INVESTIGATING THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN BOVINE TESTIS DEVELOPMENT

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of KYLE CODY CAIRES find it satisfactory and recommend that it be accepted.

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DEDICATION

This thesis is dedicated to my parents, Charles Apuna Jr. (Pops) and Marlene Caires Apuna (Mom), for their love, guidance, and support throughout my life. Your numerous sacrifices over the years have allowed me to pursue opportunities in higher education. Mom, your wisdom and positive attitude have been a constant source of encouragement for me. Pops, your demeanor and work ethic have help set the bar for the man I am, and want to become. Together you have both instilled many life-lessons and values; I am extremely grateful for the manner in which you raised me. I will never forget where I came from: Kaupakalua, Maui.

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INVESTIGATING THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN BOVINE TESTIS DEVELOPMENT

Abstract

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In 2005, products generated from the beef, dairy, and swine industries within the United States were valued at over $77 billion (USDA-ARS), thus representing a critical contribution to the US economy and production animal agriculture as a whole. Increased reproductive efficiency could have a tremendous impact on profitability in various sectors of these animal industries. Sperm production is an important, and often overlooked aspect of reproductive efficiency in the bull and boar. Further, the increasing use of artificial insemination (AI) in the dairy, beef, and swine industries argues for a better understanding of the basic mechanisms regulating sperm production and spermatogenic capacity of superior bulls and boars. However, little is known regarding the factors regulating the establishment of sperm production in livestock. A better understanding of spermatogenesis may provide new insights and approaches to increase sperm production in sires used for AI, especially when considering the increased interest in technology to sex semen (i.e. separation of X and Y sperm for development of monosex populations).

Therefore, the focus of this research has been to investigate biological factors regulating the onset and maintenance of spermatogenesis during postnatal testis development in the bull and boar. Ectopic testis tissue xenografting is a technique that provides a biological assay of germ and somatic cell proliferation, and differentiation events in response to various treatments. The procedure involves ectopically grafting donor testicular parenchyma (from a prepubertal bull or boar) under the skin, on the backs of castrated immunodeficient nude mice. This unique model allows for investigation of specific mechanisms governing the onset and maintenance of the spermatogenesis in bulls and boars, without the financial and space restrictions associated with maintaining larges numbers of domestic livestock.

Preliminary data indicated that vascular endothelial growth factor–A (VEGF), a potent endothelial cell mitogen, may be important for sperm production in the bull testis. To evaluate the role of VEGF in governing germ cell homeostasis, particularly survival and differentiation, we treated prepubertal bovine testis tissue explants in culture for a week with VEGFA-164 or vehicle, prior to xenografting. When testis grafts reached a tissue age of 32-wks, recipient mice were sacrificed and grafts were removed and analyzed for growth, survival, androgen biosynthesis, and quantitative aspects of spermatogenesis. VEGF treated grafts were not different from controls in tissue growth, androgen production or survival. Interestingly, germ cell survival and the number of differentiated germ cells were significantly higher in VEGF treated grafts when compared to controls. This suggests that VEGF is important for sperm production in bovine testis tissue.

We also conducted an experiment to critically evaluate age related changes in the biological activity of spermatogonial stem cells (SSC) and the supporting somatic cells in the neonatal porcine testis. We grafted donor testis parenchyma from 3-, 5-, 7- and 14-day old male piglets on recipient mice for 22 weeks and then evaluated the extent of germ cell differentiation within those grafts. Interestingly, when compared to all other donor ages, a significant increase in spermatogenesis was observed in testis grafts obtained from 14-day-old donors. This advocates use of 14-day-old porcine testis tissue for future xenografting studies. Just as intriguing, testis grafts obtained from 3-day-old donors were significantly larger based on weight when compared to all other donor ages, without negatively impacting germ cell survival. These data suggest the potential to deliberately impact lifetime fertility in boars through neonatal management strategies designed to (1) expedite the onset of spermatogenesis, and/or (2) increase sperm production capacity and efficiency.

The results obtained from these results provide fundamental information about biological processes regulating sperm production in bulls and boars. Understanding these processes further will be essential towards increasing male reproductive efficiency in domestic livestock species.

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CHAPTER 1

INTRODUCTION

Spermatogenesis is a highly organized and complex process that results in the production of mature spermatozoa. Spermatogenesis in adults is dependent on successful pre-pubertal testis development. During embryogenesis in the male, primordial germ cells originating from the embryonic yolk sac migrate and colonize the genital ridge, whereby they are incorporated into the primitive sex cords. The expression of the Y chromosome specific gene *sry* (Sex determining Region on Y chromosome) by the undifferentiated gonad initiates testis formation during embryogenesis. Additionally, anti-mullerian hormone (AMH) produced by fetal Sertoli cells stimulates the degeneration of the paramesonephric ducts, which otherwise would form various parts of the female reproductive tract including the cervix, oviduct, uterus, and cranial vagina.

In the normal pre-pubertal testis, developing germ cells are found in conjunction with somatic Sertoli cells, while peritubular myoid and Leydig cells are associated with the basement membrane of the seminiferous tubule and interstitium of the testis, respectively. Sertoli cells function primarily as nurse cells for differentiating germ cells and regulate the proliferation and differentiation of these cells within the seminiferous tubule. Peritubular myoid cells are located on the outside of the seminiferous tubules and have several functions including support and contractile ability. Leydig cells are located in the interstitium of the testis (between seminiferous tubules) and are responsible for the production of testosterone. During prenatal and postnatal testis development Sertoli cells proliferate as a result of stimulation by the pituitary gonadotropin, Follicle Stimulating

Hormone (FSH). This process results in an increase in Sertoli cells and the length of time proliferation occurs is variable depending on species. At some point of development, Sertoli cell proliferation ceases due to the influence of thyroid hormones acting to stimulate terminal differentiation of Sertoli cells. Terminal differentiation is necessary for Sertoli cells to support spermatogenesis in the adult. Also, within the same developmental timeline, Leydig and peritubular myoid cells also undergo proliferation and their differentiation is stimulated primarily by AMH secreted by Sertoli cells.

Completion of physiological and cellular differentiation during puberty results in a mature testis supporting spermatogenesis. This highly organized process includes all cellular transformations that occur within the seminiferous epithelium and is largely dependent on endocrine (testosterone, FSH) regulation and direct cell-to-cell interactions. Sertoli cells support the processes of germ cell proliferation, meiosis and finally morphological differentiation within the basal and adluminal compartments of the seminiferous epithelium. In addition, Sertoli cells release spermatozoa into the lumen of the seminiferous tubule. The specialized tight junctions of Sertoli cells shield haploid germ cells from autoimmune recognition and immunological attack. Spermatogonial stem cells (SSC) exist on the basement membrane of the seminiferous tubule and undergo self-renewal and differentiation. It is believed that Sertoli cells contribute to the process of spermatogonial stem cell homeostasis as well. The continual production of sperm during a male's adult life is due to SSC homeostasis followed by germ cell differentiation. Both of these processes are in part regulated and controlled by paracrine interactions with Sertoli cells and endocrine factors produced in the pituitary gland and the testis.

Due to the complexity associated with germ cell differentiation, research investigating testis biology is often restricted to whole animal experimentation. The majority of information about the cell biology associated with spermatogenesis has been gained with the use of rodent and as a result; there remains a general lack of knowledge regarding testis development and spermatogenesis in livestock. Conversely, much is known about the endocrine control and structural changes involved in testis development and spermatogenesis, but nevertheless, little information has been gained in respect to the autocrine, juxtacrine and paracrine control of these processes. *In vitro* studies have provided limited efficacy for the study of testis biology due to a variety of reasons. In culture, cell lines can mutate, and the intimate relationship between Sertoli cells and germ cells has been difficult to maintain. Also, few markers exist for SSCs and the only reliable assay for the presence of SSCs is spermatogonial stem cell transplantation.

Ectopic testis tissue xenografting is a procedure in which small pieces of donor testis tissue is grafted subcutaneously on the backs of immunodeficient nude mice recipients. Testis tissue from donor animals obtained at specific times of development has the ability to grow, mature, and produce spermatozoa after subsequent grafting. The technique has been used successfully to generate sperm in grafted tissue from several species including cats, dogs, mice, cattle, pigs, and humans.

Research with testis tissue grafting has demonstrated that grafted bovine and porcine testis tissue from pre-pubertal donors produces elongating spermatids at a developmental age similar to that in the bull and boar. It also appears that the cycle length of the seminiferous epithelium in grafted testis is similar to the *in situ* situation in both species. Additionally, microarray analysis indicates that global gene expression in

porcine testis xenografts appears comparable to testis tissue *in situ*. These findings indicate that ectopic testis xenografting is suitable model to study mammalian spermatogenesis.

There are many advantages of using this procedure when compared to cell culture and whole animal experimentation to improve the understanding of testis biology and spermatogenesis. First, this technique allows for the maintenance of the relationships between somatic cells and germ cells in the testis of the donor animal. Secondly, each recipient mouse can receive several testis grafts and this benefit is three-fold; allows for increased replication, more efficient experimental design, and more powerful statistical analysis. Third, testis tissue can be cultured or cryopreserved prior to grafting and retain the ability to produce sperm following the grafting period.

In addition to the study of spermatogenesis, ectopic testis tissue grafting could be used to maintain the germ line of individual males and to preserve the germ line of human male pre-pubertal cancer patients that must undergo chemotherapy. Researchers have successfully produced viable offspring with sperm collected from mouse grafts using intracytoplasmic spermatid injection (ICSI) and embryo transfer (ET). Another application of the technique is the production of transgenic spermatozoa using electroporation of tissue prior to grafting so that genetically modified sperm are produced in the grafted tissue.

For the study of bovine spermatogenesis, our lab indicated that eight-week-old bull calves represent the optimum donor age for ectopic testis tissue xenografting. However, the efficiency of spermatogenesis in testis grafts is relatively low. Sperm

production in bovine testis grafts is increased following treatment of the graft site with vascular endothelial growth factor (VEGF).

The ability to manipulate testis tissue prior to grafting provides a variety of avenues to investigate biological factors governing establishment of the spermatogonial stem cell niche in the mammalian testis. Therefore, the objective of the research presented here investigated how VEGF influenced sperm production in bovine testis tissue. Similarly, significant differences in the ability of pig testis tissue to produce sperm following grafting were identified. These findings along with the further characterization of factors contributing to the efficiency of spermatogenesis will allow for a better understanding of spermatogenesis in domestic livestock as a whole, and potentially, facilitate the development and implementation of assisted reproductive technologies for various agricultural, biomedical and conservation purposes.

CHAPTER 2

REVIEW OF LITERATURE

Spermatogenesis

Spermatogenesis is the process of germ cell proliferation and differentiation within the testis leading to the production of spermatozoa, the male gamete. Spermatogenesis is a highly organized process orchestrated by Sertoli cells to a great extent, and includes all cellular transformations that occur within the seminiferous epithelium. This process is largely dependent on endocrine (Zirken, 1998) and auto/paracrine regulation and direct cell-to-cell interactions (Russell and Griswold, 1993). In respect to efficiency, spermatogenesis is one of the most highly productive biological processes in animals, lending to a virtually unlimited supply of spermatozoa produced during an adult males' lifespan. This efficiency can be attributed to the continual spermatogonial stem cell (SSC) replenishment of the differentiating germ cell population in the adult testis (Oakberg, 1971). It is apparent that the dynamic molecular mechanisms involved with the onset and regulation of spermatogenesis presents a fascinating area of research for reproductive biologists to study.

Spermatogenesis can be divided into three phases of germ cell events including proliferation, meiotic divisions, and finally differentiation within the basal and adluminal compartments of seminiferous tubules. Sertoli cells support and regulate these various processes, which ultimately lead to the formation of functional spermatozoa. The developmental timeline associated with these phases is species specific.

During proliferation, spermatogonial stem cells undergo a series of mitotic divisions. In the most popular model , one spermatogonia remains a stem cell (A_s), while the other becomes committed to further successive divisions involved in spermatogenesis, of which the cell types are both A-paired (A_{pr}) and A-aligned (A_{al}) spermatogonia. These A_{pr} and A_{al} spermatogonia are characterized by the presence of intercellular bridges that connect both cell types. These intercellular bridges allow for the sharing of gene products, and are thought to promote synchronous development of cell clones and other germ cell types.

The generally accepted spermatogonial stem cell theory states the progression of the mitotic phase of spermatogonial cells as follows: A_s, A_{pr}, A_{al}, A_1, A_2, A_3, A_4, In and then B spermatogonia. Of these cell types, A_2, is the cell considered indicative to the onset of spermatogenesis. At each subsequent cell division during the proliferation phase, spermatogonia number doubles, except between the A_{al} to A_1 stage of cell differentiation where only a morphological change occurs. It has been theorized that germ cells at the A_4 cell stage can either produce In spermatogonia or retort back to A_1 spermatogonia to renew the population (De Rooji and Russell, 2000). These different germ cell types can be identified via the morphological differences in chromatin. At completion of the mitotic phase, cells have reached the B cell stage were a total of 512 germ cells can be generated from one spermatogonial stem cell (Clegg et al., 1990). The final division of B spermatogonial gives rise to preleptotene spermatocytes, the first stage of primary spermatocytes. Primary spermatocytes are the cells that undergo the first meiotic division, so it is of no surprise that they must pass through the immunopriveledged tight

junctions found between adjacent Sertoli cells; ultimately ending up in the adluminal compartment of the seminiferous tubule.

Before meiosis can occur primary spermatocytes must also experience some changes, whereby preleptotene spermatocytes become leptotene, zygotene, and pachytene spermatocytes as result of DNA synthesis. The pachytene primary spermatocytes undergo the first meiotic division to form secondary spermatocytes, which then quickly take part in the division of Meiosis II. This results in the production of four haploid round spermatids from each primary spermatocyte. It is important to note that during meiosis a large number of cells can undergo apoptosis, so it is difficult to accurately predict actual number of spermatids successfully produced during meiotic divisions, but it is stated that 4096 haploid germ cells can be produced from a single spermatogonial stem cell (or A2 spermatogonia) in the rat (De Rooij and Russell, 2000).

After meiosis has commensurated, a process known as spermiogenesis follows whereby round spermatids differentiate morphologically becoming elongated spermatozoa. Several steps including production of the acrosome, condensation of the nucleus, elongation of the spermatid with subsequent production of a flagellum, and finally the reabsorbtion of the cytoplasmic droplet characterize the process. Next, the spermatozoa are spermiated or released into the lumen of the seminiferous tubule whereby they can leave the seminiferous tubules by way of the efferent ducts. These ducts lead out to the epididymis where the sperm acquire the ability to be motile. Sperm first enter the caput or head of the epididymis and then migrate towards the cauda or tail of the epididymis where they are considered to have completed the extragonadal

maturation process. Sperm are stored here until ejaculation, in which they can be utilized through the normal mating fertilization process or other assisted reproductive methods.

Spermatogenesis is organized throughout the seminiferous tubule so that there are multiple stages of germ cell development associated with each section of the seminiferous tubule. These stages are not synchronous, so a continuous supply of sperm can be produced even though spermiation occurs only in once particular stage. The number of and length of each stage varies between species as does the arrangement, as both helical and segmental arrangements exist. In the case of the helical arrangement the process of spermatogenesis travels down and around the tubule so that in any one section of a tubule, several different stages can be found. In the case of the segmental arrangement, a wavelike release of sperm is seen as each tubule is in a synchronous stage of spermatogenesis, whereby a tubule in the second stage, will progress to the third stage, and continue until the final stage. This sequential pattern of spermatogenesis is known as the spermatogenic wave.

As germ cells progress between the Sertoli cells towards the lumen of the seminiferous tubule, the Sertoli cells regulate developmental stages of germ cells so a characteristic hierarchy of germ cells is found in the same group. In this respect, the spermatogenic cycle is formed and in bulls and boars the cycle length is about 13.5 and 8.3 days respectively. It takes approximately 4.5 and 4.7 cycles of the seminiferous epithelium in the bull and boar before spermatogenesis is complete. As a result the cycle of the seminiferous epithelium is approximately 61 days in bulls and 49 days in the boar. The cycle of the seminiferous epithelium is the time it takes for spermatogonia to complete the spermatogenesis process to the elongated spermatid stage for release.

Germ cell differentiation can be inhibited either by several mechanisms including nutritional defects (vitamin A deficiency) that arrest spermatogonial cells at the A_{al} cell stage of development. Additional regulation can occur when there is an abundance of spermatogonial cells present associated with a particular Sertoli cell. This acknowledgement of cells in an area of a tubule is referred to as the density dependant regulation. The higher the number of A_2 to A_4 spermatogonia in a region of a tubule the more likely apoptosis of cells will occur to reduce and stabilize spermatogonia numbers to a normal level. Conversely, if there this is an absence or shortage of spermatogonial cells associated with a Sertoli this particular means of germ cell regulation of germ cells may not occur (De Rooij and Russell, 2000).

The Testis

The testes are the male gonads, and in all mammals they are paired ovoid organs. The function of the male gonad is to produce steroidogenic hormones and spermatozoa, the male gamete. Each testis is encapsulated by connective tissue, namely the tunica albuginea. This layer of connective tissue functions to protect the testis from physical damage, maintain intratesticular pressure, and ultimate helping to potentiate the process of sperm transport from the testis to the epididymis. The testis is composed of two compartments, the mediastinum and the parenchyma.

The mediastinum is a fibrous connective tissue layer located in the central portion of the testis that provides structural support to the rete testis and efferent ducts. The rete testis and efferent ducts comprise the intricate tubule network that connects the

seminiferous tubules in the testis, the site of spermatogenesis, to the epididymis, the major site of extragonadal sperm maturation and storage.

The parenchyma is the major functional component of the testis and it is comprised of seminiferous tubules and interstitial tissue, whereby the interstitial tissue resides between adjacent seminiferous tubules. Localized within the interstitial tissue are blood vessels, lymphatics, nerves, immune cells, and Leydig cells. The endocrine component of the interstitium is the Leydig cells, which produce androgens in response to luteinizing hormone (LH) from the anterior pituitary. Androgens produced by the Leydig cells are important for spermatogenesis and male secondary sex characteristics.

The components of testicular parenchyma responsible for producing sperm are the seminiferous tubules. These tubules are usually arranged in lobules, thus forming highly convoluted loops. The outermost layer of these tubules consists of peritubular myoid cells and collagen. Moving inward towards the lumen, the next layer is the basement membrane; this layer provides support for Sertoli cells. Between the basement membrane and lumen, the seminiferous epithelium consisting of Sertoli and developing germ cells are found. Sertoli cells are the nurse cells that foster germ cell proliferation and differentiation, ultimately leading to the production of spermatozoa.

The seminiferous tubules are divided into two sections, the tubulus contortus and the tubulus rectus. The tubulus contortus is the portion of the seminiferous tubule where spermatogenesis occurs. Conversely, the tubulus rectus is a short transitional region of the seminiferous tubule that connects the tubulus contortus to the rete testis. This tubular network provides the pathway by which sperm must travel to leave the testis.

In all mammals, these paired organs are housed inside a scrotum, yet suspended outside the peritoneum or body wall. The primary function of the scrotum is to aid in proper temperature regulation of the testis, a process important for normal testicular function. The androgen secreted by the Leydig cells play vital roles in downstream masculinization events and in descent of the testes into the scrotum (Hutson et al., 1996).

Leydig cells

The Leydig cells of the testis are located in the interstitial space between seminiferous tubules. During embryonic development, Leydig cells appear shortly after gonadal sex differentiation, testicular cord formation, and the presence of pituitary gonadotropins in the fetal bloodstream. After formation, Leydig cells undergo two stages of proliferation and differentiation; the first being before birth, and the second occurring during the onset of puberty (Benton et al., 1995). These characteristic changes are necessary for the acquisition of functional male phenotypes and the ability to produce sperm.

Leydig cells express receptors for LH (secreted by the pituitary), and when LH binds to its G-protein coupled transmembrane receptor, Leydig cells are then stimulated to synthesize and produce androgen, mainly testosterone. Leydig cells do not store testosterone locally, but actively secrete all that they produce and as a result, they are capable of secreting the majority of testosterone found in the serum of a male. Testosterone can then diffuse into the bloodstream or into the seminiferous tubules directly or indirectly by attaching to the carrier protein albumin. Intratesticular levels of testosterone in mice have been found to normally range from 25-100 ng/ml and assist in

Sertoli cell proliferation during fetal and early neonatal life (Johnston, 2004). Testosterone has also been found to have multiple functions. First, testosterone is used in combination with the follicle-stimulating hormone (FSH) from the pituitary to assist in paracrine factor production by Sertoli cells. Secondly, testosterone works to act upon the pituitary as a negative feedback on the release of LH. Third, testosterone stimulates androgen target tissues for androgen-mediated growth. Testosterone is influential and necessary for spermatogenesis to occur in most animals (Sharpe, 1994).

