DEVELOPMENT OF CULTURE METHODS FOR SPERMATOGONIAL STEM CELLS AND ECTOPIC TESTICULAR XENOGRAFTING IN THE BULL

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of JON MICHAEL OATEY find it satisfactory and recommend that it be accepted.

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DEVELOPMENT OF CULTURE METHODS FOR SPERMATOGONIAL STEM CELLS AND ECTOPIC TESTICULAR XENOGRAFTING IN THE BULL

Abstract

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Spermatogonial stem cell transplantation, with the ability to maintain spermatogonial stem cells in culture being vital, and ectopic testicular xenografting are both methods with potential for efficient generation of transgenic animals through the male germ line. Bovine spermatogonial stem cell maintenance in vitro and the efficacy of ectopic testicular xenografting for generating transgenic bovine germ cells are evaluated in this dissertation. The biological activity of bovine spermatogonial stem cells in vitro was assessed using a cross species germ cell transplantation assay. The first study utilized an explant testis tissue culture technique and the second employed a bovine embryonic fibroblast (BEF) feeder cell for co-culture with bovine germ cells. Both experiments demonstrated a significant ($P \leq 0.05$) increase in colony numbers arising from germ cells cultured for 1wk compared to starting cell populations. With BEF co-culture, a significant decline in colony numbers was seen 2wk after culture; upon addition of exogenous GDNF in these cultures stem cell maintenance was enhanced. Data from both culture experiments demonstrate survival and proliferation of bovine spermatogonial stem cells in vitro.

Establishment of spermatogenesis and transduction of spermatogonial stem cells with a β-galactosidase transgene in bovine testicular tissue ectopically xenografted onto the backs of castrated recipient nude mouse hosts was evaluated. Elongate spermatids were observed in donor testicular tissue 24wk after grafting. Donor age related differences in the ability for spermatogenesis to become established were seen, in which testicular tissue from donor bulls at 8wk of age contained a significantly higher average percentage of tubules with elongate spermatids. Germ cell expression of β-galactosidase was observed in grafts electroporated with a LacZ expression vector prior to grafting. These data demonstrate establishment of spermatogenesis in ectopically xenografted bovine testicular tissue and transgene incorporation into the spermatogonial stem cell genome.

A fifth study was conducted to evaluate three different treatment regimens for depletion of endogenous germ cells in adult ram testes. Treatments consisted of active immunization against LHRH, localized testicular irradiation, LHRH immunization+irradiation, and non-treated control. Data demonstrated depletion of germ cells in treated rams without alteration of somatic Sertoli cell function. These treatments can now be refined and used for preparing recipient livestock animals for further development of spermatogonial stem cell transplantation in livestock.

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DEDICATION

This dissertation is dedicated to my loving wife Melissa Oatley. You have provided more understanding and support than I could have ever asked for. There are not a lot of women in this world who would have put up with all the things that I put you through during my graduate career. I'm sorry for the missed anniversary because I wanted to work instead, I promise to make it up to you. Hopefully I can continue to make you proud to be my wife.

DISSERTATION OUTLINE

This dissertation is divided into 6 self contained chapters. Chapter one provides an introductory overview of the biology, culture, cryopreservation, genetic modification and transplantation of spermatogonial stem cells; and current technologies, progress and utility of ectopic testicular xenografting. Chapters two and three focus on the evaluation of two different culture methods for their support of bovine spermatogonial stem cell survival and proliferation. Chapters four and five focus on evaluation of utilizing ectopic testicular xenografting in the bull for generation of transgenic germ cells. Chapter six examines the use of several different treatment regimens for depletion of endogenous germ cells in adult rams as a means for preparing spermatogonial stem cell transplant recipients. Two appendices are included that are aimed at enhancing the understanding of male germ cell biology and testicular function. Chapters two, three, four, five, and both appendices are written in Biology of Reproduction format. Chapter six is written in Journal of Animal Science format. At the time of this dissertation submission chapter two has been published in Biology of Reproduction (2004; Volume 70: pages 625-632), chapters three and four are in press in Biology of Reproduction, all of which are copyrighted by the Society for the Study of Reproduction Inc. Chapter five has been submitted for publication in Biology of Reproduction. Chapter six will be submitted for publication in Journal of Animal Science in the fall of 2004. The appendices are part of ongoing projects that will be finished within the next year and submitted for publication.

CHAPTER ONE

INTRODUCTION

Spermatogenesis is a highly organized and intricate process occurring in the testis that involves the interaction of both a somatic and germ cell component. This process begins with the differentiation of an undifferentiated diploid spermatogonial stem cell (SSC) to committed spermatogonia [1]. These spermatogonia then undergo several mitotic divisions, two meiotic maturation phases as spermatocytes, finally differentiating into spermatids which undergo spermiogenesis to become mature haploid spermatozoa [1]. The somatic cell contribution to this process involves structural support from Leydig and myoid cells as well as steroid biosynthesis from Leydig cells, nurse function of Sertoli cells, and hormone feedback to the hypothalamus and pituitary. The testis is an organ which contributes terminally differentiated cells in the form of spermatozoa which should lead to a depletion of germ cells overtime. However, males are able to continually produce sperm from puberty to death. The key element to this continuous production is the SSC, characterized as having the ability for self-renewal and production of differentiated daughter progeny [2]. Even though this cell type is of critical importance to male fertility, very little is known about its regulatory biology. It has been estimated that 1 in 4,000 cells in the adult mouse testis is a stem cell [3]. Due to their rarity in the adult testis, SSCs have been difficult to isolate. Likewise, their regulatory biology has been difficult to dissect since self-renewal proliferation and differentiation occurs simultaneously [2].

Spermatogonial stem cell transplantation, cryopreservation, and culture

Since SSCs are the precursor cells to spermatozoa and have the ability for self-renewal they are an ideal candidate for development of reproductive technologies and transgenesis. In

fact they are the only adult stem cell that contributes genes to the next generation. Due to their rarity, characterization of SSCs has been hampered due to lack of a functional bioassay to study them. In 1994 Brinster and colleagues [4, 5] reported a bioassay that relied on transplantation and regeneration of spermatogenesis. They demonstrated the ability to transplant a mixed germ cell population collected from a donor testis into the seminiferous tubules of a recipient's testis. Several months after transplantation donor derived spermatogenesis could be detected in recipient seminiferous tubules [4] and offspring could be produced containing the donor's genetics [5]. Since SSCs are the only cell type in the testis that can reinitiate spermatogenesis, this system was identified as a means to study their biology and termed spermatogonial stem cell transplantation. Over the past 10 years spermatogonial stem cell transplantation has been used to make critical discoveries of SSCs such as their abilities to be cryopreserved and maintained in vitro [6-9]. Also several infertile mouse models have been utilized to gain important insights into SSC biology [10-14].

Currently, the technique of spermatogonial stem cell transplantation within a species is only developed in rodents and limited in goats [15]. However, spermatogonial stem cells from other mammals including boars [16], bulls [17], and men [18] have been demonstrated to be capable of colonizing the immunodeficient mouse testis but not undergoing differentiation into spermatozoa. This cross species transplantation of germ cells into the recipient mouse testis has become a useful tool to study SSC biology in species where transplantation is currently unavailable.

Aside from its utilization as a means to study SSCs, spermatogonial stem cell transplantation has been identified as a reproductive technology applicable to domestic livestock. Transplantation between a genetically superior donor bull and a group of recipient bulls in which

donor derived spermatogenesis occurs and recipient's breed by natural service to produce offspring with donor genetics could provide a much needed advancement in the beef cattle industry. This could allow for the efficient utilization of superior genetics in a beef cattle industry that utilizes very little artificial insemination due to economic and time constraints. Moreover, transgenic technology could be applied to this technique providing an alternative system for generation of transgenic offspring through the male germ cell. The ability to cryopreserve and maintain spermatogonial stem cells in vitro are integral components of the full potential and application of this technology.

Cryopreservation of SSCs from genetically superior bulls has potential to immortalize a sire's lineage. Spermatogonial stem cells from mice, primates and humans have been cryopreserved for extended periods of time [6, 9, 18, 19]. In mouse studies a mixed population of germ cells was preserved in liquid nitrogen under relatively simple conditions and resulted in donor derived spermatogenesis in recipient testes following thawing and transplantation [6, 9]. In mice, live young have been born from sperm derived from cryopreserved spermatogonial stem cells, thus demonstrating the ability of these cells to retain full functional capability [9]. Due to the stem cell's ability for self-replicating proliferation, immortalization of these cells may be achieved with cryopreservation. Currently, the only means to preserve a male's germ cell lineage after death is through cryopreservation of semen for many species. This method provides an exhaustible resource and is therefore not ideal. Cryopreservation and transplantation of SSCs provides a much more ideal unlimited method.

In vitro culture could provide a means for expanding donor stem cell lines, investigating SSC biology, and genetic alteration of SSCs. In order for culture of SSCs to accomplish these goals a system must be devised that not only supports their survival but self-renewing

proliferation as well. Using feeder cell co-culture techniques, rodent spermatogonial stem cells have been maintained in vitro for several months [7]. However, the number of surviving SSCs decreases with time in culture [8]. The controlling mechanisms of spermatogonial stem cell self-renewal in the seminiferous epithelium has remained elusive, therefore, mimicking these conditions in vitro has been a challenge. Recently, glial cell line derived-neurotrophic factor (GDNF) has been shown to enhance SSC maintenance in cultures of murine germ cells [8]. In the same study, addition of other factors implicated as mitogens for male germ cells such as stem cell factor, leukemia inhibitory factor, and bone morphogenic protein 4 did not enhance SSC maintenance [8]. Whether GDNF enhances the maintenance of SSCs in vitro by stimulating self-renewing proliferation or inhibiting differentiation remains a topic of debate.

Over expression of GDNF in transgenic mice has demonstrated an increase in undifferentiated spermatogonia within the testis [20]. Follicle stimulating hormone (FSH) regulated expression of GDNF by Sertoli cells has been suggested in the murine testis [21]. GDNF is a member of the transforming growth factor β super family and was first identified as a factor that influences the survival and differentiation of many types of neuronal cells [22-24]. Its expression is highest during the first few weeks of postnatal life in the mouse testis [21]. During this time gonocyte to SSC conversion occurs from postnatal days 3 to 4 [25, 26] and SSC proliferation commences until the first appearance of differentiating spermatogonia on day 8 [1]. In neuronal proginator cells, GDNF has been shown to exert its effects through binding the receptor dimer of Ret tyrosine kinase and GDNF family receptor-α1 (GFRα1) [22-24]. Therefore, it has been hypothesized that spermatogonial stem cells may express the GFRα1 receptor. Both GFRα1 and Ret have been shown to be expressed by a subpopulation of undifferentiated spermatogonia in the mouse testis [20, 21]. Also, GDNF and GFRα1 expression

are upregulated in cryptorchid testes [21], which are known to be enriched for SSCs [12-14]. GDNF may be a critical component for maintaining or inducing SSC self-renewing proliferation in vitro.

The technique of spermatogonial stem cell transplantation coupled with genetic modification has potential as an alternative system to generate transgenic animals through the male germ line. Since one spermatogonial stem cell has a high proliferation potential and must undergo several rounds of proliferation and meiosis, genetic modification of SSCs could yield thousands of stably modified spermatozoa. Genetic modification of spermatogonial stem cells in large mammalian species requires the ability to maintain these cells in vitro. Also, the stable chromosomal integration of a transgene within the cellular genome requires active mitosis. Thus, development of culture methods that support proliferation of spermatogonial stem cells is fundamental. With the use of genetically modified retroviruses or lentiviruses, murine spermatogonial stem cells have been transduced with a transgene and resulted in complete spermatogenesis of these cells following transplantation [27-29]. Offspring with stably incorporated transgenes have been produced from transduction of spermatogonial stem cells followed by transplantation and natural breeding in mice [27]. Using these technologies the efficiency and cost effectiveness for generating transgenic animals could be potentially enhanced over current methods of sperm-mediated delivery and pronuclear injection. The implications of transgenic animals include generating research models to study biological systems, disease resistant livestock to increase food safety, and dairy cattle which produce therapeutic nutrients in milk.

Development of spermatogonial stem cell transplantation in any species relies on four critical points. The first being the ability to collect spermatogonial stem cells from a donor testis,

which has been achieved in a variety of species including mice [4], rats [30], bulls [17], boars [16], and primates [19]. Secondly, endogenous germ cells must be depleted from the recipient's testis to allow for the donor cells to seed the seminiferous tubules. In rodents this can be achieved by irradiation of the testis [31] or treatment of the recipient animal with chemotoxic compounds such as busulfan [4, 5]. Third, a means for injection of donor cells into recipient seminiferous tubules must be developed that does not result in harmful side effects. In rodents microinjection of germ cells into the efferent ductiles, rete testes, or seminiferous tubules has been shown to result colonization and donor derived spermatogenesis. In livestock species and primates the testicular architecture is more complex than rodents and injection into the rete testis may be the only means for successfully introducing donor spermatogonial stem cells into recipient seminiferous tubules. Lastly, a means to identify donor derived spermatogenesis in recipient testis must be in place. Transgenic animals in which germ cells express a β-galactosidase transgene have been used as donors in rodent studies [4, 5]. Donor derived spermatogenesis from colonized SSCs can be detected by incubation of recipient testes with X-Gal which serves as a substrate that is cleaved by β-galactosidase to produce a blue color. Unfortunately, comparable transgenic donors in livestock species are not available. Currently, the only means of identifying donor derived spermatogenesis in livestock species following germ cell transplantation is through DNA fingerprinting of epididymal spermatozoa. A much more functional solution would be the generation of transgenic donor animals that express a marker trasngene in their germ cells that could be easily detected after transplant such as β-galactosidase or green fluorescent protein (GFP).

Ectopic testicular xenografting

A novel method for the investigation of spermatogenesis in a variety of specific was reported by Honamarooz and coworkers in 2002 [32]. These researchers described a system by which neonatal testis tissue from pigs and goats could be grafted onto the backs of castrated recipient nude mice resulting in production of differentiated germ cells months later [32]. In 2003 Schlatt and colleagues [33] reported the birth of live young from sperm produced in mouse testicular grafts. This technique has a wide potential to study spermatogenesis in many facets and many species. As an application tool in domestic livestock, ectopic testicular xenografting could be used as an alternative means to generate transgenic offspring through the male germ cell line. Moreover, it could be used to generate donors for within species spermatogonial stem cell transplantation experiments which express marker transgenes in germ cells, thus, aiding in the evolution of spermatogonial stem cell transplantation technology in livestock. Much of the current transgenic technology in livestock relies on genetic alteration of the embryo or oocyte. These techniques have a very low success rate and high cost associations. As an alternative means, genetic alteration of SSCs could provide a much more efficient means for generating transgenic animals. Genetic alteration of one SSC using ectopic testis xenografting has the potential to generate thousands of transgenic sperm to be used for in vitro fertilization or intracytoplasmic sperm injection. Since the SSC must undergo several mitotic and meiotic cell viability checkpoints in its maturation to spermatozoa, incorporation of a transgene would most likely be stable in spermatozoa. Even though this technique has been reported in several livestock species its application to the bovine has not been reported to date.

In initial grafting studies with goats and pigs the use of neonatal donors was necessary as a source to testicular tissue that was un-programmed for spermatogenesis [32]. This essentially allowed for the setup of the seminiferous epithelium to occur following grafting. Also, an

acceleration of the first wave of spermatogenesis was demonstrated in these reports [32]. The neonatal period of testis development in the bovine extends for months during which the germ cells undergo a transition from gonocytes to differentiated spermatogonia. Likewise, the somatic cells undergo proliferation and begin maturation as well. Therefore, certain developmental stages during this process may be better than others in terms of when the tissue is most susceptible to ectopic grafting.

Generation of genetically modified animals through the male germ line stem cells is of high importance due to the potential for efficient generation of many offspring with stably expressed transgenes. Methods developed in the past have relied on genetic manipulation at the level of the embryo such as sperm-mediated transfection, pronuclear injection, and embryonic stem cell manipulation [34-]. With sperm-mediated delivery of transgenes into oocytes, the success of stable chromosomal incorporation is low, usually resulting in a mean range of 0-8% of the embryos expressing the transgene [34-40]. When using methods applied to the embryo, such as pronuclear injection or embryonic stem cell manipulation, the success rate is also low at 1-8% [41-43]. Co-injection of pretreated sperm with a reporter gene into oocytes has been more successful resulting in 64-94% of embryos expressing the transgene, but only an average of 40% of the offspring expressed the transgene [44]. Use of this method is not optimal for stable transgene insertion since the first developmental checkpoint occurs after fertilization where the expression of transgenes with unstable incorporation are suppressed later in development or embryo viability is lost. If successful these methods are still limited to the number of stably modified offspring they could produce since the dependent factor is the embryo which most likely would only result in one offspring.

Several methods have been evaluated for their abilities to stably transduce spermatogonial stem cells including electroporation and retroviral infection. Electroporation of whole testis with a vector often results in somatic cell incorporation or transient germ cell expression [45-46]. Retroviral infection has been shown to be capable of stably incorporating a transgene into rodent spermatogonial stem cells, although at a low efficiency [27-29]. Insertion of a transgene into the SSCs within testis tissue prior to grafting could result in hundreds of spermatozoa with stable integration. In order to achieve the best success of SSC transduction a neonatal donor would be of benefit since the germ cell population would be enriched for gonocytes or SSCs. The road to efficient generation of genetically modified animals containing stably incorporated transgenes may lie through the spermatogonia stem cell. Both techniques of spermatogonia stem cell transplantation and ectopic testicular xenografting have potential to achieve this goal.

