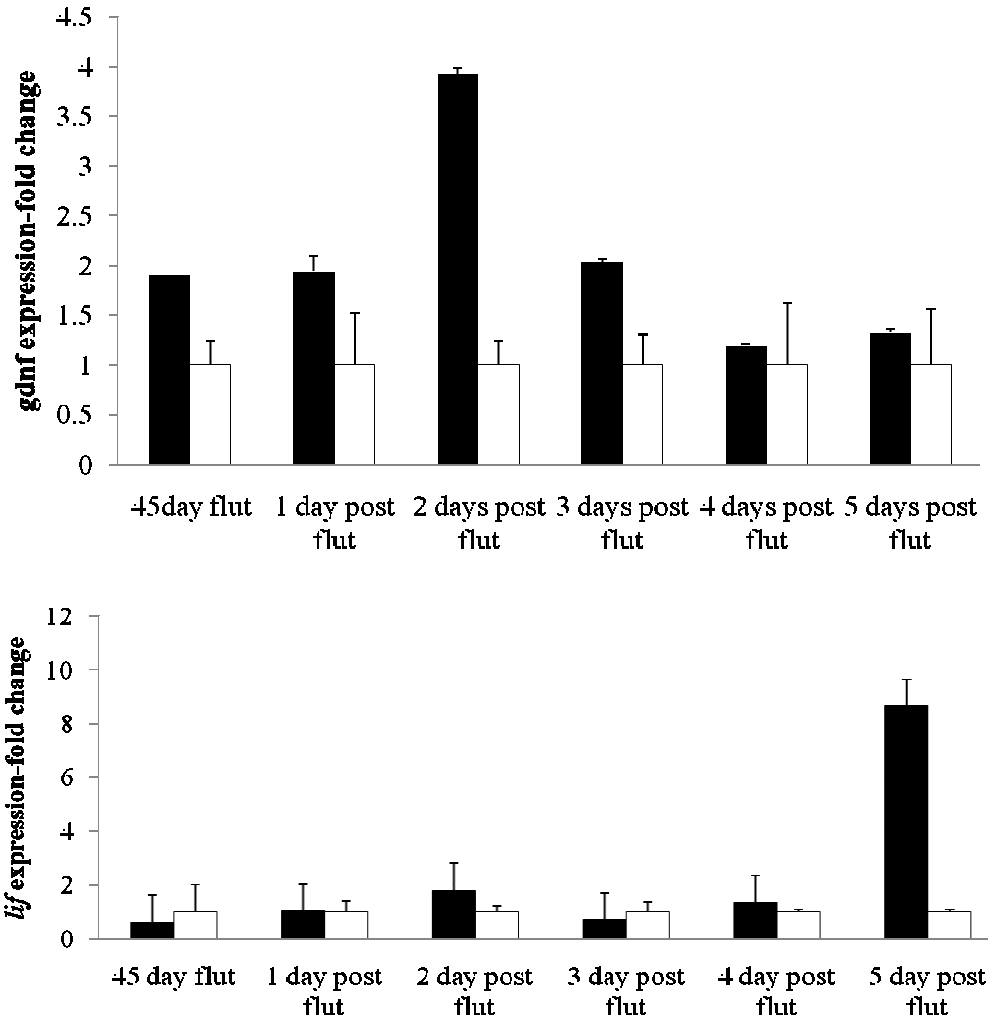


Figure 2. Testis gene expression during and following flutamide treatment of mice. Flutamide was injected daily into mice for 45 days and mice were sacrificed for five days after treatment. (A) The expression of *gdnf* following flutamide treatment (sold bar) or corn oil only (open bar). (B) The expression of *lif* following flutamide treatment (sold bar). Bars are mean + SEM, * $p<0.05$ comparing to the control.

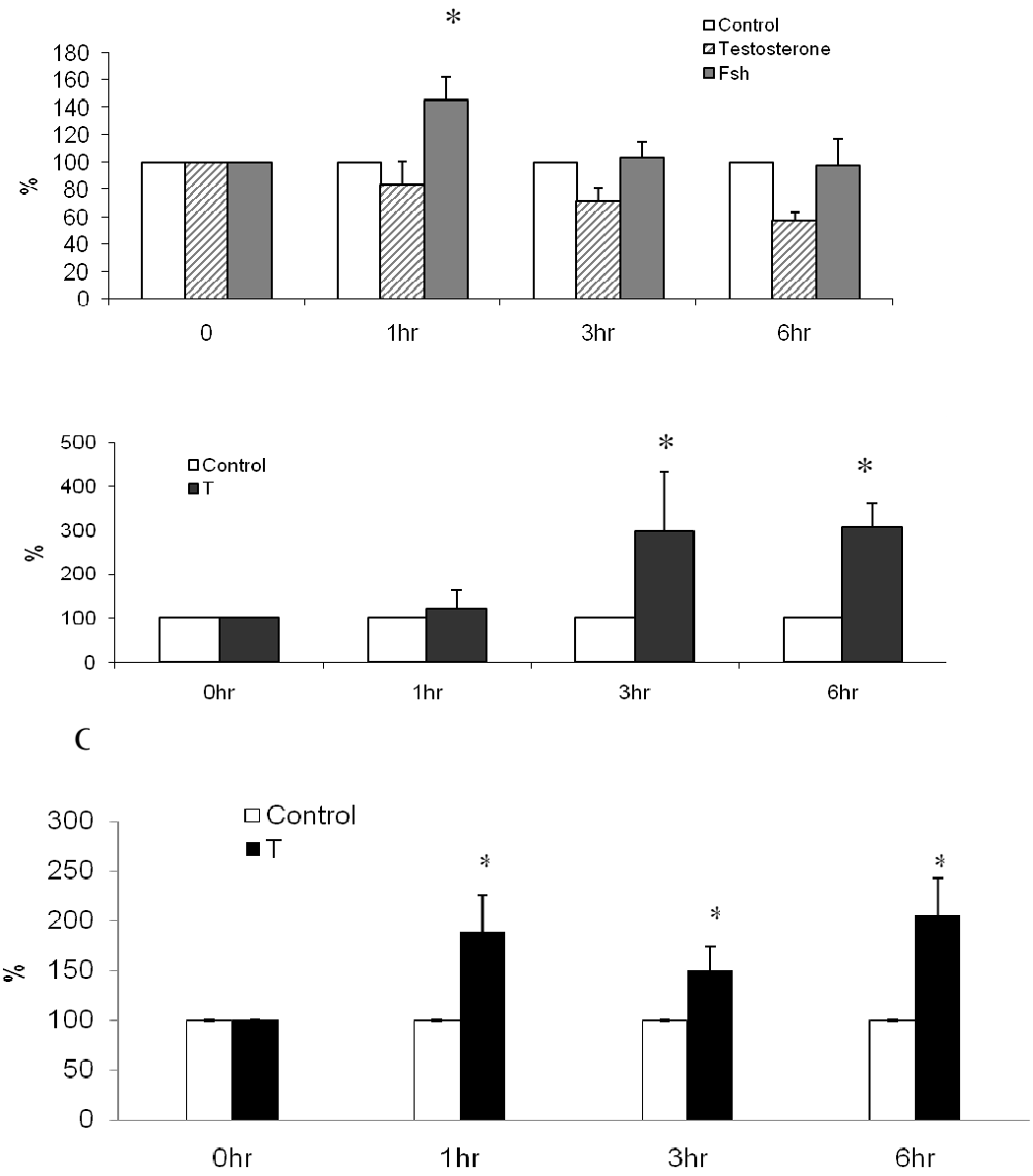
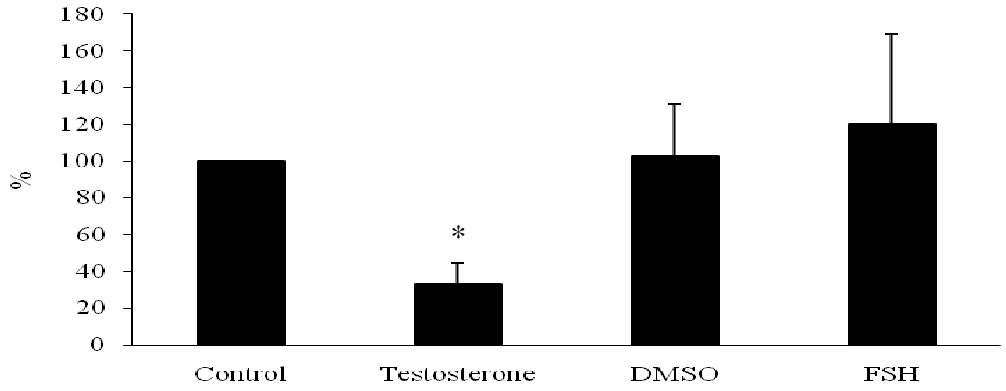


Figure 3. Gene expression in cultured Sertoli cells and cultured peritubular myoid (PM) cells treated with FSH (Sertoli cells) or testosterone (Sertoli and PM cells). (A) *gdnf* expression in testosterone and FSH treated cultured Sertoli cells. (B) *gdnf* expression in testosterone treated PM cells. (C) *lif* expression in testosterone treated PM cells. Bars are mean + SEM, *$p<0.05$.