Finally, in respect to embryogenesis, testosterone secreted by primitive Leydig cells into circulation, crosses the blood-brain barrier to disable the functionality of the preovulatory center of the hypothalamus in fetal male brain, so that LH will be secreted tonically and not cyclically in the post natal male.

Sertoli cells

The Sertoli cells are the sustenticular cells of the testis that maintain spermatogenesis. In terms of morphology, these somatic cells were first described as long columnar cells having a base that is attached to the basement membrane, and an apical end that protrudes into the lumen of the seminiferous tubule (Sertoli, 1865). These cells have been characterized as having many arm-like projections that wrap around and interact with developing germ cells in the seminiferous epithelium. The Sertoli cells structure is convoluted in nature so it can be very difficult to identify them from germ cells, but nonetheless, these pale colored cells have a large nucleus, and a tripartite nucleolus, which allow them to be differentiated from germ cells following histological analysis. The recent discovery of a Sertoli cell specific transcription factor, GATA-4,

allows for the quantification of both Sertoli cell gene expression, and the morphological identification of Sertoli cells with the use of immunohistochemistry (McCourd et al., 2003a). GATA-4 expression is constant, and is not affected by FSH treatment (Zhang et al., 2003).

Sertoli cells have many functions in the testis, and this includes supporting developing germ cells of which they are in intimate association with. Sertoli cells are the primary (avoid jargon) regulators of germ cells. It is known that Sertoli cells regulate the maturation of some germ cells, and recently it has been shown that they also modulate the apoptosis of selected germ cells, making them destined for programmed cell death (Barone, et al., 2004). Sertoli cells also phagocytose cellular material within the seminiferous epithelium. This function becomes critical during the final stages of spermatogenesis when much of the cytoplasm is removed from elongating spermatids, and also for the extrusion of apoptotic germ cells.

Another critical function of Sertoli cells includes their active role in maintenance of the blood testis barrier. Sperm are haploid cell types and as a result, are recognized as foreign by the male immune system. If an immune response was triggered, autoimmune attack would commence, germ cell populations would be depleted, and infertility would likely be the global affect. As a result, it is important that immune cells be restricted from the seminiferous epithelium. A network of tight junctions between adjacent Sertoli cells along with peritubular myoid cells provide a restrictive barrier to prevent immune responses from occurring from within the seminiferous tubule. The presence of tight junctions between adjacent Sertoli cells ultimately partitions the seminiferous tubule into two vertical compartments, the basal compartment (between tight junction and the

basement membrane) and adluminal compartment (between the tight junction and the lumen). This restrictive structure also serves to prevent the backflow of lumen fluids to lymph, and only allows factors such as testosterone to freely pass. It is also known that the breakdown of the blood testis barrier results in male infertility.

Sertoli cell tight junctions are also involved in the transport of spermatocytes from the basal to adluminal compartment. As one adluminal tight junction begins to break down, another tight junction on the basal end has already being produced. This process facilitates the transport of germ cells towards the lumen, and because the tight junctions are so restrictive, Sertoli cells must also secrete nutrients to the more advanced germ cells in the peripheral adluminal compartment. Sertoli cells also function in spermiation, whereby elongate spermatids are released from the peripheral adluminal compartment into the lumen of the seminiferous tubule.

Prenatal developmental aspects of the Sertoli cell

For spermatogenesis to be successful in the adult male, efficient Sertoli cell proliferation and differentiation must occur. During embryonic development, the primitive Sertoli cells are the first cells to recognizably differentiate in the indifferent fetal gonad, and as a result are the primary regulators of testicular cord formation and testis development. If during this time, Sertoli cells do not undergo proper proliferation and differentiation a variety of disorders may arise (Sharpe et al., 2003). Primitive Sertoli cells likely originate from the mesonephros must undergo proliferation early in the sexually dimorphic embryo before the formation of the indifferent male gonad can occur. This proliferative event coincides with the production of SRY and is necessary for

normal testis development in the male (Schmahl et al. 2003). SRY is a transcription factor that functions as the testis-determining gene, and when it is expressed testis formation occurs at the genital ridge. The first stages of Sertoli cell differentiation during this time period results in testicular cord formation whereby primitive Sertoli cells and primordial germ cells (PGC) form aggregate bodies, and in respect to time this process is highly species specific (Frojman, et al. 1989). Shortly after cord formation, the basement membrane and other primitive cell types can be observed readily, whereas there is an absence of a lumen (Ichihara et al., 1993).

Besides testis formation alone, Sertoli cells are also involved in male differentiation. SRY functions to regulate formation and development of testicular cords. Additionally, Sertoli cells also produce anti-Mullerian hormone (AMH) throughout embryogenesis, and this factor causes the regression of the paramesonephric ducts in the developing male reproductive tract, which otherwise, like in the female would ultimately form female reproductive organs. It is also believed, that AMH may be involved in regulating the mitotic divisions of PGCs primarily involved with testicular cord formation (Vigier et al., 1988). It also appears that Sertoli cells play an active role is organizing germ cell arrangement within the testicular cord prior to birth. This organization has not been characterized specifically, but it is likely to involve a variety of regulatory molecular signal mechanisms likely related to cytoplasmic bridge and gap junction formation between PGCs. It has also been postulated that Sertoli cells regulate Leydig cell development as a result of producing AMH and regulation the formation of the testicular cord (Byskov, 1986).

Sertoli cells function primarily in the adult mammals to support developing germ cells and stimulate meiotic divisions. During embryogenesis, Sertoli cell function rather conversely by acutely inhibiting meiotic activity and instead are responsible for promoting mitotic activity. Sertoli cells produce a meiosis preventing substance (MPS) prenatally and the activity of this substance is present throughout much of the prespermatogenic period, with a decline to reach low levels at the onset of spermatogenesis (Gondos et al., 1996). Findings also demonstrate that Sertoli cells secrete a meiosis activating substance (MAS) shortly after birth, whereby greatest activity for MAS is associated with the onset on spermatogenesis (Gondos et al., 1996).

Several studies strongly suggest that the fetal proliferation of Sertoli cells is most likely mediated via the actions of follicle stimulating hormone (FSH). Removal of FSH either by decapitation or immunological mechanisms against gonadotropin hormone releasing hormone (GnRH) of developing fetuses prevents Sertoli cell proliferation (Orth, 1984). It has been demonstrated that both FSH and cyclic AMP (cAMP) are both stimulatory in the signal cascade leading to Sertoli cell proliferation. In this case, it is likely that the actions of FSH are mediated via cAMP in activating the PI3-K/PDK1 signal transduction pathway (Richards, 2001). During the time of Sertoli cell proliferation directed via FSH, FSH receptor expression is also up regulated (Warren et al., 1984). This proliferation of Sertoli cells can also be negatively regulated by β-endorphins secreted b nearby Leydig cells in the interstitium (Orth et al., 1990). Besides stimulating proliferation of Sertoli cells, *in-vitro* studies have shown that FSH can also have inhibitory actions on Sertoli cell differentiation. This action was demonstrated by a reduction in AMH and transferrin secretion by Sertoli cells treated in culture with FSH.

The in vivo evidence for this finding was consistent; when fetuses were decapitated in utero they showed elevated transferrin and AMH levels (Migrenne et al., 2003).

Postnatal developmental aspects of the Sertoli cell

Postnatal Sertoli cell development is important for the establishment of adult spermatogenesis. It has been demonstrated that daily sperm production is directly related to the number of adult Sertoli cells in several species (Johnson et al., 1984; Ford et al., 1999). After birth, Sertoli cells continue to proliferate for a period of time, which is highly species specific, before undergoing marked terminal differentiation. This terminal differentiation consists of cell growth and major morphological changes necessary before spermatogenesis can occur as Sertoli cells must acquire the ability to support developing germ cells. Morphologically, the Sertoli cells are small round cells (similar in size to germ cells) with variable cell surface characteristics during the proliferative stage.

In mammals, most of the proliferation of Sertoli cells occurs early in the postnatal period, but continues at a lesser rate until puberty is reached. As previously mentioned, FSH and cAMP have been shown to be responsible for controlling this proliferation (Griswold et al., 1977). In the boar and bull, the most rapid proliferation of Sertoli cells has commenced by 13 and 30 weeks respectively (Putra et al., 1985; Curtis and Amann, 1981). The rat is characterized by having maximal Sertoli cell proliferation between 10 and 15 days of age (Wang et al., 1989). In comparison to the rat, the age of maximum Sertoli cell proliferation is much later in the bull and boar. As expected when examining the testis of bulls and boars Sertoli cell density is greatest towards the end of maximal proliferation, and begins to decline before the number of Sertoli cells have stabilized.

During differentiation, Sertoli cells grow in size, become more columnar in shape, and undergo marked nuclear changes. In the ram testis, once Sertoli cell number stabilized (Sertoli cell proliferation was complete), a subsequent increase in both cellular and nuclear sizes increased by a factor of 3 and 1.5 respectively (Monet-Kuntz et al., 1984) and in some species a 3 fold increase of nuclear diameter is seen (Ramos et al., 1979).

The small round nuclei seen in Sertoli cells shortly after birth in the neonate become elongate and translocate to the basal side of the Sertoli cell. During those processes, a distinct change in the nucleolus is also observed as they transform from a very obscure complex morphology, to a complex tripartite structure (Griswold et al., 2001). The increases in nuclear diameter are associated with increase RNA production by the mature Sertoli cell (Lamb et al., 1982).

The majority of the increase in cell volume is attributed to increases in cytoplasm. This is a result of Sertoli cells elongating, and extending apically towards the center of the testicular cords. Lateral arms form as the cytoplasm extends tangentially, and this change in shape is a direct result of the changes in morphology by the nearby germ cells. Many organelles develop and increase in size within the cytoplasm, of which the most extensive changes are in respect to the smooth endoplasmic reticulum (ER), the Golgi apparatus, and the mitochondria.

Tight junctions are also formed, and these changes coincide with the developmental timeline relative to cycle of the seminiferous epithelium and tubule formation in the testis. As previously mentioned, tight junctions between adjacent Sertoli cells are the most restrictive component of the blood testis barrier. These tight junctions

are first seen between Sertoli cells during the first round of spermatogenesis when spermatocytes are first present. A population of gap junctions inhabits the proximity between adjacent Sertoli cells as tight junctions are in manufacture, but ultimately regresses as the formation of tight junctions reaches completion. It is unclear as to what regulatory mechanisms are responsible for the formation of tight junctions, but work with hamsters suggests two postulates to explain the dynamics of junction complexes in the testis. It is suggested that the gap junctions either mediate the production of tight junctions or serve as a temporary placeholder until tight junctions replace them (Vignon et al., 1987).

Biochemical activity of Sertoli cells

The biochemical activity of postnatal Sertoli cells lend towards many functional aspects of male reproduction. Prenatal Sertoli cells produce and secrete AMH during embryogenesis, but this substance has also been detected in postnatal bulls and neonatal rats (Donahoe et al., 1970; Picon, 1970). The function of AMH during embryogenesis is known, but its effects in postnatal mammals still remain unclear.

Androgen binding protein (ABP) is also secreted by Sertoli cells. It was first detected in the early 1970s, when a protein with a similar steroid-binding activity to the androgen receptor was detected in rat testis (French and Ritze´n, 1973). This glycoprotein functions by binding androgens (produced by nearby Leydig cells) with high affinity and transports them to the epididymis (Westphal, 1986). It has been demonstrated that ABP is similar in chemistry to the sex hormone-binding globulin (SHBG) produced by the liver, which functions to bind steroids in circulation (Musto et al., 1980; Danzo et al., 1980;

Cheng et al., 1984). ABP is present in the intermediate and late stages of differentiation of rats, in a developmental matter necessary for testosterone transport to the epididymis for proper development and function of that organ.

During early postnatal Sertoli cell differentiation, estrogen synthesis is high (resulting from high levels of aromatase activity), but rapidly declines during as differentiation commences (Armstrong et al., 1977). This pattern coincides very similarly with the expression of the FSH receptor (Thanki et al., 1978; Hiendel et al., 1977). It is also important to note that Sertoli cells are the only known cells in the male to contain a receptor for FSH (Griswold, 1999). The concentration of FSH receptor expressed as binding per unit of weight decreases gradually with age, while there is an actual increase in the number of binding sites per testis from birth to puberty (Gondos, 1996). Androgen receptor (AR) is produced by Sertoli cells in highest levels when the Sertoli cell's responsiveness to FSH is lowest.

Hypothalamic-Pituitary-Gonadal Axis in the Male

Hormone secretion has essential roles in the activation of many cells and processes in the body. Two of the major organs that are involved in hormone regulation are the hypothalamus and the pituitary. These organs are located in close proximity of each other in the lower portion of the brain and through a coordinated effort regulate many body functions. The hormones that are released by the hypothalamus are transported directly to the pituitary by way of the hypothalamic-hypophyseal portal system (HHPS). Part of this blood flow system that travels out of the anterior pituitary is directed past or in close conjunction with the hypothalamus so that hormones of pituitary

origin can directly mediate the actions of negative feedback on the hypothalamus. This has been demonstrated as both β-endorphin and the pituitary gonadotropins (LH and FSH), negatively feed back to regulate the release of the hypothalamic hormone gonadotropin-releasing hormone, GnRH (Hafez and Hafez, 2000).

Gonadotropin Releasing Hormone (GnRH)

GnRH is a decapeptide released from the hypothalamus in response to a variety of environmental and endocrine factors. These factors depend of species and sex and a variety of other factors including, melatonin, blood glucose and fatty acid levels, leptin, steroid hormones, and various erotogenic stimuli. GnRH is secreted from peptidergenic neurons in the hypothalamus into the HHPS and travels to the adenohypophysis to stimulate the release the release of pituitary gonadotropins, FSH and LH.

Luteinizing Hormone (LH)

Luteinizing hormone (LH) is a glycoprotein hormone that is secreted by luteotrophic gonadotroph cells in the adenohypophysis in response to GnRH. In the male, the primary function of LH is to stimulate acute testosterone production by Leydig cells. This occurs after LH binds to its G-coupled protein receptor to activate the cAMP and PI3-kinase signal transduction pathways thus inducing testosterone synthesis. It has been suggested that LH may stimulate the proliferation of primitive Leydig cells, and actually upregulate the expression of the LH receptor on Leydig cells. Some evidence also states that LH may upregulate FSH receptor expression on Sertoli cells.

Follicle Stimulating Hormone (FSH)

FSH is both secreted from folliculotrophic gonadotrophs cells in the adenohypophysis in response to GnRH. In the male, the primary function of FSH is to regulate testicular development and spermatogenesis. Some species such as humans and primates require FSH for both quantitative and qualitative (to a lesser extent) spermatogenesis, whereas in the case of rodents systems, FSH is only required for quantitative spermatogenesis. It is important to note that FSH is important for Sertoli cell function during all stages of life. Researchers have used both in-utero decapitation and treatment with an anti-FSH antibody effectively to prove that FSH is the primary regulator of Sertoli cell proliferation in rodents (Orth et al., 1984). Also when neonatal boars are hemi-castrated, additional FSH stimulated Sertoli cell proliferation occurs in the contra-lateral testis (Lunstra et al, 2003). A number of *in vitro* studies support these findings, and interestingly when Sertoli cells are treatment with FSH in culture, the period of proliferation is extended by over 10 days (Buzzard et al., 2002). Additionally, when rodents immunized against GnRH were treated with FSH both with and without the presence of LH and testosterone, Sertoli cell proliferation and differentiation commenced normally (Haywood et al., 2003). However, in the absence of testosterone, there was a great reduction in the number of post meiotic germ cells, meaning that both FSH and testosterone are required for quantitative and qualitative spermatogenesis (Haywood et al., 2003). FSH is also critical for the formation of tight junctions found between adjacent Sertoli cells which function to maintain the blood testis barrier (Sluka et al., 2006). The

development of an FSH null mouse model has also been extremely useful for validating many of these results in vivo (Baker et al., 2003).

Inhibin

In the male, Inhibin is a protein hormone that is produced mainly by the Sertoli cells, and to a lesser extent by Leydig cells. Inhibin belongs to the transforming growth factor β (TGF-β) super family and is a heterodimeric glycoprotein which contains one α subunit and either β_A or β_B forming inhibin A or B. The function of Inhibin B in the male is to inhibit FSH secretion in a negative feedback manner at the level of the hypothalamus. It also functions to play roles in germ cell differentiation and regulating the LH sensitivity of Leydig cells (Hsueh et al., 1987).

Testosterone

In the male, testosterone is an androgenic steroid hormone produced by the Leydig cells of the testis in response to LH. It is the principal male sex hormone and is involved in the development and maintenance of the male reproductive tract and accessory sex glands, while also being greatly responsible for mediating male sexual behavior, spermatogenesis, and immune function. Also, in respect to embryogenesis, testosterone secreted by primitive Leydig cells into circulation, crosses the blood-brain barrier and acts to disable the functionality of the preovulatory center of the hypothalamus in fetal male brain, so that LH will be secreted tonically and not cyclically in the post natal male.

For testosterone synthesis to be initiated, LH must first bind to its g-protein, coupled transmembrane receptor on the Leydig cell. Following this event, cholesterol will be converted to testosterone through the activation of several steroidogenic enzymes within the mitochondria and cytoplasm of the Leydig cell. Once testosterone has been synthesized it can now exert its biological actions.

The effects of testosterone in humans and other vertebrates occur by way of two main mechanisms: by activation of the androgen receptor (directly or as DHT), and by conversion to estradiol and activation of certain estrogen receptors. Free testosterone is transported into the cytoplasm of target tissue cells, where it can bind to the androgen receptor, or can be reduced to 5α-dihydrotestosterone (DHT) by the cytoplasmic enzyme 5α-reductase. DHT binds to the same androgen receptor even more strongly than T, so that its androgenic potency is about 2.5 times that of T. The T-receptor or DHT-receptor complex undergoes a structural change that allows it to move into the cell nucleus and bind directly to its hormone response elements, which are specific nucleotide sequences of the chromosomal DNA. Once this occurs, testosterone can mediate its effects on spermatogenesis by specifically activating the transcriptional activity of certain genes, leading to the production of important proteins involved in regulating Sertoli cell function.

Ectopic Testis Xenografting

Ectopic grafting of testicular tissue is a method in which a portion of testicular parenchyma from a donor animal is placed ectopically on a foreign area of a recipient animal. This recipient animal is usually an immunodeficient nude male mouse (nu+/nu+).

The nu/nu mouse strain lacks a thymus and hence does not have the characteristic immune system T-cells that could mount an immune response and ultimately rejecting donor-derived testis tissue.

Scientists have been experimenting with testis transplantation since the late 1800's (Turner, 1938). The resulting efficacy of the technique involved great variability between differences researchers. The majority of these differences can most likely be attributed to the site or region whereby grafted tissue is placed, as grafts placed inside the body cavity were relatively non-progressive when compared to the successful grafts located in the scrotal pouch. The large ranges in the efficiency of spermatogenesis in these cases are likely due to the differences in temperature between grafting regions. Other areas successfully tested in relation to testis tissue grafting include the anterior chamber of the eye, dorsal back region, behind the ear and under the kidney capsule. From these previous grafting studies and the body of knowledge surrounding endocrinology, it seems logical that by castrating the recipient mouse prior to grafting results in an ideal environment for foreign testis tissue to thrive.

Ectopic tissue xenografting provides a useful means for researchers to investigate factors and mechanisms important to germ cell differentiation, Sertoli cell populations, and production of sperm in various livestock species without maintaining those bulls and boars respectively. For example, in bovine testis tissue grafts only about 10% of the seminiferous tubules are capable of undergoing complete spermatogenesis after a 24-week grafting period. As a result of this relatively low efficiency, factors that up-and/or-down regulates Sertoli cell proliferation and germ cell differentiation in the testis tissue grafts can be more easily observed.

Before grafting it is obvious that the removal of the epididymis and other accessory sex glands must occur. Additionally, both the Sertoli and Leydig cells need access to the recipient mouse's blood supply so as a result, the tunica albuginea must be removed. The testicular parenchyma is then placed in Hanks Buffered Salt Solution (HBSS) until the time of grafting. In order to both minimize the discomfort to the recipient mouse and maximize blood supply to the tissue, testis grafts are placed subcutaneously (on the backs) of the recipient mice. Mice and the site of the graft are monitored and a small protuberance of testis tissue under the skin can be observed at the site of the graft. Following grafting, spermatids retrieved from mouse, goat and pig testis xenografts have successfully stimulated the resumption of meiosis in mouse oocytes with the use of ICSI.