Spermatogonial stem cell transplantation, with the ability to maintain spermatogonial stem cells in culture being vital, and ectopic testicular xenografting are both methods with potential for efficient generation of transgenic animals through the male germ line. Bovine spermatogonial stem cell maintenance in vitro and the efficacy of ectopic testicular xenografting for generating transgenic bovine germ cells are evaluated in this dissertation. The global objective of this research is to develop models for generating transgenic animals through the male germ line. The first aim of this dissertation will cover the evaluation of bovine spermatogonial stem cell survival and proliferation in two separate culture systems. The objective of the first study was to evaluate spermatogonial stem cell survival and proliferation in a testis tissue explant culture system. In the second study the objective was to evaluate spermatogonial stem cell proliferation in a single cell co-culture system and investigate the

effects of GDNF on that proliferation. In order to achieve this objective we developed a bovine embryonic fibroblast cell line to serve as a feeder cell for a single cell suspension of bovine germ cells. In both these studies cross species germ cell transplantation into immunodeficient mouse testes was used to evaluate spermatogonial stem cell biological activity.

The second aim of this dissertation focuses on evaluating the establishment of spermatogenesis in neonatal bovine testicular tissue following ectopic xenografting and the utility of this technique for generating transgenic spermatozoa and will be covered by two separate experiments. The objectives of the first study were to evaluate the development of spermatogenesis in ectopically grafted neonatal bovine testicular tissue and investigate the utility of using electroporation to stably transfect spermatogonial stem cells within grafts with a foreign β-galactosidase transgene. The second study was aimed at evaluating differences in the establishment of spermatogenesis in bovine testicular tissue at different testicular developmental stages of postnatal life to optimize differentiated germ cell production.

The third aim of this dissertation is to evaluate depletion of endogenous germ cells in ram testes using several different methods. This experiment was conducted to provide initial development of a spermatogonial stem cell recipient model in a livestock species. This experiment will hopefully yield a model to aid in the development of spermatogonial stem cell transplantation technology in livestock.

In the appendix portion of this dissertation two studies are covered that were aimed at providing a better understanding of spermatogenesis in the mammalian testis. In the first study, gonadotropin influence on spermatogonial stem cell biology was investigated in the adult mouse testis using active immunization against LHRH and spermatogonial stem cell transplantation. In

the second study, expressions of key genes in the early developing bovine testis were investigated using real time RT-PCR analysis.

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CHAPTER TWO

Testis tissue explant culture supports survival and proliferation of bovine spermatogonial stem cells[1*]

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Abstract

The present study was designed to evaluate the survival and proliferation of bovine spermatogonial stem cells in an explant culture system over a 2wk period. Explants of calf testicular parenchyma were placed on 0.45μm pore membranes in culture and maintained for 1-2wk. Histological examinations of fresh (t0) and cultured tissues revealed morphologically normal seminiferous tubules. Germ cell numbers/tubule increased ($P \leq 0.05$) during culture when compared to t0, yet germ cell differentiation was not observed. Testosterone was present in medium throughout the culture period, indicating functional Leydig cells. Sertoli, spermatogonial, and spermatogonial stem cell viability was evaluated by RT-PCR for cell specific gene expression of stem cell factor, protein gene product 9.5, and GFRα1, respectively. Results demonstrated the expression of all genes at t0, 1wk, and 2wk of culture. Single cell suspensions were prepared from the testicular tissues at t0 and during culture and transplanted into nude mouse testes to investigate spermatogonial stem cell viability. One-month after transplantation, colonies of round bovine cells were identified in all mouse testes analyzed, indicating survival of spermatogonial stem cells. The average number of resulting colonies in recipient testes was significantly ($P \leq 0.05$) higher following 1wk of culture compared to t0, and was numerically higher at 2wk of culture compared to t0. This increase in colony numbers over time in culture indicates spermatogonial stem cell proliferation in vitro. This explant culture system appears to provide an environment that supports survival and proliferation of bovine spermatogonial stem cells.

Introduction

Sustained sperm production in the male relies on spermatogonial stem cells having the ability to undergo dual actions of (1) production of daughter progeny committed to the spermatogenic pathway and (2) self-replicating proliferation which allows for continuous sperm production throughout the life time of a male. Theoretically, one cell has the ability to give rise to 4,096 mature spermatozoa in the rat [1], although less occurs due to apoptosis. These cells have gained attention because of their demonstrated ability to restore fertility in infertile testes following transplantation in rodents [2,3]. In mice, spermatogonial stem cells have been genetically modified in vitro and resulted in transgenic offspring being sired from transplanted recipients [4]. Thus the genetic alteration of a single spermatogonial stem cell could give rise to thousands of modified spermatozoa after transplantation into a recipient testis.

In the mouse testis, gonocytes migrate to the basement membrane and undergo a conversion to stem cells from day 0-5 postnatally, where spermatogonial stem cell niches are formed from which all differentiating germ cells will arise [5]. In the bovine, this migration, conversion, and niche assembly occurs over a period of months; at 4wk of age gonocyte migration occurs and differentiated spermatogonia are not present until 8wk of age [6]. Therefore, within this period of 4-8wk of age spermatogonial stem cell conversion and niche assembly must occur. Previously, we have demonstrated that transplantation of germ cells from bull calves older than 4wk of age results in the best colonization in recipient mouse seminiferous tubules [7], further indicating that spermatogonial stem cell conversion is not complete until after 4wk of age in the bovine.

Knowledge of spermatogonial stem cells and mechanisms regulating biological activity has been hindered due to their rarity in the testis and lack of methods to isolate them. Currently,

spermatogonial stem cells can only be unequivocally identified on a functional basis by their ability to colonize recipient seminiferous tubules following transplantation. Recently, through the use of spermatogonial stem cell transplantation glial cell-line derived nerved growth factor (GDNF) has been demonstrated to play a role in regulating spermatogonial stem cell activity in mice [8,9]. In several systems including many types of neurons, GDNF has been shown to act on progenitor cells by binding to the receptor dimer of Ret tyrosine receptor kinase and GDNF family receptor-α1 (GFRα1) [10-12]. Therefore, it has been hypothesized that spermatogonial stem cells express the GFRα1 receptor.

Development of in vitro culture techniques that support the survival, proliferation and differentiation of spermatogonial stem cells will aid in the ability to study them as well as modify their genome. In vitro culture of rodent spermatogonial stem cells has been demonstrated using feeder layers for periods extending up to 4 mo [13]. However, culture of bovine germ stem cells has been met with challenges [7,14]. Maturation of bovine gonocytes through meiosis has been reported in vitro using calcium-alginate encapsulation [15], but stem cell activity using this method has not been investigated. The ability to stably modify bovine spermatogonial stem cells would be facilitated by use of an in vitro culture system that supports their survival and proliferation.

To date, spermatogonial stem cell transplantation between bulls is not possible; therefore, a bioassay model to evaluate bovine cells has been developed. Fresh bovine germ cells are capable of colonization and proliferation in recipient immunodeficient mouse seminiferous tubules [7,14]. This bioassay can be used to evaluate culture and transfection techniques with bovine spermatogonial stem cells. The present study was designed to evaluate the survival and proliferation of bovine spermatogonial stem cells in an explant culture system over a 2wk period.

We hypothesized that bovine spermatogonial stem cells would proliferate in explant cultures as tested by cross species transplantation.

Materials and Methods

Testicular tissue collection and culture

The Washington State University Institutional Animal Use and Care Committee approved all animal procedures. Testes removed from prepubertal bull calves (1-2 mo, n=3) served as sources of donor testicular parenchyma. Pieces (10-20 mg) were removed from the testes and cultured on 0.45-μm pore membranes (Millipore, Bedford, MA) within 6-well culture plates individualized by donor. Ten sections of tissue from each donor were placed on an individual membrane in a single well and 12 wells or 2 plates were used for each donor, equaling 120 sections per donor. Cultures were maintained for 1-2wk in culture medium (DMEM containing 10% FBS, 30 mg/ml penicillin, and 50 mg/ml streptomycin) at 32^{o}C in an atmosphere of 5% CO_2 in air. Media was collected every other day, stored at –20^{o}C, and fresh media replaced. Fresh (t0) samples were used as a representation of the starting cell populations.

Histology and testosterone RIA

Twenty tissue samples from each donor were fixed in Bouin's solution overnight at 4^{o}C at t0, 1, and 2wk of culture. Tissues were subsequently dehydrated in 70% ethanol, embedded in paraffin, and sectioned at 6 microns. Sections were stained with hemotoxylin to visualize cell nuclei. Slides were evaluated under inverted light microscopy and digital images were captured (Coolsnap Pro, MediaCybernetics, Carlsbad, CA). Average germ cell nuclei/tubule were quantified by calculating the average number of germ cell nuclei in 20 round tubules per sample.

Germ cell nuclei could be differentiated from somatic Sertoli cell nuclei by the distinct morphological differences between the two cell types. Round stained nuclei were considered to be germ cell while cells without round nuclear morphology and a single distinctive nucleolus were considered Sertoli cells and not included in the overall germ cell counts. Average tubule diameter was measured using an eyepiece micrometer on 20 tubules per sample. Medium samples were assayed for testosterone concentration using a commercial kit (DSL-400; Diagnostic System Laboratories, Webster, TX; detection limit of 0.05 ng/ml). Control medium was culture medium before addition to the explant cultures.

RT-PCR for stem cell factor, protein gene product 9.5, and GFRα1

At t0, 1, and 2wk of culture total cellular RNA was collected from 20 tissue sections of each donor using the Trizol method (Invitrogen, Carlsbad, CA). Complimentary DNA for each sample was synthesized by oligo(d)T primed reverse transcription using M-MLV reverse transcriptase (Invitrogen). Samples were then analyzed by PCR for expression of the Sertoli cell specific and spermatogonial specific expression of stem cell factor (SCF) and protein gene product 9.5 (PGP 9.5), respectively. Samples were also assessed for possible spermatogonial stem cell survival by investigating the expression of GFRα1. All samples were also assayed for expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), to ensure the quality of the template cDNA. All primer sets were designed from available GenBank sequences for the bovine (SCF, GAPDH, GFRα1) and horse (PGP 9.5), using ABI Primer Express 3 (Applied Biosystems, Foster City, CA). Primer sequences were as follows; 5'-ATTGGTGGCAAATCTTCCCA-3', 5'-TGCACTCCACAAGGTCATCAA-3' (SCF), 5'-ACCCCGAGATGCTGAACAAAG-3', 5'-CCCAATGGTCTGCTTCATGAA-3' (PGP 9.5), 5'-

ACGGCACAGTCAAGGCAGAG-3', 5'-GTGATGGCGTGGACAGTGGT-3' (GAPDH), and 5'-CCACCAGCATGTCCAATGAC-3', GAGCATCCCATAGCTGTGCTT-3' (GFRα1). Reactions of 25µl were performed containing 2mM $MgCl_2$, 0.25 mM dNTPs, 1X PCR buffer, 5 pmol of each primer, and 1U of *Taq* DNA polymerase. Reaction conditions were 94°C denaturation for 5 min followed by 35 cycles of 94°C for 30s, 58°C for 30s, and 72°C for 30s, with a final extension of 72°C for 10 min. Products were separated and visualized by 0.9% agarose gel electrophoresis with Gel Star staining (Molecular Probes, Eugene, OR).

Transplantation and colonization analysis

At t0, 1, and 2wk of culture single cell suspensions were collected from 20 testicular tissue sections of each donor by two-step enzymatic digestion [7]. Cells were then diluted to $2X10^7$ cells/ml, assessed for cell survival by trypan blue exclusion, and labeled with the fluorescent dye PKH26 (PKH26 Red Fluorescent Membrane Linker Dye; Sigma, St. Louis, MO). The labeling stably incorporates the fluorescent dye along with long aliphatic tails into regions of lipids of the cell membrane and has been used in germ cell transplantations between livestock species [16,17]. Cells were then washed extensively in DMEM by centrifugation to insure there was no residual dye in the suspension. Cells were again assessed for survival by trypan blue exclusion, diluted to $1X10^7$ cells/ml, and visualized by UV light microscopy with a TRITC filter to ensure adequate staining. Approximately 10µl of labeled cell suspension was microinjected into the seminiferous tubules of 8-10wk old recipient nude mice (n=3 mice/donor/time point, totaling 9 mice/time point) that had been treated with busulfan (33mg/kg body weight) 4wk prior to deplete endogenous germ cells [7].

Bovine germ cells were injected into the testes of recipient mice as described [7]. Briefly, recipient mice were anesthetized with a combination of ketamine (3.3µg) and xylazine (150µg) in sterile physiological saline. A mid-line incision was then made through the abdomen and the testes were exteriorized; a small hole was then made in the connective tissue surrounding the efferent bundle and a glass micro needle containing 10µl of cell suspension was inserted into the rete testis. Cells were then infused through the rete testis into the seminiferous tubules, the testes replaced, the incision closed with suture, and the animal allowed to recover. In most recipients, one testis was injected while the contralateral one served as a negative control. One month after transplantation recipient mice were killed by CO_2 inhalation and the testes were removed. Seminiferous tubules were dispersed with collagenase (1mg/ml) in Hanks balanced salt solution (HBSS) at 37^{o}C for 15 min with gentle agitation. Isolated tubules were then spread on glass slides and mounted with a cover slip under HBSS. Samples were visualized by UV light microscopy with a TRITC filter to identify donor bovine colonies. The numbers of colonies in the tubules from each recipient testis were counted and digital images were captured.

Statistical analysis

The experiments were replicated using three different donor animals. All statistical analyses were conducted using the Proc GLM function of SAS. Differences were determined by analyzing the data using a Duncan's test for significance between means. The main effects used in the model were time in culture, colony number, tubule diameter, average germ cell nuclei, and testosterone concentration. The level of significance was set at $P \leq 0.05$. Data in text and figures are presented as the mean$\pm$SEM.

Results

Histological analysis and testosterone production

Histological assessment of t0, 1, and 2wk-cultured tissues was conducted to evaluate seminiferous epithelium morphology. Microscopic visualization revealed seminiferous tubules were morphologically intact throughout the culture period and germ cells could be observed in tubules (Figure 1). Quantification of the average number of germ cell nuclei/tubule revealed an increase of germ cells ($P \leq 0.05$) in cultured tissue compared to t0 (Figure 2). In contrast, seminiferous tubule diameter decreased in cultured tissues compared to t0 (Figure 3). Although germ cell numbers increased during the culture period, no germ cell differentiation (i.e. meiotic cells) was observed.

To investigate Leydig cell viability during the culture period, medium collected on d7 and 14 from testis tissue explant cultures were assayed for production of testosterone. Testosterone was detected in the collected medium throughout the culture period (Figure 4); concentrations peaked after wk1 of culture (6.4 ± 2.7 ng/ml) and then declined during wk2 (3.3 ± 1.3 ng/ml). Testosterone concentration in the control medium (culture medium before addition to testis tissue explant cultures) was below the detectable limit of the assay (0.05 ng/ml).

Sertoli cell, spermatogonial, and spermatogonial stem cell specific gene expression

The function of Sertoli cells in the testis tissue explant cultures was assessed by evaluating the expression of SCF at each time point. The expression of SCF was detected by RT-PCR at t0, 1wk, and 2wk of culture (Figure 5). Similarly, the presence of spermatogonia was assessed by RT-PCR for the expression of PGP 9.5. Like the expression of SCF, PGP 9.5

expression was detected at t0, 1wk, and 2wk of culture (Figure 5). Spermatogonial stem cell survival was assessed by examination of the expression of GFRα1 in the fresh and cultured tissues. Expression of GFRα1 was detected in all tissues examined at t0, 1wk, and 2wk of culture (Figure 5). Thus, Sertoli cells, spermatogonia, and spermatogonial stem cells were present and functional in the cultured tissues.

Transplantation

To investigate spermatogonial stem cell survival and proliferation during explant culture, fluorescently labeled single testicular cell suspensions (Figure 6A) collected at t0, 1, and 2wk of culture were transplanted into busulfan treated recipient immunodeficient mouse testes. Fluorescently labeled colonies of round donor bovine cells were identified in all recipient testes analyzed 1mo after transplantation (Table 1). Microscopic visualization of donor testes revealed clusters or chains of donor cells in multiple areas throughout recipient seminiferous tubules (Figure 6; Table 1). Negative control testes, those not injected, showed no fluorescent cell colonies. The number of resulting colonies from 1 wk cultured tissues was higher ($P \leq 0.05$) than t0 colony numbers (Figure 7). Colony numbers arising from 2 wk-cultured tissues were numerically higher than those from t0; however, this difference was not statistically significant (Figure 7).

Discussion

In this study, a culture system was devised where Sertoli, Leydig, and spermatogonial stem cell interaction would be maintained. Results demonstrated that Sertoli cells remain viable at least 2wk during tissue explant culture based on their ability to express SCF. This ligand

secreted by Sertoli cells has been demonstrated to be essential in regulating spermatogonial proliferation in the testis through action on c-kit (SCF receptor) present on differentiated spermatogonia [18]. Interstitial Leydig cell testosterone production has been clearly demonstrated to be obligatory for spermatogenesis on both a qualitative and quantitative level [19-21]. The requirements of testosterone on spermatogonial stem cell actions are currently unknown, but undoubtedly play a role. Demonstration of testosterone production throughout the culture period in this study indicates survival of Leydig cells for at least 2wk. Both stem cell factor and testosterone secretion in the testis are known to be under control of FSH and LH, respectively. The source of FSH and LH in the culture system used came from the serum added to the media (confirmed by RIA, LH 0.54 ng/ml and 38.13 ng/ml FSH). Immunohistochemical evaluation of the bovine testis has revealed the spermatogonial specific expression of PGP 9.5 [22]; however, the exact spermatogonial cell type expressing it is unknown. Expression of this gene throughout the culture period indicates the survival of spermatogonia for at least 2wk.