A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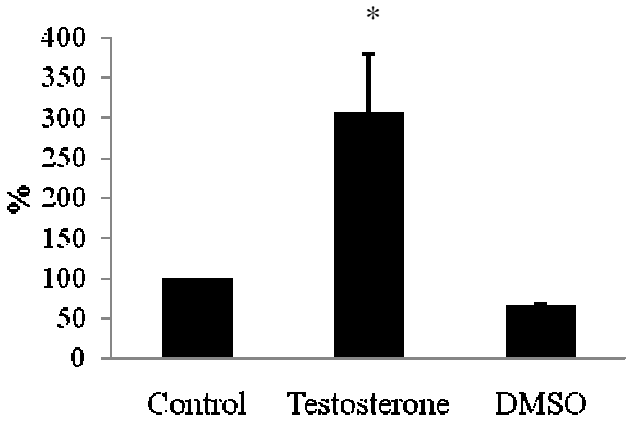
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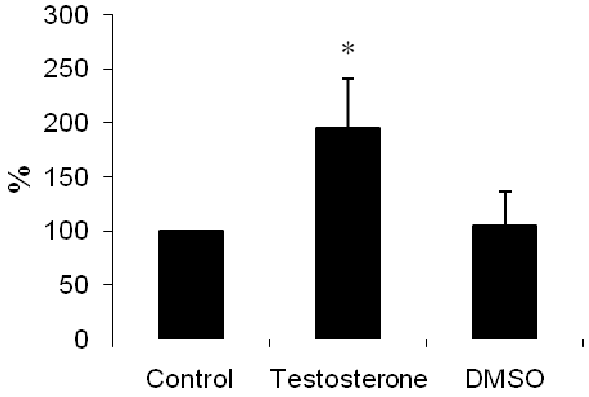
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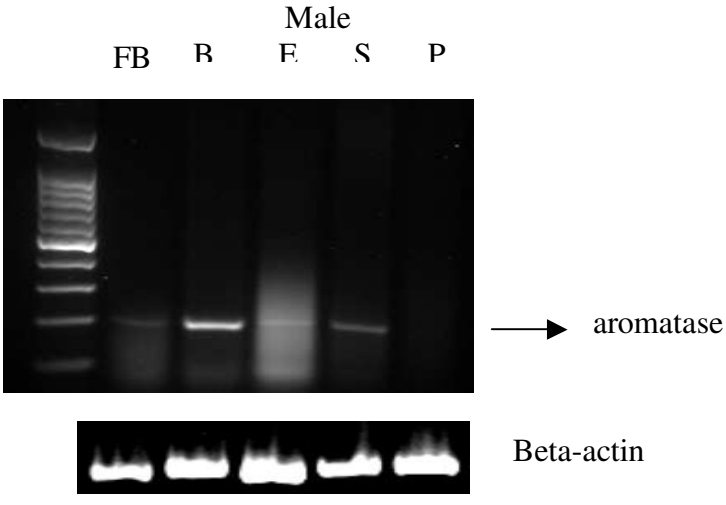
Male
FB
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E
S
P
aromatase
Beta-actin

Figure 4. Gene expression in Sertoli and peritubular myoid (PM) cells following treatment with FSH and/or testosterone for 24 hours. (A) *gdnf* expression in cultured Sertoli cells 24 hours treatment, (B) *gdnf* expression in testosterone treated PM cells. (C) *lif* expression in testosterone treated PM cells. Bars are mean + SEM, *$p<0.05$. (D) Representative agarose gel showing the lack of aromatase expression in cultured PM cells (P). Brain tissues (FB in female, B in male), epididymis (E) and Sertoli cell (S) are positive controls for aromatase expression. PCR reactions with primers for beta-actin were used as a positive control for cDNA integrity.

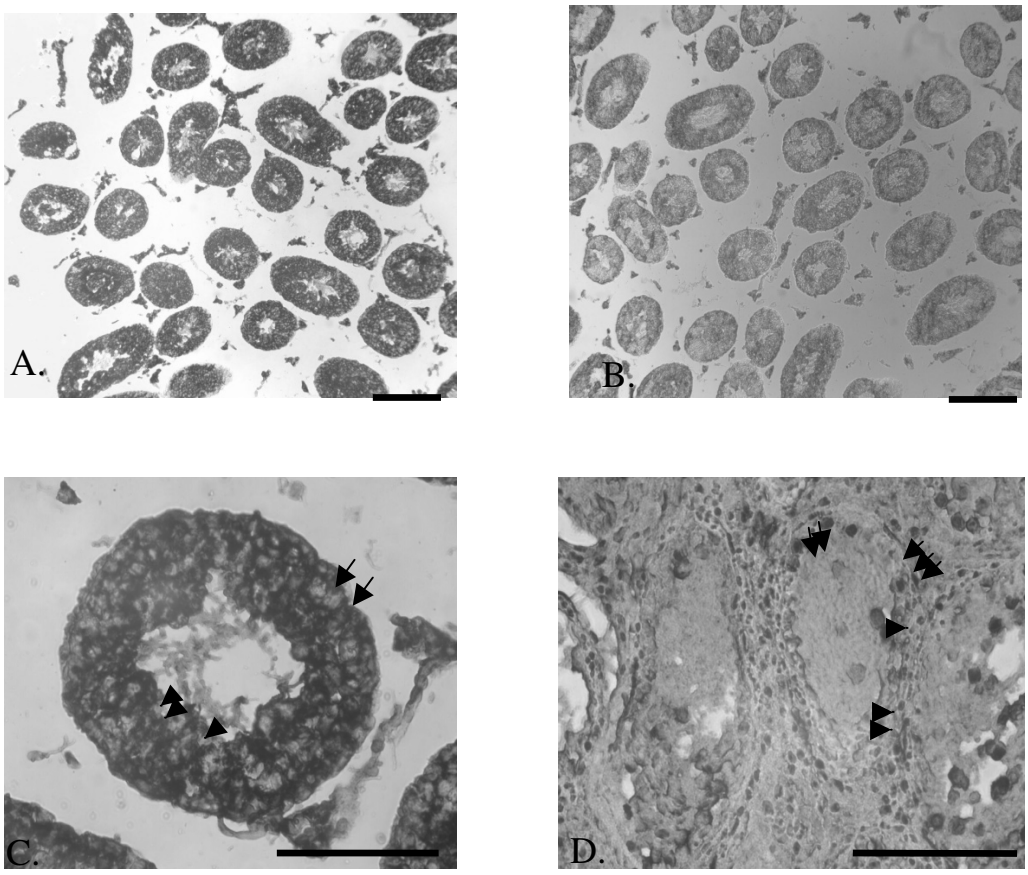


Figure 5. Testis expression of *gdnf* mRNA in adult wild type mice and *tfm* mutant mice. (A) *in situ* hybridization of *gdnf* antisense probe in adult wild type testis. (B) *in situ* hybridization of *gdnf* sense probe in testis of adult wild type mouse serving as a negative control (C) *in situ* hybridization of *gdnf* antisense probe in the seminiferous tubule of adult wild type mouse testis. Note positive signal in Sertoli cells (arrowheads), germ cells (arrowheads) and peritubular myoid cells (arrows). (D) *in situ* hybridization of *gdnf* antisense probe in the seminiferous tubule of a testis from a *tfm* mutant mouse. Note positive signal in some germ cells (arrows) and peritubular myoid cells (arrows). Bar= 50 μm.

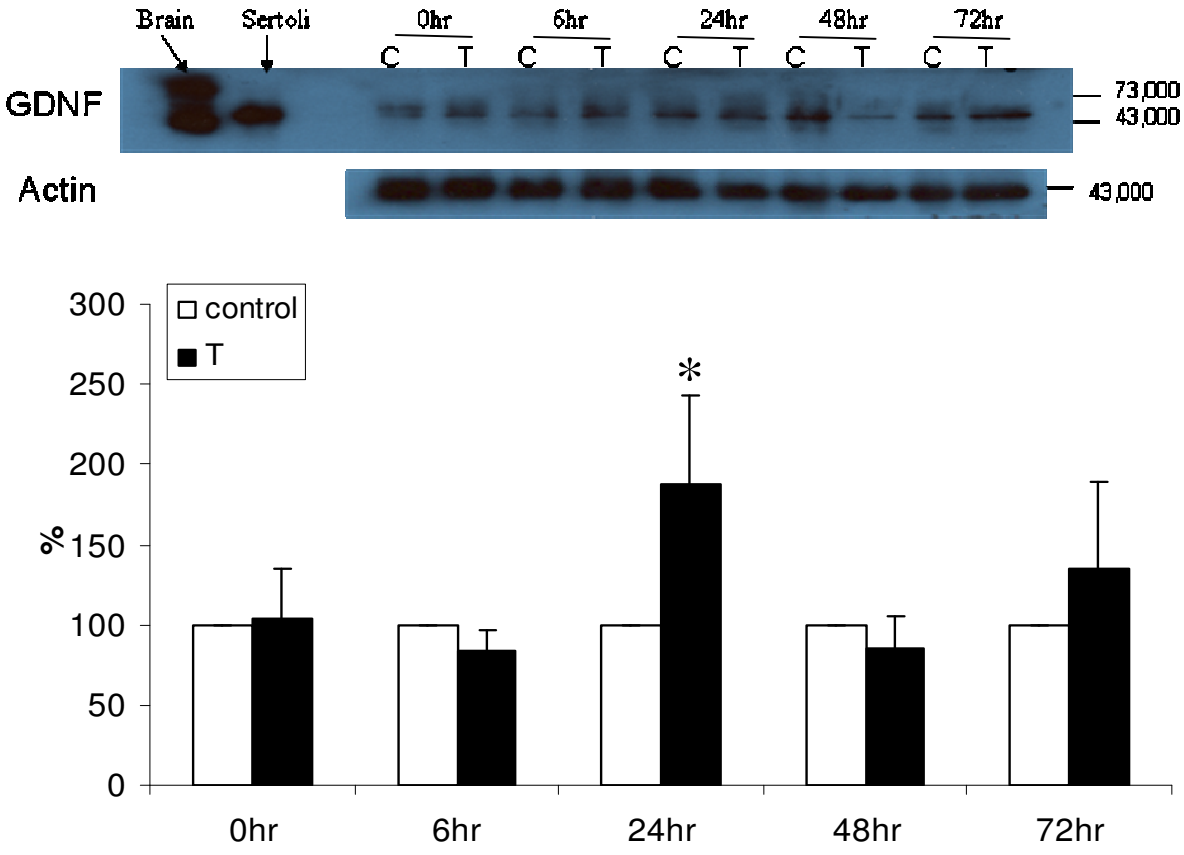
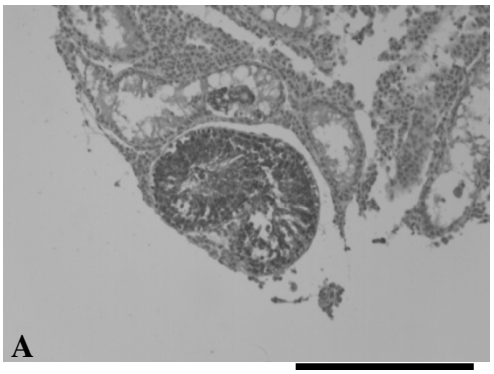