Development between testis tissue xenografts is similar to the *in situ* testis in boars. This supports the ability of murine gonadotropins to support spermatogenesis of higher order species and that exogenous administration of hormones is not needed. In another study performed where hamster testis were grafted to mice, the testicular samples from adult animals whether regressed or not, displayed a lower incidence of spermatogenic recovery when compared to immature donors. This implicates that grafting immature testicular tissue appears to be more successful relative to spermatogenesis then adult testis, and our findings support this.

Manipulation of testis tissue prior to xenografting

The success of bovine testis grafts depends on many factors including: donor age, endocrine environment of the recipient mouse, and endogenous treatments of testis tissue

prior to (or during) the grafting period with factors that may potentially regulate testis function. Also, no differences in the establishment of spermatogenesis between testis tissue explant cultured on floating filters for 5-7 days (Oatley, 2004a) when compared to freshly grafted tissue. Thus, treating testis tissue explants in culture may provide a useful means to determine the direct effects of factors regulating on germ cell and Sertoli cell survival in ectopic testis tissue grafts. Schmidt et. al. (2006) treated testis tissue at the time of grafting with 1 μg/ml of vascular endothelial growth factor (VEGF), a potent angiogenic factor. The hypothesis was that testis tissue treated with VEGF prior to grafting would significantly increase angiogenesis in the respected grafts, leading to improved graft survival and production of more elongate spermatids. When compared to controls, VEGF treatment significantly increased graft weight and spermatogenesis in grafted tissue; interestingly blood vessel numbers were not different. This suggests VEGF may have an effect on somatic and germ cell proliferation and/or differentiation in the testis. Other researchers have localized VEGF and its receptors, Flt-1 and KDR, to both the Leydig and Sertoli cells in the testis. VEGF receptors are differentially expressed in germ cells, whereby Flt-1 is present on elongate spermatids and KDR is present on spermatogonia. This suggests that VEGF has non-endothelial targets in the testis and that it may regulate Sertoli cell proliferation. As a result of these data, the overall goal of my research is to test the hypothesis that VEGF has a positive effect on proliferation and germ cell differentiation, and ultimately the efficiency of spermatogenesis in bovine testis xenografts.

Vascular Endothelial Growth Factor (VEGF)

The action of VEGF on endothelial cells is well documented and much is known about their mechanisms of action in this cell type. VEGF receptors are receptor tyrosine kinases (RTK), which are enzymes that can transfer a phosphate groups (via autophosphorylation) to a tyrosine residue in a protein (on c-terminus end of receptor) following ligand binding and dimerization. Receptor tyrosine kinases possess an extracellular ligand binding domain, a transmembrane domain and an intracellular catalytic domain. The transmembrane domain anchors the receptor in the plasma membrane, while the extracellular domains bind growth factors. Following ligand binding, tyrosine residues are rapidly autophosphorylated, and various signal transduction cascades can be triggered. Characteristically, the extracellular domains of VEGF receptors are comprised of immunoglobulin-like domain structural motifs (Shibuya et al., 2006).

Flt-1, which occurs in trans-membrane and soluble forms, negatively regulates angiogenesis during early embryogenesis, but it also acts as a positive regulator of angiogenesis and inflammatory responses in postnatal life, playing a role in several human diseases such as rheumatoid arthritis and cancer. The soluble VEGFR1 is over expressed in placental trophoblast cells (Shibuya et al., 2006). VEGFR2 has critical functions in physiological and pathological angiogenesis through distinct signal transduction pathways regulating proliferation and migration of endothelial cells (Shibuya et al., 2006). The downstream targets of both receptors include activation of many signaling pathways (PI3K/Akt, Ras/Raf-MEK/Erk, eNOS/NO, and IP3/Ca2+,

PKC, PKA) that leads to gene transcription responsible for its functions as an endothelial cell mitogen and vascular permeability factor (Namiecinska et al., 2005).

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References

Anand RJ, Paust HJ, Altenpohl K, Mukhopadhyay AK. Regulation of vascular endothelial growth factor production by Leydig cells in vitro: the role of protein kinase A and mitogen-activated protein kinase cascade. Biol Reprod. 2003 May;68(5):1663-73. Epub 2002 Nov 27.

Aslam I, Fishel S, Moore H, Dowell K and Thornton S (2000) Fertility preservation of boys undergoing anti-cancer therapy: a review of the existing situation and prospects for the future. Hum Reprod 15,2154–2159

Aubard Y (2003) Ovarian tissue xenografting. Eur J Obstet Gynecol Reprod Biol 108,14–18.

Avarbock MR, Brinster CJ and Brinster RL (1996) Reconstitution of spermatogenesis from frozen spermatogonial stem cells. Nat Med 2,693–696.

Bahadur G and Ralph D (1999) Gonadal tissue cryopreservation in boys with paediatric cancers. Hum Reprod 14,11–17.

Baker PJ, Pakarinen P, Huhtaniemi IT, Abel MH, Charlton HM, Kumar TR, O'Shaughnessy PJ. Failure of normal Leydig cell development in follicle-stimulating hormone (FSH) receptor-deficient mice, but not FSHbeta-deficient mice: role for constitutive FSH receptor activity. Endocrinology. 2003 Jan;144(1):138-45.

Barone F, Aguanno S, D'Alessio A, D'Agostino A. Sertoli cell modulates MAA-induced apoptosis of germ cells throughout voltage-operated calcium channels. FASEB J. 2004 Feb;18(2):353-4. Epub 2003 Dec 4.

Benton L, Shan LX, Hardy MP. Differentiation of adult Leydig cells. J Steroid Biochem Mol Biol. 1995 Jun;53(1-6):61-8.

Blanchard KT, Boekelheide K. Adenovirus-mediated gene transfer to rat testis in vivo. Biol Reprod 1997 56:495-500

Brinster RL and Zimmerman JW (1994) Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci USA 91,11298–11302.

Buzzard, Wreford NG, Morrison JR. R Marked extension of proliferation of rat Sertoli cells in culture using recombinant human FSH. Reproduction. 2002 Nov;124(5):633-41.

Byskov AG. Differentiation of mammalian embryonic gonad. Physiol Rev. 1986 Jan;66(1):71-117. Review

Chan F, Allison JE, Stanley AJ, Gumbreck LG. Reciprocal Transplantation of Testis Between Normal and Pseudohermaphroditic Male Rats. Fertility and Sterility 1969; 20(3): 482-494.

Clegg ED, Ettlin RA, Sinha Hikim AP, Russell LD. Histological and Histopathological Evaluation of the Testis. Clearwater FL: Cache River Press; 1990: 1-40.

Collin O, Bergh A. Leydig cells secrete factors which increase vascular permeability and endothelial cell proliferation. Int J Androl. 1996 Aug;19(4):221-8.

De Rooij DG and Russell LD. All you wanted to know about spermatogenesis but were afraid to ask. Journal of Andrology 2000; 21:776-798.

Deanesly R. Spermatogenesis and Endocrine Activity in Grafts of Frozen and Thawed Rat Testis. Journal of Endocrinology 1954; 11: 201-206.

Dobrinski I., Germ cell transplantation and testis tissue xenografting in domestic animals. Anim Reprod Sci. 2005 Oct;89(1-4):137-45. Review.

Dufour JM, Gores P, Hemendinger R, Emerich DF, Halberstadt CR., Transgenic Sertoli cells as a vehicle for gene therapy. Cell Transplant. 2004;13(1):1-6. Review.

Ehmcke J, Simorangkir DR, Schlatt S. Identification of the starting point for spermatogenesis and characterization of the testicular stem cell in adult male rhesus monkeys. Hum Reprod. 2005 May;20(5):1185-93. Epub 2005 Feb 10.

Ergun S, Kilic N, Fiedler W, Mukhopadhyay AK. Vascular endothelial growth factor and its receptors in normal human testicular tissue.Mol Cell Endocrinol. 1997 Jul 4;131(1):9-20.

Ergun S, Kilic N, Harneit S, Paust HJ, Ungefroren H, Mukhopadhyay A, Davidoff M, Holstein AF. Microcirculation and the vascular control of the testis. Adv Exp Med Biol. 1997;424:163-80. Review.

Fujita K, Ohta H, Tsujimura A, Takao T, Miyagawa Y, Takada S, Matsumiya K, Wakayama T and Okuyama A (2005) Transplantation of spermatogonial stem cells isolated from leukemic mice restores fertility without inducing leukemia. J Clin Invest doi:10.1172/JCI24189.

Gook DA, Edgar DH, Borg J, Archer J and McBain JC (2005) Diagnostic assessment of the developmental potential of human cryopreserved ovarian tissue from multiple patients using xenografting. Hum Reprod 20,72–78.

Gosden RG, Boulton MI, Grant K, Webb R. Follicular development from ovarian xenografts in SCID mice. J Reprod Fertil. 1994; 101: 619 -623.

Haggstrom Rudolfsson S, Johansson A, Franck Lissbrant I, Wikstrom P, Bergh A. Localized expression of angiopoietin 1 and 2 may explain unique characteristics of the rat testicular microvasculature. Biol Reprod. 2003 Oct;69(4):1231-7. Epub 2003 May 28. Haider SG., Cell biology of Leydig cells in the testis. Int Rev Cytol. 2004;233:181-241. Review.

Halberstadt C, Emerich DF, Gores P., Use of Sertoli cell transplants to provide local immunoprotection for tissue grafts. Expert Opin Biol Ther. 2004 Jun;4(6):813-25. Review.

Hibbitt O, Coward K, Kubota H, Prathalingham N, Holt W, Kohri K, Parrington J. In vivo gene transfer by electroporation allows expression of a fluorescent transgene in hamster testis and epididymal sperm and has no adverse effects upon testicular integrity or sperm quality. Biol Reprod. 2006 Jan;74(1):95-101. Epub 2005 Sep 14.

Honaramooz A, Li M-W, Penedo CT, Meyers SA, Dobrinski I. Accelerated maturation of primate testis by xenografting into mice. Biol Reprod. 2004; 70: 1500 -1503.

Honaramooz A, Snedaker A, Bolani M, Scholer H, Dobrinski I, Schlatt S. Sperm from neonatal mammalian testes grafted in mice. Nature 2002; 418: 778-781.

Huang Z, Tamura M, Sakurai T, Chuma S, Saito T, Nakatsuji N. In vivo transfection of testicular germ cells and transgenesis by using the mitochondrially localized jellyfish fluorescent protein gene. FEBS Lett 2000 487:248-251

Hum Reprod Update. 2004 Nov-Dec;10(6):525-32. Epub 2004 Aug 19. Review.

Huminiecki L, Chan HY, Lui S, Poulsom R, Stamp G, Harris AL, Bicknell R. Vascular endothelial growth factor transgenic mice exhibit reduced male fertility and placental rejection. Mol Hum Reprod. 2001 Mar;7(3):255-64. Erratum in: Mol Hum Reprod 2001 May;7(5):495.

Hutson JC, Garner CW, Doris PA. Purification and characterization of a lipophilic factor from testicular macrophages that stimulates testosterone production by Leydig cells. J Androl. 1996 Sep-Oct;17(5):502-8.

Ichihara I, Kawamura H, Pelliniemi LJ. Ultrastructure and morphometry of testicular Leydig cells and the interstitial components correlated with testosterone in aging rats. Cell Tissue Res. 1993 Feb;271(2):241-55.

Jahnukainen K, Hou M, Petersen C, Setchell B and Söder O (2001) Intratesticular transplantation of testicular cells from leukemic rats causes transmission of leukaemia. Cancer Res 61,706–710.

Johnson L, Falk GU, Suggs LC, Henderson DJ, Spoede GE, Brown SW, McGowen TA, Meguerditchian H, Barnard JJ. Heterotopic transplantation as a model to study the

regulation of spermatogenesis; some histomorphological considerations about sperm decline in man. Contracept Fertil Sex. 1997 Jul-Aug;25(7-8):549-55.

Johnson L, Suggs LC, Norton YM, Welsh Jr. TH, Wilker CE. Effect of Hypophysectomy, Sex of Host and or Number of Transplanted Testes on Sertoli Cell Number and Testicular Size of Syngeneic Testicular Grafts in Fisher Rats. Biology of Reproduction 1996; 54: 960-969.
Kilic N, Fiedler W, Holstein AF, Ergun S. Expression of VEGF and its receptors and capillary density in Leydig cell tumors of the human testis. Adv Exp Med Biol. 1997;424:181-2.

Kilinc F, Kayaselcuk F, Aygun C, Guvel S, Egilmez T, Ozkardes H., Experimental varicocele induces hypoxia inducible factor-1alpha, vascular endothelial growth factor expression and angiogenesis in the rat testis. J Urol. 2004 Sep;172(3):1188-91.

Korpelainen EI, Karkkainen MJ, Tenhunen A, Lakso M, Rauvala H, Vierula M, Parvinen M, Alitalo K. Overexpression of VEGF in testis and epididymis causes infertility in transgenic mice: evidence for nonendothelial targets for VEGF. J Cell Biol. 1998 Dec 14;143(6):1705-12.

Kubota H, Hayashi Y, Kubota Y, Coward K, Parrington J. Comparison of two methods of in vivo gene transfer by electroporation. Fertil Steril. 2005 Apr;83 Suppl 1:1310-8.

Lagercrantz J, Farnebo F, Larsson C, Tvrdik T, Weber G, Piehl F. A comparative study of the expression patterns for vegf, vegf-b/vrf and vegf-c in the developing and adult mouse. Biochim Biophys Acta. 1998 Jun 16;1398(2):157-63.

Langer CJ, Natale RB. The emerging role of vascular endothelial growth factor receptor tyrosine kinase inhibitors. Semin Oncol. 2005 Dec;32(6 Suppl 10):23-9.

LeCouter J, Ferrara N. EG-VEGF and the concept of tissue-specific angiogenic growth factors. Semin Cell Dev Biol. 2002 Feb;13(1):3-8. Review.

LeCouter J, Lin R, Tejada M, Frantz G, Peale F, Hillan KJ, Ferrara N. The endocrine-gland-derived VEGF homologue Bv8 promotes angiogenesis in the testis: Localization of Bv8 receptors to endothelial cells. Proc Natl Acad Sci U S A. 2003 Mar 4;100(5):2685-90. Epub 2003 Feb 25.

Liu X, Yang Y. Effect of VEGF on the angiogenesis in male reproduction system. Zhonghua Nan Ke Xue. 2004 Jan;10(1):49-51. Review. Chinese.

Loveland KL, Hedger MP, Risbridger G, Herszfeld D, De Kretser DM. Identification of receptor tyrosine kinases in the rat testis. Mol Reprod Dev. 1993 Dec;36(4):440-7.

Lyttle DJ, Fraser KM, Fleming SB, Mercer AA, Robinson AJ. Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. J Virol. 1994 Jan;68(1):84-92.

M. A. Bedell and A. M. Zama., Genetic Analysis of Kit Ligand Functions During Mouse Spermatogenesis. J Androl, March 1, 2004; 25(2): 188 - 199.

M. Kanatsu-Shinohara, H. Miki, K. Inoue, N. Ogonuki, S. Toyokuni, A. Ogura, and T. Shinohara. Germline niche transplantation restores fertility in infertile mice. Hum. Reprod., September 1, 2005; 20(9): 2376 - 2382.

Ma P, Ge Y, Wang S, Ma J, Xue S, Han D. Spermatogenesis following syngeneic testicular transplantation in Balb/c mice. Reproduction. 2004 Aug;128(2):163-70. Management and Welfare of Farm Animals. 3rd ed. UFAW (Universities Federation for Animal Welfare). 1988. London: Bailliere Tindall. 260 pp.

Marshall GR, Plant TM. Puberty occurring either spontaneously or induced precociously in rhesus monkey (Macaca mulatta) is associated with a marked proliferation of Sertoli cells. Biol Reprod. 1996;54: 1192 -1199.

Marti HH, Risau W. Systemic hypoxia changes the organ-specific distribution of vascular endothelial growth factor and its receptors. Proc Natl Acad Sci U S A. 1998 Dec 22;95(26):15809-14.

McCoard SA, Wise TH, Lunstra DD, Ford JJ. 2003a. Stereological evaluation of Sertoli cell ontogeny during fetal and neonatal life in two diverse breeds of swine. J Endocrinol. 178:395-403.

McLean DJ., Spermatogonial stem cell transplantation and testicular function. Cell Tissue Res. 2005 Oct;322(1):21-31. Epub 2005 Nov 3. Review.

Mezquita J, Mezquita B, Pau M, Mezquita C. Characterization of a novel form of angiopoietin-2 (Ang-2B) and expression of VEGF and angiopoietin-2 during chicken testicular development and regression.

Nagano M, Patrizio P, Brinster RL. Long-term survival of human spermatogonial stem cells in mouse testes. Fertil Steril. 2002 Dec;78(6):1225-33.

Nalbandian A, Dettin L, Dym M, Ravindranath N. Expression of vascular endothelial growth factor receptors during male germ cell differentiation in the mouse. Biol Reprod. 2003 Sep;69(3):985-94. Epub 2003 May 28.

Namiecinska M, Marciniak K, Nowak JZ. VEGF as an angiogenic, neurotrophic, and neuroprotective factor. Postepy Hig Med Dosw (Online). 2005;59:573-83.

Neill JD., The Physiology of Reproduction: Section 3, Volumes 1 and 2, Raven Press, 2003.

Oatley JM, de Avila DM, Reeves JJ, McLean DJ., Spermatogenesis and germ cell transgene expression in xenografted bovine testicular tissue. Biol Reprod. 2004 Aug;71(2):494-501. Epub 2004 Apr 7.

Oatley JM, Reeves JJ, McLean DJ., Establishment of spermatogenesis in neonatal bovine testicular tissue following ectopic xenografting varies with donor age. Biol Reprod. 2005 Feb;72(2):358-64. Epub 2004 Oct 6.

Ohta H and Wakayama Y (2004) Full-term development of offspring using round spermatids produced ectopically from fetal male germ cells. J Reprod Dev 50,429–437.

Ohta H, Yomogida K, Dohmae K, Nishimune Y. Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF. Development 2000 127:2125-2131

Olive V, Cuzin F., The spermatogonial stem cell: from basic knowledge to transgenic technology. Int J Biochem Cell Biol. 2005 Feb;37(2):246-50. Review.

Orwig KE, Schlatt S., Cryopreservation and transplantation of spermatogonia and testicular tissue for preservation of male fertility. J Natl Cancer Inst Monogr. 2005;(34):51-6. Review.

Ozeki M, Tabata Y. In vivo promoted growth of mice hair follicles by the controlled release of growth factors. Biomaterials. 2003 Jun;24(13):2387-94.

Parvinen P. Cyclic function of Sertoli cells. In: Russell LD, Griswold MD (eds.), The Sertoli Cell. Clearwater, FL: Cache River Press; 1993: 331–347

pcDNA3.1 Manual. Catalog nos. V790-20 and V795-20, respectively. Version I, 081401, 28-0104. Invitrogen. 200

Plant TM. Puberty in primates. In: Knobil E, Neill JD, eds. The Physiology of Reproduction. 2nd ed. New York: Raven Press, 1994: 453-485.

Pukazhenthi BS, Wildt DE, Howard JG. The phenomenon and significance of teratospermia in felids. J Reprod Fertil Suppl. 2001;57: 423 -433.

Reis MM, Tsai MC, Schlegel PN, Feliciano M, Raffaelli R, Rosenwaks Z, Palermo GD. Xenogeneic transplantation of human spermatogonia. Zygote. 2000 May;8(2):97-105.

Rudolfsson SH, Wikstrom P, Jonsson A, Collin O, Bergh A. Hormonal regulation and functional role of vascular endothelial growth factor a in the rat testis. Biol Reprod. 2004 Feb;70(2):340-7. Epub 2003 Oct 15.

Russell LD, Ettlin RA, Sinha-Hikim AP, Clegg ED. Histological and histopathological evaluation of the testis . Vienna, Ill: Cache River Press; 1990.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition. (Plainview, New York: Cold Spring Harbor Laboratory Press).

Samson M, Peale FV Jr, Frantz G, Rioux-Leclercq N, Rajpert-De Meyts E, Ferrara N. Human endocrine gland-derived vascular endothelial growth factor: expression early in development and in Leydig cell tumors suggests roles in normal and pathological testis angiogenesis. J Clin Endocrinol Metab. 2004 Aug;89(8):4078-88.