Development of in vitro culture systems for bovine spermatogonial stem cells has been a challenge due to the lack of a functional system to evaluate their presence in a cell suspension. Cross species transplant of bovine cells into mouse testes is the only functional method for evaluating the survival and proliferation of bovine spermatogonial cells in culture [7,14].

The demonstration in this study that explant cultured bovine cells could form round cell colonies in recipient mouse seminiferous tubules indicates the survival of spermatogonial stem cells. The round cell morphology observed of the colonized bovine cells is consistent with spermatogonial stem cell colonization as previously observed in other cross species transplantations into recipient mouse testes [14,23,24]. Also, this observation is inconsistent with the appearance of somatic cell colonization in recipient testes as has been previously

demonstrated in rodent transplantations [25-27]. Donor bull germ cells used in this study were from animals at a developmental stage in which the testis germ cell population consists solely of undifferentiated gonocytes or spermatogonial stem cells. Therefore, it is unlikely that the colonizing donor cells in recipient mouse seminiferous tubules were differentiated germ cells or somatic cells.

The further demonstration that resulting colony numbers increased compared to t0 or fresh cells indicates that these cells were proliferating during the culture period. This observation in explant cultures of bovine testis is in contrast to feeder cell co-cultures of mouse spermatogonial stem cells, in which the stem cells decreased in a time dependent manner over a 7 day culture when compared to the fresh cell suspensions [8]. The observation that colony numbers were significantly higher in 1wk cultured tissue compared to t0 but not statistically significant ($P \geq 0.05$) at 2wk of culture indicates that the spermatogonial stem cells are losing viability at 2wk of explant culture. Another possible explanation of this observed effect is that the numerical and statistical difference between 1 and 2wk of culture were minimal and could be a cause of a steady state level of proliferation and apoptosis of the stem cells.

The exact mechanism that stimulated this proliferation remains unknown, but speculation could be made that Sertoli and interstitial cells are inducing proliferation through paracrine actions in response to lack of meiotic germ cells in the seminiferous epithelium. Recently, it has been demonstrated that glial cell-line derived nerve growth factor (GDNF) induces proliferation of mouse spermatogonial stem cells [9]. Addition of GDNF to mouse testis germ cell co-cultures has been demonstrated to enhance maintenance of spermatogonial stem cells [8]. It is possible that enhanced GDNF expression in the cultured bovine tissues induced spermatogonial stem cell proliferation, thus increasing the numbers of cells present compared to the fresh cell suspension.

In neuronal progenitor cells, GDNF has been demonstrated to act on a receptor dimmer consisting of Ret tyrosine kinase and GFRα1. With the demonstration of GDNF actions on spermatogonial stem cells, it has been hypothesized that spermatogonial stem cells may express GFRα1. In this study we were able to show the expression of the gene for GFRα1 throughout the culture period, thus supporting the conclusion of spermatogonial stem cell survival for at least 2wk of explant testis tissue culture.

Another explanation of spermatogonial stem cell proliferation is related to the age of the donor animal, at 1-2 mo of age in the bovine, gonocytes have just migrated to the basement membrane and are likely undergoing conversion to stem cells and forming niches. Removal of the tissue at this age could trigger spontaneous stem cell conversion and proliferation in vitro. Culture of gonocytes from neonatal bull calves in a calcium alginate encapsulation system has been demonstrated to support the maturation of round spermatids by 10 weeks of culture [15]. This maturation is at an accelerated rate compared to the in vivo situation of a normal bull, in which haploid germ cells are not present until 24-28 weeks of age [6]. This accelerated rate of in vitro maturation in the bovine supports the theory that removal of the germ cells and somatic cells from a prepubertal animal stimulates spontaneous germ cell actions in vitro. Likewise, in vivo the testis is maintained at higher temperature than the culture temperature in this study. Many reports have demonstrated an inhibition of spermatogenesis at higher temperatures in bulls and rodents due to cryptorchidism [28-30]. The lowered temperature at which the tissue explants were cultured may have triggered stem cell proliferation, which would not normally be seen at 1-2mo of age in the intact bull calf.

Histological examination of the cultured tissues revealed maintenance of seminiferous tubular structure and a significant increase in germ cell nuclei/tubule when compared to fresh

tissue. The presence of meiotic cells in the cultured tissues was not observed, indicating spermatogonial proliferation and/or differentiation. Tubular diameter decreased and interstitial area appeared to increase in cultured tissues compared with t0. Similar to the apparent proliferation of spermatogonia, the exact reasons of these observations are elusive. Speculation could be made that the concentrations of FSH and testosterone in the medium were reduced compared to intertesticular concentrations in vivo. Sertoli and Leydig cells appeared to retain their functional abilities to produce growth factors and steroids, respectively. Taken together, increase in germ cell nuclei/tubule and increased colonization of cultured cells supports the conclusion that spermatogonial stem cells proliferated during explant culture. In vivo, maturation of male germ cells in the testis is dependent on interaction with Sertoli cells. It has been well established that both testosterone and FSH are necessary for qualitative and quantitative spermatogenesis [31,32]. However, the exact mechanisms that control spermatogonial stem cell activity in the testis are unknown.

The actions of the spermatogonial cell population govern the overall outcome of spermatogenesis, that is, the number of spermatozoa produced daily in the testis. Spermatogonial stem cell proliferation is the determining factor of how many differentiated type A spermatogonia will be available to further mature, undergo meiosis, and transform into spermatozoa. Spermatogonial stem cells undergo both self-replicating proliferation and production of daughter progeny to become type A spermatogonia. These important aspects of spermatogonial stem cells have gained them much interest for use in reproductive technologies such as spermatogonial stem cell transplantation and transgenesis. Stable genetic modification of one male germ stem cell has the potential to provide a system by which thousands of genetically modified sperm could be produced. Moreover, in vitro culture of these cells has the ability to

provide a model system to unravel the mechanisms controlling their actions in vivo. The ability to culture spermatogonial stem cells may give us a new avenue through male gametes to introduce transgenes into domestic animals.

The ultimate application of spermatogonial stem cell cultures and transplantation technology in livestock may be the ability to generate transgenic offspring. In order to accomplish this several challenges must be overcome. One of these is the ability to culture spermatogonial stem cells from livestock animals. The current study demonstrates the survival and proliferation of bovine spermatogonial stem cells during explant testis tissue culture over a 2wk period. This culture system could now be tested in conjunction with in vitro genetic modification techniques to alter bovine spermatogonial stem cells such as electroporation or retroviral transfection, with subsequent transfer into a recipient host's testis.

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Table 1. Colonization of recipient mouse testes by culture bovine germ cells.

Time in Culture	Colonized recipient testes/Recipient testes injected	AVG # of colonies/recipient testis
t0	7/7	32.9
1wk	10/10	56.8
2wk	9/9	51.6

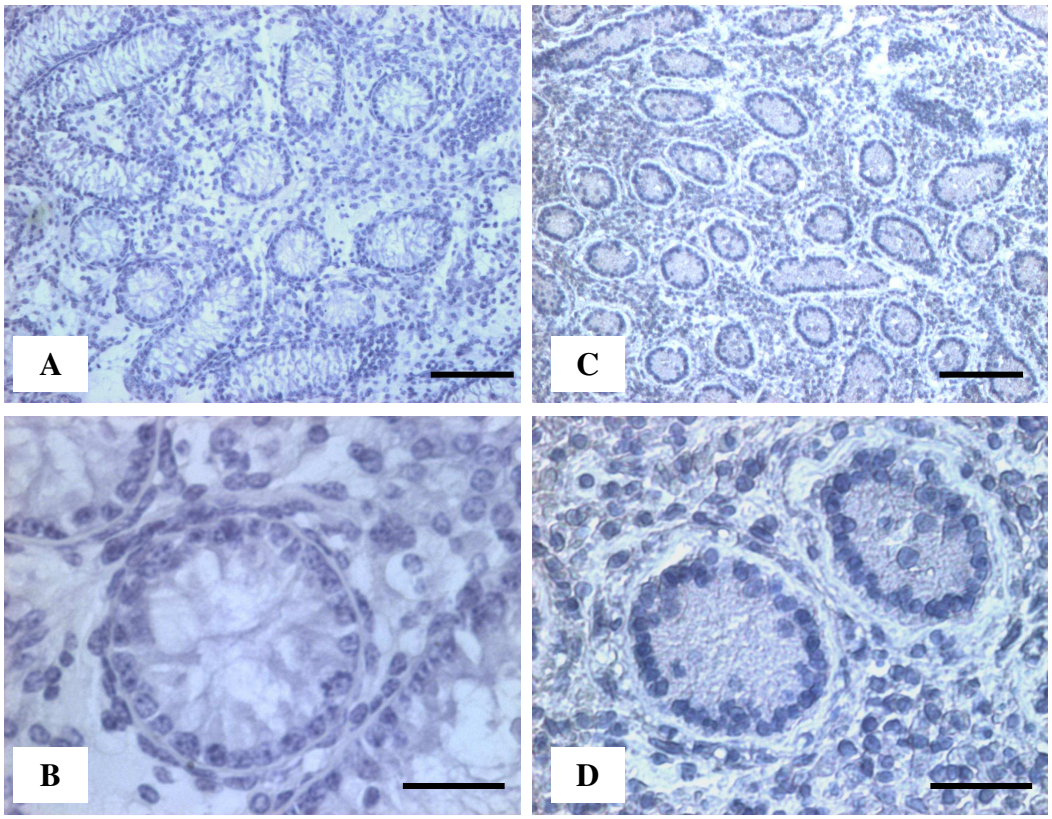


Figure 1. Photomicrographs showing cross sections of A/B) t0 testicular parenchyma, and C/D) 2wk cultured testicular parenchyma. Cultured tissue had a significant increase in numbers of germ cells . Bar = 100µm (A and C) and 30µm (B/D).

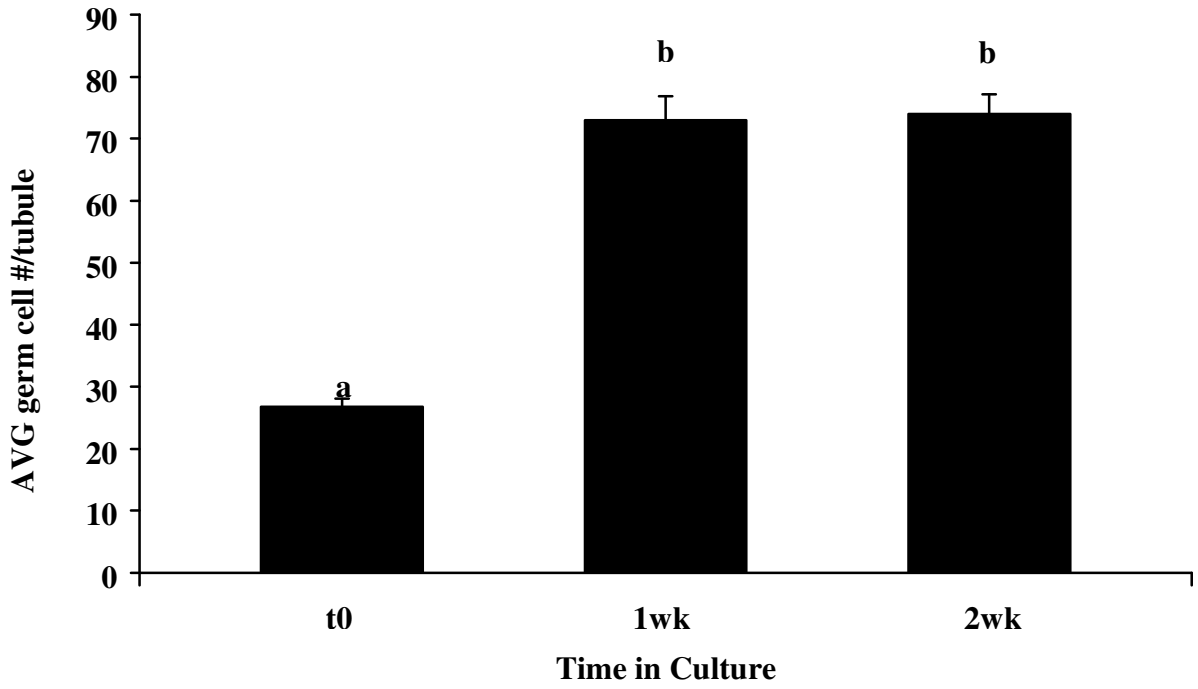


Figure 2. Average germ cell numbers/tubule in t0 and cultured tissues. Data are presented as the mean$\pm$SEM. Bars with different letters are different at $P \leq 0.05$.

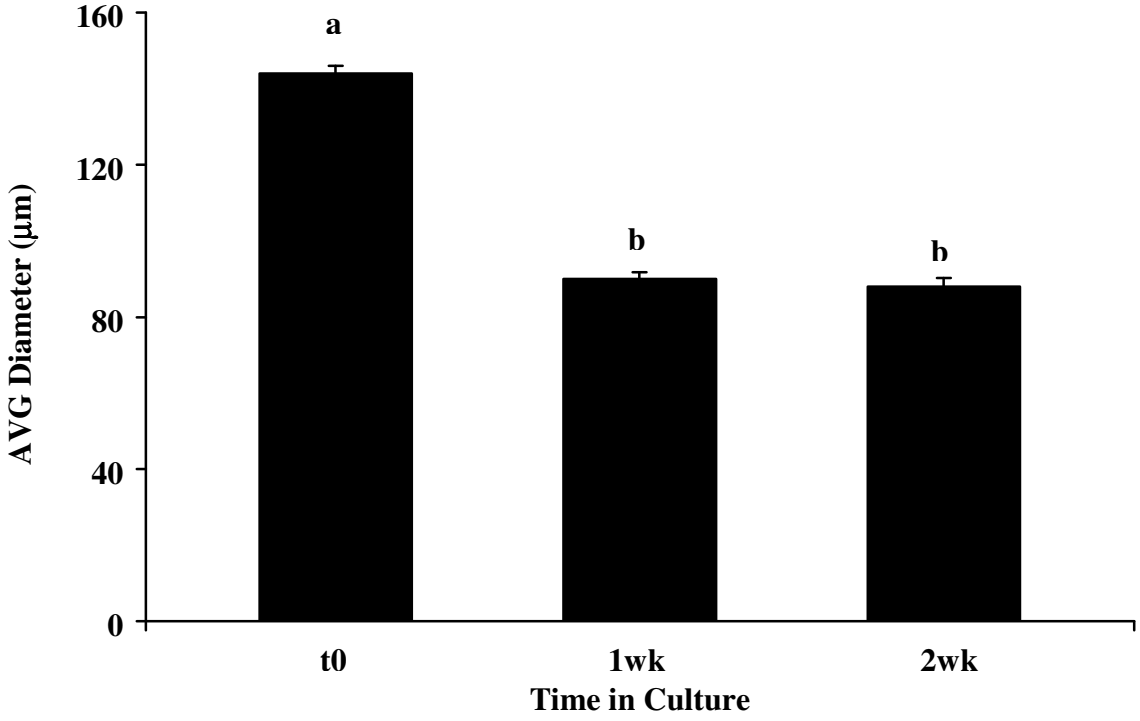


Figure 3. Average seminiferous tubule diameter in t0 and cultured tissue. Data are presented as the mean±SEM. Bars with different letters are different at P≤0.05.

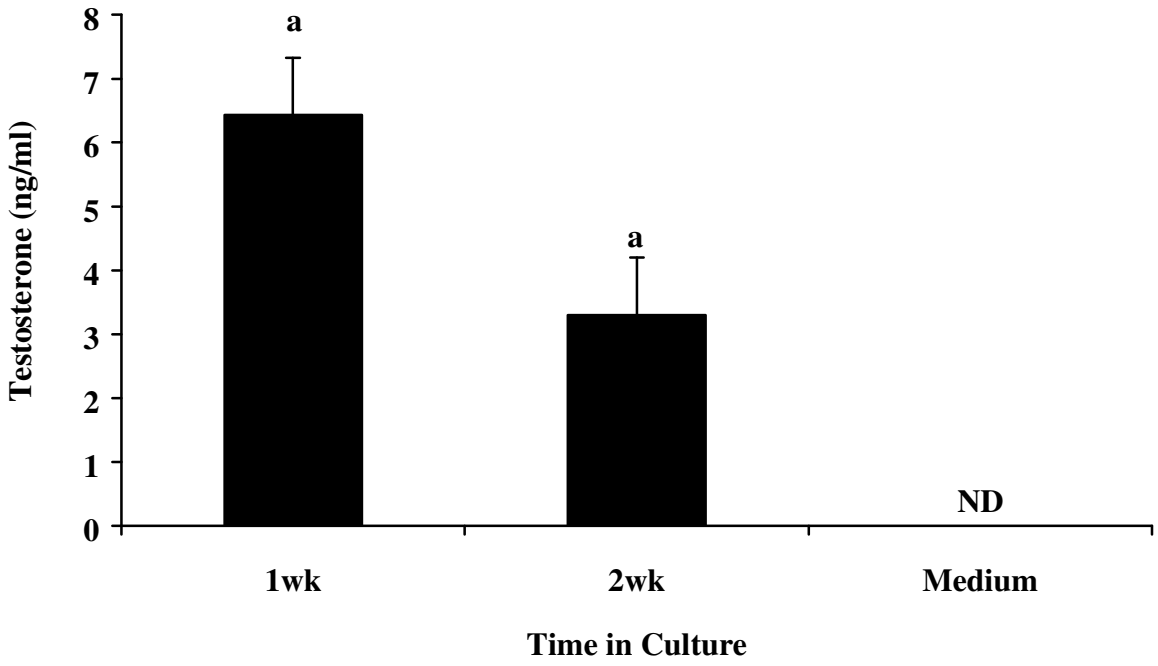


Figure 4. Testosterone concentration in tissue culture medium throughout a 2wk period. Data are presented as the mean±SEM.