Figure 6. GDNF protein expression in cultured Sertoli and peritubular myoid (PM) cells. (A) A representative Western blot of GDNF protein expression in male brain, Sertoli cells and cultured PM cells. An antibody to beta-actin was used as a loading control and to normalize GDNF protein expression within each group at each time point.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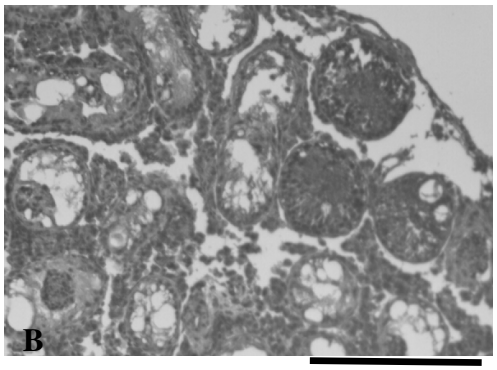
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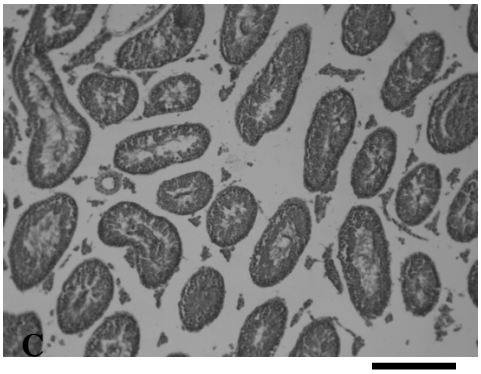
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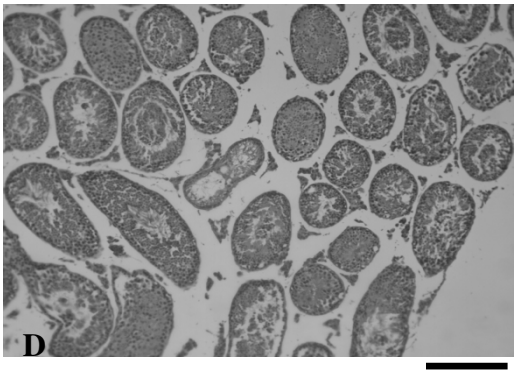
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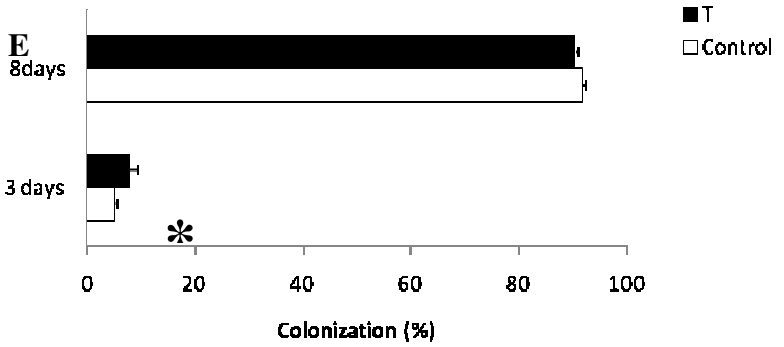
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8days
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Colonization (%)
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Figure 7. Colonization efficiency of spermatogonial stem cells after co-cultured with peritubular myoid cells. Spermatogonial stem cells co-cultured with PM cells were transplanted into the testes of W/Wv mice. Spermatogonial stem cells were maintained in co-culture with PM cells for 3 or 8 days and cultures were treated to testosterone or vehicle. (A) Photomicrographs of seminiferous tubules of a testis from a recipient W/Wv mouse injected with spermatogonial stem cells co-cultured with PM cells for 3 days treated with vehicle and (B) treated with testosterone. (C) Photomicrographs of testis from a recipient W/Wv mouse injected with spermatogonial stem cells co-cultured with PM cells for 8 days treated with vehicle or (D) with testosterone treatment. (E) Graph representing colonization efficiency of spermatogonial stem cells co-cultured with PM cells. Bars are mean + SEM, *$p<0.05$. Scale bars = 50 μm.

CHAPTER FIVE

Regulation of GDNF expression in the peritubular myoid and Sertoli cells in mice

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Short title: GDNF testis transcriptional network

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) is important for spermatogonial stem cell (SSC) proliferation and self-renewal in the testis. Sertoli cells and peritubular myoid cells (PM cell) in the testis express and secrete GDNF. In Sertoli cells follicule-stimulating hormone (FSH) stimulates GDNF expression while in PM cells testosterone induces GDNF expression. However, the subsequent signaling and transcription factors downstream of FSH and testosterone stimulation that leads to GDNF expression in PM and Sertoli cells is not known. The objective of this study was to investigate transcriptional network and additional signaling factors that regulate GDNF expression in Sertoli and PM cells. We demonstrate different trawnscription factors are involved in GDNF expression in PM and Sertoli cells. In Sertoli cells, *eya1* and *pax2* expression increases following FSH treatment. Both of these transcription factors are involved in GDNF expression in the embryonic kidney. In PM cells, testosterone stimulates expression of *six2;* another gene involved in GDNF expression in the embryonic kidney, but does not induce *eya1* and *pax2* expression. Interstingly, pregnenolone, a steroid hormone produced by Sertoli cells and Leydig cells, also enhances *six2* expression in PM cells. In contrast, pregnenolone represses *eya1* and *pax2* expression in PM cells suggesting that testosterone and pregnenolone regulate GDNF expression through different mechanisms. We hypothesized that pregnenolone is involved in the regulation of GDNF production in the testis. The progesterone receptor can be activated by progesterone and pregnenolone and is expressed by PM cells. GDNF mRNA and protein levels are supressed by pregnenolone treatment in the cultured PM cells. Likewise, pregnenolone decreases LIF expression in PM cells. These data suggest that pregnenolone plays an important role in spermatogonial stem cells (SSCs) homeostasis by balancing the induction of GDNF by

testosterone and FSH. Likewise, these data contribute to our understanding of the endocrine and transcription factors that define the spermatogonial stem cell niche.

Introduction

Spermatogonial stem cells (SSC), surrounded by Sertoli cells and peritubular myoid cells in a microenvironment called the SSC niche, are the source of differentiating germ cells for sperm formation in mammalian testes [1]. The SSC niche has been described to be important in regulating SSC homeostasis by creating an environment in which factors such as glial cell line-derived neurotrophic factor (GDNF) are secreted [1, 2]. In addition, Sertoli cells secrete factors that stimulate spermatogonial differentiation such as kit ligand, also known as stem cell factor. During spermatogonial differentiation into sperm, kit, the receptor for kit ligand, has been described as a differentiation marker [3-6]. In contrast, GDNF has been reported to trigger SSC self-renewal mechanisms to maintain the SSC population in the testes [7-9]. GDNF, secreted from Sertoli cells and peritubular myoid cells. Follicle stimulating hormone (FSH) stimulates Sertoli cells to secrete GDNF while testosterone induces cultured perritubular myoid cells to produce GDNF mRNA and protein. GDNF binds to rearranged during transformation (RET) receptor and glial cell line-derived neurotrophic factor receptor alpha 1 (GFR alpha 1) to activate the Akt pathway potentially inducing SSC proliferation [8, 10-16].

Pregnenolone is a precursor steroid for testosterone and estrogen synthesis in mammals [17, 18]. In male, the majority of testosterone synthesis occurs in Leydig cells in the interstitial space located adjacent to the seminiferous tubule in the testes [19, 20]. Interestingly, Sertoli cells also can synthesize pregnenolone [21, 22]. Both Leydig and Sertoli cells contain steroidogenic acute regulatory protein (StAR) and cytochrome P450 side chain cleavage (P450scc) but 17-alpha-hydroxylase and 3-beta-hydroxysteroid dehydrogenase are only expressed in the Leydig cells [22, 23]. Therefore, the conversion of pregnenolone to testosterone only occurs in Leydig cells. Ford *et. al.* [21] demonstrated that Sertoli cells only produce pregnenolone following FSH

stimulation. Pregnenolone binds to the progesterone receptor (P4r) to influence cell biological function. P4r has three subtypes (A-, B- and C-form) which also can be divided into genomic (nuclear receptor, A- and B-form) and nongenomic (cell membrane receptor, C-form) receptors [24, 25, 26]. In the testis, Leydig cells [27] and differentiating sperm [28] express P4r. In sperm, activated P4r enhances the acrosomal reaction in capacitated sperm obtained from the epididymis [29]. The activated C-form P4r induces testosterone production in adult rat Leydig cell [30]. However, activated P4r nuclear subtypes decreases steroidogenesis in Leydig cells [31].