Sato M, Ishikawa A, Kimura M. Direct injection of foreign DNA into mouse testis as a possible in vivo gene transfer system via epididymal spermatozoa. Mol Reprod Dev. 2002 Jan;61(1):49-56.

Saunders PT., Germ cell-somatic cell interactions during spermatogenesis. Reprod Suppl. 2003; 61:91-101. Review.

Schlatt S, Honaramooz A, Boiani M, Schöler HR, Dobrinski I. Progeny from sperm obtained after ectopic grafting of neonatal mouse testes. Biol Reprod. 2003; 68: 2331 - 2335.

Schlatt S, Kim SS and Gosden R (2002) Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and heterotopic grafting to castrated hosts. Reproduction 124,339–346.

Schlatt S, Rosiepen G, Weinbauer GF, Rolf C, Brook PF and Nieschlag E (1999) Germ cell transfer into rat, bovine, monkey and human testes. Hum Reprod 14,144–150.

Schmahl J, Capel B. Cell proliferation is necessary for the determination of male fate in the gonad. Dev Biol. 2003 Jun 15;258(2):264-76.

Schwarzenbach H, Chakrabarti G, Paust HJ, Mukhopadhyay AK. Gonadotropin-mediated regulation of the murine VEGF expression in MA-10 Leydig cells. J Androl. 2004 Jan-Feb;25(1):128-39.

Senger PL., Pathways to Pregnancy and Parturition, 2nd edition. Current Conceptions, Inc., 2003.

Sharpe R. Experimental evidence for Sertoli-germ cell and Sertoli-Leydig cell interaction. In: Russell LD, Griswold MD (eds.), The Sertoli Cell. Clearwater, FL: Cache River Press; 1993: 391–418

Sharpe RM. The Physiology of Reproduction Second Edition. New York NY: Raven Press Ltd; 1994: 1363-1434.

Shibuya, M. and Claesson-Welsh L. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. Experimental Cell Research. V312, Issue 5 , 10 March 2006, Pages 549-560

Shifren JL, Doldi N, Ferrara N, Mesiano S, Jaffe RB. In the human fetus, vascular endothelial growth factor is expressed in epithelial cells and myocytes, but not vascular endothelium: implications for mode of action. J Clin Endocrinol Metab. 1994 Jul;79(1):316-22.

Shigekawa, K., and Dower, W. J. (1988). Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells. BioTechniques 6, 742-751.

Shinohara T, Inoue K, Ogonuki N, et al. Birth of offspring following transplantation of cryopreserved immature testicular pieces and invitro microinsemination. Hum Reprod. 2002; 17: 3039 -3045.

Shinohara T, Inoue K, Ogonuki N, Kanatsu-Shinohara M, Miki H, Nakata K, Kurome M, Nagashima H, Toyokuni S, Kogishi K, Honjo T, Ogura A. Birth of offspring following transplantation of cyropreserved immature testicular pieces and in-vitro microinsemination. Human Reproduction 2002; 17(12): 3039-3045.

Silva AR, Morato RG, Silva LDM. The potential for gamete recovery from non-domestic canids and felids. Anim Reprod Sci. 2004; 81: 159 -175.

Skinner M K and Griswold M D, (eds.). Sertoli Cell Biology. 494 pp. Elsevier Academic Press, San Diego, 2005.

Sluka P, O'donnell L, Bartles JR, Stanton PG. FSH regulates the formation of adherens junctions and ectoplasmic specialisations between rat Sertoli cells in vitro and in vivo. J Endocrinol. 2006 May;189(2):381-395.

Sugiyama N, Obinata M, Matsui Y. Bcl-2 inhibits apoptosis of spermatogonia and growth of spermatogonial stem cells in a cell-intrinsic manner. Mol Reprod Dev. 2001 Jan;58(1):30-8.

Tournaye H, Goossens E, Verheyen G, Frederickx V, De Block G, Devroey P, Van Steirteghem A.,Preserving the reproductive potential of men and boys with cancer: current concepts and future prospects.

Tunckiran A, Cayan S, Bozlu M, Yilmaz N, Acar D, Akbay E. Protective effect of vascular endothelial growth factor on histologic changes in testicular ischemia-reperfusion injury. Fertil Steril. 2005 Aug;84(2):468-73.

Turner CD. Intra-ocular Homotransplantation of prepuberal testes in the rat. The American Journal of Anatomy 1938; 63(1): 101-159.

Uchida K, Takahashi A, Miyao N, Takeda K, Tsutsumi H, Satoh M, Tsukamoto T. Juvenile hemangioma of the testis: analysis of expression of angiogenic factors. Urology. 1997 Feb;49(2):285-6.

Umemoto Y, Sasaki S, Kojima Y, Kubota H, Kaneko T, Hayashi Y, Kohri K. Gene transfer to mouse testes by electroporation and its influence on spermatogenesis. J Androl. 2005 Mar-Apr;26(2):264-71.

Viglietto G, Romano A, Maglione D, Rambaldi M, Paoletti I, Lago CT, Califano D, Monaco C, Mineo A, Santelli G, Manzo G, Botti G, Chiappetta G, Persico MG. Neovascularization in human germ cell tumors correlates with a marked increase in the expression of the vascular endothelial growth factor but not the placenta-derived growth factor. Oncogene. 1996 Aug 1;13(3):577-87.

Wagener A, Blottner S, Goritz F, Fickel J. Detection of growth factors in the testis of roe deer (Capreolus capreolus). Anim Reprod Sci. 2000 Dec 1;64(1-2):65-75.

Weiss, L., Cell and Tissue Biology: A Textbook of Histology, 6th Edition, Urban and Schwarzenberg, Baltimore, 1988.

Widlak W, Scieglinska D, Vydra N, Malusecka E, Krawczyk Z. In vivo electroporation of the testis versus transgenic mice model in functional studies of spermatocyte-specific hst70 gene promoter: A comparative study. Mol Reprod Dev. 2003 Aug;65(4):382-8.

Wistuba J, Luetjens CM, Wesselmann R, Nieschlag E, Simoni M, Schlatt S. Meiosis in Autologous Ectopic Transplants of Immature Testicular Tissue Grafted to Callithrix jacchus. Biol Reprod. 2005 Dec 21;

Wistuba J, Mundry M, Luetjens CM and Schlatt S (2004) Cografting of hamster (Phodopus sungorus) and marmoset (Callithrix jacchus) testicular tissues into nude mice does not overcome blockade of early spermatogenic differentiation in primate grafts. Biol Reprod 71,2087–2091

Yamazaki Y, Fujimoto H, Ando H, Ohyama T, Hirota Y, Noce T. In vivo gene transfer to mouse spermatogenic cells by deoxyribonucleic acid injection into seminiferous tubules and subsequent electroporation. Biol Reprod 1998 59:1439-1444

Yamazaki Y, Yagi T, Ozaki T, Imoto K. In vivo transfer to mouse spermatogenic cells using green fluorescent protein as a marker. J Exp Zool 2000 286:212-218

Yomogida K, Ohtani H, Harigae H, Ito E, Nishimune Y, Engel JD, Yamamoto M. Developmental stage- and spermatogenic cycle-specific expression of transcription factor GATA-1 in mouse Sertoli cells. Development 1994 120:1759-1766

Yomogida K, Yagura Y, Nishimune Y. Electroporated transgene-rescued spermatogenesis in infertile mutant mice with a sertoli cell defect. Biol Reprod. 2002 Sep;67(3):712-7.

Young KA, Nelson RJ. Short photoperiods reduce vascular endothelial growth factor in the testes of Peromyscus leucopus. Am J Physiol Regul Integr Comp Physiol. 2000 Sep;279(3):R1132-7.

Zhang H, Toyofuku T, Kamei J, Hori M. GATA-4 regulates cardiac morphogenesis through transactivation of the N-cadherin gene. Biochem Biophys Res Commun. 2003 Dec 26;312(4):1033-8

CHAPTER 3

VASCULAR ENDOTHELIAL GROWTH FACTOR-A PROMOTES GERM CELL SURVIVAL AND DIFFERENTIATION IN BOVINE TESTIS XENOGRAFTS

Abstract

Ectopic testis tissue xenografting provides a useful model for investigating factors that regulate germ cells and the supporting somatic cells in the mammalian testis. The role for VEGF as an endothelial cell mitogen has been well documented and recent work has suggested non-endothelial cell targets for VEGF-A in testicular germ cells. In addition, VEGF treatment of bovine testis xenografts at the time of grafting resulted in larger testis grafts that had more spermatids when compared to controls. Furthermore, the number of blood vessels in VEGF treated grafts was not significantly greater than controls. The objective of this study was to determine how VEGF regulates germ cell differentiation in the bovine testis. To accomplish this objective, we used the experimental approach of treating bovine testis tissue in culture with VEGF prior to grafting to determine if VEGF actions on germ cell differentiation were independent of blood vessel growth into the grafted tissue. We hypothesized that treating bovine testis tissue with VEGF in explant cultures prior to grafting would result in more differentiating germ cells in the testis tissue. Testicular parenchyma obtained from 4- and 8-wk bovine donors were placed in explant culture on floating filters for 5days prior to grafting, and treated every two days with recombinant mouse VEGF-164 (rmVEGF; 0, 100, 200 ng/ml) with media change. Following culture and treatment, testis tissues were grafted onto the backs of immunodeficient mice. Recipient mice were sacrificed when testis xenografts reached a tissue age of 32 weeks. Following sacrifice, grafts were weighed and evaluated for the extent of germ cell differentiation. Mouse vesicular gland weights were recorded as a bioassay for androgen synthesis by testis grafts. No differences existed between donor age and grafting period so these datasets were pooled.

Seminiferous tubules of testis grafts treated with VEGF contained more differentiating germ cell as evident by a greater percentage of meiotic germ cells and spermatids when compared to controls ($p < 0.05$). However, no differences were detected in the growth and androgen biosynthesis of testis grafts. These data provide direct evidence that VEGF has a functional role in promoting germ cell survival and differentiation in bovine testis xenografts. The underlying mechanisms responsible for the VEGF-induced germ cell differentiation are the focus of future experiments.

Introduction

Spermatogenesis includes the proliferation, meiotic division, and morphological differentiation of germ cells within the basal and adluminal compartments of seminiferous tubules of the testis leading to the production of spermatozoa, the male gamete. The regulation of germ cell differentiation is mainly orchestrated by cell-cell interactions with Sertoli cells [1] and extracellular signals including endocrine [2] and locally produced factors [3]. This process is initiated around puberty and results in sustained sperm production and potential fertility throughout the lifespan of an adult male. A key component of this process is the continual maintenance of the spermatogonial stem cell (SSC) niche in the seminiferous tubule to provide differentiating spermatogonia [4,5]. However, the mechanisms governing the self-renewal and differentiation of SSCs remain unclear.

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a potent angiogenic factor involved in promoting endothelial cell proliferation, survival, and differentiation [6]. The VEGF family is encoded by five different genes:

VEGFA, VEGFB, VEGFC, VEGFD, and placenta growth factor (PlGF) of which, VEGF-A has the most profound effects on tissue neovascularization and appears to be the only pro-angiogenic factor expressed throughout tumor growth and metastasis [6]. The VEGFA gene can undergo alternative splicing to form five different isoforms: VEGFA-205, VEGFA-188, VEGFA-164, VEGFA-144, and VEGFA-120 [7]

VEGFA isoforms stimulate endothelial cells through two membrane-bound receptor tyrosine kinases, Flt-1 (also known as VEGFR1) and KDR/Flk-1 (also known as VEGFR2) that share cognate cytoplasmic signaling domains. However, their downstream signals elicit distinctive cellular responses following binding of VEGF-A in endothelial cells. VEGFA binding to FLT-1 stimulates cell-cell interactions and capillary tube formation [8,9] whereas VEGFA binding to KDR initiates classical signaling involved in proliferation, survival, and differentiation process [8,9]. The majority of VEGF-A functions are mediated through KDR binding that results in receptor autophosphorylation and intracellular signaling [10].

VEGFA regulates cell processes outside of tissue and tumor neovascularization. For example, research has demonstrated the importance of VEGF-A, Flt-1, and KDR in biological processes such as embryogenesis [11], folliculogenesis [12] and seminiferous cord formation in the embryonic testis [13]. Data also suggests potential functional roles for VEGFA in the post-natal testis, as KDR and Flt-1 are expressed in testicular germ cells in humans [14], rats [15], and mice [16], while somatic Sertoli and Leydig cells produce VEGFA [17]. Furthermore, several studies have shown detrimental affects on male fertility in transgenic mice overexpressing VEGFA-120 [18] and VEGFA-165 [19]. Mutant mice lacking KDR or Flt-1 have provided little insight to the role of VEGFA in

the testis because embryonic lethality (E9–10) occurs before testis development [19, 20]. Accordingly, targeted inactivation of a single VEGF allele also results in embryonic death *in utero* [20]. A study in our lab revealed that subcutaneously treating bovine testis tissue at the ectopic graft site with exogenous VEGFA-164, the most potent isoform of VEGFA, resulted in larger testis grafts that had more spermatids when compared to controls [21].

The objective of the current study was to determine if VEGFA, and its receptors, are important in regulating germ cell survival and differentiation in the bovine testis. To accomplish this objective, we used the experimental approach of treating bovine testis tissue in culture with VEGFA prior to grafting to determine if VEGF actions on germ cell physiology were independent of blood vessel growth into the grafted tissue. We hypothesized that treating bovine testis tissue with VEGF in explant cultures prior to grafting would result in (1) more germ cell survival, and (2) a greater number of differentiating germ cells in the testis tissue when compared to controls. Both outcomes will be validated utilizing ectopic testis tissue xenografting [22, 23] as an assay for germ cell differentiation associated with the onset of pubertal age in bulls, 32 weeks.

Here, we examine the role of VEGFA in the regulation of germ cell survival and differentiation; two processes essential for sperm production, and in this approach, explore mechanisms involved with the paracrine regulation of spermatogenesis in the bull.

Materials and Methods

Chemicals and Reagents

All reagents were purchased from Sigma (www.sigmaaldrich.com) unless otherwise stated.

Donor Bulls and Recipient Mice

The Washington State University Animal Care and Use Committee approved all animal procedures. Angus bull calves of known parentage and birthdates were obtained from the Washington State University Beef Center. Recipient immunodeficient NCr nude mice (Taconic, http://www.taconic.com; CrTac:NCR-Fox1<nu>) were raised under normal conditions and standard rodent chow provided ad libitum. Immunodeficiency in this mouse strain stems from an abnormal thymus due to homozygosity of the autosomal recessive nude gene (nu/nu).

Tissue Collection

Testis tissue samples were obtained from ten 4-wk-old bull calves and ten 8-wk-old bull calves using standard castration protocols. Testes were immediately placed in Hanks Balanced Salt Solution (HBSS) on ice. In the laboratory, the testes were detunicated and parenchymal tissue was cut into 3 mg pieces and maintained in HBSS until culture. In addition, pieces of testicular parenchyma were placed in Bouin's fixative for 4 h at 4°C followed by dehydration and storage in 70% ethanol for eventual morphological evaluation and comparison.

Testicular Tissue Explant Culture

Testicular parenchyma obtained from 4- and 8-wk bovine donors were placed in explant culture on 0.45-μm pore floating filter membranes (Millipore, Bedford, MA) for 5 days prior to grafting. Tissue explants were cultured at 32°C in Dulbecco modified Eagle medium [DMEM] containing 10% fetal bovine serum, 30 mg/ml penicillin, and 50 mg/ml streptomycin including recombinant mouse VEGF-164 (rmVEGF164; R & D Systems, http://www.rndsystems.com); at 0 (control), 100, or 200 ng/ml. Media was changed every 2 days including fresh addition of rmVEGF. Eight grafts per donor (n = 13) were treated with rmVEGF164 (24 grafts/donor) at each concentration and each experiment was conducted in duplicate (Figure 1).

Ectopic Testis Tissue Xenografting

After 5 days in culture, four pieces of testis tissue were ectopically grafted onto castrated immunodeficient nude mice. A total of six recipient mice were grafted with testis tissue from each donor. Briefly, mice were anesthetized with ketamine (0.1 mg/kg body weight [BW]) and xylazine (0.5 mg/kg BW) castrated using surgical procedures as previously described [22]. The incisions were closed using absorbable suture (Ethicon, http://www.novartis.com) and the mice were allowed to recover before being returned to their cages.

Analysis of Donor Grafts and Recipient Mice

Recipient mice were sacrificed using CO2 inhalation and cervical dislocation when grafted bovine testis reached a tissue age of 32-wks which was determined using

the birth dates of donor calves. Testis grafts were removed, weighed and fixed in Bouin fixative at 4°C for 4 h prepared for histology, blocked in paraffin and sectioned at 8 μm. Sections were affixed, deparaffinized, rehydrated, stained with hematoxylin and eosin and evaluated using light microscopy at 400x magnification. Tissues were visually evaluated to determine the average number of grafts containing seminiferous tubules with spermatogonia, meiotic germ cells, differentiating spermatids, or only Sertoli cells. Digital images were captured using a Leica DFC 280 camera and a Leica DME compound microscope (Leica Microsystems Imaging Solutions Ltd., http://www.leica-microsystems.com) at 400x magnification. Seminiferous tubule cross-section number and graft weight were recorded as measure for tissue growth by testis grafts. In addition, at sacrifice, blood was collected from the recipient mice by cardiac puncture; serum was isolated for later use. Mouse vesicular glands were removed, weighed, and recorded as a bioassay for androgen synthesis by testis grafts.

Statistical Analysis

Germ cell proportion and seminiferous tubule cross-section number was averaged per mouse to prevent unequal weighting due to variation in these traits. All statistical comparisons were performed using the Statistical Analysis System (SAS) software, version 9.13 (SAS Institute, Cary, North Carolina, USA). Data are presented as the mean ± SEM; non-parametric data are also presented as Wilcoxon mean scores. For all datasets, differences between treatments were considered significant at the $P \leq 0.05$ level. No differences existed between donor age (4- vs. 8-wk) and grafting period so these datasets were pooled.

Analysis of differences between means for testicular tissue graft growth, spermatogenesis, survival, and recipient mouse vesicular gland weight were performed using the Proc GLM function of SAS. Pairwise comparisons were evaluated between groups using the Duncan's Multiple Range test for significance. The datasets for germ cell proportion did not meet two of the statistical assumptions required for analysis of variance (ANOVA; normality and homogeneity of variance), and standard transformations did not result in normality or homogeneity. Therefore, a nonparametric ANOVA using the Proc NPAR1WAY function of SAS (Kruskal-Wallis test) was used to determine the significance of treatment effect on germ cell proportions within seminiferous tubule cross-sections and bovine testis xenografts. If the latter test was significant, pairwise comparisons between groups (e.g., individual tests of VEGF treatment level and germ cell proportion) were evaluated using Mann-Whitney tests.

Results

Testicular parenchyma obtained from bovine donors was cultured as tissue explants for 5 days. Testis tissue explants were treated with rmVEGF164 (VEGF) at 100 or 200 ng/ml. Control testis tissue cultures were treated with vehicle only. Following culture, testis tissue was grafted onto nude mice and removed when testis tissue age equaled 32 wks. Recovered bovine testis tissue grafts were categorized as functional based on evidence of active germ cell differentiation in seminiferous tubules visualized by microscopy. All data reporting germ cell numbers and the proportion of seminiferous tubules containing specific germ cell populations were obtained from testis tissue grafts characterized as functional. Analysis of the seminiferous epithelium in each graft to

determine the presence of specific germ cell types and other analysis criteria was conducted using the largest cross section of each testis graft tissue. Additionally, the number of seminiferous tubule cross sections containing only Sertoli cells was used as a functional assay of germ cell survival within bovine testis grafts.

VEGF treatment of bovine testis tissue explants prior to ectopic xenografting

Testis tissue growth and survival was the first criteria use to evaluate the effect of VEGF treatment on the bovine testis xenografts (Fig. 1 and 2). No differences were observed in the number of functional grafts recovered between testicular parenchyma cultured in the presence or absence of VEGF (Fig. 2). Additionally, VEGF had no effect on graft growth, as no differences were observed in graft weight (Fig. 3A) or seminiferous tubule cross-section number (Fig. 3B). To determine the effect of VEGF on Leydig cell function, we removed and weighed recipient mouse vesicular glands to assess the ability of bovine testis grafts to produce biologically active testosterone. No significant differences in vesicular gland weights were observed between treatment groups indicating that VEGF did not affect androgen biosynthesis by testis tissue grafts (Fig. 4).