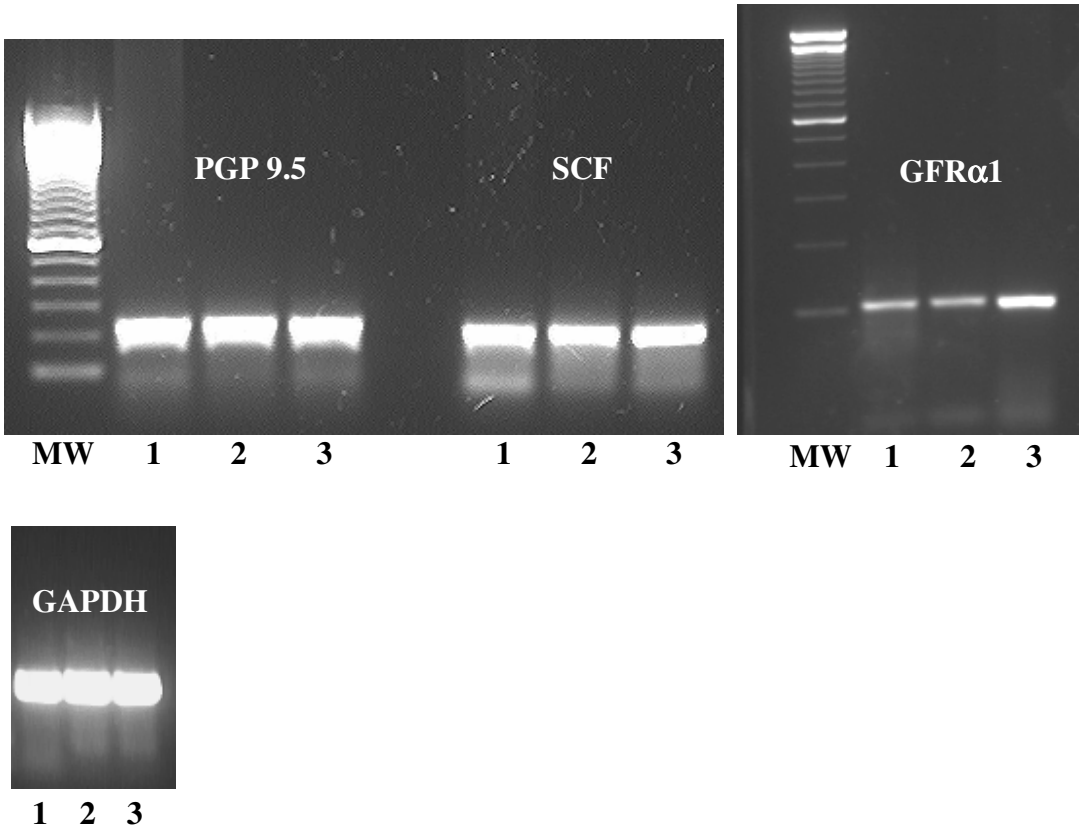
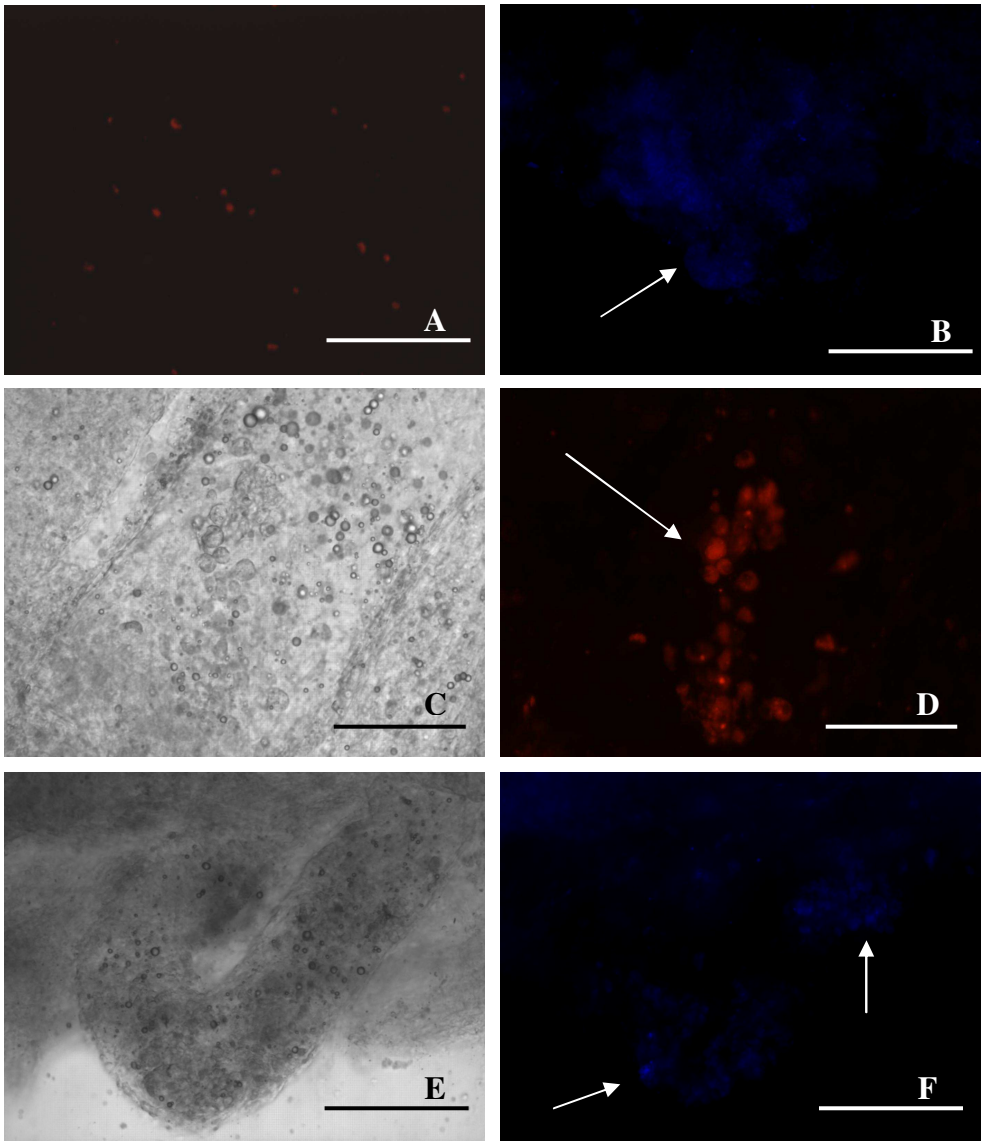


Figure 5. Expression of PGP 9.5, SCF, GFRα1, and GAPDH genes throughout the culture period. **MW:** 100 bp DNA ladder, **1:** t0, **2:** 1wk culture, and **3:** 2wk culture.

Figure 6. Transplantation of bovine testis germ cells into mouse seminiferous tubules. Bovine germ stem cells that colonized recipient mouse seminiferous tubules formed chains or colonies of cells (arrows). A) Cultured single cell suspension after fluorescent staining prior to transplant (100X magnification), B) Colonized recipient tubules 1mo after transplant with 2wk cultured bovine cells (40X magnification), C/D) Colonized recipient tubule 1mo after transplantation with 2wk cultured bovine cells (200X magnification, (C) bright light view (D) corresponding fluorescent view), and E/F) Colonized recipient tubule 1mo after transplant with 1wk cultured bovine cells (100X magnification, (E) bright light view (F) corresponding fluorescent view). Bar = 100 μm (A, B, E, F) and 40 μm (C, D).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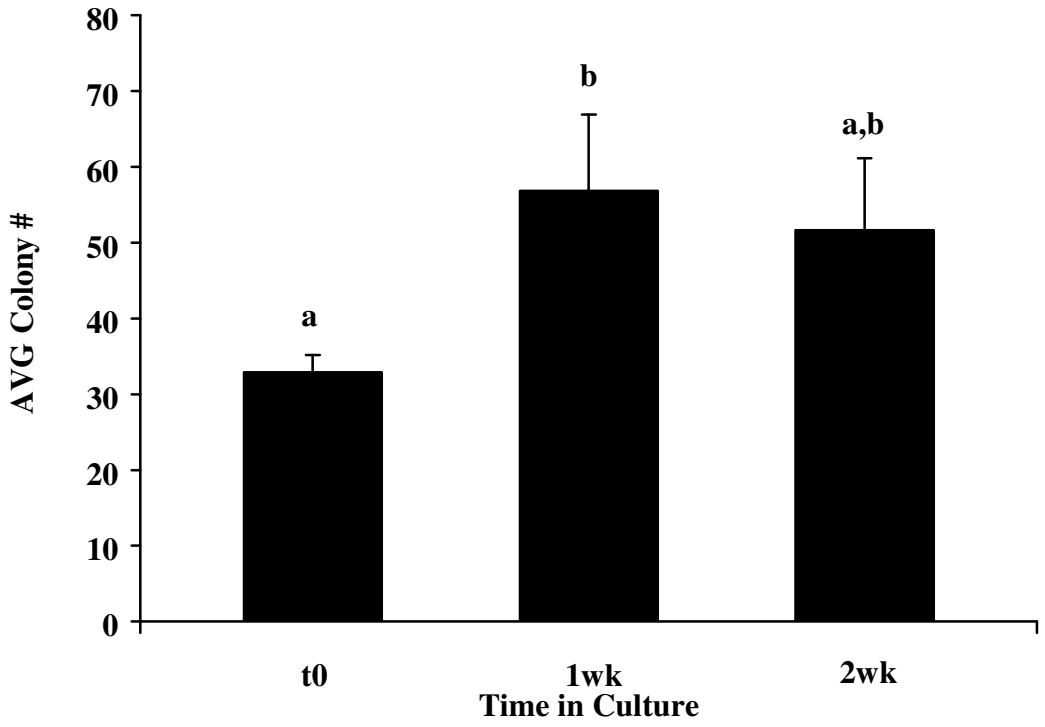

Figure 7. Number of donor bovine colonies in recipient mouse seminiferous tubules 1 mo after transplantation with fresh and cultured bovine testicular germ cells. Data are presented as the mean$\pm$SEM. Bars with different letters are different at $P \leq 0.05$.

CHAPTER THREE

Biological activity of cryopreserved bovine spermatogonial stem cells during in vitro culture*

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Abstract

Spermatogonial stem cell functional roles in spermatogenesis are self-renewing proliferation and production of differentiated daughter progeny. The ability to recapitulate these actions in vitro is important for investigating their biology and inducing genetic modification which could potentially lead to an alternative means of generating transgenic animals. The objective of this study was to evaluate the survival and proliferation of frozen-thawed bovine spermatogonial stem cells in vitro and investigate the effects of exogeneous GDNF. In order to accomplish this objective we developed a bovine embryonic fibroblast feeder cell line, termed BEF, to serve as feeder cells in a co-culture system with bovine germ cells. Bovine spermatogonial stem cell survival and proliferation in vitro was evaluated by xenogeneic transplantation into the seminiferous tubules of immunodeficient mice. Bovine germ cells co-cultured for 1wk resulted in significantly more round cell donor colonies in recipient mouse testes compared to donor cells transplanted just after thawing. Bovine germ cells co-cultured for 2wk had fewer colony forming cells than the freshly thawed cell suspensions or cells cultured for 1wk. Characterization of the feeder cell line revealed endogenous expression of GDNF mRNA and protein. Addition of exogenous GDNF to the culture medium decreased the number of stem cells present at 1wk of co-culture, but enhanced stem cell maintenance at 2wk compared to cultures without added GDNF. These data indicate that frozen-thawed bovine spermatogonial stem cells survive cryopreservation and can be maintained during co-culture with a feeder cell line in which the maintenance is influenced by GDNF.

Introduction

Spermatogenesis is a highly organized complex process that relies on undifferentiated spermatogonial stem cells to undergo self-replication and production of differentiated daughter cells to provide a continual supply of spermatozoa. Transplantation of spermatogonial stem cells from a donor mouse testis into the seminiferous tubules of a recipient mouse testis has been shown to result in donor derived spermatogenesis [1, 2]. Long-term culture and cryopreservation of rodent spermatogonial stem cells has also been demonstrated [3-6]. Use of in vitro culture techniques, cryopreservation and transplantation has opened a new avenue to study the biology of spermatogonial stem cells with hope of better understanding male germ cell biology and regulatory factors of male fertility.

Another area of application for spermatogonial stem cell culture, cryopreservation and transplantation is domestic livestock. Spermatogonial stem cell transplantation can potentially be utilized in domestic livestock as a means to preserve a male's germ cell line, increase genetic gain in domestic herds, and generate transgenic animals. In vitro culture and cryopreservation of spermatogonial stem cells are integral parts for the application of this technology. A system that supports the proliferation and maintenance of spermatogonial stem cells in vitro could be used to preserve and expand spermatogonial stem cell numbers as well as aid in genetic modification. Spermatogonial stem cell transplantation has been demonstrated in rodents [1-2] and goats [7] and bovine spermatogonial stem cells have been demonstrated to be capable of colonizing recipient mouse seminiferous tubules, but not undergo differentiation into spermatozoa [8-10].

Over the past 10 years all definitive reports of maintenance of spermatogonial stem cells in vitro have relied on co-culture with a feeder cell monolayer [4, 6]. In initial studies with the mouse, co-culture with a STO cell feeder line was demonstrated to support the survival of

spermatogonial stem cells for several months [4]. Investigation of mouse spermatogonial stem cell maintenance during the first 7 days of co-culture has shown a decline in stem cell number compared to the starting cell population [6]. Addition of exogenous glial cell line-derived neurotrophic factor (GDNF) was able to enhance the maintenance of spermatogonial stem cells within these co-cultures [6]. Over expression of GDNF in mice resulted in an accumulation of undifferentiated spermatogonia [11]. Both of these reports have implicated GDNF as a survival and proliferation factor for spermatogonial stem cells.

In recent years maintenance of bovine germ cells in vitro without feeder cells has been demonstrated. Lee et al. [12] used a calcium alginate encapsulation method and demonstrated the differentiation of bovine gonocytes to haploid cells; however, spermatogonial stem cell maintenance was not evaluated. Izadyar et al. [13] used a gel matrix to support the proliferation and differentiation of bovine spermatogonia in vitro; however, analysis of spermatogonial stem cell survival was limited due to lack of a labeling method for the donor cells prior to transplantation. We have previously demonstrated the survival and proliferation of bovine spermatogonial stem cells over a 1wk period using a testis tissue explant culture system [10]. This system involves the culture of small pieces of testicular tissue and is limited because tissue infrastructure breakdown begins shortly after 1wk of culture. Explant culture is a promising method for inducing short term increases in spermatogonial stem cell numbers, but limited for long-term use and genetic modification of stem cells. A system that allows for the culture of a single cell suspension of bovine spermatogonial stem cells would be more beneficial for long-term survival and genetic modification.

Cryopreservation can be used to preserve a male spermatogonial stem cell line. Culture and cryopreservation in combination could be used to immortalize a male's genetic line through

the germ cells due to the spermatogonial stem cells ability for self-replication. The efficacy of this approach with livestock spermatogonial stem cells has not been clearly demonstrated. The objective of the current study was to evaluate the maintenance of frozen-thawed bovine spermatogonial stem cells during in vitro culture with and without addition of exogenous GNDF. To accomplish this objective we developed a bovine embryonic fibroblast feeder cell line for co-culture with bovine germ cells and hypothesized that bovine spermatogonial stem cell maintenance could be sustained in this system and enhanced by GDNF addition.

Materials and Methods

Donor bull germ cell collection and cryopreservation

All animal procedures were approved by the Washington State University Institutional Animal Care and Use Committee. Testicular germ cells were collected from donor bull calves (1-2 mo of age, n=3) by enzymatic digestion of testicular parenchyma as previously described [10]. Cells were diluted to $1X10^7$ cells/ml and suspended in freeze medium (DMEM containing 10% FBS, 30mg/ml penicillin, 50 mg/ml streptomycin, and 10% DMSO). The suspensions were placed into freeze vials at 1ml volumes and kept at -80^oC for 24hr. Vials were then transferred to liquid nitrogen for long-term storage. Approximately 10 mo after being cryopreserved the cell suspensions were thawed in a 37^oC water bath and cells were diluted in DMEM. Cells were then collected by centrifugation and resuspended in DMEM containing 10% FBS. Cell concentration and survival was determined using a hemocytometer and trypan blue exclusion, respectively. Collected cells were then used for transplantation into recipient testes immediately or in vitro culture.

Bovine Embryonic Fibroblast feeder cell co-culture

A bovine embryonic fibroblast feeder cell line (BEF-JMO#5) was created for use in a co-culture system with cryopreserved bovine germ cells. This cell line was created from a day 35 bovine embryo following previously described procedures for creation of embryonic fibroblast feeder cell lines [14]. Briefly, a day 35 bovine embryo was collected into physiological saline and chopped into small sections with a sterile razor blade. Chopped tissue was placed into a 10ml solution of Hanks balanced salt solution (HBSS) containing 0.05% trypsin and 1mM EDTA and incubated at 37^{o}C for 15min. Tissue was then allowed to settle on ice for 2min followed by passing the suspension through a 14-gauge needle several times. Another 10ml of enzyme solution was added and incubated again at 37^{o}C for 15 min. The suspension was again allowed to settle on ice for 2min and the supernatant removed for cell collection by centrifugation at 300 xg for 5min. Collected cells were resuspended in culture medium (DMEM with 10% FBS, 30 mg/ml penicillin, and 50 mg/ml strepromycin) and grown in culture at 37^{o}C and frozen stocks were created.