GDNF is expresseed in both PM cell and Sertoli cells [32]. The importance of GDNF for SSC self-renewal and proliferation suggests that expression is tightly regulated. However, the mechanism of this regulation is not known. In addition, it is not known if the availability of GDNF in certain regions of the seminiferous tubule is limited. Yoshida *et. al.* [33] demonstrated that there are two apparent types of SSCs in the testis, one is associated with vascular area which is the active SSCs and the other is dormant SSCs. The active SSCs can initiate differentiation and also migrate to the non-vascular associated area to continue the supply of differentiated spermatogonia for spermatogenesis. These data illustrate that GDNF concentration might be different between the vascular and non-vascular areas to initiate SSC proliferation and prevent apoptosis. We hypothesize that SSCs in the vascular associated area are exposed to higher concentrations of GDNF because the Sertoli and PM cells are in direct contact to FSH and other serum factors. Thus, the Sertoli cells in this location synthesize pregnenolone in response to FSH that regulates GDNF production from PM cells to maintain SSCs homeostasis in the testis niche.

We demonstrated that testosterone could enhance GDNF expression in PM cells. Sertoli cells under the influence of FSH also produce GDNF. Thus we are developing a model of the cells

and factors that regulate GDNF production in the testis to create a microenvironment for SSC homeostasis. In addition, we are interested in determining the intracellular transcriptional network that stimulates GDNF expression in Sertoli and PM cells. We hypothesized that several transcription factors that are critical for GDNF expression in the embryonic kidney are expressed in Sertoli and PM cells following hormone treatment [34, 35]. Several transcription factors have been identified as key regulators to regulate GDNF expression during renal development, such as *paired boxes gene* 2 (Pax 2), *eyes absent* 1 (eya1) and *sine oculis* 2 (six2) [36]. Based on these results, we analyzed the expression of these transcription factors in Sertoli and PM cells after hormonal treatment. Results indicate that different transcription factors appear to regulate GDNF expression in the Sertoli and PM cells. These data suggest that in addition to different external signals stimulating GDNF expression in Sertoli and PM cells, these signals activate distinct pathways to stimulate and repress GDNF expression. Thus, the regulation of GDNF expression to support SSC self-renewal may be regulated at multiple levels to prevent uncontrolled SSC proliferation and maintain SSC function under rigid constraints.

Materials and Methods

Mice

C57Bl/6NCrj (C57) mice were purchased from the Jackson Laboratory. Animal maintenance and surgery protocols followed NIH guidelines and were approved by the Washington State University Institutional Animal Care and Use Committee.

Cell culture

All reagents were purchased from Sigma unless otherwise noted. Sertoli cells and PM cells were isolated from C57Bl/6NCrJ mice testes and cultured in DMEM/F12 medium (1:1). Sertoli cells and PM cells were cultured in DMEM/F12 (1:1) and PM cell culture conditions included 10% fetal bovine serum. For PM cell isolation testes were digested with 0.5% collagenase type IV in HBSS at 37^{o}C for 30 minutes and washed three times with Hanks Buffered Salt Solution (HBSS) to remove interstitial cells. Tissue was further digested with 0.5% collagenase type IV and 1% hyaluronidase in HBSS digestion buffer at 37^{o}C for 20 min to release PM cells. The supernatant was collected and applied on the top of discontinuous Percol gradient (20% ~ 50%) and centrifuged at 800 x*g* for 20 min. Four distinct layers of cells are present after the centrifugation and the PM cell enriched fraction is the third layer from the bottom of the tube. The PM cell enriched fraction was diluted 1:2 with HBSS followed by centrifugation at 40 x*g* for 10 min at 4C. The cell pellet was suspended in culture medium and the cells diluated such that $1x10^4$ cells were distributed in each well. The purity of the cell population was assessed by staining cells with alpha-actin antibody and showed that approximately 95% of the cells were PM cells consistently across cell preparations. PM cells were cultured in the DMEM/F12 with

10% FBS for one week, and then treated with 10^{-5}M testosterone (Sigma) in serum free DMEM/F12 medium for assaying GDNF gene and protein expression.

The Sertoli cell isolation was a modification of other protocols. Testes were digested in HBSS containing 2.5g/L trypsin, 1g/L collagnase type IA, 1g/L hyaluronidase and 10mg/ml DNaseI (Sigma) at 37^{o}C for 10 minutes. The tube was placed on ice and Sertoli cells were collected by centrifugation at 150 x*g* for 5 minutes. A total of 10^{6} cells were cultured per well in DMEM/F12 with 10µg/ml insulin and 5µg/ml transferrin medium at 34 ^{o}C for 2 days to allow cells to attach to the culture plate. After two days of culture, cells were treated with 20mM Tris-HCl (Invitrogen) at room temperature for 2.5 minutes to release germ cells. To recover Sertoli cells before treatment, cells were cultured in DMEM/F12 medium supplement with 0.1% bovine serum albumin (DMEM/F12-BSA) at 34 ^{o}C for 24 hours. Sertoli cells were treated with either 10^{-5}M testosterone or 20ng/ml FSH in the DMEM/F12-BSA medium for detecting GDNF mRNA expression.

RNA purification, quantitative RT-PCR (qRT-PCR)

Total RNA was purified with the use of Trizol reagent (Invitrogen, Carlsbad, CA). The isolation procedure was described previously [34] and cDNA was synthesized from 5µg total RNA and by M-MLV reverse transcriptase (Progema) using an oligo(dT)12-18 primer (Invitrogen) in a 20µl reaction solution. A total of 2µl cDNA was used as template for qRT-PCR. Taqman primers (Applied Biosystems) for *gdnf* (Mm00599849_m1), *lif* (Mm00434762_g1), *pax2* (Mm01217938_m1), *eya1* (Mm01239749_m1), *six2* (Mm00807058_m1) and mitochondrial ribosomal protein S2 (Mm00475529_m1) were used to assess gene expression

following the manufacture's protocol. Analysis of real time PCR data was conducted using the Qgene method [37].

Immunofluorescence

Cells fixed with 4% paraformaldehyde (w/v in PBS) at room temperature for 20 minutes. After fixation, the cells were washed three times with PBS and incubated with primary antibody diluted in PBS (1:200, rabbit anti-PR) at room temperature for 1 hour. After antibody incubation, three washes with PBS were used to wash unbound antibody. Secondary antibody (1:500, rat anti-rabbit-cy5, a gift from Dr. B. Dan Rodgers) was applied and incubated a room temperature for one hour. Following three washes with PBS, the cells were incubated in the Hoechst 33342 (1:1000 dilution, Invitrogen) at room temperature for 10 minutes. A final series of two PBS washes were applied and the images captured with the use of a fluorescent microscope.

Protein assay and Western blotting

Cultured cells were rinsed with ice-cold TSE buffer (10mM Tris, 250 mM sucrose and 0.1mM EDTA) and collected into 20ml Pyrex grinder for fifty grinds at 4 ^{o}C to disperse cell membrane. The protein pellet was collected following 10,000 X *g* centrifugation and the concentration was determined by Lowry method (Bio-Red). GDNF, LIF and progesterone receptor expression was determined by Western blot. A total of 5µg of protein/well was loaded into 12.5% bis-acrylamine (Bio-Red) gel. After electrophoresis, the protein was transferred into PVDF membrane (Invitrogen) for protein detection. The membrane was incubated with 10% Casein-PBS buffer (Sigma) for blocking nonspecific protein at 4^{o}C overnight. The primary antibody of GDNF, LIF and progesterone receptor (1:200, Santa Cruz Biotechnology) or actin-HRP

(1:10,000, Santa Cruz Biotechnology) was applied and the blot incubated at room temperature for one hour. The membrane was washed with a 0.05% PBS-Tween followed by incubation with the secondary anti-rabbit-HRP antibody for GDNF antibody and anti-goat-HRP antibody for LIF antibody (1:10,000) for one hour at room temperature. Following three washes with PBS-Tween buffer, luminal reagent (Santa Cruz Biothchnology) was applied and the antibody-protein complex was visualized by Kodak image station 2000MM system.

Radioimmunoassay (RIA)

For measure pregnenolone concentration, Sertoli cells were treated with 20ng/ml FSH and the medium was collected to measure pregnenolone concentration. The component of RIA for pregnenolone was purchased from MP Biomedicals (Ohio, USA) and the protocol followed a previously published paper [38].

Statistical analysis

The Qgene analytical method was used to normalize gene expression [37]. The statistical difference was determined by SPSS software with Student t test analysis and the P-value less than 0.05 was considered significant.

RESULTS

FSH stimulates pregnenolone secretion from cultured Sertoli cells and progesterone receptor (P4r) protein expresses in PM cells.

Sertoli cells were treated were 20ng/ml FSH for 24 hours to determine pregnenolone production by assaying the pregnenolone concentration in the culture medium. The result of the RIA showed that pregnenolone concentration in the medium was significantly higher than controls six hr after treatment (Fig 1A). In contrast, 24 hr after treatment, pregnenolone production was less than controls (Fig. 1A). To determine if cultured PM cells expressed progesterone receptor, PM cells were fixed and incubated with a fluorescently labeled P4r antibody. The A- and B-form of P4r are expressed in the PM cells (Fig. 1B).

GDNF and LIF expression in pregnenolone treated PM cells.