VEGF treatment increased the proportion of functional bovine testis grafts undergoing complete germ cell differentiation.

To determine if culturing bovine testis tissue with VEGF prior to grafting had an effect on quantitative spermatogenesis we evaluated the number of germ cells in each testis graft and converted this to germ cell proportions in functional testis grafts.

Regardless of treatment, all testis xenografts contained spermatogonia and meiotic germ cells. Interestingly, bovine testis tissue cultured with VEGF had significantly more ($P<0.05$) seminiferous tubules containing differentiating germ cells compared to controls (Fig. 5). VEGF treatment at 100 ng/ml resulted in 36% of testis grafts to support complete germ cell differentiation as determined by the presence of haploid spermatids, significantly more ($P<0.05$) when compared to controls (4.8%). Similarly, a total of 40.9% of grafts in the 200 ng/ml VEGF treatment group contained spermatids. Establishment of spermatogenesis was similar in testis tissue treated with VEGF at 100 ng/ml and 200 ng/ml respectively (Fig. 5).

VEGF treatment increases germ cell differentiation in bovine testis xenografts.

In order to determine the effect of VEGF treatment on germ cell survival and differentiation within bovine testis xenografts, seminiferous tubules of each graft were evaluated. Determination of germ cell survival was based on the presence of spermatogonia or only Sertoli cells within each testis tissue graft. The number of seminiferous tubule cross-sections containing spermatogonia was significantly increased ($P<0.05$) in testis tissue cultures treated with VEGF (Fig 6A) when compared to vehicle. In contrast to controls (55.2%), VEGF treatment at 100 ng/ml resulted in 23.6% more tubules containing spermatogonia in testis grafts. Similarly, a 32.9% increase in germ cell survival was observed in testis tissue treated with VEGF at 200 ng/ml when compared to vehicle. The inverse relationship was observed for the proportion of seminiferous tubule cross sections containing only Sertoli cells (Fig. 6B). Hence, germ cell death is significantly reduced ($P<0.05$) in bovine testis tissue cultured with VEGF prior to ectopic

grafting (Fig 6C and 6D). Photomicrograph cross-sections comparing germ cell survival is represented by the presence of no germ cells (Fig. 7A) or spermatogonia (Fig. 7B).

To assess the extent of germ cell differentiation between control and treatment groups, seminiferous tubule cross-sections containing spermatogonia were classified based on the existence of meiotic germ cells (primary and/or secondary spermatocytes) and haploid spermatids (round, elongating, and/or elongate spermatids) as can be seen in Figure 7C. Compared to controls, seminiferous tubules of bovine testis grafts cultured with VEGF contained more differentiated germ cells as evident by a greater percentage of meiotic germ cells and spermatids when compared to controls ($P < 0.05$; Fig. 8A, 8B). Comparison of germ cell types within testis grafts demonstrated that accelerated germ cell differentiation occurred in VEGF treated testis tissue when compared to vehicle alone ($P < 0.05$; Fig. 8C, 8D).

Discussion

The purpose of this research was to investigate the role of VEGF in the regulation of germ cell survival and differentiation; two processes essential for sperm production. Previous work in our lab demonstrated increased spermatogenesis in bovine testis tissue treated with VEGF. In that study, testis xenografts treated with 1.0 μg of VEGF at the ectopic graft site of the recipient mouse were larger in size, with a higher percentage of seminiferous tubules containing elongating spermatids [21]. No significant difference was observed in blood vessel number in treated and control samples. Therefore, we concluded that VEGF was influencing the survival and/or differentiation of germ cells in the testis tissue. To determine the specific action of VEGF on testicular cells to stimulate

germ cell differentiation, we used the experimental approach of treating bovine testis tissue in culture with VEGF or vehicle prior to grafting. This allowed us to accomplish the objective of determining if VEGF effects on germ cell homeostasis were independent of blood vessel growth into the grafted tissue.

To determine the effect of VEGF on germ cell production in bovine testes, we grafted testis tissue that was treated with VEGF in cultured on the backs of immunodeficient mice. Ectopic testis tissue xenografting is a technique that provides a biological assay of germ and somatic cell proliferation, and differentiation events in response to various treatments [22, 23, 25, 26]. Relationships between somatic cells and germ cells are maintained and development is highly conserved between tissue grafts and *in situ* testis [27,28], this is a representative model to study factors involved with the onset and maintenance of spermatogenesis in various livestock species. Also, as seminiferous tubules within grafts are finitely organized due to blockages in the tubule network, SSC colonies cannot theoretically repopulate the entirety of the testis graft, as in the *in vivo* testis [27]. Thus, ectopic testis tissue grafting is a good model to investigate germ cell survival throughout postnatal testis development.

Culturing bovine testis tissue explants with VEGF prior to grafting did not affect growth, survival or androgen biosynthesis by the testis tissue when compared to controls. Culturing testis tissue as explants prior to grafting results in smaller grafts, with fewer seminiferous tubules when compared to tissue grafted shortly after collection [21]. This is likely due to tissue necrosis during the 5-day culture period. The results of this study of testis tissue graft growth following 5-day culture were consistent with previous explant culture experiments [21]. Likewise, the number of seminiferous tubule cross-sections

within testis grafts treated with VEGF or control did not differ. By contrast, there was a difference in graft weight between VEGF-treated and control grafts. The number of seminiferous tubules was similar in treated and control grafts. Thus, the variation in graft weight could be due to unaccounted differences in mouse fibroblast infiltration or somatic cell proliferation. This contrast is not surprising as our current approach aimed to minimize the affect of VEGF on neovascularization within the graft site of the recipient mouse.

Our previous findings that VEGF increased spermatogenesis in testis grafts are extended by this study [21]. All bovine testis tissue xenografts contained spermatogonia and meiotic germ cells. However, culturing bovine testis explants with VEGF treatment at either 100 or 200 ng/ml resulted in greater than 7-fold increase ($p<0.05$) in the number of grafts producing haploid spermatids. Evaluating pooled distributions of seminiferous tubule cross-sections with or without germ cells suggest that VEGF treatment significantly increased ($p<0.05$) quantitative germ cell survival. The number of differentiated germ cells within distributions of seminiferous tubule cross-sections containing spermatogonia were enumerated based on the presence of meiotic germ cells and haploid spermatids. VEGF treatment considerably increased the extent of germ cell differentiation, evident by more meiotic germ cells and spermatids ($P < 0.05$), when compared to controls.

VEGF signaling is mediated through KDR and Flt-1, two receptor tyrosine kinases (RTK). Upon VEGF binding to KDR, tyrosine residues are autophosphorylated, allowing receptor association with a variety of signal transduction pathways [9, 10]. In endothelial cells (EC), KDR activation rapidly induces several signaling cascades

including PI3K, PLC-γ, MAPK, and Src involved in promoting cellular proliferation, survival, and the differentiation events [7-9]. Ligand binding to Flt-1 is can regulate cell-cell interactions and attenuate downstream signaling events mediated by KDR. A soluble variant of Flt-1 is also produced as a result of alternative splicing and functions primarily as a decoy receptor, acting to sequester VEGF and block signal transduction by KDR [7,10].

Signaling pathways stimulated by VEGF have been implicated in germ cell homeostasis [37] and several studies have demonstrated the importance of VEGF for male fertility [13, 18-19]. Accordingly, testicular germ cells of several species express KDR and Flt-1 [14-16] and although these receptors are expressed in EC, no active angiogenesis takes place within the postnatal testis. Leydig and Sertoli cells both produce VEGF [17]. VEGF synthesis by Leydig cells is promoted in response to hCG/LH [15] and recent evidence suggest that FSH triggers Sertoli cell production of VEGF [17, 24, 41]. Interestingly, FSH has also been shown to stimulate proliferation of spermatogonia [34], thus FSH could be acting downstream to regulate germ cell proliferation by regulating synthesis and secretion by VEGF. Another Sertoli cell product synthesized in response to FSH is kit-ligand, and upon binding to spermatogonia expressing *c-kit* (RTK in the same family as KDR) triggers a PI3K cascade leading to proliferation of those cells [37-39]. However, germ cell transplants from mutant mice lacking *c-kit* receptor have demonstrated that c-kit/kit-ligand signal transduction is not involved in the initial step of SSC differentiation [40]. Glial cell line-derived neurotrophic factor (GDNF), also secreted by Sertoli cells, is known to regulates the fate of spermatogonial stem cell (SSC) populations to either self-renew or differentiate [30].

GDNF binds to the RET/GFRα1 tyrosine kinase receptor complex on the surface of SSCs [31], resulting in downstream activation of *Bcl6b,* a repressor protein critical for maintenance of the stem cell niche in rodents [32] and germ cell survival in bulls [33]. Signal transduction by RET and GFRα1 is highly comparable to KDR activated cascades following ligand binding. Moreover, GDNF and VEGF share functional roles for promoting astrocyte survival in neuronal tissue [35-36].

VEGF binding to KDR initiates a variety of signaling cascades similar to those exerted by GDNF and c-kit in the postnatal testis, two factors of known importance for regulating germ cell proliferation, survival and differentiation [30-34, 37-40]. Further, VEGF produced locally by Sertoli cells [41] is in close association with spermatogonia expressing KDR in the cytoplasm [16]. These data taken together with our recent observations in bovine testis grafts support the hypothesis that VEGF produced by Sertoli cells in the testis could act in a paracrine manner to regulate germ cell homeostasis and extends the potential for exogenous manipulate testis tissue during explant culture prior to ectopic grafting.

The increased potential of VEGF-cultured testis tissue grafts to undergo quantitative spermatogenesis could result from an increased population of spermatogonia in the testis tissue as a result of increased proliferation, decreased apoptosis, or both as in the case of endothelial cells. Thus, evaluation of SSC populations during explant culture in regard to 5-bromodeoxyuridine (BrdU) incorporation and positive TdT-mediated dUTP Nick-End Labeling (Tunnel) staining should be conducted. Accordingly, the underlying mechanisms responsible for VEGF-promoted germ cell differentiation in the postnatal testis should also be the focus of future experiments.

Knowledge regarding how VEGF regulates germ cell homeostasis in the postnatal testis will improve the current understanding of male reproductive biology, and lead to the development of novel reproductive strategies to optimize sperm production in bulls. Also, since VEGF promotes germ cell survival our data advocates improvements in the efficiency for male germ-line preservation. Cryopreservation of bovine SSCs could foster the ability to continually produce sperm from high genetic merit bulls long after death. Further, because VEGF is important for male fertility, we suggest that selectively blocking VEGF signaling in the testis may provide a novel means for effect hormone-independent male contraception.

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References

1. Russell LD, Griswold MD, eds. *The Sertoli Cell*. Clearwater, Fla: Cache River Press; 1993:577 -595.

2. Zirkin BR. Spermatogenesis: its regulation by testosterone and FSH. Semin Cell Dev Biol. 1998 Aug;9(4):417-21. Review.

3. Parvinen M, Ventela S. Local regulation of spermatogenesis: a living cell approach Hum Fertil (Camb). 1999;2(2):138-142.

4. Mclean DJ. Spermatogonial stem cell transplantation and testicular function. Cell Tissue Res 2005; 322:21–31.

5. Oakberg EF. Spermatogonial stem-cell renewal in the mouse. Anat Rec. 1971 Mar;169(3):515-31.

6. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol. 2005 Feb 10;23(5):1011-27. Epub 2004 Dec 7. Review.

7. Yamazaki Y, Morita T. Molecular and functional diversity of vascular endothelial growth factors. Molecular Diversity (2006) 10: 515–527

8. Tammela T, Enholm B, Alitalo K, Paavonen K. The biology of vascular endothelial growth factors. Cardiovasc Res. 2005 Feb 15;65(3):550-63. Review.

9. Takahashi H, Shibuya M. The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. Clin Sci (Lond). 2005 Sep;109(3):227-41. Review.

10. Mac Gabhann F, Popel AS. Differential binding of VEGF isoforms to VEGF receptor 2 in the presence of neuropilin-1: a computational model. Am J Physiol Heart Circ Physiol. 2005; 288(6):H2851-60.

11. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996; 380:435–439. 29)

12. Shimizu T. Promotion of ovarian follicular development by injecting vascular endothelial growth factor (VEGF) and growth differentiation factor 9 (GDF-9) genes. J Reprod Dev. 2006 Feb;52(1):23-32.

13. Bott RC, McFee RM, Clopton DT, Toombs C, and Cupp AS. Vascular Endothelial Growth Factor and Kinase Domain Region Receptor Are Involved in Both Seminiferous

Cord Formation and Vascular Development During Testis Morphogenesis in the Rat. Biol Reprod. 2006; 75:56-67.

14. Ergun S, Kilie N, Fiedler W, Mukhopadhyay AK. Vascular endothelial growth factor and its receptors in normal human testicular tissue. Mol Cell Endocrinol. 1997; 131:9-20.

15. Rudolfsson SH, Wilkstrom P, Jonsson A, Collin O, Bergh A. Hormonal regulation and functional role of vascular endothelial growth factor A in the rat testis. Biol Reprod. 2004; 70:340-347.

16. Nalbandian A, Dettin L, Dym M, Ravindranath N. Expression of vascular endothelial growth factor receptors during male germ cell differentiation in the mouse. Biol Reprod.2003; 69:985-994.

17. Liu X, Yang Y. Effect of VEGF on the angiogenesis in male reproduction system Zhonghua Nan Ke Xue. 2004 Jan;10(1):49-51. *Chinese*

18. Huminiecki L, Chan HY, Lui S, Poulsom R, Stamp G, Harris AL, Bicknell R. Vascular endothelial growth factor transgenic mice exhibit reduced male fertility and placental rejection. Mol Hum Reprod. 2001 Mar;7(3):255-264

19. Korpelainen EI, Karkkainen MJ, Tehunen A, Lakso M, Rauvala H, Vierula M, Parvinen M, Alitalo K. Overexpression of VEGF in the testis and epididymis causes infertility in transgenic mice: evidence for nonendothelial targets for VEGF. J Cell Biol. 1998; 143:1705-1712

20. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996; 380:439–442.

21. Schmidt JA, de Avila JM, McLean DJ. Effect of vascular endothelial growth factor and testis tissue culture on spermatogenesis in bovine ectopic testis tissue xenografts. Biol Reprod. 2006 Aug;75(2):167-75.

22. Oatley JM, Reeves JJ, McLean DJ. Establishment of spermatogenesis in neonatal bovine testicular tissue following ectopic xenografting varies with donor age. Biol Reprod. 2005; 72:358-364.

23. Schmidt JA, de Avila JM, McLean DJ. Grafting period and donor age affect the potential for spermatogenesis in bovine ectopic testis xenografts. Biol Reprod. 2006 Aug;75 (2):160-6.

24. Reisinger K, Baal N, McKinnon T, Munstedt K, Zygmunt M. The gonadotropins: Tissue-specific angiogenic factors? Mol Cell Endocrinol. 2007 Apr 15;269(1-2):65-80.

25. Schmidt JA, de Avila JM, McLean DJ. 2007. Analysis of gene expression in bovine testis tissue prior to ectopic testis tissue xenografting and during the grafting period. Biol Reprod. 2007 Feb 21; [Epub ahead of print]

26. Oatley JM, de Avila DM, Reeves JJ, McLean DJ. Establishment of spermatogenesis and the seminiferous epithelium in ectopically xenografted bovine testicular tissue. Biol Reprod. 2004, 71:494-501.

27. Zhang X, Ebata KT, Nagano MC. Genetic analysis of the clonal origin of regenerating mouse spermatogenesis following transplantation. Biol Reprod. 2003 Dec;69(6):1872-8.

28. Zeng W, Avelar GF, Rathi R, Franca LR, Dobrinski I. The length of the spermatogenic cycle is conserved in porcine and ovine testis xenografts. J Androl. 2006 Jul-Aug;27(4):527-33.

29. Zeng W, Rathi R, Pan H, Dobrinski I. Comparison of global gene expression between porcine testis tissue xenografts and porcine testis in situ. Mol Reprod Dev. 2007 Jun;74(6):674-9.

30. Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H,

Saarma M, Sariola H. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science. 2000 Feb 25;287(5457):1489-93

31. Naughton CK, Jain S, Strickland AM, Gupta A, Milbrandt J. Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biol Reprod. 2006 Feb;74(2):314-21.

32. Payne C, Braun RE. Glial cell line-derived neurotrophic factor maintains a POZ-itive influence on stem cells. Proc Natl Acad Sci USA. 2006 Jun 27;103(26):9751-2.

33. Aponte PM, Soda T, van de Kant HJ, de Rooij DG. Basic features of bovine spermatogonial culture and effects of glial cell line-derived neurotrophic factor. Theriogenology. 2006 Jun;65(9):1828-47.

34. De Rooij DG, Van Dissel-Emiliani FM, Van Pelt AM. Regulation of spermatogonial proliferation. Ann N Y Acad Sci. 1989;564:140-53. Review.

35. Zheng SX, Zhou LJ, Chen ZL, Yin ML, Zhu XZ. Bilobalide promotes expression of glial cell line-derived neurotrophic factor and vascular endothelial growth factor in rat astrocytes. Acta Pharmacol Sin. 2000 Feb;21(2):151-5.

36. Miyajima H, Osanai M, Chiba H, Nishikiori N, Kojima T, Ohtsuka K, Sawada N. Glyceraldehyde-derived advanced glycation end-products preferentially induce VEGF expression and reduce GDNF expression in human astrocytes. Biochem Biophys Res Commun. 2005 May 6;330(2):361-6.

37. Dolci S, Pellegrini M, Di Agostino S, Geremia R, Rossi P. Signaling through extracellular signal-regulated kinase is required for spermatogonial proliferative response to stem cell factor. J Biol Chem. 2001 Oct 26;276(43):40225-33.

38. Rossi P, Sette C, Dolci S, Geremia R. Role of c-kit in mammalian spermatogenesis. J Endocrinol Invest. 2000 Oct;23(9):609-15.

39. Ohta H, Yomogida K, Dohmae K, Nishimune Y.Development. Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF. 2000 May;127(10):2125-31.

40. Ohta H, Tohda A, Nishimune Y. Proliferation and differentiation of spermatogonial stem cells in the w/wv mutant mouse testis. Biol Reprod. 2003 Dec;69(6):1815-21.

41. Marti HH, Risau W. Systemic hypoxia changes the organ-specific distribution of vascular endothelial growth factors and its receptors. Proc Natl Acad Sci U S A 1998 95:15809-15814

Table 1. Summary of Mean (SEM) Tissue Data in Bovine Testis Tissue Grafts

VEGF treatment	Graft Survival* (%)	Graft Weight (mg)	ST Number (sections)	VG Weight (mg)
0 ng/ml	53.9% (11.0%)	12.7 (2.1)	91.7 (16.2)	184.6 (35.6)
100 ng/ml	64.1% (15.8%)	14.3 (2.1)	80.0 (11.1)	210.3 (28.2)
200 ng/ml	61.5% (9.9%)	11.7 (1.9)	78.7 (15.3)	244.4 (35.7)

SEM, standard error of mean.
ST, seminiferous tubules within each graft.
VG, recipient mouse vesicular glands.
* Total functional testis grafts divided by total recovered grafts

Table 2. Summary of Germ Cell Data in Bovine Testis Tissue Grafts

VEGF treatment	Establishment of Spermatogenesis Mean (SEM)	Germ Cell Survival **Ω** (% of tubules)	Meiotic Germ Cells **Ω** (% of tubules)	Haploid Spermatids **Ω** (% of tubules) **Ψ**
0 ng/ml	4.8% (4.8%) [a]	55.2% [a]	16.4 [a]	2.4% [a]
100 ng/ml	36.0% (10.9%) [b]	68.2% [b]	25.8 [b]	5.1% [b]
200 ng/ml	40.9% (5.5%) [b]	74.3% [b]	28.8 [b]	6.0% [b]

SEM, standard error of mean.
Means affected by different letters show significant differences ($p<0.05$) for treatment

Ω Parametric means are presented although comparisons were made using a nonparametric ANOVA with Wilcoxon Rank Sums for differences between means.