After thawing, bovine germ cells were diluted to provide 1 X 10^5 live cells/cm^2 and placed into 25-cm^2 tissue culture flasks containing a BEF feeder cell monolayer that was mitotically arrested with mitomycin-C [4]. Bovine germ cells were co-cultured for 1 or 2wk at 32^{o}C in an atmosphere of 5% CO_2 in air. Culture medium consisted of DMEM containing 10% FBS, 1X Gibco vitamin solution, 30mg/ml penicillin, and 50mg/ml streptomycin.

Xenogeneic transplantation of cryopreserved and co-cultured bovine germ cells and colonization analysis

Bovine testis cells were collected just after thawing from cyropreservation or after 1 and 2wk of co-culture with BEF cells by trypsin-EDTA digestion and diluted to $1X10^7$ live cells/ml in HBSS. Cells were then labeled with the fluorescent PKH26 dye following manufacturer instructions (Sigma; St. Louis, MO) and microinjected into the seminiferous tubules of recipient nude mice (n=3 mice/donor/time point) that had been treated with busulfan (30mg/kg b.w.) to deplete endogenous germ cells as previously described [10]. Breifly, approximately 10µl of cell suspension was microinjected through a beveled glass needle that was inserted into a small hole that was previously made in the connective tissue surrounding the efferent bundle. The cell suspension was then infused through the rete testis into the seminiferous tubules. With all recipient mice, one testis was transplanted with bovine germ cells and the contralateral testis used as a non-injected negative control.

One month after transplantation recipient nude mice were killed by CO_2 inhalation followed by cervical dislocation. The testes were removed, placed in HBSS and detunicated. The seminiferous tubules were then manually dispersed, spread on a glass microscope slide and mounted under a cover slip with HBSS. The slides were then analyzed using fluorescent microscopy for detection and quantification of donor bovine colonies at 100X magnification. The total number of fluorescent donor bovine colonies within the seminiferous tubules of each recipient mouse testis was manually counted and digital images were captured with a CoolSnap *cf* digital camera (Media Cybernetics; Silver Spring, MD).

RNA isolation and RT-PCR

Total cellular RNA was collected from co-cultures of bovine germ and BEF cells at 1 and 2wk, and cultures of mitomycin-C treated BEF alone using the Trizol method

(Invitrogen, Carlsbad, CA). Isolated RNA was reverse transcribed to cDNA using oligo(d)T priming and M-MLV reverse transcriptase. Samples were then analyzed by PCR for the expression of GDNF family receptor-α1 (GFRα1). A partial characterization of BEF feeder cells was conducted by analyzing samples of mitomycin-C treated BEF cells for the expression of the growth factors leukemia inhibitory factor (LIF), stem cell factor (SCF), and GDNF. All samples were also analyzed for the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), to ensure the quality of template cDNAs. Primer sets were designed from available GenBank or TIGR sequences of the bovine genes using ABI Primer Express 3 (Applied Biosystems; Foster City, CA). The primer sequences and GenBank accession numbers are as follows; GFRα1 (5'-CCACCAGCATGTCCAATGAC-3', GAGCATCCCATAGCTGTGCTT-3'; from TIGR gene index TC213602), LIF (5'-CTGGTTCTCCACTGGAAACACG-3', 5'-TGCACAGCTTGTCCAGGTTGT-3'; from GenBank NM173931), SCF (5'-ATTGGTGGCAAATCTTCCCA-3', 5'-TGCACTCCACAAGGTCATCAA-3'; from GenBank NM174375), GDNF (5'-GCAGCCGAAACAATGTACGA, 5'-AAGGCGATGGGTCTGCAA-3'; from GenBank AY382559), and GAPDH (5'- ACGGCACAGTCAAGGCAGAG-3', 5'-GTGATGGCGTGGACAGTGGT-3'; from GenBank U85042). Reactions of 25μl were performed containing 2mM $MgCl_2$, 0.25 mM dNTPs, 1X PCR buffer, 5 pmol of each primer, and 1U of *Taq* DNA polymerase. Reaction conditions were 94°C denaturation for 5 min followed by 35 cycles of 94°C for 30s, 58°C for 30s, and 72°C for 30s, with a final extension of 72°C for 10 min. Products were separated and visualized by 0.9% agarose gel electrophoresis with Gel Star staining (Molecular Probes, Eugene, OR). Cultures of mitomycin-C treated BEF cells maintained for 2 wk in vitro were also used as negative control samples for the expression of the germ cell specific GFRα1.

Immunohistochemisty for GDNF production by BEF cells

Mitomycin-C treated BEF cells maintained in culture for 1 or 2wk were fixed with Acetone:Methanol (1:1) for 10min at 4^{o}C followed by washing in PBS. Non specific antibody binding was then blocked by incubating samples with 10% non immune goat serum for 15min at room temperature. Samples were then incubated at 4^{o}C overnight with primary antibody (monoclonal mouse anti human GDNF; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:250 in PBS. The next day samples were washed with three changes of PBS and incubated at 37^{o}C for 1hr with horse-radish peroxidase (HRP) labeled secondary antibody (goat anti mouse IgG; BioRad; Hercules, CA) diluted 1:100 in PBS. Samples were again washed with three changes of PBS, stained for HRP with an aminoethyl carbazole (AEC) kit following manufacturer's instructions (AEC Substrate Kit; Santa Cruz Biotechnology). Counterstaining was done with hematoxylin, samples were evaluated using light microscopy, and digital images were captured at 100X magnification. Controls consisted of cells incubated with the omission of primary antibody, secondary antibody, and AEC chromogen.

Exogenous GDNF addition to cultures

In order to evaluate the effect of exogenous recombinant GDNF on bovine spermatogonial stem cell survival and proliferation during BEF co-culture, recombinant human GDNF was added to the cultures (PeproTech Inc.; Rocky Hill, NJ). Bovine cells were maintained in BEF co-culture as described above in DMEM with 10% FBS, 1X Gibco vitamin solution, 30 mg/ml penicillin, 50 mg/ml streptomycin, and 100 ng/ml GDNF was added.

Cultures were maintained for 1-2 wk followed by RNA isolation or trypsinization and transplant into recipient mouse seminiferous tubules as described.

Statistical analyses

All data were analyzed using the SAS system software with the Proc GLM function. Differences between means were determined using Duncan's test for significance for average donor bovine colonies arising from transplants of frozen-thawed germ cells, 1 and 2wk BEF co-cultured germ cells, and 1 and 2wk BEF co-cultured germ cells with exogenous GDNF. Data were considered significantly different at $P \leq 0.05$. In all figures data are presented as the mean$\pm$SEM.

Results

Colonization of cryopreserved bovine spermatogonial stem cells cultured on BEF feeder cells

To investigate the ability of bovine spermatogonial stem cells to survive during cryopreservation, donor bovine germ cells were collected, cryopreserved for approximately 10mo then transplanted into recipient mouse seminiferous tubules. One month after transplantation colonies of round donor bovine cells were identified within the seminiferous tubules of all recipient mouse testes (Figure 1A). The average number of donor bovine colonies per testis was 25.9$\pm$1.8 (Figure 2).

The maintenance of bovine spermatogonial stem cells during BEF co-culture was also evaluated by cross species testicular germ cell transplantation. Total cell populations from 1 and 2wk cryopreserved bovine germ cells cultured on BEF feeder cells were transplanted into the seminiferous tubules of recipient nude mice to investigate spermatogonial stem cell maintenance.

One month after transplantation colonies of round donor cells were detected in all recipient mouse testes analyzed (Figure 1B). The average number of colonies from 1wk cultures (49$\pm$5.6) was significantly higher than the average number of colonies from cells immediately transplanted after thawing. The average number of colonies resulting from 2wk cultured cells (10.6$\pm$3.6) was significantly lower than those arising from both frozen-thawed and 1wk cultured germ cells (Figure 2). Negative control recipient testes showed no fluorescent colonies of cells.

RT-PCR for germ cell specific and growth factor genes

Isolated RNA from testis cell co-cultures at 1 and 2wk were assayed by RT-PCR for expression of GFRα1, which has been shown to be expressed by undifferentiated spermatogonia and suggested as a spermatogonial stem cell marker [11, 15]. The presence of germ cells in both 1 and 2wk co-cultures of testis cells on BEF feeder cells was confirmed by expression of GFRα1 (Figure 3), also suggesting the presence of spermatogonial stem cells. Control cultures of BEF cells alone showed no expression of GFRα1 (Figure 3).

GDNF addition to cultures

The factor GDNF has been shown to enhance the maintenance of murine spermatogonial stem cells in vitro [6]. The BEF feeder cells created in this study endogenously express GDNF (Figure 4), suggesting this factor also enhances bovine spermatogonial stem cell maintenance in vitro. In order to investigate the effects of exogenous GNDF on maintenance of bovine spermatogonial stem cells during in vitro BEF co-culture, a recombinant GDNF was added to the culture media at a concentration of 100 ng/ml. The average number of bovine spermatogonial stem cell colonies in recipient seminiferous tubules arising from 1wk cultured cells with GDNF

was significantly lower than 1wk cultures without exogenous GDNF (Figure 2). In contrast, the average number of resulting colonies from 2wk cultured bovine germ cells with GNDF was significantly higher than 2wk cultured cells without the exogenous GDNF (Figure 2).

Characterization of BEF feeder cell line

Microscopic evaluation of the BEF cell line created in this study revealed a fibroblast morphology (Figure 4). In order to partially characterize the cell line, RT-PCR was used to investigate the expression of several growth factors suggested to act as mitogens on male germ cells (LIF, SCF, and GDNF). Of the three factors investigated by RT-PCR only GDNF was found to be expressed by the BEF feeder cells (Figure 4A). Immunohistochemical labeling of GDNF showed production of the protein by BEF cells as well, with intense cytoplasmic distribution (Figure 4B).

Discussion

An in vitro system that supports spermatogonial stem cell survival and proliferation is of benefit for enhancement of stem cell number and genetic modification. Based on previous studies in rodents we believe that culture of bovine spermatogonial stem cells as a single cell suspension must be done on a feeder cell monolayer.

In this study, germ cell suspensions from donor bulls were cryopreserved in liquid nitrogen for approximately 10mo prior to thawing for culture and transplantation. Colonization of frozen-thawed cells in recipient mouse seminiferous tubules demonstrates the survival of these cells in a relatively simple cryopreservation system. The round cell morphology of colonized bovine cells in mouse seminiferous tubules was consistent with previous reports of other cross

species testicular transplantations [8, 9, 10, 16, 17]. This observation was in contrast to previous reports of somatic cell colonization in a recipient testis [18-20]. The donor bulls used in this study were 4-8wk of age, a development stage in which gonocytes or spermatogonial stem cells are the only cell type present within the testis. Therefore, it is highly likely that any germ cell colonization in recipient mouse testes was undifferentiated spermatogonial stem cells rather than differentiated germ cell types. Long-term cryopreservation of mouse and human spermatogonial stem cells has also been reported using a similar system [3, 17]. Survival of bovine type A spermatogonia during cryopreservation has been reported using a very similar method to the one employed in this study; however, evaluation of spermatogonial stem cell survival was again limited due to lack of a means for identifying donor cells following transplantation [21]. Similarly, bovine gonocytes have been demonstrated to be capable of survival during cryopreservation and undergoing differentiation in vitro post thaw [12]. It appears that spermatogonial stem cells from many species are quite robust and capable of survival during cryopreservation in relatively simple conditions.

Culture of bovine germ cells on BEF feeder cells for 1wk resulted in a significant increase in round cell colonies following transplantation compared to frozen-thawed germ cells transplanted prior to culture. This observation provides a strong indication of bovine spermatogonial stem cell survival and proliferation over a 1wk period. Mouse spermatogonial stem cells have been shown to decline during the first 7 days of culture on feeder cells compared to the starting cell population [6]. However, the addition of GDNF to the medium enhanced spermatogonial stem cell numbers in these cultures, implicating GDNF as a factor for spermatogonial stem cell maintenance [6]. Other studies have also indicated the proliferative effects of GNDF on undifferentiated spermatogonia in the mouse [11]. Evaluation of the BEF

feeder cell developed in this study demonstrated the expression of endogenous GDNF mRNA and protein production, which may have contributed to the enhanced stem cell maintenance in 1wk cultures.

A significant observation in this study was the decrease in bovine colony numbers in recipient mouse testes arising from 2wk cultured bovine germ cells. This would indicate that spermatogonial stem cell death or stem cell differentiation to committed spermatogonia occurred over the 2wk period therefore decreasing the percentage of stem cells within the overall germ cell population.

The number of bovine round cell colonies in the seminiferous tubules of recipient mice when germ cells were co-cultured with BEF cells and exogenous GDNF was significantly lower compared to cultures without exogenous GDNF at 1wk. This observation indicates that a higher concentration of GDNF above what was endogenously produced by the feeder cells had a negative impact on stem cell maintenance. Similar studies with porcine spermatogonia have also shown dose dependent effects of exogenous growth factor addition on cell survival and proliferation in vitro, in which a negative effect on cell survival was seen past a threshold concentration [22]. The FBS added to the culture medium may have also contained GNDF, therefore, further addition of a recombinant GDNF along with endogenous BEF production may have again passed a threshold and had a negative impact on spermatogonial stem cell maintenance.

The addition of exogenous GDNF enhanced stem cell maintenance in 2wk cultures of bovine germ cells in this study. A 2-fold increase of surviving stem cells was seen with exogenous GDNF compared to 2wk cultures without exogenous GDNF. Currently, the means of action that GDNF has on spermatogonial stem cell maintenance is unknown. Whether it inhibits

stem cell differentiation or promotes stem cell proliferation remains debatable. If stem cell differentiation was occurring during the 2wk period, a higher stem cell pool may have been maintained compared to cultures without added exogenous GDNF. The maintenance of this pool may have been through inhibition of stem cell differentiation as proposed by Nagano et al. [6] or by inhibition of cell death.

If GDNF action on stem cells works through inhibiting differentiation, then stem cell numbers would increase due to autonomous self renewal. This theory would support the results obtained in this study for 1wk cultured cells without exogenous GDNF and the loss of stem cells at 2wk of culture could be attributed to cell death over that period. The enhanced stem cell maintenance at 1wk of culture may have resulted in a sub-group of stem cells whose viability was short lived and could not be maintained throughout the 2wk period. The appearance of colonies from 2wk cultured cells may have resulted from another sub-group of stem cells that arose from enhanced maintenance at 1wk that may not have been as short lived and could be maintained for an extended period of time. Proliferative spermatogonial stem cells in vitro and in vivo may be a short lived subpopulation in which cell death occurs if differentiation to committed spermatogonia is not supported or initiated. Another subpopulation of spermatogonial stem cells whose proliferation is not quite as robust, but have a more vigorous long-term viability, may also be present.

Addition of exogenous GNDF above the endogenous BEF production and FBS concentration may have passed a threshold concentration that resulted in cell death at 1wk of culture or resulted in a steady state level of proliferation and cell death. This would explain the decreased number of stem cells in 1wk cultures with added GNDF. At 2wk of culture the enhanced maintenance of stem cells from cultures with added GDNF may have resulted from an

increased percentage of long term viable cells at 1wk that were still present at 2wk. It appears that GDNF may play an integral role in spermatogonial stem cell proliferation, inhibition of differentiation, and viability.

Based on expression of GFRα1, germ cell survival and viability was demonstrated throughout the 2wk culture period. Also, GFRα1 expression suggested spermatogonial stem cell presence. In neuronal cells, GDNF has been shown to exert its effects on a target cell through the receptor dimer of Ret tyrosine kinase and GFRα1 [23-25]. Therefore, it has been hypothesized that spermatogonial stem cells may express the GFRα1 receptor. Both these receptors have also been shown to be expressed by a subpopulation of undifferentiated spermatogonia in the mouse testis [15].

Development of in vitro culture and cryopreservation techniques for livestock spermatogonial stem cells is necessary for the application of spermatogonial stem cell transplantation. This technology has potential for use as a reproductive tool to enhance genetic gain and generate transgenic animals through the male germ line. In this study a BEF feeder cell co-culture system was developed that supports the maintenance of bovine spermatogonial stem cells. The BEF co-culture system reported here has potential to be used as a means for accomplishing stable genetic modification and investigation of factors regulating the biological activity of spermatogonial stem cells.