To determine if pregnenolone regulates GDNF expression in PM cells, an experiment to determine the dose respose of pregnenolone treatment for six hours was conducted. Pregnenolone did not significantly increase the expression of GDNF at any concentration compared to vehicle treated controls. In fact at treatment of PM cells at 10^{-8} M and 10^{-10} M caused a significant decrease in GDNF expression in the cultured PM cells (Fig. 2A). Therefore, PM cells were treated at 10^{-6} and 10^{-8} M pregnenolone for subsequent analysis to determine the effect of pregnenolone on GDNF expression in PM cells. Over the course of 48 hr, pregnenolone does not effect *gdnf* expression in PM cells. However, after 72 hr of pregnenolone treatment, *gdnf* expression is significantly repressed in PM cells when compared to vehicle treated controls (Fig 2B). Evaluation of GDNF protein expression in PM cells following pregnenolone treatment

demonstrated that the amount of GDNF protein in the cultured PM cells is significantly less 48 hr after treatment compared to vehicles controls but not different from controls at other timepoints (Fig. 2C).

Another factor that is important for SSC maintenance in vitro that is also expressed by PM cells is leukemia-inhibiting factor (*lif*). Therefore we investigated the regulation of *lif* in PM cells after pregnenolone treatment. Interestingly, pregnenolone decreased *lif* expression three and six hours after treatment compared to vehicle treated controls (Fig. 3A). Similarly, the amount of LIF protein present in PM cells after pregnenolone treatment is significantly less 24, 48 and 72 hr after treatment compared to controls (Fig 3B).

Pax2, six2 and eya1 expression in cultured Sertoli and PM cells after hormone treatment.

GDNF is essential for the development of the embryonic kidney. Investigation of the transcription factors that regulate GDNF expression has resulted in the development of a small but important network of genes that influence GDNF exrpression in this system. Based on the hypothesis that similar transcription factors regulate GDNF expression in the testis, cultures of primary Sertoli cand PM cells were treated with FSH for Sertoli cells and testosterone and pregnenolone for PM cells to define the expression pattern of six2, eya1 and pax2. In PM cells, testosterone induces six 2 expression three hours after treatment compared to controls (Fig 4A) but has no effect on eya1 and pax2 expression (data not shown). Pregnenolone treatment of PM cells induces six2 expression at three, 24 and 48 hours and pax2 expression at 72 hours after treatment (Fig. 4B). In contrast, pregnenolone significantly suppresses both eya1 expression six hours and pax2 expression six, 24 and 48 hours after treatment in the cultured PM cell (Fig. 4C

and D). These data indicate that testosterone and pregnenolone treatment in PM cells regulate GDNF expression through different transcription factors.

In cultured Sertoli cells, FSH stimulates GDNF expression. Treatment of Sertoli cells with FSH resulted in an increase of eya1 and pax2 expression 24 to 48 hr after treatment (Fig 4E and F) but does not induce six2 (data not shown). These data indicate that steroid hormones and FSH stimulate different PM and Sertoli cells utilize different transcriptional factor networks to regulate GDNF expression.

Discussion

What is the role of PM cell in the SSC niche in the testis? Recently, Zhang *et al*. [39] created AR-PM cell knockout mice to investigate the role of PM cells to support spermatogenesis. In addition, these data also suggest the importance of PM cells to support the SSC niche. We have demonstrated that testosterone can enhance PM cell express GDNF to maintain SSCs in the SSC niche. GDNF is secreted from not only Sertoli cells but also PM cells. Therefore, multiple cells and factors appear to be involved in the regulation of the SSC population in the testis. Investigation of the factors that regulate GDNF is important because dysregulation of GDNF expression leads to either the overproduction of SSCs and testicular cancer or loss of SSCs and sterility im males.

What factors regulate PM and Sertoli cells to maintain GDNF concentration in the SSC niche? To expand our knowledge base about regulation of the GDNF expression in the testis, we investigated the expression of transcription factors in PM and Sertoli cells that are known to regulate GDNF expression in other cell systems including the embryonic kidney and neural cells. In addition, negative regulation of the production of GDNF is likely important to prevent over-production of undifferentiated spermatogonia leading to testicular germ cell related tumors. Therefore, we hypothesized that factors in addition to FSH and testosterone regulate GDNF production in the testis. Pregnenolone is produced by Sertoli cells for the potential conversion to testosterone in Leydig cells. Prior to difussing from the seminiferous epithelium to the Leydig cells, pregnenolone must pass through the PM cells. The second objective of this study was to determine if pregnenolone regulates GDNF expression in PM cells.

The concentration of GDNF is important for SSC proliferation [7, 10, 40]. In the GDNF $^{-/+}$ mice, the SSC number gradually decreases during in adults [7]. GDNF is secreted from Sertoli

cells through the regulation of FSH [32]. He et. al. [11] detected GDNF in neonatal cultured Sertoli cell medium at a concentration of 0.1-0.2 ng/ml. Therefore, Kubota et. al. [40] demonstrated that 1 ng/ml GDNF in culture induces neonatal SSC proliferation and self-reneal. These results indicate that GDNF concentration is very important for SSC maintenance and proliferation. In addition, it suggests that GDNF might not only come from Sertoli cells but also PM cells to support SSC proliferation.

During SSC proliferation, the GDNF concentration needs to be maintained in the SSC niches in the seminiferous tubules. The cycle of seminiferous epithelium also demonstrates this concept [41]. The mRNA expression of FSH and androgen receptors shows different expression pattern during spermatogenesis [42]. In the rat, the highest amount of FSH receptor mRNA is present during stages XII to I. These stages are when undifferentiated spermatogonial proliferation ouccrs and coincide to a low level of androgen receptor expression compared to other stages. We have reported that testosterone can repress GDNF expression in Sertoli cells. During these stages, we believe that FSH initiates and maintains GDNF production from Sertoli cells to promote SSC proliferation in the SSC niche.

During stages III to XI, the androgen receptor expression increases but FSH receptor expression decreases compared to other stages [43, 44]. Tadokoro *et. al.* [32] reported that the number of FSH receptors is correlated with the amount of GDNF. In Sertoli cells, FSH receptor is a key regulator for the GDNF expression. What are other cells that secrete GDNF to support SSC survival? We demonstrated that testosterone enhances GDNF expression from PM cells. During the period of time the FSH receptor expression is low in the seminiferous tubules, we hypothesize that testosterone maintains GDNF production to support SSC survival. In addition,

testosterone may also support SSCs survival in other areas of the seminiferous tubule were low levels of FSH is present due to distance from the vasculature.

Results of Yoshida *et. al.* [33] illustrate that undifferentiated spermatogonia are associated to regions of the seminiferous tubules that are close to blood vessels. They demonstrated that during differentiation, A-*aligned* cells could migrate into a peripheral area away from the vasculature where a germ cell cohort develops and spermatogenesis initiates in that seminiferous tubular section. These results also suggest SSCs in the non-vascular area are dormant and may not need a high concentration of GDNF to maintain their survival. From our data, it indicates that, during SSC proliferation, FSH stimulates GDNF and pregnenolone secretion. The pregnenolone decreases GDNF expression from the PM cells in the vascular associated niche to balance the stimulation provided by FSH. Therefore, in the non-vascular associated area, testosterone can maintain a low level of GDNF secretion from PM cells to support SSC survival. In the vascular and non-vascular area, the concentration of FSH may be different. However, more experimentation is needed to determine FSH concentration in these areas.

During nephrogenesis, a group of transcription factors that are upstream of GDNF have been identified with the use of mouse knockout models. These transcription factors include p*aired box* 2 (Pax2), *eyes absent* 1 (eya1) and *sine oculis* 2 (six2) [36]. Pax2 is a transcriptional factor of the paired-box family and is involved in the function of the midbrain-hindbrain region, the optic nerve regulation and kidney development [45]. In the pax2 total knockout mouse embryo, *gdnf* expression is completely lost and this results in embryonic death. Other transcription factors that are upstream of GDNF expression are eya1 and six2. Eya1 is a cytosolic protein that needs six2 to transport into nucleus to regulate GDNF expression. In the PM cell, we demonstrated that

pax2 expression is decreased by pregnenolone from six to 48 hours. Similarly, pregnenolone suppressed *eya1* expression six hours after treatment. Interestingly, *six2* expression was induced by pregnenolone treatment at three, 24 and 48 hours (Fig. 4B). Thus, in PM cells pregnenolone regulated suppression of eya1 may be involved in the regulation of GDNF expression. Similarly, *pax2* expression is suppressed by pregnenolone in PM cells so these factors known to be upstream of *gdnf* expression in the embryonic kidney may be the proteins that suppress *gdnf* in PM cells after pregnenolone treatment.

In the testosterone treated cultured PM cells, only *six2* is significantly increased following treatment for six and 12 hours. We obtained similar data in pregnenolone treated PM cells suggesting that *six2* might regulate by other genes, such as *Hox11* leading to the activation of GDNF expression. Inactivation of *Hox11* results in losing *six2* and *gdnf* expression. Threrefore, pregnenolone might act through *Hox11* to influence *six2* to regulate *gdnf* expression. In contrast, the inhibition of *eya1* and *pax2* may be dominant over the expression of *six2* to regulate *gdnf* expression. Suppression of the *eya* and *pax2* expression may be more important than induction of *six2* for gdnf expression. This is supported by the data demonstrating that PM cells in culture express low levels of *gdnf* without exogenous hormone treatment. Threrefore, investigation of protein-protein interactions may be important for determining the critical aspects of the regulatory network enhancing or repressing gdnf expression in the somatic cells of the testis, and thus future experimentation will be conducted to evaluate this hypothesis.