Ψ Histological analysis of differentiated germ cell populations in bovine testis tissue cultured with vehicle only identified a single mouse (out of 20) containing testis grafts containing haploid spermatids. Similar analysis identified the occurrence of complete graft germ cell differentiation in 9 mice (out of 25) receiving bovine testis tissue cultured with VEGF at 100 ng/ml prior to grafting. Accordingly, complete germ cell differentiation was seen in 9 mice (out of 22) grafted with bovine testis tissue cultured with VEGF at 200 ng/ml at the rate

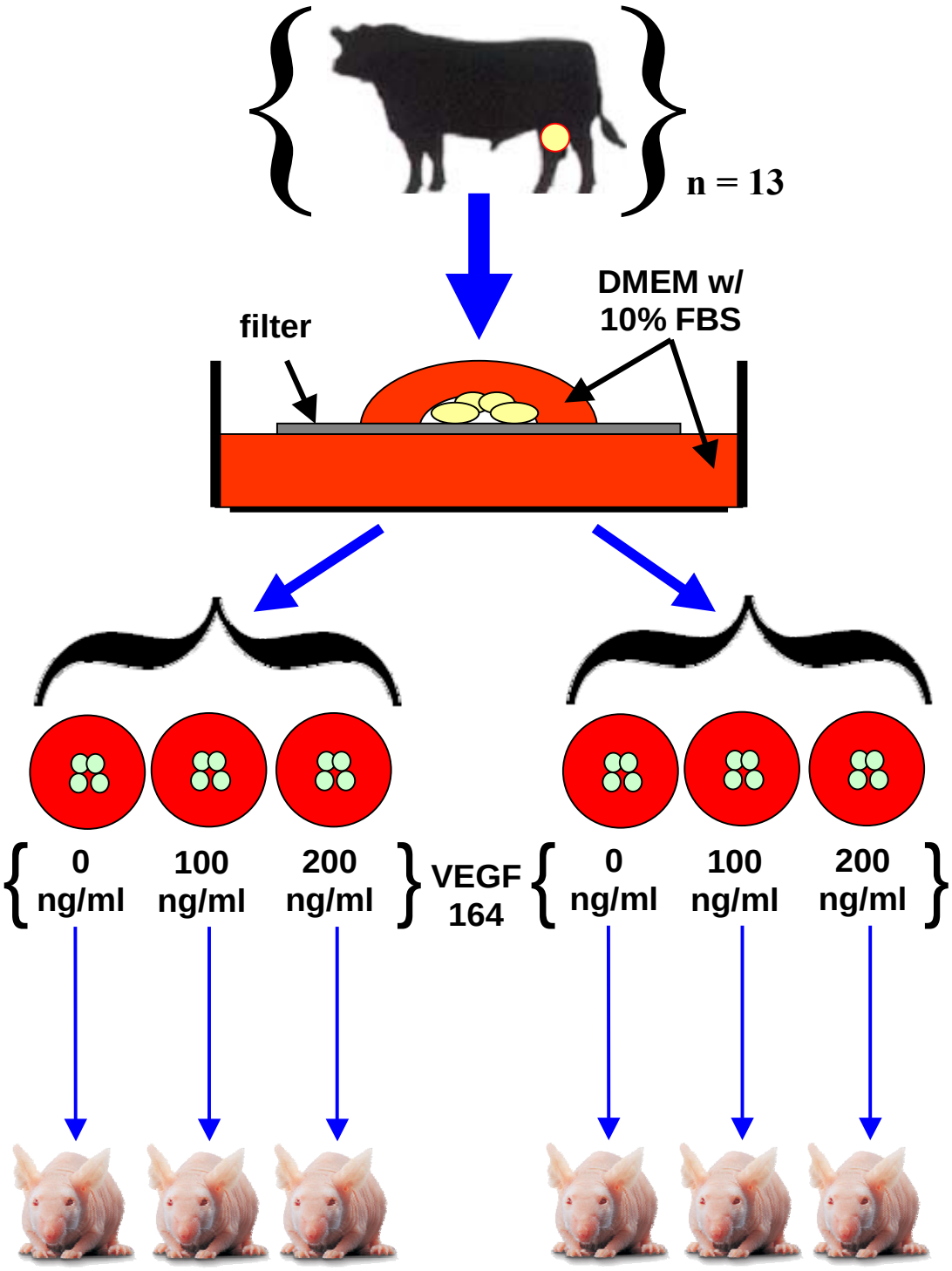

Figure 1. Diagrammatic representation of the experimental design implemented in the current study.

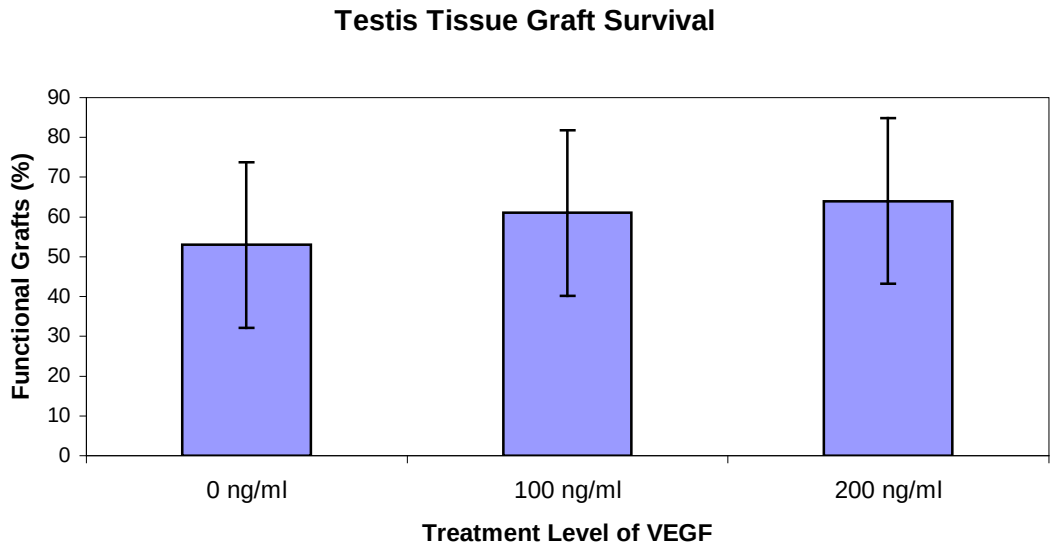

Figure 2. Comparison of survival rates in bovine testis tissue grafts cultured with VEGF or vehicle following the ectopic grafting period, determined by multiplying recovery rate and proportion of functional grafts. There were no significant differences (P>0.05) between means.

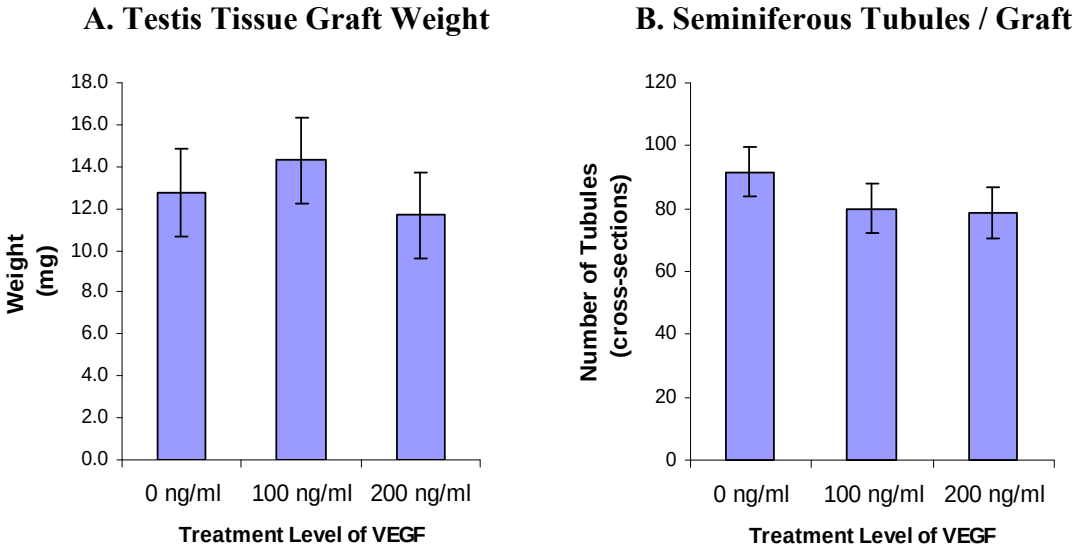

Figure 3. Growth analysis of functional bovine testis tissue grafts cultured with VEGF or vehicle following the ectopic grafting period. A) Average weight of individual testis grafts. B) Number of seminiferous tubule cross-sections per testis graft (pooled average / mouse). There were no significant differences (P>0.05) between means.

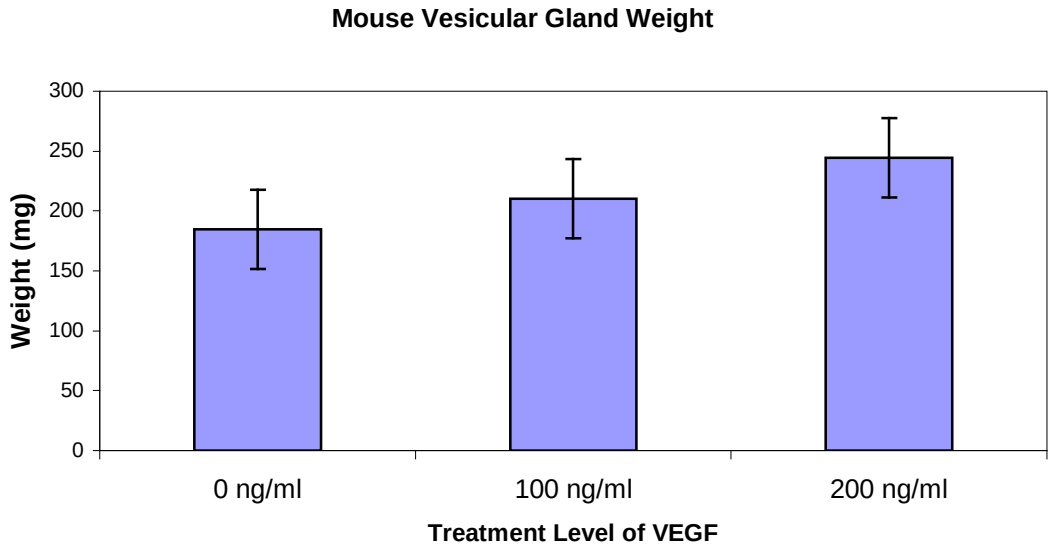

Figure 4. Comparison of androgen biosynthesis within bovine testis tissue grafts cultured with VEGF or vehicle following the ectopic grafting period as determined by paired vesicular gland weights of recipient mice supporting functional grafts. There were no significant differences (P>0.05) between means.

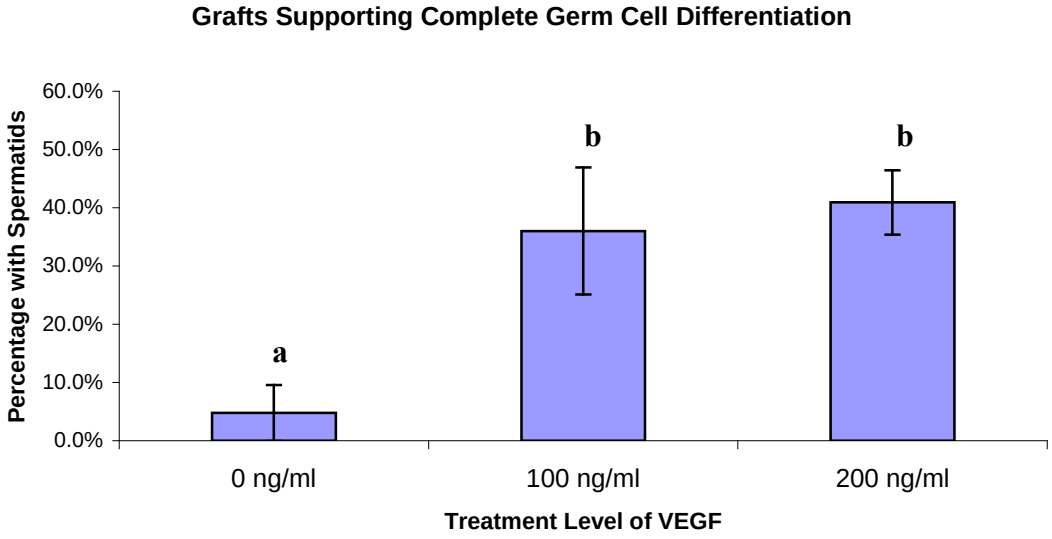

Figure 5. Differences in the ability of functional bovine testis tissue grafts cultured with VEGF or vehicle to support production of haploid spermatids following the ectopic grafting period. Bars affected by different letters show significant differences (p<0.05) between means.

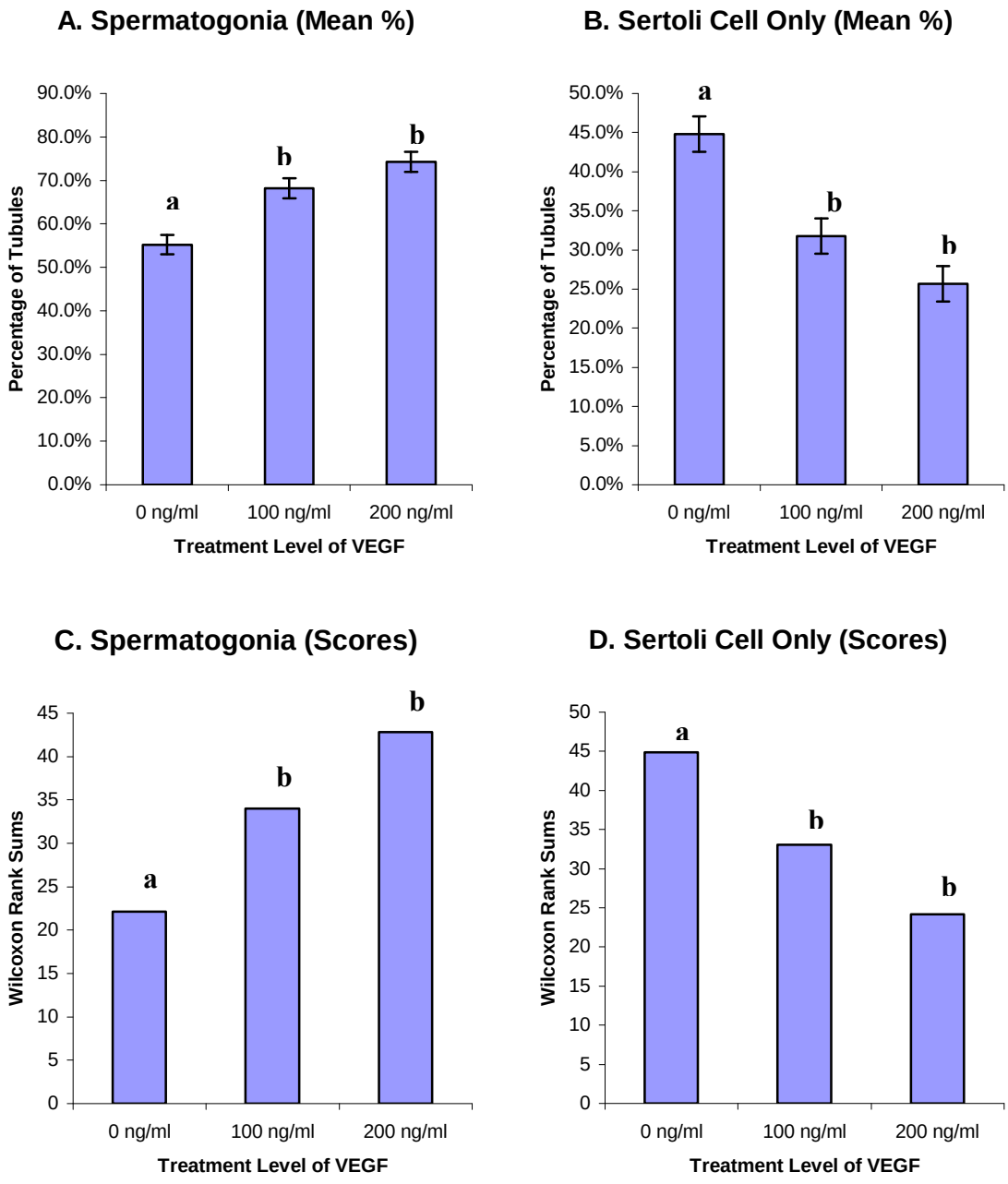

Figure 6. Analysis of germ cell survival in functional bovine testis tissue grafts cultured with VEGF or vehicle following the ectopic grafting period. Percentage of seminiferous tubules classified by presence of spermatogonia (A) or only Sertoli cells (C). Nonparametric analysis of treatment effects on graft survival is also reported for proportions of tubules containing spermatogonia (B) or only Sertoli cells (D). Bars affected by different letters show significant differences ($p<0.05$) between means.

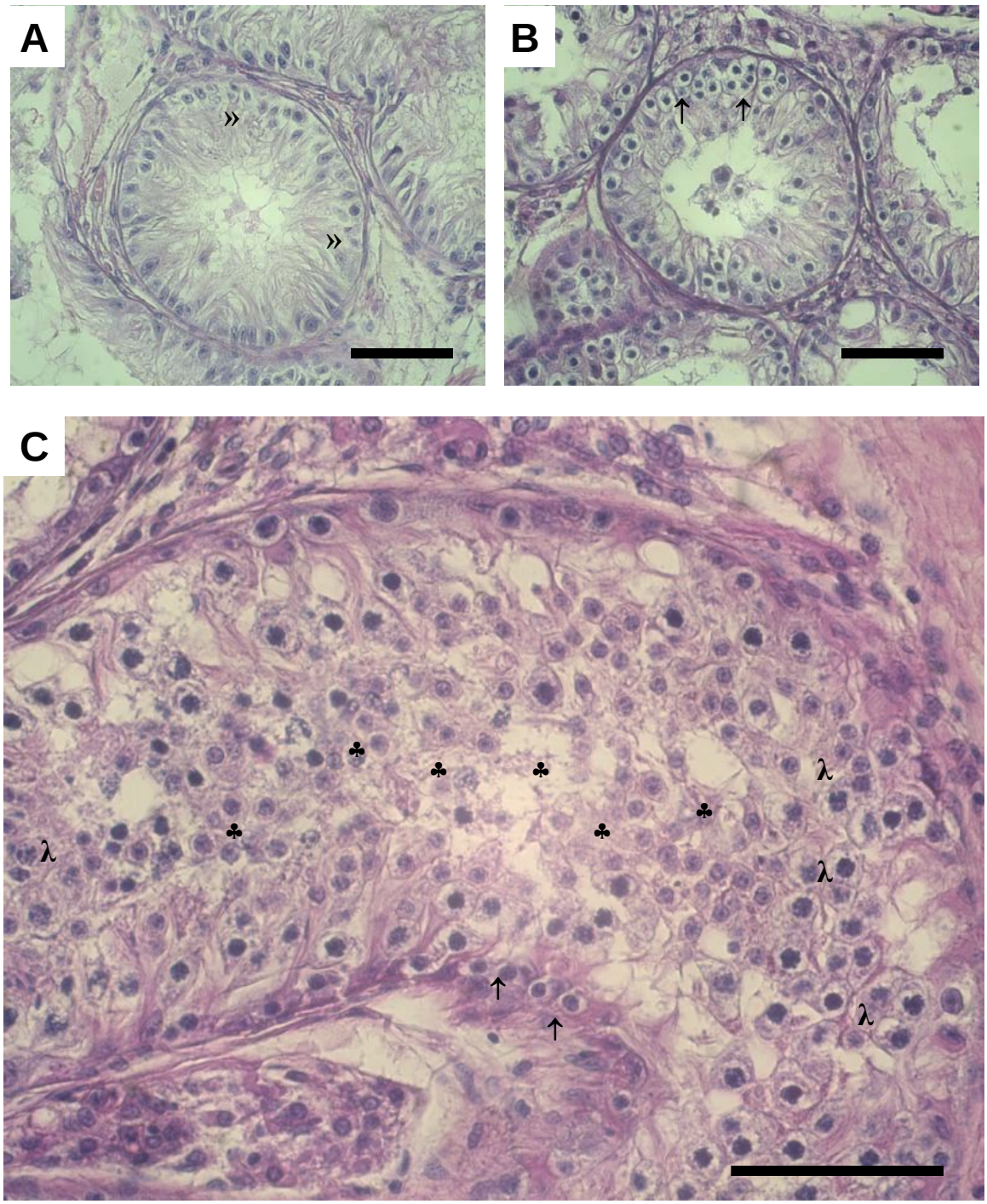

Figure 7. Morphological analysis comparing germ cell survival in bovine testis tissue grafts treated with VEGF or vehicle. (A) Germ cell survival = presence of spermatogonia or germ cells within seminiferous tubule cross-sections. (B) Germ cell death = presence of only Sertoli cells within seminiferous tubule cross-sections. (C) Establishment of spermatogenesis leading to the production of spermatids. Indicators of testicular cell types are as follows: Sertoli cells (»), spermatogonia (↑), meiotic germ cells (λ), and spermatids (♣). Scale bar is 50 μm.