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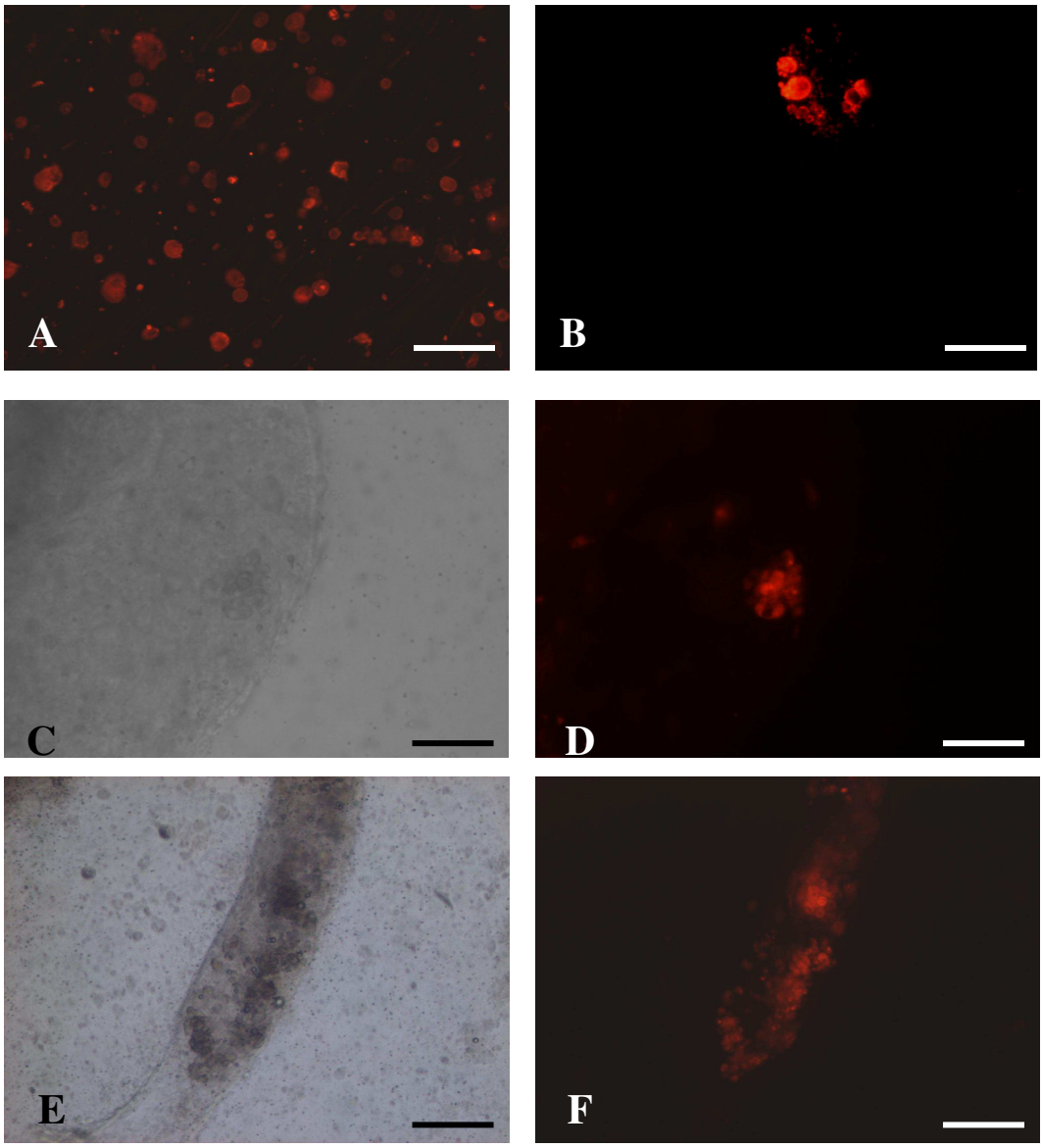
Figure 1. Fluorescent images of PKH26 labeled bovine germ cells. **A:** Frozen-thawed donor cells prior to transplantation or in vitro culture, **B:** 1wk BEF co-culture, **C/D (light field/fluorescent field):** Colonized cryopreserved bovine cells 1 month after transplantation into recipient mouse seminiferous tubules, **E/F (light field/fluorescent field):** Colonized BEF co-culture bovine cells 1 month after transplantation into recipient mouse seminiferous tubules. Bar = 100 μm (A, B, E, F) and 40 μm (C, D).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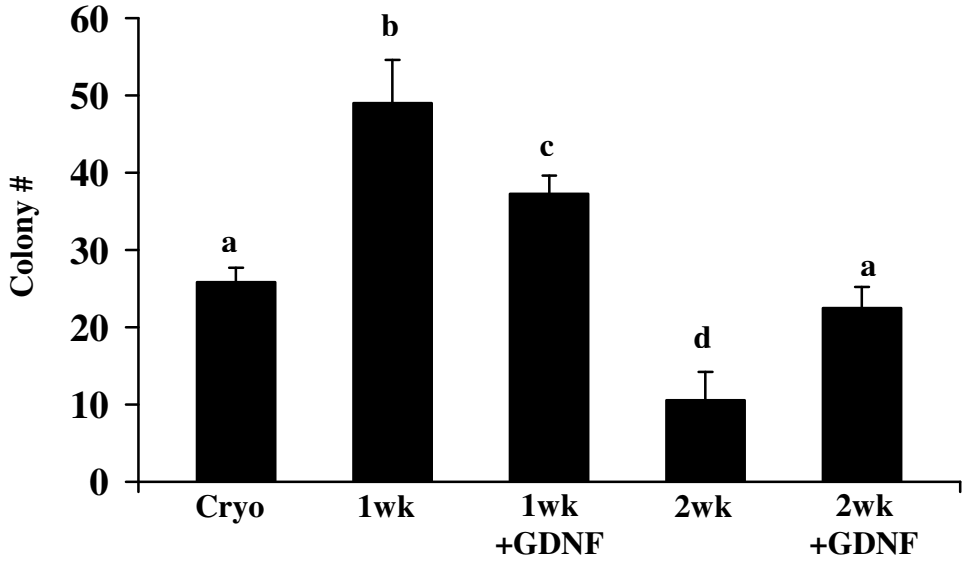


Figure 2. Average number of round germ cell donor colonies in recipient mouse seminiferous tubules 1 month after transplantation with frozen-thawed bovine germ cells or germ cell BEF cell co-cultures. Bars with different letters are significantly different at $P \leq 0.05$.

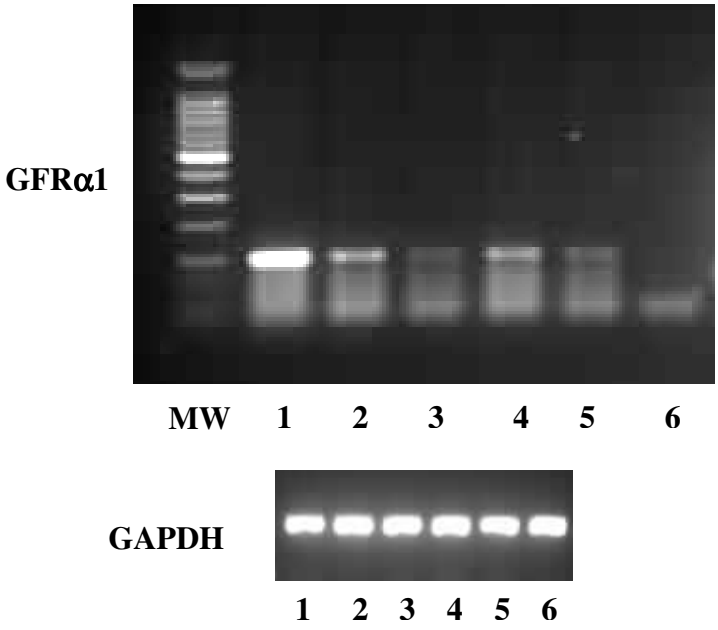
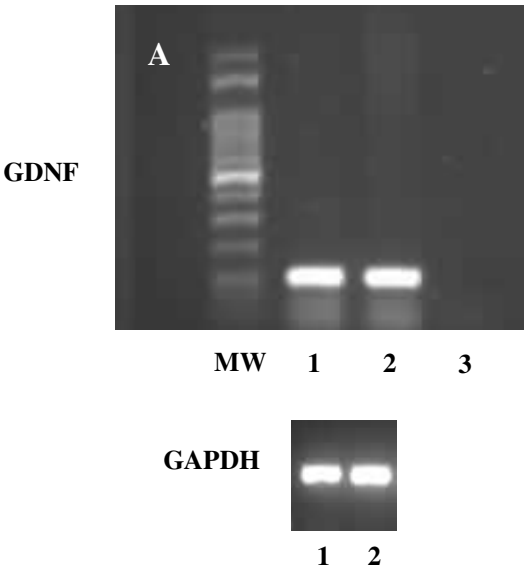


Figure 3. Detection of germ cell specific expression of GFRα1 by RT-PCR in co-cultures of frozen-thawed bovine germ cells on BEF cells. **MW:** 100 bp DNA ladder, **1:** bovine testis cDNA (positive control), **2 and 3:** 1wk co-cultures of bovine germ cells on BEF cells, **4 and 5:** 2 wk co-cultures of bovine germ cells on BEF cells, **and 6:** Mitomycin-C treated BEF cells (negative control).

Figure 4. Identification of GDNF expression by BEF feeder cells. **A:** mRNA expression detected by RT-PCR; MW: 100bp DNA ladder, 1: bovine testis cDNA (positive control), 2: BEF cDNA template, and 3: H_2O negative control (-template cDNA). **B:** protein expression detected by immunohistochemistry, fibroblast morphology with distinct GDNF staining within the cytoplasm, **C:** negative control (omission of primary antibody) showed no GDNF specific staining. Bar = 100 μm.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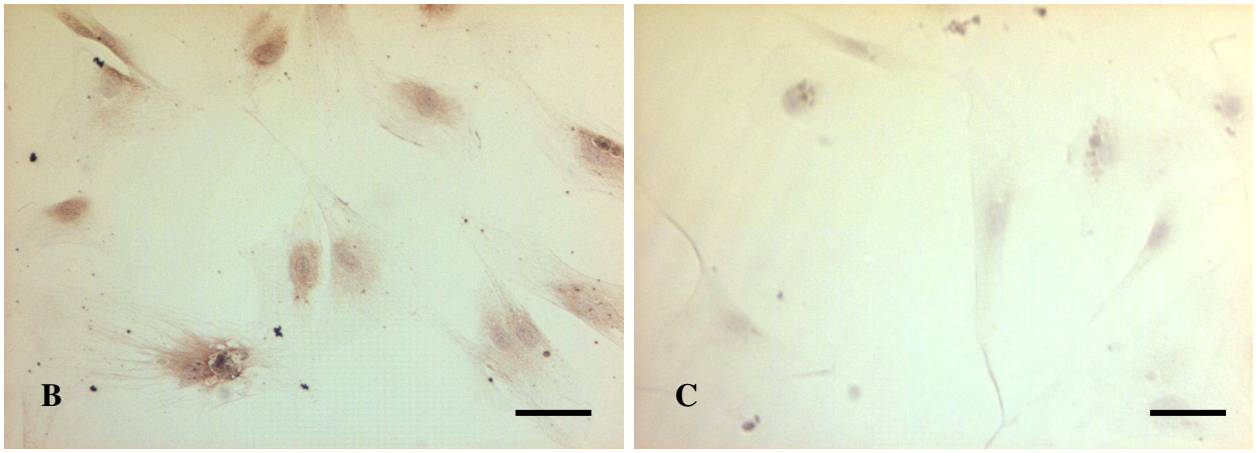
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CHAPTER FOUR

Spermatogenesis and germ cell transgene expression in xenografted bovine testicular tissue*

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Abstract

The present study was conducted to evaluate the development of spermatogenesis and utility of using electroporation to transfect germ cells with the β-galactosidase gene in neonatal bovine testicular tissue ectopically xenografted onto the backs of recipient nude mice. Bull testicular tissue from 4wk donor calves, which contains a germ cell population consisting solely of gonocytes or undifferentiated spermatogonia, was grafted onto the backs of castrated adult recipient nude mice. Testicular grafts significantly increased in weight throughout the grafting period and the timing of germ cell differentiation in grafted tissue was consistent with postnatal testis development in vivo relative to the bull. Seminiferous tubule diameter also significantly increased with advancing time after grafting. At 1wk after grafting, gonocytes in the seminiferous cords completed migration to the basement membrane and differentiated germ cell types could be observed 24wk after grafting. The presence of elongating spermatids at 24wk confirmed that germ cell differentiation occurred in the bovine tissue. Leydig cells in the grafted bovine tissue were also capable of producing testosterone in the castrated recipient mice from 4wk to 24wk after grafting at concentrations that were similar to levels in intact non grafted control mice. The testicular tissue that had been electroporated with a β-galactosidase expression vector showed tubule specific transgene expression 24wk after grafting. Histological analysis showed that transgene expression was present in both Sertoli and differentiated germ cells but not interstitial cells. The system reported here has potential to be used for generation of transgenic bovine spermatozoa.

Introduction

Cross species ectopic testicular grafting is a unique approach for the investigation of testicular biology. Furthermore, this technology also has the potential to generate transgenic spermatozoa. When testicular tissue from neonatal donor mice, pigs, goats or primates is ectopically grafted onto a castrated recipient mouse host, production of spermatozoa commences at an accelerated rate [1-3]. It has been demonstrated that offspring can be produced using sperm collected from syngeneic mouse grafts and embryos produced from fertilization of oocytes with sperm produced in xenogeneic pig grafts [1, 2]. When prepubertal bovine testicular tissue was grafted onto the backs of intact recipient nude mice, differentiated germ cells were observed 20wk later, without any acceleration of germ cell differentiation observed [4]. Genetic manipulation of the germ line stem cells in ectopically grafted donor testis tissue could provide a novel means by which to generate transgenic offspring through the male germ cell.

In previous reports of successful sperm production in ectopic grafting, the use of neonatal donors and castration of recipient mice were necessary [1-3]. This success was attributed to an immature testicular cell population, consisting of gonocytes as the only germ cells present and a recipient host environment that resembled a neonatal phase in terms of the concentrations of circulating hormones. In contrast to published reports with several other species, the only report using the bovine as a donor has shown the capability of germ cell differentiation using a 19wk old prepubertal donor animal and intact recipient nude mouse host [4].

In the bovine testis, postnatal developmental assembly of the seminiferous epithelium and establishment of spermatogenesis occur over a period of months. Postnatally, at 2wk of age

gonocytes are found within the center of seminiferous cords and have begun migration to the basement membrane [5]. At 4wk of age the majority of gonocytes have completely migrated and differentiating spermatogonia first appear at 12wk of age [5, 6]. Within this 4-12wk period gonocyte conversion to spermatogonial stem cells and the establishment of stem cell niches must occur. It is not until 16wk of age that meiotic spermatocytes first appear in the seminiferous epithelium and elongating spermatids can be seen at 28wk [6]. Access to a germ cell population enriched for undifferentiated spermatogonial stem cells is beneficial for genetic manipulation of male germ cells since one genetically altered spermatogonial stem cell could yield thousands of transgenic spermatozoa [7]. At 19wk of age in bulls differentiated spermatogonia are clearly present along with primary spermatocytes and the ratio of differentiated germ cells to spermatogonial stem cells is higher compared to earlier age points [6]. Therefore, use of neonatal tissue has the advantage of containing less differentiated germ cells and a more enriched spermatogonial stem cell population.

Previous attempts to transduce germ cells in the mammalian testis with a transgene have been met with challenges [8-12]. Electroporation of testes following injection of a transgene containing vector into the seminiferous tubules has resulted in somatic Sertoli and Leydig cell expression or transient germ cell expression [12]. Direct transfection of spermatozoa in vitro often results in transient rather than stable chromosomal incorporation [8, 9]. The most successful method to date for stable transduction of male germ cells has been through the use of retroviral infections of spermatogonial stem cells [13-15]. This technique has resulted in the production of transgenic spermatozoa that are capable of fertilization and vertical transmission of the transgene to offspring in rodents [15]. Production of transgenic spermatozoa using retroviral transfection of spermatogonial stem cells has thus far required the use of a spermatogonial

transplantation technique, which at this time is only applicable to rodents and limited in domestic livestock.

The objectives of this study were to evaluate the development of spermatogenesis in ectopically grafted neonatal bovine testicular tissue and investigate the utility of using electroporation to stably transfect spermatogonial stem cells within grafts with a foreign β-galactosidase transgene.

Materials and Methods

Recipient mice and donor bulls

The Washington State University Animal Care and Use Committee approved all animal procedures. Donor testicular parenchyma was collected from bulls at 4wk of postnatal age (n=6). Sections of donor testicular parenchyma approximately 10mg in weight were collected into Hanks Balanced Salt Solution (HBSS) and kept on ice until grafting. Six pieces of donor tissue were ectopically grafted onto the backs of each castrated adult immunodeficient recipient nude mouse (n=18 mice/donor) (Taconic; Germantown, NY). Recipient mice were anesthetized with a combination of ketamine (0.1 mg/kg b.w) and xylazine (0.05 mg/kg b.w.) in sterile physiological saline. Mice were castrated before small incisions were made through the skin on the backs of the animal where the muscle was scored in individual areas, and six 10mg pieces of donor bull testis placed individually within the scored muscle areas of each recipient mouse. The incisions were closed with suture and the animals allowed to recover.

Electroporation

Prior to grafting three of the six pieces of donor testicular tissue were electroporated with a linearized β-galactosidase expression vector. The construct was created by restriction enzyme digestion and ligation of a 4.5 kb fragment of LacZ into a pMSCV vector (BD Biosciences Clontech; Palo Alto, CA). The vector was suspended at a concentration of 1μg/μl in Opti MEM (100μl) and pipetted into the center of each piece of testicular tissue. Tweezer trodes were used to electroporate each of the 3 pieces with one of three different voltages, 25, 50, or 75mV (BTX Harvard Apparatus; Holliston, MA). The tissues were then placed on ice in HBSS until the time of grafting. Testicular tissue from the donor testis prior to grafting (T0) was used as a representation of the starting material.

Donor graft analysis

Recipient mice were killed at 1, 4, 12, or 24wk after grafting by CO_2 inhalation followed by cervical dislocation (n=9 mice/time point). Grafts were removed, fixed in Bouin's solution for 4hr at 4°C and subsequently dehydrated and stored at 4°C in 70% ethanol. Each donor graft was weighed individually following fixation and dehydration. Samples were then embedded in paraffin, sectioned at 6 μm, and stained with hemotoxylin. Electroporated grafts were fixed in 4% PFA for 2hr at 4°C followed by incubation with X-Gal to detect any β-galactosidase expression. Samples were subsequently dehydrated, embedded in paraffin, sectioned at 10 microns, and counterstained with eosin.