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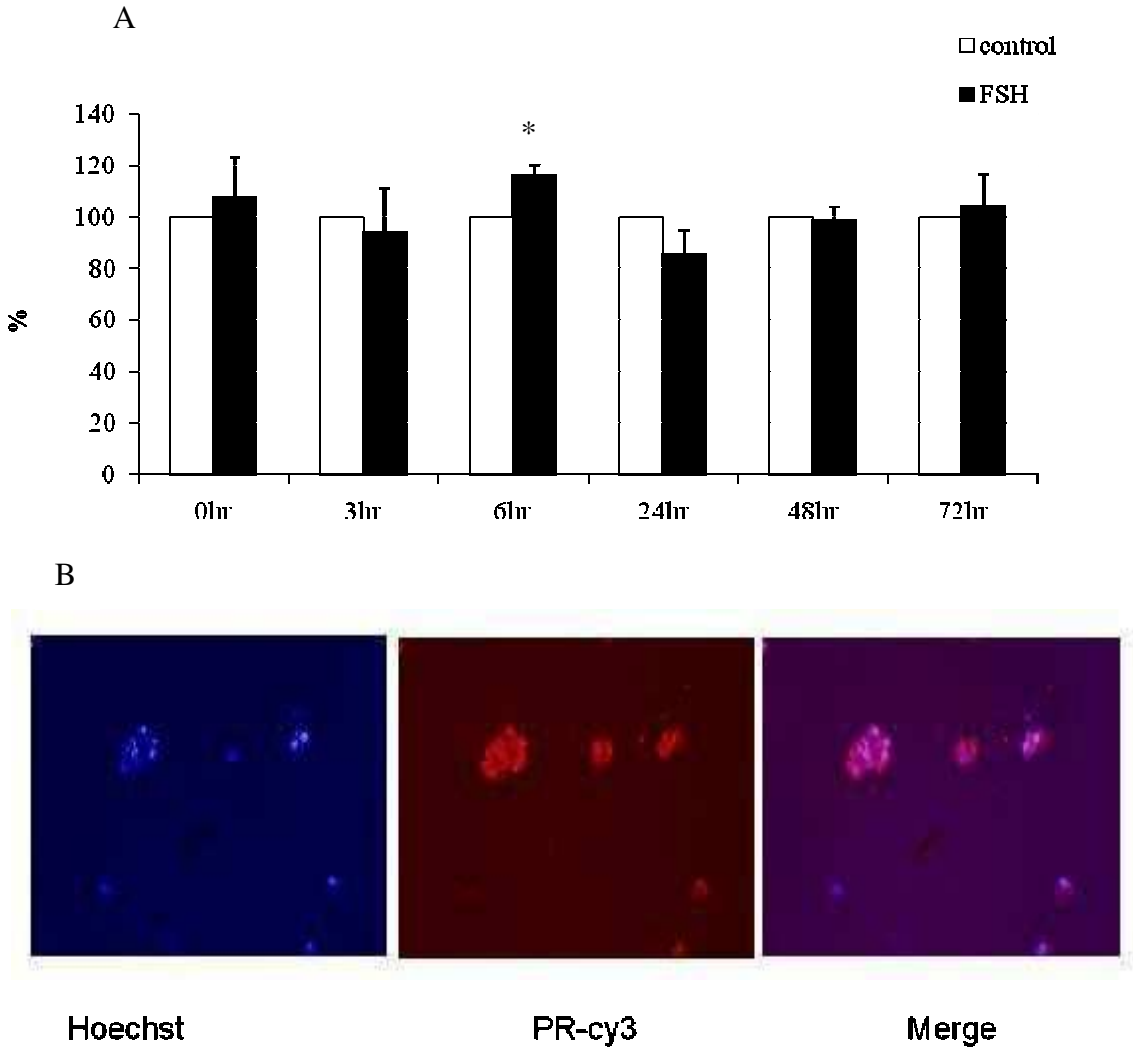

Figure 1. P**regnenolone synthesis in FSH treated cultured Sertoli cells and progesterone receptor expressed in the PM cells.** (A) Sertoli cells were treated with 20ng/ml FSH for 0 to 72 hr. The medium was collected and used to measure pregnenolone concentration. In the six hours treatment, pregnenolone concentration increased 15% comparing to the control. *, $P<0.05$. (B) Progesterone receptor (PR) expression in the cultured PM cells. The result of immunohistochemistry shows that PR expresses in the PM cells.

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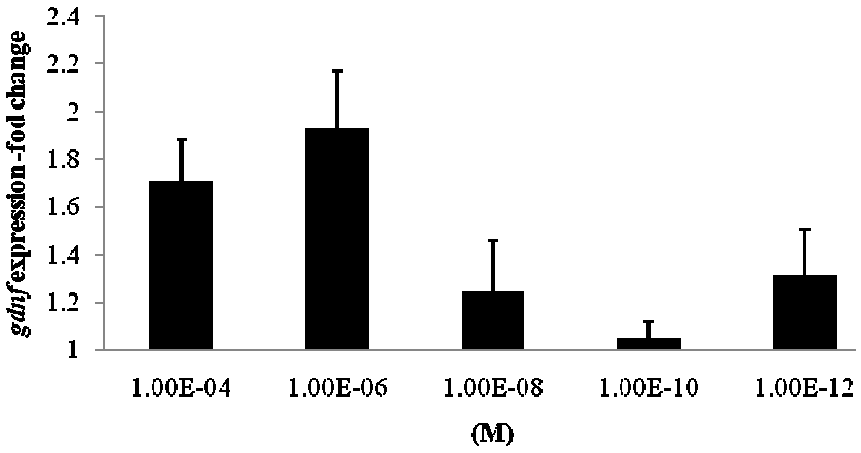
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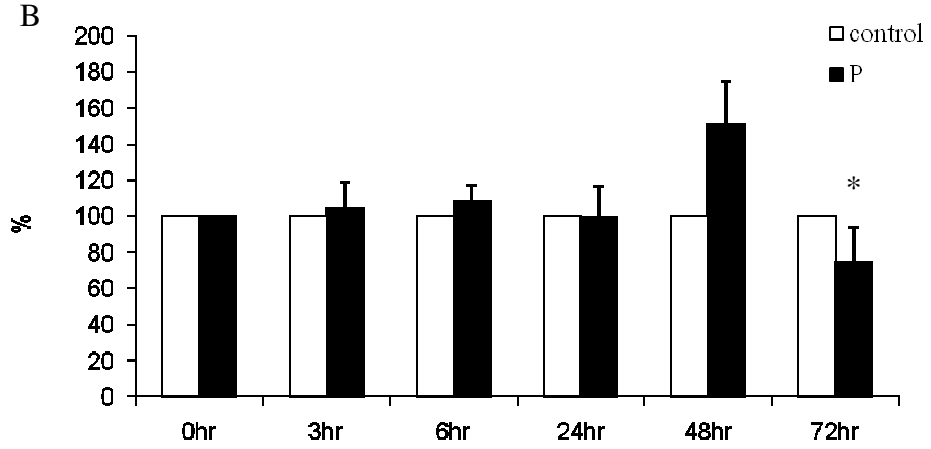
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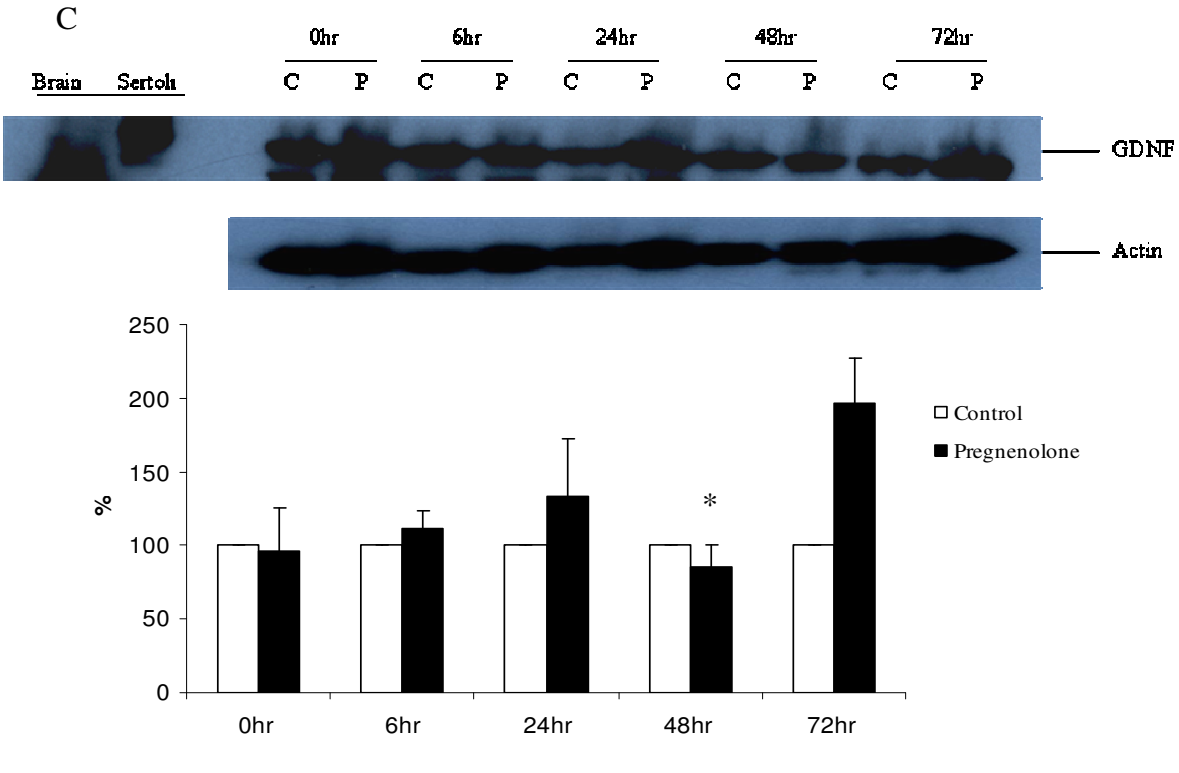
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Figure 2. **GDNF mRNA and protein expression in pregnenolone treated PM cells.** (A) The dose effect of *gdnf* expression in pregnenolone treated PM cells. The data was presented fold change comparing to the control. (B) The *gdnf* transcript expression in PM cells treated with 10^{-6} M pregnenolone. (C) GDNF protein expression following pregnenolone treatment. In B and C all data are represented versus vehicle treated control at the same time point. Asterisks indicate significant differences at $p < 0.05$.