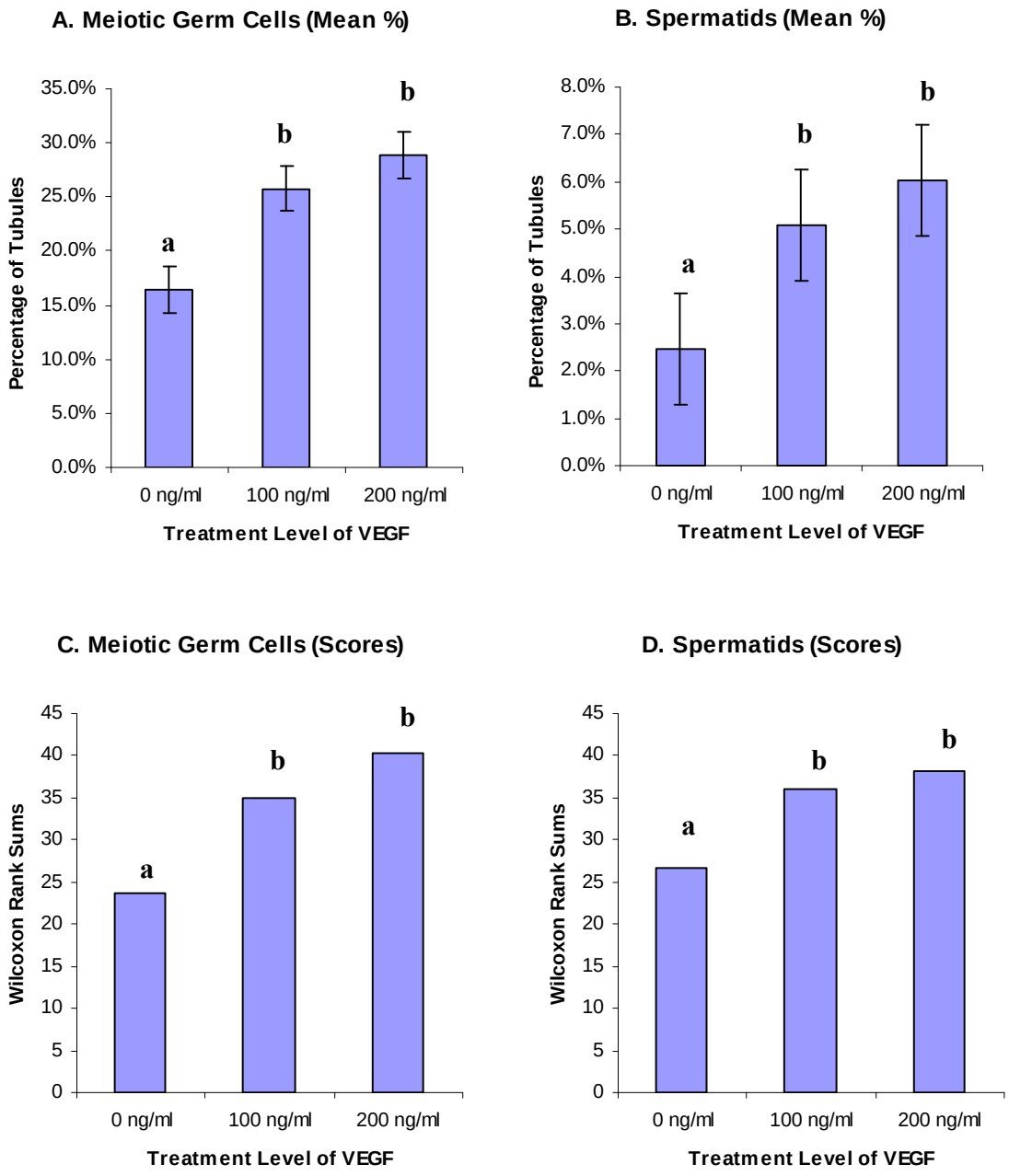

Figure 8. Comparison of differentiating germ cell types in functional bovine testis tissue grafts cultured with VEGF or vehicle following the ectopic grafting period. Bars affected by different letters show significant differences ($p<0.05$) between means.

APPENDIX

Establishment of Spermatogenesis in Neonatal Porcine Testicular Tissue Following Ectopic Xenografting Varies with Donor Age

Abstract

Somatic and germ cell maturation precedes the start of spermatogenesis and is coordinated so efficient spermatogenesis will occur in the adults. The present study was conducted to critically evaluate age-related differences on the establishment of spermatogenesis in neonatal boars with the use of ectopic testis tissue xenografting. Ectopic testis tissue xenografting affords investigation of testicular germ and somatic cell proliferation and differentiation events in many species. Testis tissue obtained from 3, 5, 7 and 14-day-old piglets were ectopically xenografted onto castrated, immunodeficient nude mice. Grafts were removed 22-wk later and evaluated for growth, the establishment of spermatogenesis and recipient mouse testosterone biosynthesis and FSH concentration as assayed. Testis tissue graft growth was significantly greater in testis grafts from 3-day donor tissue, when compared to all other ages; 5, 7, and 14-day-old donor tissue weight was not significantly different at removal. FSH concentrations in recipient mice supporting testis grafts from 5, 7, and 14-day old donor tissue did not differ and were similar to normal physiological levels in age matched, intact nude mice. Serum FSH levels were significantly lower in recipient mice supporting testis grafts from 3-day old donor tissue. Biological assay of vesicular gland weight indicated no differences in testosterone production by testis grafts of varying donor age. Porcine testis tissue obtained from 3, 5, 7 and 14-day-old neonatal boars were all capable of producing round and elongate spermatids after 22 weeks of grafting, but testis grafts from 14 day old donors had a significantly higher (8-fold) percentage of seminiferous tubules with spermatids compared to all other donor ages ($p < 0.05$). These data demonstrate age-related differences in the ability of donor testicular tissue from neonatal boars to develop

and establish spermatogenesis following ectopic testicular xenografting. Future research will investigate age-related variation in somatic and germ cell populations responsible for testis graft differences in the establishment of spermatogenesis and how this is associated with efficient sperm production in boars.

Introduction

Spermatogenesis is an organized process requiring many systemic and local factors [1, 2] and includes all cellular transformations that occur within the seminiferous epithelium leading to the production of mature spermatozoa in the seminiferous tubule of the testis. However, germ cell interactions with nearby Sertoli cells are necessary for germ cells to undergo mitosis, meiosis, and spermiogenesis [2, 3]. Moreover, it is believed that Sertoli cells contribute to the process of spermatogonial stem cell (SSC) renewal [4-9], allowing for continual production of sperm during a male's adult lifespan. Leydig and Sertoli cells also account for the majority of testosterone and estrogen synthesis in the male [10], and secrete factors exerting negative feedback on the pituitary gland; both processes promoting normal male fertility [11-13].

The majority of information about the cell biology associated with spermatogenesis has been gained with the use of rodent models and as a result; there remains a general lack of knowledge regarding testis development and sperm production in livestock. Further, due to the complexity involved with germ cell differentiation, most research investigating testis biology is often restricted to *in vitro* studies. Although insightful, the intimate relationship between Sertoli cells and germ cells are often lost, and cell lines can mutate during culture. Ectopic testis tissue xenografting is a procedure

whereby small pieces of testicular parenchyma from a donor animal are grafted under the skin of an immunodeficient nude mouse. This technique maintains interactions between germ and somatic cells in the grafted tissue and thus provides a biological assay for the establishment of spermatogenesis in a variety of species [15-17]. Additionally, multiple studies have demonstrated that testis tissue xenografts are representative of *in vivo* testis development [16, 18-19].

The onset of puberty is commonly associated with the completion of the first wave of spermatogenesis in a variety of species [20]. It is known that Sertoli cell number established before puberty determines adult testis size [21-27] and mature spermatogenic capacity [28-29] in various mammalian species. Puberty in most domestic pig breeds occurs around 20 weeks of age [30] and researchers have investigated the establishment of the spermatogenesis in the postnatal boar testis during between 1 month of age and adulthood [29-36], but little is known about relationships between somatic and germ cells in the neonatal testis as previously mentioned. Although porcine Sertoli cells still express markers of proliferating cells around 4 months of age [31], several reports suggest the importance of Sertoli cell proliferation in the boar testis during the first 2 weeks of life [32,37]. This represents several candidate physiological stages in development that may govern the spermatogenic capacity of the mature boar testis.

In 2005 the United States Department of Agriculture (USDA) reported that products generated from the domestic swine industry accounted for more than $97 billion in total receipts adding $34.5 billion to the country's gross national product, hence pork production is a vital part of the United States (US) economy. Profit-potential for various industry sectors is largely determined by reproductive efficiency. Despite the extensive

use of boars for natural service and artificial insemination within the swine industry, improving male reproduction has been greatly overlooked. Currently, little is known about the basic mechanisms regulating testis biology in boars. As a result, a better understanding of sperm production in boars could tremendously impact profitability in the swine industry.

The objective of the present study was to evaluate age-related differences in the establishment of spermatogenesis in testicular tissue obtained from 3, 5, 7, and 14-day old neonatal boars after 22 weeks of grafting. We also investigated endocrine related differences associated with the normal male fertility in recipient mice supporting porcine testis tissue of varying donor age.

Porcine Sertoli cell numbers increase 4 fold during the first two weeks of neonatal life, thus we hypothesized that testicular tissue from 14-day-old boars would have the greatest ability to establish and support spermatogenesis following grafting when compared to other donor ages. We also predicted no differences in testis graft androgen biosynthesis or recipient mouse serum FSH would exist between the donor ages. Completion of these objectives will provide a better understanding of the mechanisms regulating neonatal germ and somatic cell development associated with establishing future spermatogenic capacity in the boar testis.

Materials and Methods

Chemicals and Reagents

All reagents were purchased from Sigma (www.sigmaaldrich.com) unless otherwise stated.

Donor Animals and Tissue Collection

The Washington State University Animal Care and Use Committee approved all animal procedures. Neonatal testis was obtained from 3, 5, 7 and 14-day-old white line composite boars at the Washington State University Swine Facility using standard castration protocols. A minimum of 3 boars was randomly chosen to represent each donor age. Testes were immediately placed in Hanks Balanced Salt Solution (HBSS) on ice and transported to the laboratory, where visceral and parietal tunics were removed.

Ectopic Testis Xenografting

Testicular parenchyma tissue was cut into 5 mg pieces and ectopically xenografted onto castrated, recipient immunodeficient NCr nude mice (Taconic, http://www.taconic.com; CrTac:NCR-Fox1<nu>) or placed in Bouin's fixative for 4 h at 4°C followed by dehydration and storage in 70% ethanol for eventual morphological evaluation and comparison. Briefly, mice were anesthetized with ketamine (0.1 mg/kg body weight [BW]) and xylazine (0.5 mg/kg BW) castrated using surgical procedures, and managed as previously described [22].

Analysis of Donor Grafts and Recipient Mice

Recipient mice were sacrificed using CO2 inhalation followed by cervical dislocation after 22-wks of grafting. Testis grafts were removed, weighed, and processed as previously described [17] for histological analysis of establishment of spermatogenesis. Seminiferous tubule cross-section number and graft weight were

recorded as measure for tissue growth by testis grafts. At sacrifice, mouse vesicular gland weights were recorded as a bioassay for testosterone, and blood was also collected from recipient mice by cardiac puncture and serum was isolated later evaluated for [FSH] by radioimmunoassay. The extent of germ cell differentiation in testis tissue grafts was visually evaluated by comparing the average number of seminiferous tubules cross-sections containing spermatogonia, meiotic germ cells, differentiating spermatids, or only Sertoli cells within the largest center section of each tissue sample. Digital images were captured using a Leica DFC 280 camera and a Leica DME compound microscope (Leica Microsystems Imaging Solutions Ltd., http://www.leica-microsystems.com) at 400x magnification.

Statistical Analysis

Germ cell proportions were averaged per mouse to prevent unequal weighting due to variation in these parameters. All statistical comparisons were performed using the Proc GLM function of Statistical Analysis System (SAS) software, version 9.13 (SAS Institute, Cary, North Carolina, USA), and differences were considered significant at the $p \leq 0.05$ level. Pairwise comparisons were evaluated between donor ages using the Duncan's Multiple Range test for significance. Data are presented as the mean ± SEM and differences between donor ages were considered significant at the $P \leq 0.05$ level.

Results

Porcine Testis Tissue Graft Growth, Androgen Biosynthesis and Recipient Mouse Serum FSH Concentration

Growth of testicular tissue from neonatal boars could be identified during the grafting period by visually increase in protuberance size under the skin on the backs of recipient mice. To assess age related differences in growth potential of porcine donor tissue at removal, testis graft weights were measured and the numbers of seminiferous tubule cross-sections were determined. Analysis of growth potential indicated donor-age related variation in testis tissue graft weight and tubule number. Both parameters for graft growth were significantly higher ($P < 0.05$) in testis grafts from 3-day donor tissue, when compared to all other ages (Fig. 1 and 2). In contrast, donor testis tissue from 5, 7, and 14-day-old neonatal boars had similar growth rates as no differences ($P > 0.05$) were detected in graft weight (Fig. 1) or seminiferous tubule number (Fig. 2).

To assess the ability for testis tissue grafts to synthesize biologically active testosterone, recipient mouse vesicular gland weights were compared between donor groups. Vesicular gland weights indicated no differences ($P > 0.05$) in the ability of testis tissue grafts to produce testosterone despite variation in donor age (Fig. 3). We also investigated the ability of porcine testis tissue xenografts to produce factors that exert negative feedback on pituitary follicle stimulating hormone (FSH) synthesis when compared to normal, intact mice of similar age and strain by radioimmunoassay. FSH concentrations in recipient mice supporting testis grafts from 5, 7, and 14-day old donor tissue did not differ ($P > 0.05$) and were similar to normal physiological levels in age matched, intact nude mice (Fig. 4). However, serum FSH levels were significantly lower ($P < 0.05$) than normal in recipient mice supporting testis grafts from 3-day old donor tissue.

Degree of Germ Cell Differentiation in Porcine Testis Tissue Grafts

The extent of spermatogenesis in testis tissue from neonatal boars of different ages was determined after 22 weeks of grafting. Porcine testis tissue obtained from 3, 5, 7 and 14-day-old neonatal boars were all capable of producing round and elongate spermatids after 22 weeks of grafting (Fig. 5). Testis grafts from 14-day-old donors contained a significantly greater ($p < 0.05$) percentage of seminiferous tubules with spermatids compared to all other donor ages (Fig. 5). In contrast, spermatogenesis was established at similar rates ($P > 0.05$) in donor testis tissue from 3, 5, and 7-day-old neonatal boars (Fig. 5). The average percentage of tubules containing spermatids in testis tissue from 14-day-old donors was approximately 8-fold higher (45.4%) when compared to other donor ages averaging 5.4%. Photomicrograph cross-sections representing the degree of germ cell differentiation within porcine testis grafts can be found in Figure 6.

Discussion

Porcine testis tissue obtained from donor ages were all capable of complete germ cell differentiation after 22 weeks of grafting, but testis grafts from 14-day-old donors had significantly more (8-fold) seminiferous tubules with spermatids than all other donor ages ($p < 0.05$). Thus, differences exist in neonatal boar testis development associated with the establishment of the first wave of spermatogenesis in porcine testis tissue xenografts. These data suggest the optimum age for manipulating lifetime spermatogenic efficiency in boars should occur at two weeks of age, and may explain why previous attempts to increase spermatogenic capacity and testis size in boars did not result in larger testes or more sperm production [31]. Further, because grafting 14-day porcine testis tissue results in the greatest graft germ cell differentiation, this donor age may provide

the best tissue for germ-line genetic manipulation of cells, potentially facilitating a novel means to produce transgenic boar spermatozoa.

Although androgen biosynthesis was similar between donor ages, differences existed in the ability for testis grafts to grow and exert negative feedback on pituitary FSH production. Testis grafts from 3-day donor tissue were on average 2-fold larger when compared to testis tissue obtained from 5, 7, and 14-day-old neonatal boars. Testis grafts from 5, 7, and 14-day old donor tissue were of similar size and recipient mice supporting these testis grafts contained serum concentrations of FSH in similar to normal physiological levels in age matched, intact nude mice. Serum FSH levels were significantly lower than controls ($P < 0.05$) in recipient mice supporting testis grafts from 3-day old donor tissue. Interestingly, vesicular gland weight demonstrated no donor-age-related differences in testosterone synthesis, suggesting increased potential for testis grafts from 3-day donors to negatively regulate pituitary production of FSH, due to more inhibin production from a larger population of Sertoli cells. This effect may also be due to germinal aplasia [40], or failure of germ cells to develop, but this appears unlikely based on the data demonstrating the establishment of spermatogenesis was similar in testis tissue grafts from 3, 5, and 7-day neonatal donors.

During prenatal and postnatal testis development, it is established that FSH stimulates Sertoli cells to proliferate and in response to thyroid hormones, Sertoli cells cease proliferation and initiate terminal differentiation [38, 41-42]. The timeline for Sertoli cell proliferation and differentiation events are highly species dependant, occur before puberty and are essential for spermatogenesis in the adult. *In vivo* studies have suggested the potential to manipulate establishment of Sertoli cell populations in non-

human primates [25], rodents [28, 37] bulls [39] and boars [29, 33]. However, in an attempt to increase Sertoli cell number and pubertal testis size, researchers' induced hypothyroidism in postnatal boars starting at 21 days of age [31]. However, postnatal hypothyroidism did not affect testis development or sperm production in boars.

We demonstrated significant differences in the establishment of spermatogenesis in testis grafts from 14-day old donors, even though all donor tissue completed the first round of spermatogenesis associated with pubertal age. Although porcine Sertoli cells still express markers of proliferating cells around 4 months of age [31] our findings report age-related differences in growth potential of neonatal testis tissue when grafted on immunodeficient mice, and indicate important physiological changes in Sertoli cell homeostasis during the neonatal testis development. This conclusion is supported by *in vivo* evidence [32, 35-37], and since postnatal boar testis developmental is conserved between grafted and *in vivo* testis tissue, we suggest future efforts to impact lifetime spermatogenic capacity in boars should occur during the first two weeks of life
While germ cells in the neonatal boar testis may have contributed to the observed variation in the establishment of spermatogenesis, they exist as gonocytes in the lumen of the seminiferous tubule, and do not migrate to the basement membrane of until after 14 days of age. Thus, future research should investigate factors regulating physiological changes in Sertoli cell populations responsible for the dramatic increase in the extent of spermatogenesis seen in testis grafts from 14-day-old donors. Information regarding critical changes in gene and protein expression involved with neonatal Sertoli cell homeostasis in boars will allow for a better understanding of male fertility and infertility,

and potentially facilitate novel management strategies improve male reproductive biology for various agricultural, biomedical and conservation purposes.

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References:

1. Zirkin BR. Spermatogenesis: its regulation by testosterone and FSH. Semin Cell Dev Biol. 1998 Aug;9(4):417-21. Review.

2. Parvinen M, Ventela S. Local regulation of spermatogenesis: a living cell approach. Hum Fertil (Camb). 1999;2(2):138-142.

3. Russell LD, Ettlin RA, Sinha Hikim AP, Clegg ED. Mammalian spermatogenesis. In: Russell LD, Ettlin RA, Sinha Hikim AP, Clegg ED (eds.), Histological and Histopathological Evaluation of the Testis. Clearwater: Cache River Press; 1990: 1–40

4. Sharpe RM, Regulation of spermatogenesis. In: Knobil K, Neil JD, (eds.) The Physiology of Reproduction, vol. 1, 2nd ed New York: Raven Press 1994 1363-1434

5. Oakberg EF. Spermatogonial stem-cell renewal in the mouse. Anat Rec. 1971 Mar;169(3):515-31.

6. Huckins C. The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation, and maturation. Anat Rec 1971; 160:533-558

7. Meistrich ML, van Beek MEAB. Spermatogonial stem cells. In: Desjardins C, Ewing LL (eds.), Cell and Molecular Biology of Testis. New York, Oxford: Oxford University Press; 1993: 266–295

8. Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 1994; 91:11298-11302

9. Mclean DJ. Spermatogonial stem cell transplantation and testicular function. Cell Tissue Res 2005; 322:21–31.

10. Waites GM, Speight AC, Jenkins N. The functional maturation of the Sertoli cell and Leydig cell in the mammalian testis. J Reprod Fertil 1985 75:317-326 (review)

11. Gondos B, Berndtson WE. Postnatal and pubertal development. In: Russell LD, Griswold MD (eds.), The Sertoli Cell. Clearwater, FL: Cache River Press; 1993: 116–153

12. Padmanabhan V, Sharma TP. Neuroendocrine vs. paracrine control of follicle-stimulating hormone. Arch Med Res. 2001 Nov-Dec;32(6):533-43. Review.