Samples were evaluated using light microscopy and digital images captured with a CoolSnap *cf* digital camera (Media Cybernetics; Silver Spring, MD). Analysis of non electroporated cross sections included assessment of the average seminiferous tubule diameter which was calculated by measuring 20 round tubules per sample at each time point using a slide

micrometer as a reference. The average percentage of tubules containing spermatogonia, pachytene spermatocytes, round spermatids and elongating spermatids was also calculated for each non-electroporated sample by counting the total number of round tubules/three microscopic fields and the number of round tubules containing each germ cell type within the same three fields. Analysis of electroporated samples included assessment of the average number of seminiferous tubules with β-galactosidase expression which was calculated by counting the number of tubules/three microscopic fields and the number of tubules containing any blue staining within these fields. The average number of tubules with germ cell β-galactosidase expression was also determined by counting the number of tubules with blue stained germ cells within the three microscopic fields.

Recipient mouse analysis

Blood was collected from recipient mice at the time of sacrifice by cardiac puncture and serum was subsequently collected by centrifugation. Two populations of control mice were also used, intact adult males and adult males castrated 2d prior to sacrifice. Serum samples were then analyzed for testosterone concentration using a commercial kit (DSL-400; Diagnostic Systems Laboratory Inc., Webster, TX).

Statistical analyses

Data were analyzed using the SAS systems software with the Proc GLM function. Differences between means were determined for tubule diameter, graft weight, gonocyte migration, % of tubules with different germ cell types, recipient testosterone concentration, % of tubules with blue staining, and % of tubules with blue stained germ cells using Duncan's test for

significance. Data were considered significantly different at $P \leq 0.05$. In all figures data are presented as the mean$\pm$SEM, bars with different letters are significantly different.

Results

Donor graft analysis

Bovine testis tissue grafts were weighed at each time point after removal from nude mice to provide an indication of the proliferation of testicular somatic cells and progression of spermatogenesis in the bovine tissue. Average graft weight showed a significant increase in a time dependent manner beginning at 4wk after grafting (Figure 1A). The average weight of 1wk grafts compared to T0 was not different ($P \geq 0.05$), whereas the average weight of grafts at 4 and 12wk were significantly ($P \leq 0.05$) higher than T0 and 1wk but not different from each other. Graft weights at 24wk were significantly higher than any other time point. Seminiferous tubule diameter was measured to assess Sertoli and germ cell proliferation. Average seminiferous tubule diameter showed a significant increase with increasing time after grafting beginning between 1 and 4wk (Figure 1B).

Bovine testicular tissue removed at 1, 4, 12, and 24wk after grafting was examined histologically to evaluate the establishment of spermatogenesis. Tubular morphology was maintained in all grafts throughout the 24wk grafting period (Figure 2). At T0 the tubules resembled seminiferous cords with apparent gonocytes in the center of the cords (Figure 2A). Testis tissue grafts 1wk after grafting had seminiferous tubules very similar in diameter to T0 (Figure 1B). However, the percentage of tubules with complete gonocyte migration to the basement membrane was significantly increased in 1wk-grafted tissue compared to T0 (Figure 3). The majority of cords at T0 had gonocytes still present in the center of the cords, whereas at

1wk after grafting gonocyte migration to the basement membrane was complete in the majority of cords (Figure 3).

Bovine testis tissue 4wk after grafting was characterized by a germ cell population predominated by gonocytes without the presence of any differentiated germ cell types (Figure 2C, Table 1). Transition of gonocytes to spermatogonia was apparent at 12wk after grafting and the structurally intact seminiferous tubules were present (Figure 2D). Meiotic germ cell development, as determined by the presence of pachytene spermatocytes, was not present until 24wk after grafting (Figure 2E, Table 1). At 24wk after grafting the germ cell population consisted of spermatogonia, pachytene spermatocytes, round spermatids, and elongate spermatids (Figure 4, Table 1). The average percentage of tubules containing pachytene spermatocytes was 49.9%; whereas 92.9% of the total tubules contained spermatogonia (Figure 5). Germ cell progression to the round spermatid stage was seen in an average of 18.3% of the tubules (Figure 5). The average percentage of tubules containing elongate spermatids was low, comprising only 10.9% of the total tubules (Figure 5). The appearance of tubules containing Sertoli cells only was evident at 24wk after grafting (Figure 2F), comprising 7.1% of the total tubules (Figure 5).

Recipient mouse testosterone analysis

Serum testosterone concentration was measured to evaluate the Leydig cell function in grafted bovine testicular tissues. Testosterone concentration was significantly increased in recipient serum at 4, 12, and 24wk after grafting compared to 1wk (Figure 1C). The serum concentrations of testosterone at 4, 12, and 24wk after grafting were at a physiological concentrations consistent with what was present in intact adult male mouse controls (1-4 ng/ml).

Serum from control male mice two days after castration had a testosterone concentration below the low standard of the assay (<0.05 ng/ml).

Electroporated graft analysis

Donor bovine testicular tissue was electroporated with a vector containing the β-galactosidase gene prior to grafting to investigate the ability to stably transfect spermatogonial stem cells with a transgene and possibly generate transgenic differentiated germ cells. Since the efficiency of electroporation to transduce a cell can be variable at different voltages three different voltages of 25, 50, and 75mV were tested. All grafts removed at 4, 12, and 24 wk after grafting showed positive staining for β-glactosidase (Figure 6A). Based on whole mount observations of the grafts, expression appeared to be seminiferous tubule specific and no discernable differences could be detected between the different voltages prior to histological analysis. Since the most significant germ cell development was not observed until 24wk after grafting, only those results will be presented. Cross sections of 24wk electroporated grafts showed seminiferous tubule specific β-galactosidase expression within both Sertoli and germ cells, whereas no interstitial cell expression was observed (Figure 6B-D). The 50mV tissues showed a significantly higher average number of tubules with β-galactosidase expression than the 25 and 75mV tissues (Figure 7A). Likewise, the 50mV tissues had a significantly higher number of tubules with germ cell β-galactosidase expression compared to the other voltages (Figure 7B). The 25 and 75mV tissues were significantly different from the 50mV tissues based on both the average number of tubules with any β-galactosidase expression and average number of tubules with germ cell expression. Transgene expression was observed in differentiated germ cells within the 25 and 50mV electroporated tissues (Figure 6). The most advanced germ cell

type observed in the 75mV electroporated tissues were spermatogonia and nearly all β-galactosidase expression was within Sertoli cells. It appeared that the 75mV electroporation had a negative effect on germ cell development within the grafts.

Discussion

Spermatogenesis requires a complex and finely organized sequence of events to support the differentiation and maturation of spermatogonia into sperm. The cells involved in this process are capable of producing sperm even when testicular tissue is removed from an animal and placed at locations distal from the testis [1, 2]. In this study, we demonstrate that differentiated germ cells can be produced in bovine testicular tissue transplanted from the testes of 4wk old calves under the skin on the backs of castrated adult nude mice. Most notably, 24wk after grafting the tissue, elongate spermatids were observed in the transplanted testicular tissue. These data also indicate that mouse gonadotropins stimulate the proliferation of bovine somatic Sertoli cells and production of testosterone by Leydig cells in the bovine tissue. In contrast to previous studies of testis xenografting in other domestic livestock species, our data indicates there is no or marginal acceleration in the differentiation of germ cells in grafted neonatal bovine testicular tissue. In this study we also demonstrate that a transgene can be stably incorporated into spermatogonial stem cells following electroporation and result in production of differentiated germ cells that express the transgene following ectopic grafting.

Survival and development of grafted testis tissue was monitored by histological analysis and measuring the weight of grafts from 1 to 24wk after transplantation. A significant increase in tissue weight occurred 4wk after grafting. The weight increase and histological examination of tissue at 4wk indicted somatic cell proliferation. In addition, graft weight increased throughout

the 24wk period including a significant increase between 12 and 24wk after grafting. Tubule diameter was also significantly different between 12 and 24wk after grafting, which was most likely due to germ cell development and changes in Sertoli cell morphology. It is well established that Sertoli cell proliferation in the testis occurs neonatally in a variety of mammalian species. In rats, Sertoli cell proliferation occurs at the start of seminiferous cord formation and ceases at approximately day 16 of postnatal life with first appearance of meiotic cells [16, 17]. In bulls, the time frame of postnatal Sertoli cell proliferation is not firmly established, but the data available indicates that it ceases around 28wk of postnatal age [6]. Previous reports have demonstrated changes in Sertoli cell size and morphology with advancing germ cell development in the rat testis [18, 19]. Graft weight and seminiferous tubule diameter data collected in this study demonstrates growth and proliferation of neonatal bovine testicular tissue and cells when ectopically xenografted onto recipient mice in a pattern that is similar to normal maturation of the intact bull calf testis.

The average percentage of total tubules prior to grafting at T0 (4wk old donor) that contained gonocytes in the center of the seminiferous cords was 62.9%, whereas only 37.1% of the cords had complete gonocyte migration. In contrast, at 1wk after grafting, comparable to a 5wk old bull calf, only 20.1% of cords had gonocytes still present in the center and 79.9% had complete migration. In the developing postnatal mammalian testis, gonocytes migrate from the center of seminiferous cords to the basement membrane for the differentiation to spermatogonia or spermatogonial stem cells and the establishment of stem cell niches [20]. In the mouse testis, this occurs from postnatal days 0-5 [21, 22], however, in the bovine this process is much longer. Gonocyte migration begins at 2wk of postnatal life and is complete by 8wk in the bovine testis [5]. Within the window of 2 to 12wk of postnatal life, spermatogonial stem cell conversion and

niche assembly must occur. The data in this study indicate that gonocyte migration is accelerated in grafted neonatal bovine testicular tissue. This initial period during 1wk after grafting could potentially be used for manipulation to increase the efficiency of spermatogonial stem cell conversion and niche assembly, which may correlate to increased and accelerated sperm production in grafted testicular tissues.

In this study castrated adult nude mice were used as recipients to provide an environment for the neonatal donor testicular tissue that was deficient in testosterone. In an in vivo situation testosterone concentration in the serum of a neonatal male is low and does not become elevated until later in germ cell development. Therefore, providing grafted neonatal donor testicular tissue with an environment that has low testosterone concentration after grafting has similarities to the neonatal period and may be beneficial for establishment of that tissue in a recipient host. Likewise, spermatogonial stem cell functionality has been shown to be enhanced in a low testosterone environment [23, 24]. Therefore, for gonocyte to stem cell conversion and niche assembly to occur in grafted neonatal testicular tissue, a low testosterone environment may be essential. In a previous report differentiated germ cell production occurred using 19wk old pubertal bulls as donors and intact recipient mice [4]. This approach thus provided a recipient host environment that had high concentrations of circulating testosterone, which mimicked the environment present in an intact bull at the same developmental age. In the work reported here, testosterone concentration in the serum of castrated recipient mice 4 to 24wk after grafting was found to be at a level similar to intact adult male mouse controls (1-4ng/ml), demonstrating vascularization and functionality of the donor bovine Leydig cells. However, at 1wk after grafting, the recipient testosterone concentration was low (0.2 ± 0.04ng/ml), possibly due to inability of the immature Leydig cells to initially respond to LH. In the intact bull calf,

testosterone concentration in the serum begins to rise at 18 wk of age [25]. Our data suggests that Leydig cell differentiation is accelerated in grafted testis tissue. It is also possible that the high LH concentration in recipient mouse serum initiated functionality of the bovine Leydig cells earlier than would be normally seen in an intact bull at the same developmental stage.

In vivo, meiotic germ cells are first apparent in the developing bovine testis at 16wk of age [6]. In this study, pachytene spermatocytes were first seen in the grafted testicular tissue at 24wk after grafting, which is comparable to a 28wk old intact bull calf testis. These data indicate that spermatogenesis is not accelerated to the point of meiotic germ cell development when using a 4wk old donor bull. However, the exact time point between 12 and 24wk after grafting when meiotic cells were present cannot be determined from this study. At 24wk after grafting the average percentage of the total tubules that contained pachytene spermatocytes was only 49.9%, unlike in the in vivo situation where a much higher percentage of tubules containing meiotic germ cells at a comparable developmental age would be expected.

The presence of elongating spermatids was used to indicate production of differentiated germ cells in the grafted bovine testicular tissues. Spermatids were observed at 24wk after grafting which was comparable to a 28wk old intact bull. In an intact bull, elongating spermatids are not seen until 28wk of age [6]. Only an average of 11% of the total tubules contained elongate spermatids at 24wk after grafting, indicating a low number of tubules had supported germ cell differentiation in grafted neonatal bovine testicular tissue. In contrast to reports of accelerated germ cell development in ectopically grafted neonatal mouse, pig, goat and primate testis, no comparable acceleration of germ cell differentiation was seen with neonatal bovine testis in this study.

This is the first report of transducing spermatogonial stem cells of bulls with a foreign β-galactosidase transgene through electroporation of neonatal bovine testis followed by grafting onto recipient mice for further growth, maturation and evaluation. This technique has potential advantage over sperm-mediated transgenesis and pronuclear injection because it could result in a higher success rate of transgene chromosomal incorporation. The multiple mitotic and meiotic cell divisions and checkpoints that a male germ cell undergoes before becoming mature spermatozoa, would ensure that expression of a transgene in spermatozoa would likely be stable if it was incorporated at the spermatogonial stem cell stage. With sperm-mediated delivery of transgenes into oocytes, the success of stable chromosomal incorporation is low, usually resulting in a mean range of 0-8% of the embryos expressing the transgene [26-28]. When using methods applied to the embryo, such as pronuclear injection or embryonic stem cell manipulation, the success rate is also low at 1-8% [29-31]. Co-injection of pretreated sperm with a reporter gene into oocytes has been more successful resulting in 64-94% of embryos expressing the transgene, but only an average of 40% of the offspring expressed the transgene [32]. Use of this method is not optimal for stable transgene insertion since the first developmental checkpoint occurs after fertilization where the expression of transgenes with unstable incorporation are suppressed later in development or embryo viability is lost. If successful these methods are still limited to the number of stably modified offspring they could produce since the dependent factor is the embryo which most likely would only result in one offspring. The potential exists to use genetic modification of germ cells in grafted testicular tissue along with intracytoplasmic sperm injection (ICSI) to generate substantial numbers of transgenic offspring. Genetic manipulation of one spermatogonial stem cell in grafted testicular tissue has potential to yield hundreds of stably modified spermatozoa for use in ICSI. Thus, the efficiency of generating transgenic embryos

could be enhanced compared to current methods of sperm-mediated transgenesis, pronuclear injection or co-injection of pretreated sperm and transgene DNA.

Over the 24wk period, electroporated grafts grew and developed in a similar pattern to the non electroporated grafts. A significant observation in this study was the localized expression of β-galactosidase to the seminiferous tubules without interstitial cell expression. The pMSCV-β-galactosidase vector used in this study contains retroviral murine stem cell virus (MSCV) LTR regions that are designed for high stem cell expression. In order for chromosomal integration to occur following electroporation, a cell must be mitotically active. Histological evaluation of non-electroporated grafts indicates that interstitial cells are not mitotically active shortly after grafting. Therefore, Sertoli and germ cell mitosis shortly after grafting could explain the tubule specific incorporation of the vector.

At 4wk in the bovine male, gonocytes or spermatogonial stem cells are the only germ cell types histologically detectable in the testis. Therefore, these data demonstrate the ability to transduce spermatogonial stem cells with a β-galactosidase transgene for later expression in differentiated germ cell types 24wk later. Based on the observation of differentiated germ cell expression of the transgene, both 25 and 50mV were capable of transduction of the donor spermatogonial stem cells. In contrast, differentiated germ cells were not observed in any of the 75mV tissues. Based on the average percentage of total tubules with germ cell expression of the transgene, 50mV was significantly better than the 25 or 75mV samples. These data indicate that a range between 25 and 50mV is the most efficient means for transfection of spermatogonial stem cells with a foreign transgene in neonatal bovine testicular tissue by electroporation.

As an application tool in the livestock industry, ectopic testicular grafting could be used as part of a novel technique for generation of transgenic livestock. Genetic manipulation of

undifferentiated germ cells in donor testicular tissue prior to grafting has potential to result in production of transgenic spermatozoa that could be used for in vitro fertilization (IVF) or ICSI to generate transgenic embryos. As concern over food safety increases, interest in transgenic livestock animal production has been suggested as a means for production of food animals resistant to particular pathogens that result in harmful diseases. As a research tool, genetic manipulation of germ or somatic cells in donor testicular tissue prior to grafting has potential for use in increasing the understanding of spermatogenesis at the molecular level in gene knockout and over expression studies. The systems demonstrated in this study can now be tested for their ability to produce transgenic spermatozoa that can be used for IVF or ICSI as an alternative means for generating transgenic embryos.

In this study, fully developed spermatozoa were not observed, but elongated spermatids were present. Allowing the donor testis more time after grafting could yield fully developed sperm capable of fertilization using ICSI. Even with the production of spermatids in the grafted tissue, oocyte fertilization could occur through procedures such as round spermatid injection [33]. Manipulation of the donor tissue or the recipient animal could enhance the efficacy of this procedure by increasing the percentage of tubules in which complete spermatogenesis occurs or by accelerating and enhancing germ cell development in tubules. Transgenic animal production has many applications in the livestock industries and the generation of transgenic animals may be accelerated through the male germ line stem cell.

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Table 1. Appearance of specific germ cell types in grafted bovine testicular tissue over a 24wk period.