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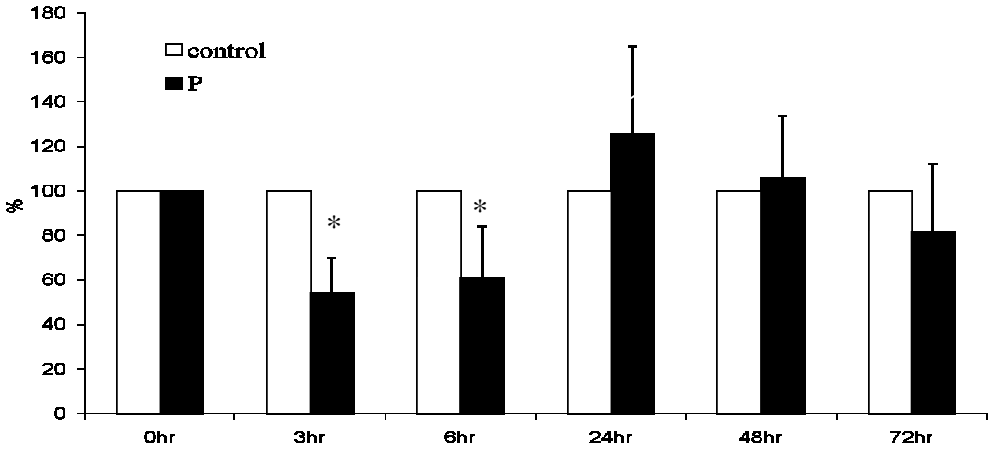
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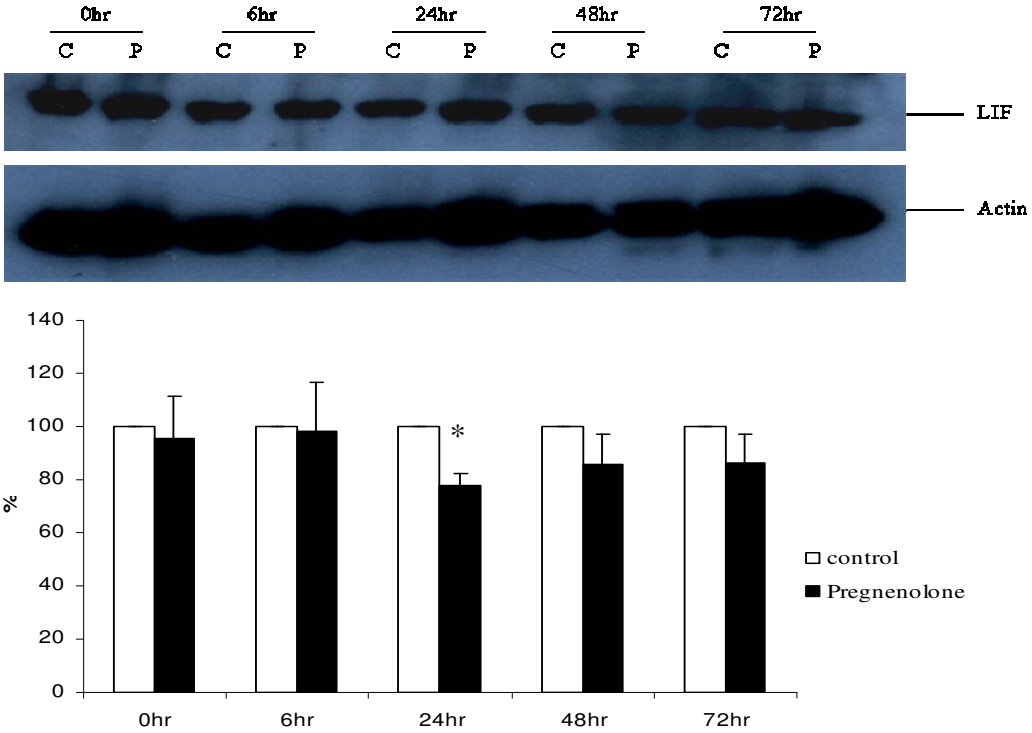
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Figure 3. **LIF mRNA and protein expression in pregnenolone treated PM cells.** (A) *lif* transcript expression in PM cells six to 72 hr following treatment with 10^{-6} M pregnenolone. (B) LIF protein expression six to 72 hr after pregnenolone treatement. All data are represented versus vehicle treated control at the same time point. Asterisks indicate significant differences at $p < 0.05$.

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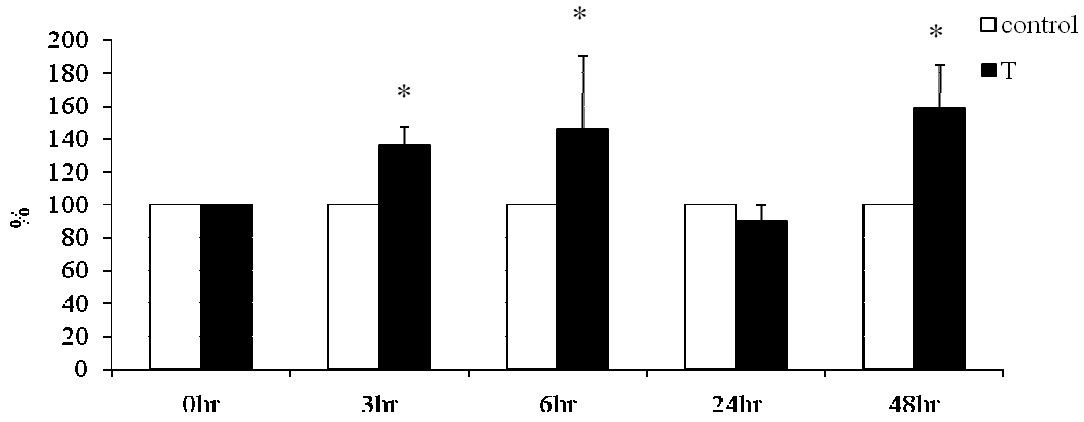
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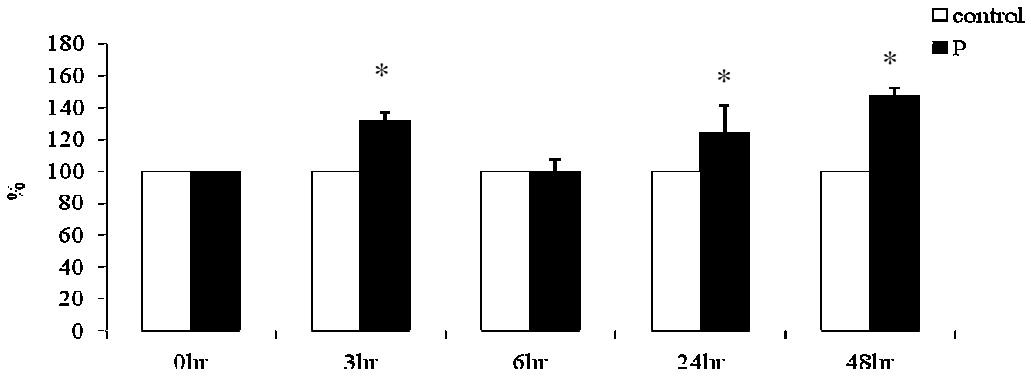
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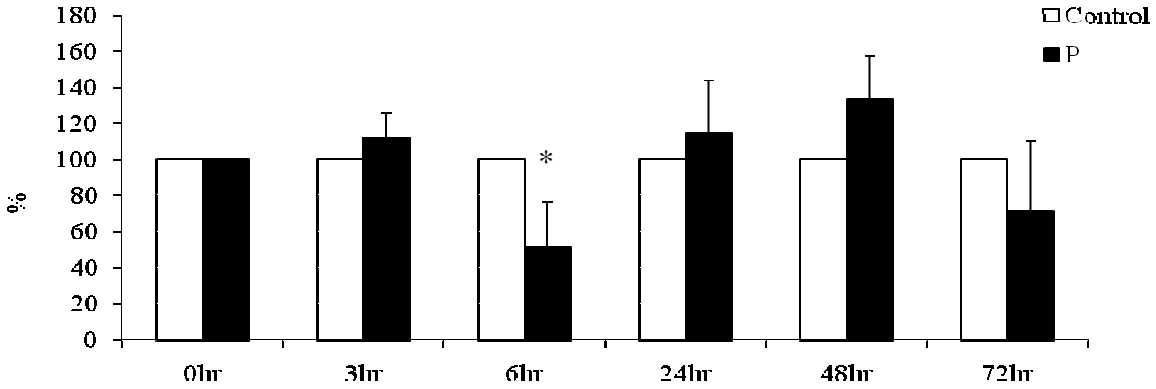
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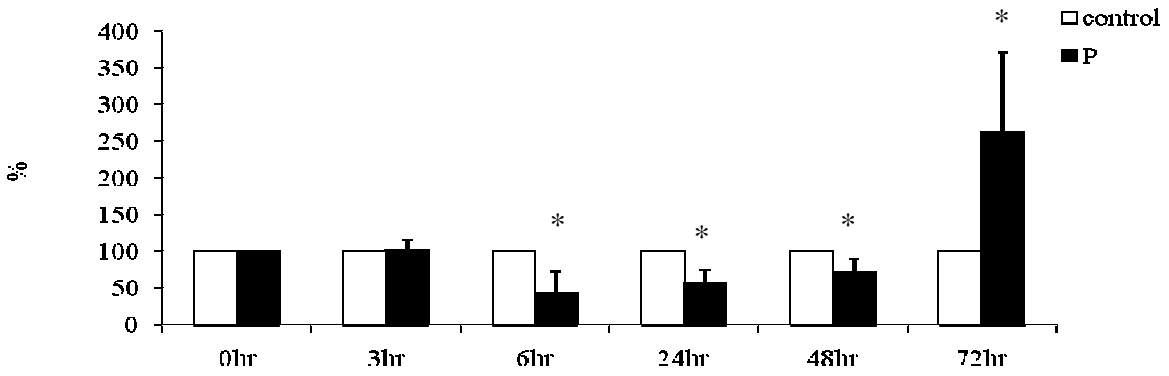
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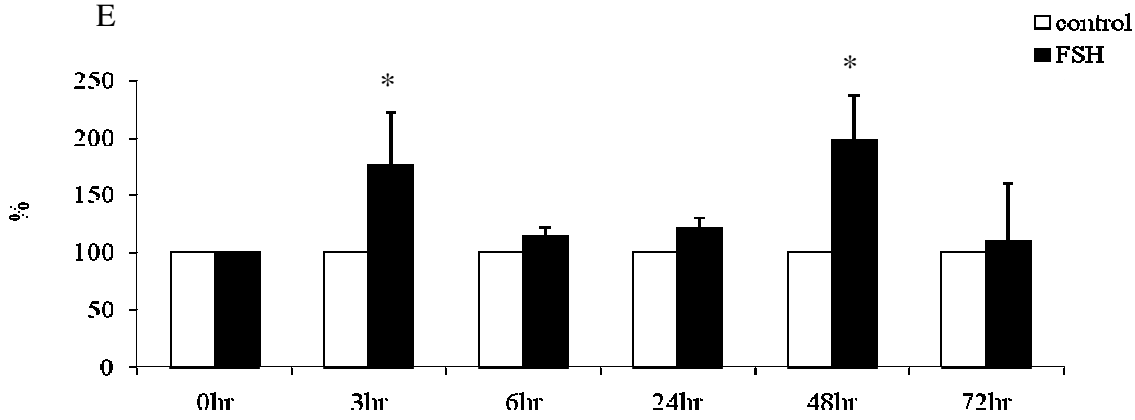
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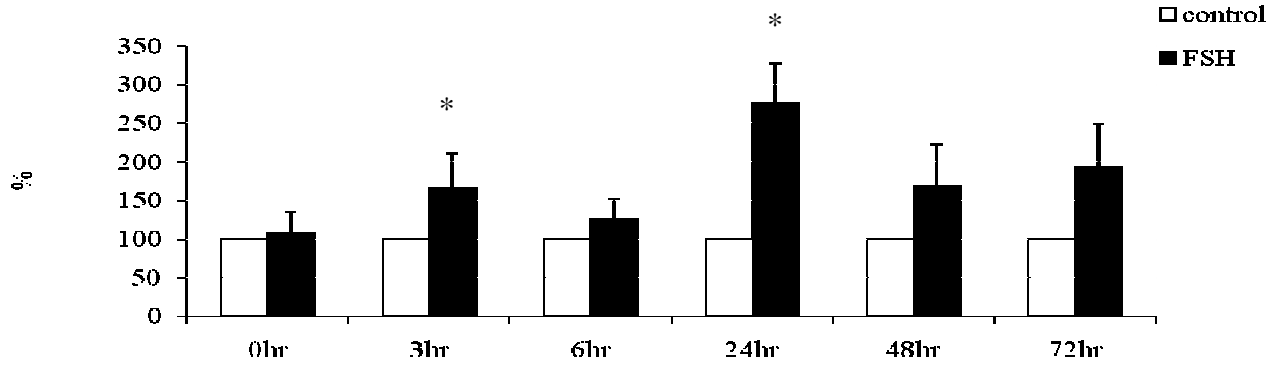
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Figure 4. **Pax2, six 2 and eya1 expression in PM cells and Sertoli cells after hormonal treatment.** (A and B) Six2 expression in the PM cells after testosterone (A) and pregnenolone (B) treatment in the different time points. Six2 expression was increased by testosterone treatment at three and six hours but decreased at 24 hours. Pregnenolone induces six2 expression at three, 24 and 48 hours. (C) Pregnenolone decreases eya1 expression in the PM cells at the six hours comparing to the control. (D) Pregnenolone decreases pax2 expression at six, 24 and 48 hours but increasing at 72 hour in the PM cells. (E and F) In the Sertoli cell, FSH induces pax2 expression at 24 and 48 hours (E) and eya1 expression at 24 and 72 hours (F). *, $P<0.05$.