13. Plant TM, Marshall GR. The functional significance of FSH in spermatogenesis and the control of its secretion in male primates. Endocr Rev. 2001 Dec;22(6):764-86. Review.

14. Meachem SJ, Nieschlag E, Simoni M. Inhibin B in male reproduction: pathophysiology and clinical relevance.

15. Honaramooz A, Snedaker A, Boiani M, Scholer H, Dobrinski I, Schlatt S, Sperm from neonatal mammalian testes grafted into mice. Nature 2002 418:778-781

16. Oatley JM, de Avila DM, Reeves JJ, McLean DJ. Establishment of spermatogenesis and the seminiferous epithelium in ectopically xenografted bovine testicular tissue. Biol Reprod. 2004, 71:494-501.

17. Schmidt JA, de Avila JM, McLean DJ. 2006. Grafting period and donor age affect the potential for spermatogenesis in bovine ectopic testis xenografts. Biol Reprod, 75(2):160-6.

18. Zeng W, Avelar GF, Rathi R, Franca LR, Dobrinski I. The length of the spermatogenic cycle is conserved in porcine and ovine testis xenografts. J Androl. 2006 Jul-Aug;27(4):527-33.

19. Zeng W, Rathi R, Pan H, Dobrinski I. Comparison of global gene expression between porcine testis tissue xenografts and porcine testis in situ. Mol Reprod Dev. 2007 Jun;74(6):674-9.

20. Amann RP. Sperm production rates. In: Johnson AD, Gomes WR, VanDemark NL (eds.), The Testis, vol. 1. New York: Academic Press; 1970; 433–482

21. Berndtson WE, Igboeli G, Pickett BW. Relationship of absolute numbers of Sertoli cells to testicular size and spermatogenesis in young beef bulls. J Anim Sci 1987 64:241-246

22. Berndtson WE, Igboeli G. Numbers of Sertoli cells, quantitative rates of sperm production and the efficiency of spermatogenesis in relation to the daily sperm output and seminal quality of young beef bulls. Am J Vet Res 1989 50:1193-1197

23. Russell LD. Form, dimensions and cytology of mammalian Sertoli cells. In: Russell LD, Griswold MD (eds.), The Sertoli Cell. Clearwater, FL: Cache River Press; 1993: 1–37

24. Johnson L, Varner DD, Tatum ME, Scrutchfield WL. Season but not age affects Sertoli cell number in adult stallions. Biol Reprod 1991 45:404-410

25. Sharpe RM, Walker M, Millar MR, Atanassova N, Morris K, McKinnell C, Saunders PT, Fraser HM. Effect of neonatal gonadotropin-releasing hormone antagonist administration on Sertoli cell number and testicular development in the marmoset: comparison with the rat. Biol Reprod 2000 62:1685-1693

26. Johnson MH. The role of the pituitary in the development of the blood-testis barrier in mice. J Reprod Fertil 1973 32:509-511

27. Chubb C. Genes regulating testis size. Biol Reprod 1992 47:29-36

28. Orth JM, Gunsalus GL, Lamperti AA. Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. Endocrinology 1988 122:787-794

29. Lunstra D.D,, T.H. Wisea, and J.J. Ford. Sertoli Cells in the Boar Testis: Changes During Development and Compensatory Hypertrophy after Hemicastration at Different Ages

30. Lunstra DD, Ford JJ, Christenson RK, Allrich RD. Changes in Leydig cell ultrastructure and function during pubertal development in the boar. Biol Reprod. 1986; 34: 145 –158.

31. Klobucar I, Kosec M, Cebulj-Kadunc N, Majdic G. Postnatal hypothyroidism does not affect prepubertal testis development in boars. Reprod Domest Anim. 2003 Jun;38(3):193-8.

32. McCoard SA, Wise TH, Ford JJ. Endocrine and molecular influences on testicular development in Meishan and White Composite boars. J Endocrinol. 2003 Sep;178(3):405-16.

33. Nonneman D, Rohrer GA, Wise TH, Lunstra DD, Ford JJ. A variant of porcine thyroxine-binding globulin has reduced affinity for thyroxine and is associated with testis size. Biol Reprod. 2005 Jan;72(1):214-20.

34. Almeida FF, Leal MC, Franca LR. Testis morphometry, duration of spermatogenesis, and spermatogenic efficiency in the wild boar (Sus scrofa scrofa). Biol Reprod. 2006 Nov;75(5):792-9

35. At-Taras EE, Kim IC, Berger T, Conley A, Roser JF. Reducing endogenous estrogen during development alters hormone production by porcine Leydig cells and seminiferous tubules. Domest Anim Endocrinol. 2006 Dec 18

36. At-Taras EE, Berger T, McCarthy MJ, Conley AJ, Nitta-Oda BJ, Roser JF. Reducing estrogen synthesis in developing boars increases testis size and total sperm production. J Androl. 2006 Jul-Aug;27(4):552-9.

37. McCoard SA, Lunstra DD, Wise TH, Ford JJ. Specific staining of Sertoli cell nuclei and evaluation of Sertoli cell number and proliferative activity in Meishan and White Composite boars during the neonatal period. Biol Reprod 2001 64:689-695

38. Holsberger DR, Cooke PS. Understanding the role of thyroid hormone in Sertoli cell development: a mechanistic hypothesis. Cell Tissue Res. 2005 Oct;322(1):133-40. Epub 2005 Nov 3. Review.

39. Majdic G, Snoj T, Horvat A, Mrkun J, Kosec M, Cestnik V. Higher thyroid hormone levels in neonatal life result in reduced testis volume in postpubertal bulls. Int J Androl. 1998 Dec;21(6):352-7.

40. Zarate A, Garrido J, Canales ES, Soria J, Schally AV. Disparity in the negative gonadal feedback control for LH and FSH secretion in cases of germinal aplasia or Sertoli-cell-only syndrome. J Clin Endocrinol Metab 1974 38:1125-1127

41. Orth JM. The role of follicle-stimulating hormone in controlling Sertoli cell proliferation in testes of fetal rats. Endocrinology 1984 115:1248-1255

42. Davies AG. Gonadotropin induces changes in the Sertoli cells of the immature mouse testis. J Reprod Fertil 1976 47:83-85

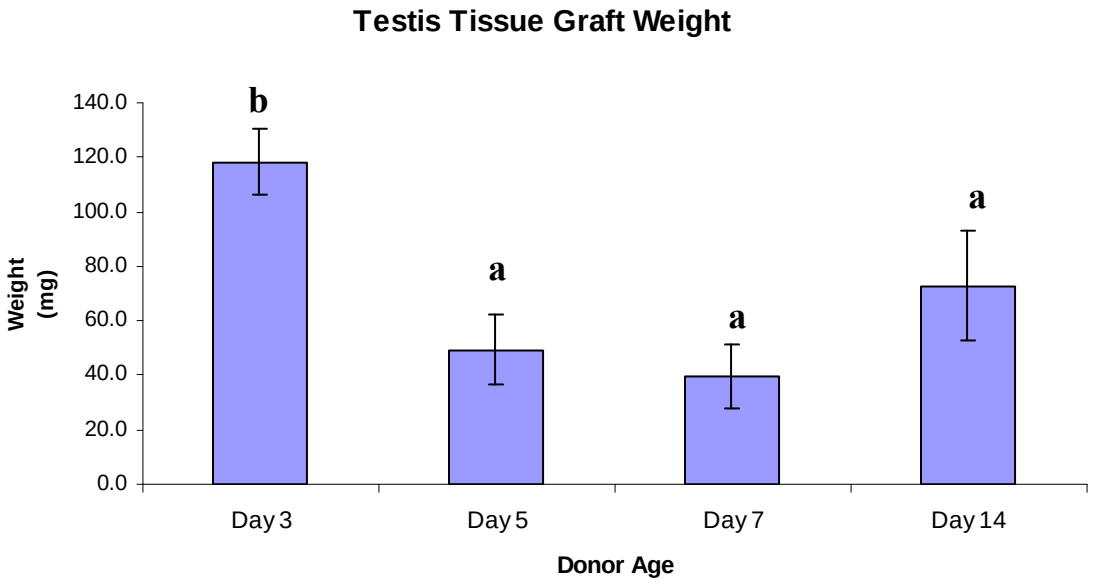

Figure 1. Growth analysis of functional porcine testis tissue grafts of varying donor age following the ectopic grafting period as determined by mean weight of individual testis grafts. Bars affected by different letters show significant differences ($p<0.05$) between means.

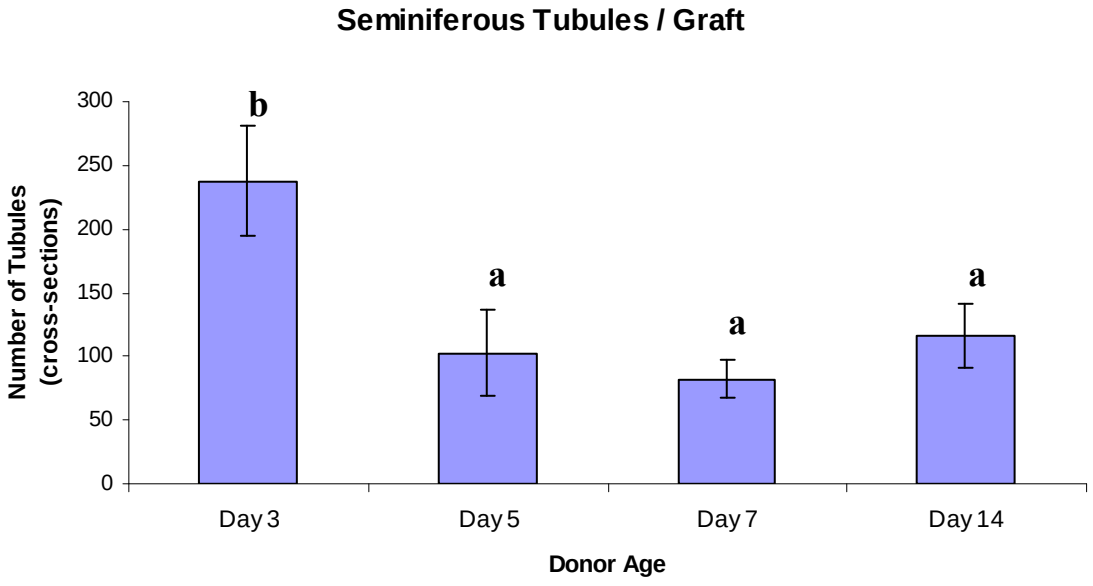

Figure 2. Growth analysis of functional porcine testis tissue grafts of varying donor age following the ectopic grafting period as determined by number of seminiferous tubule cross-sections per testis graft. Bars affected by different letters show significant differences ($p<0.05$) between means.

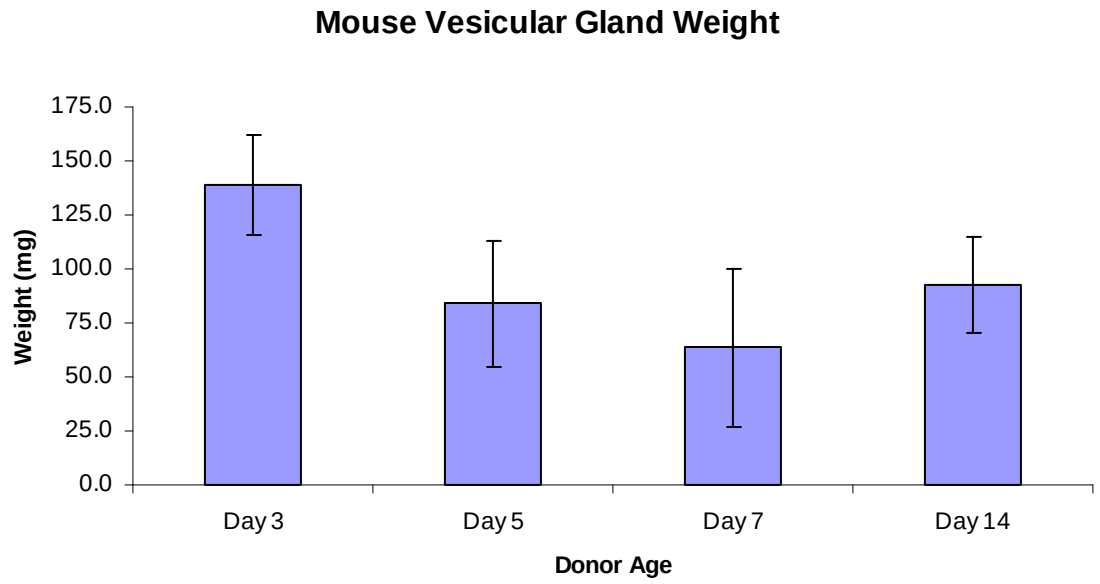

Figure 3. Comparison of androgen biosynthesis within porcine testis tissue grafts of varying donor age. There were no significant differences (P>0.05) between means.

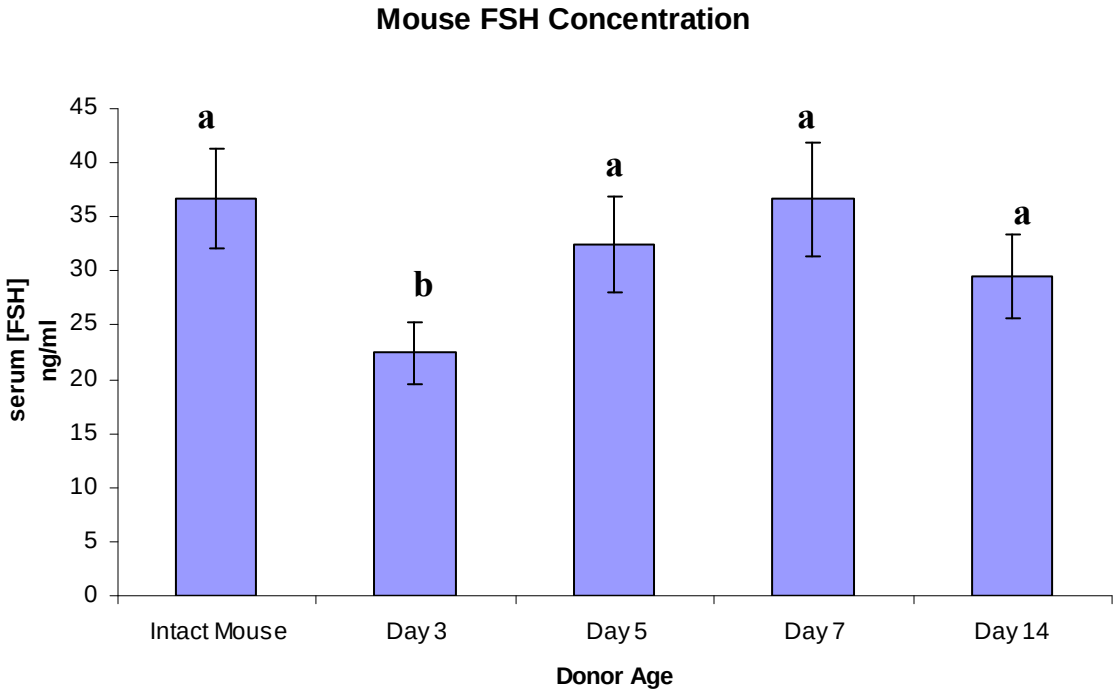

Figure 4. Comparison of serum FSH concentration in non-grafted intact mice, and recipient mice supporting porcine testis tissue grafts of varying donor age. Bars affected by different letters show significant differences (p<0.05) between means.

Figure 5. Percentage of seminiferous tubules containing round and elongating spermatids within porcine testis tissue grafts of varying donor age. Bars affected by different letters show significant differences ($p<0.05$) between means.

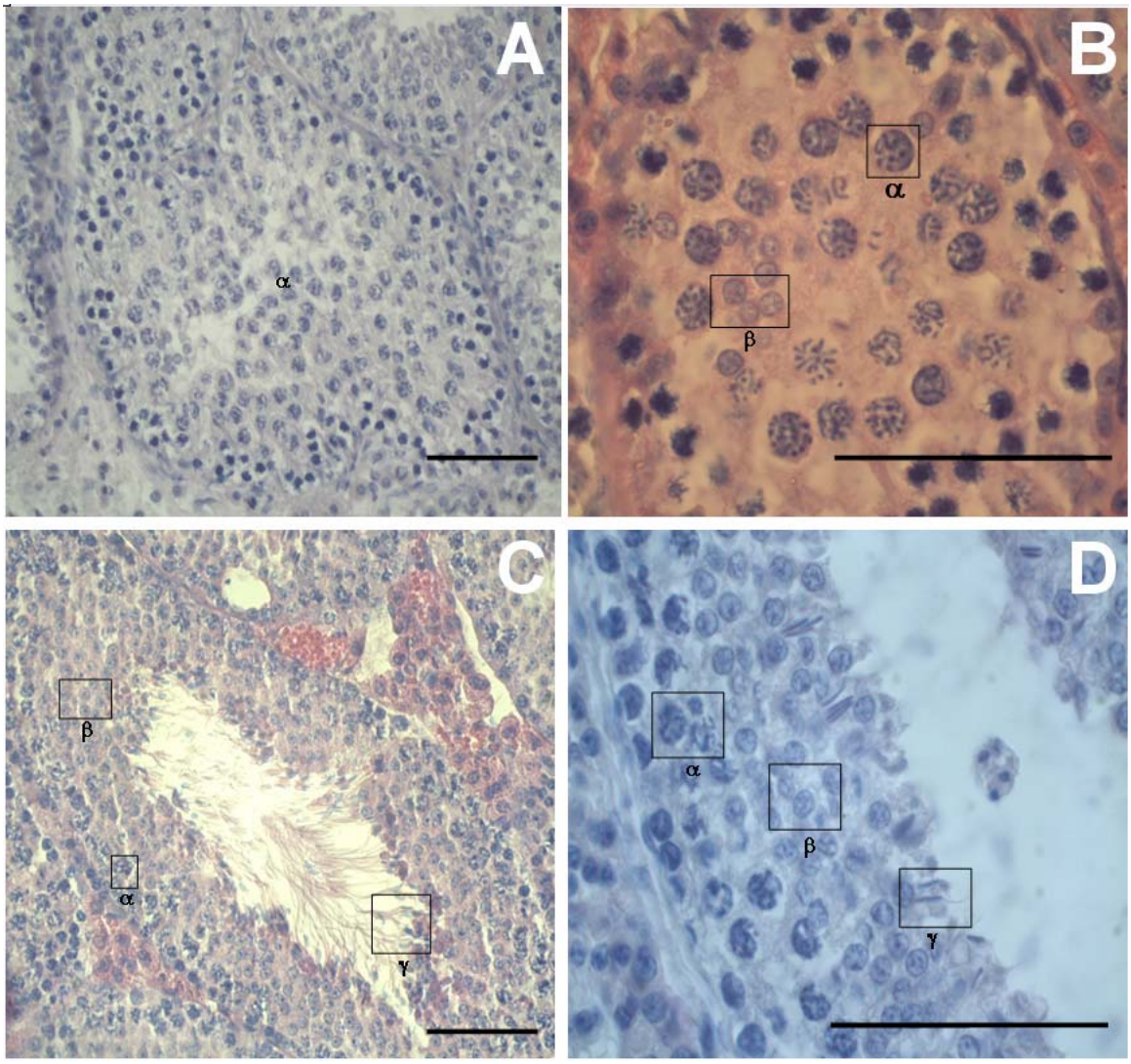

Figure 6. Morphological analysis comparing germ cell populations in porcine testis tissue grafts of varying donor age. Tubule cross-sections representing most advanced germ cell type: (A) spermatocytes **α**, (B) round spermatids **β**, (C & D) elongating spermatids **γ.** Scale bars = 50 μm.