CHAPTER SIX

CONCLUSION

The spermatogonial stem cell (SSC) niche in the testis, also known as the SSC microenvironment, plays important roles in the maintenance of SSC homeostasis. The complexity of the system regulating SSCs is illustrated by the fact that removal of physiological signals provided through circulation can negatively impact the SSC population and factors such as GDNF that are regulated at the cell to cell level can also impair SSC renewal. For example, androgens through the androgen receptor regulate not only testes formation in the embryo stage but also spermatogenesis and SSC survival. We used GnRH immunized and flutamide-treated mouse models to investigate testosterone regulation of SSC fucntion. In GnRH immunized mice, after testosterone propionate (TP) injection, SSCs were transplanted into recipient mice testes at specific time points. The result showed that SSC forming colony number increased in the recipient mice testis indicating testosterone can directly or indirectly influence SSCs proliferation. Next, we detected *gdnf*, *c-ret* and *lif* expression with real time RT-PCR to determine possible mechanisms of testosterone regulation of SSC activity. The results demonstrated that the expression of these factors increased in the TP injection group compared to controls. The similar result also shows in the flutamide-treated mice. These data support the hypothesis that testosterone might influence SSC proliferation through *gdnf* expression in the Sertoli cell. To test this hypothesis, cultured Sertoli cells were treated with testosterone, however this treatment did not increase *gdnf* expression so we refined the hypothesis to expand the somatic cell types we were investigating.

Peritubular myoid (PM) cells express androgen receptor and are in close proximity to the SSCs and the SSC niche. Therefore we tested the hypothesis that testosterone is regulating

GDNF expression in PM cells. PM cells do not express aromatase, the enzyme that converts testosterone to estrogen so this nature of any effect stimulated by testosterone was specific to this hormone. Overall, results show that PM cells play an important role in the SSCs homeostasis regulation mechanism. To illustrate testosterone regulation of *gdnf* expression in the PM cell, PM cells were co-cultured with SSC in the medium with testosterone and then those cells were transplanted into W/W^v mice to determine if testosterone treatment would increase the number of SSCs in the cultures. The results showed that, at the early culture time, the colony number was increased, but it did not show significantly different at the longer culture time. These results demonstrate that testosterone enhance *gdnf* expression to support either maintaining SSC proliferation or survival.

Sertoli cells and PM cells both express *gdnf* but the mechansisms of this regulation is not knownw. To further refine the biological system regulating SSC homeostasis in the testis, we investigated other factors that regulate Sertoli and PM cells. FSH stimulates steroidogenesis in the Sertoli cell resulting in the production of pregnenolone. Pregnenolone diffuses from Sertoli cells to Leydig cells and must pass through PM cells during this process. Therefore, PM cell response to pregnenolone was investigated. FSH significantly increased the production of pregnenolone in Sertoli cells and PM cells express the progesterone receptor that is also activiated by pregnenolone. To determine the time dependent effect of progeneolone in the *gdnf* and *lif* expression, PM cells were treated with pregnenolone through a time course experiment. Pregnenolone suppressed gdnf expression in PM cells over the course of 48 to 72 hr. Likewise, pregnenolone decreased *lif* expression in PM cells. These data suggest that FSH, testosterone and pregnenolone provent a regulatory loop to maintain GDNF at a concentration that stimulates SSCs to differentiate for spermatogenesis but not for too much proliferation so that germ cell

origin tumors form. Similalry, pregnenolone suppresses the expression of LIF expression to prevent SSCs over-proliferation that occurs if GDNF concentration is too high.

Overall, the data indicates that testosterone can stimulate GDNF secretion to contribute to the SSC testis niche. Until now, GDNF has been identified only being secreted from the Sertoli celll. These data support the hypothesis that PM cells also contribute GDNF to the SSC niche and the Sertoli cell secretion of GDNF, especially in adult mice, is not sufficient to support SSC proliferation and spermatogenesis.

Next, both Sertoli cell and PM cell secrete GDNF but how these two cells regulate *gdnf* expression is unknown. During nephrogenesis, transcription factors upstream of GDNF have been identified, such as *paired box* 2 (Pax 2), *eyes absent* 1 (eya1) and *sine oculis* 2 (six2). Pax2 is a transcriptional factor of the paired-box family. Eya1 is a cytosolic protein that needs six2 to transport into nucleus to regulate GDNF expression. The data shows that, in the Sertoli cell, *eya1* and *pax2* expression is increased by FSH treatment. In the PM cell, testosterone may enhance GDNF expression through *six2* transcription factor. Interestingly, pregnenolone decreases *eya1* and *pax2* expression that indicates that testosterone and pregnenolone may act through different transcription factors to regulate GDNF expression. Potentially PM cells might have an independent regulatory mechanism to regulate *gdnf* suppressing key factors induced by pregnenolone to suppress GDNF expression. In the future, we will detect and expand the number of transcription factors expressed in order to determine what transcriptional networks regulate *gdnf* expression in the Sertoli cell and PM cell.