	T0	1wk	4wk	12wk	24wk
Gonocytes	X	X	X		
Spermatogonia				X	X
Pachytene spermatocytes					X
Round spermatids					X
Elongating spermatids					X
Sertoli cell only				X	X

Figure 1. Average weight (**A**) and seminiferous tubule (**B**) diameter of bovine testicular tissue at T0, 1, 4, 12, and 24wk after grafting. **C:** Average serum testosterone concentration in castrated recipient nude mice at 1, 4, 12, and 24wk after grafting with neonatal bovine testicular tissue and control mice 2d after castration. Data are presented as the mean±SEM.

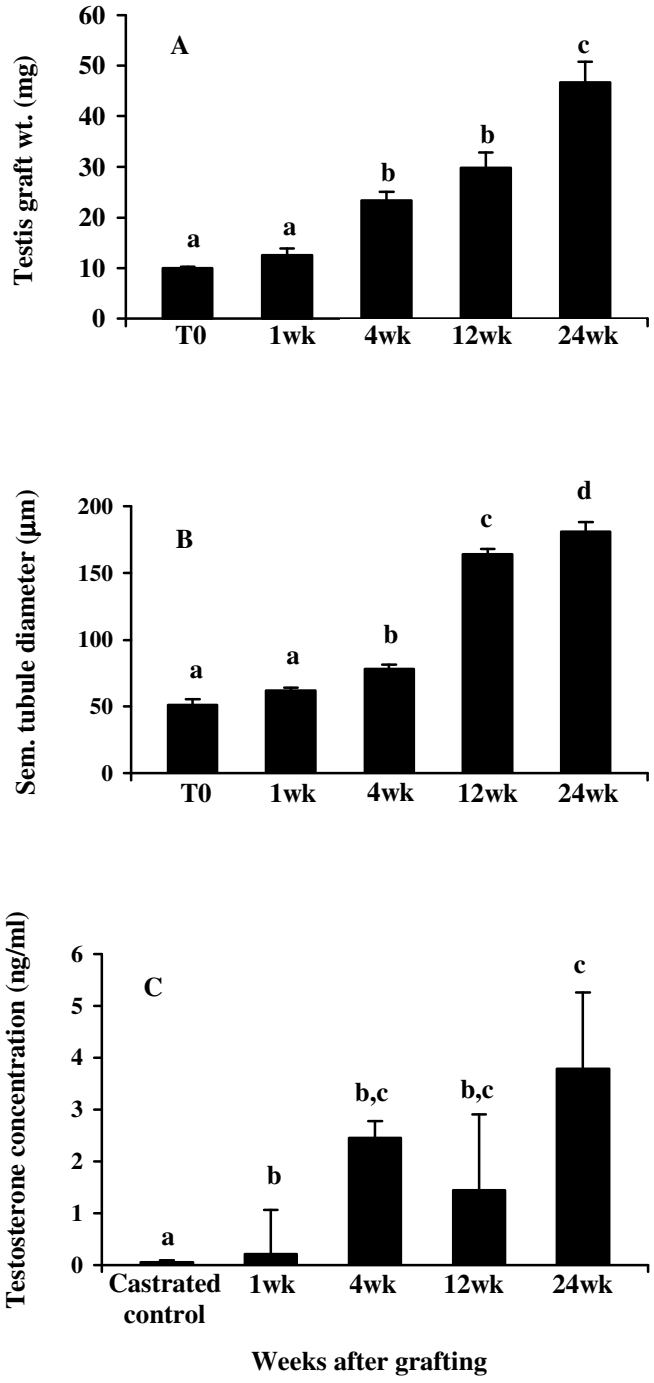
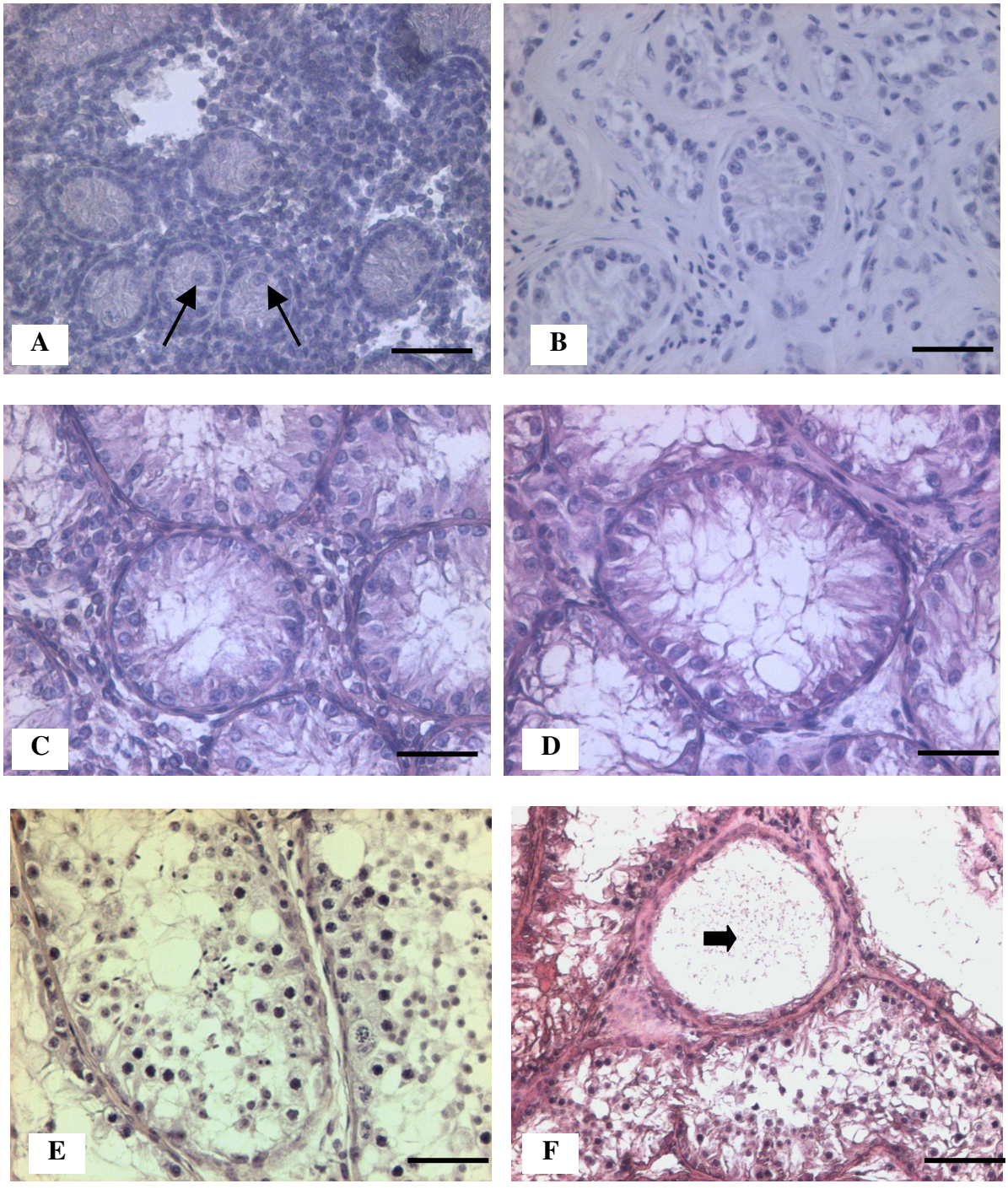

A
Testis graft wt. (mg)
60
50
40
30
20
10
0
a
a
b
b
c
T0
1wk
4wk
12wk
24wk
B
Sem. tubule diameter (µm)
200
150
100
50
0
a
a
b
c
d
T0
1wk
4wk
12wk
24wk
C
Testosterone concentration (ng/ml)
6
5
4
3
2
1
0
a
b
b,c
b,c
c
Castrated control
1wk
4wk
12wk
24wk
Weeks after grafting

Figure 2. Micrographs of grafted bovine seminiferous tubule morphology after grafting. **A:** 4wk old donor calf prior to grafting, gonocytes in the center of seminiferous tubules were apparent (arrows); **B:** 1wk after grafting, demonstrating gonocyte migration to the basement membrane was complete; **C:** 4wk after grafting; **D:** 12wk after grafting; **E:** 24wk after grafting, elongating spermatids were observed; and **F:** 24wk after grafting in which Sertoli cell only tubules were observed (arrow). Bar = 40 μm for each figure.


A
B
C
D
E
F

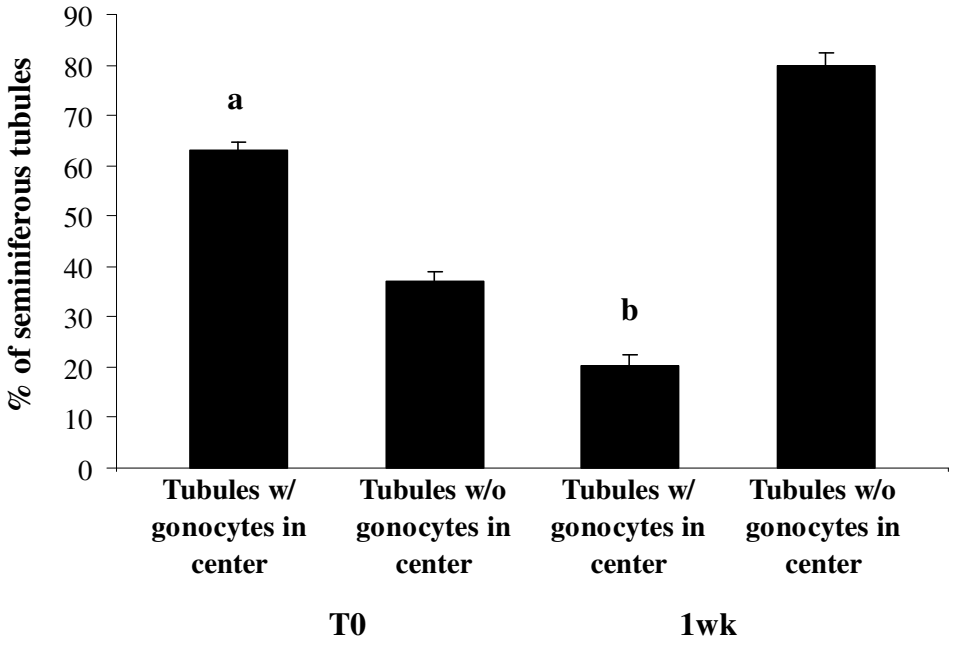
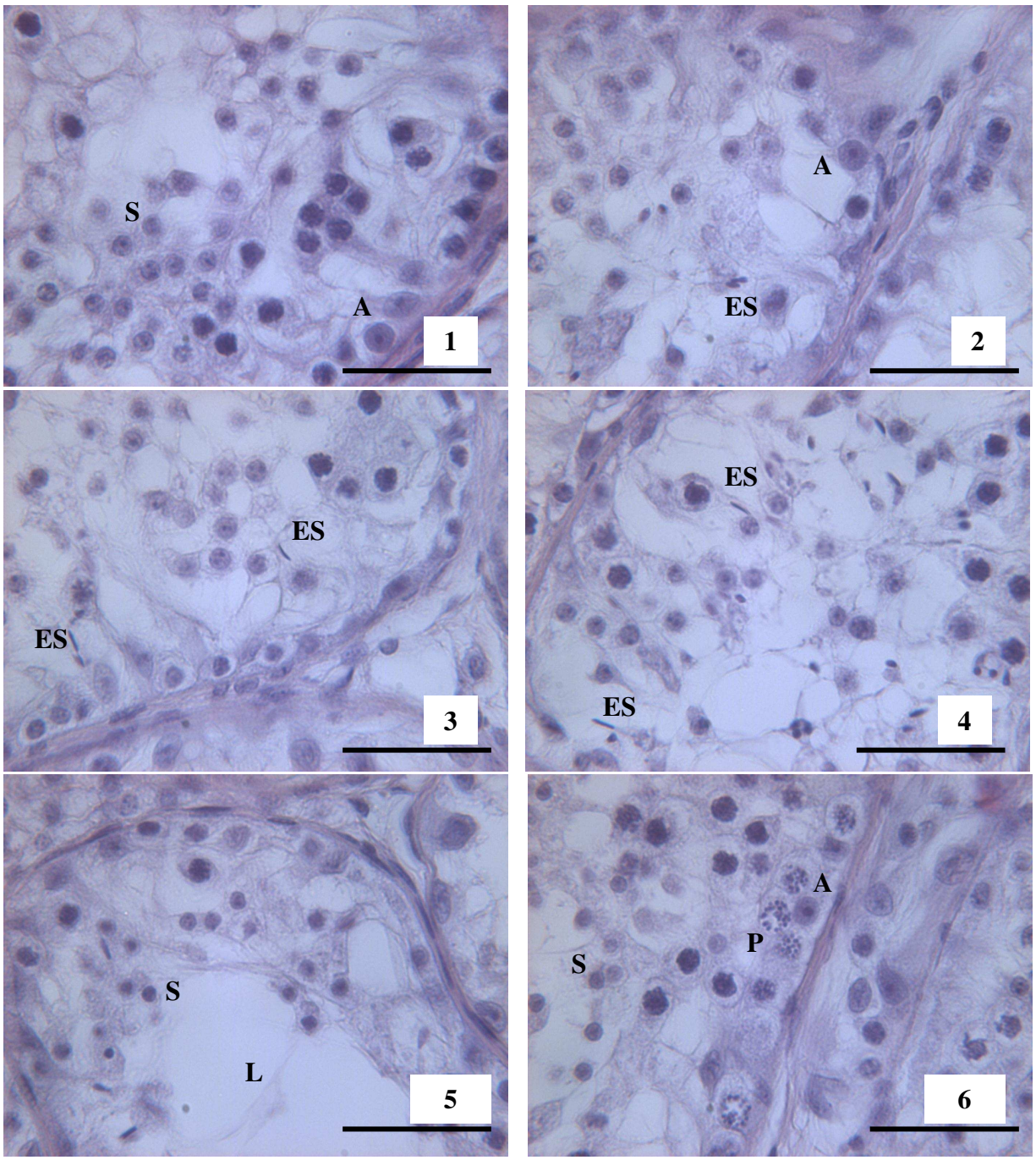


Figure 3. Average percentage of seminiferous cords containing gonocytes in the center and tubules containing fully migrated gonocytes in T0 and 1wk grafted bovine testicular tissue. Data are presented as the mean±SEM.

Figure 4. Micrographs of seminiferous tubules from bovine testicular tissue 24wk after grafting onto the backs of mice. **1:** Tubule showing A spermatogonia (A) and round spermatids (S). **2:** Tubule showing an A spermatogonium (A) and elongating spermatids (ES). **3 and 4:** Tubules showing elongating spermatids (ES). **5:** Tubule showing round spermatids (S) and the lumen (L). **6:** Tubule showing an A spermatogonium (A) and pachytene spermatocytes (P). Bar = 60 μm for each figure.


S
A
1
A
ES
2
ES
ES
3
ES
ES
4
S
L
5
A
P
S
6

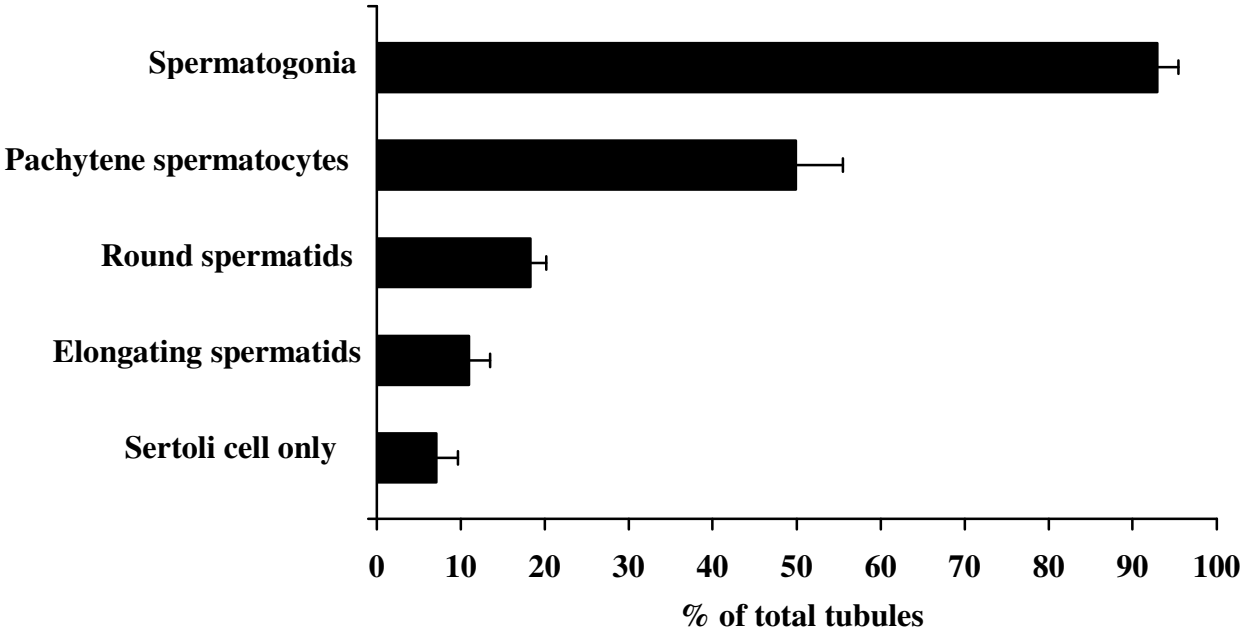


Figure 5. Average distribution of tubules containing certain germ cell types in bovine testicular tissue 24wk after grafting. Data are presented as the mean±SEM.

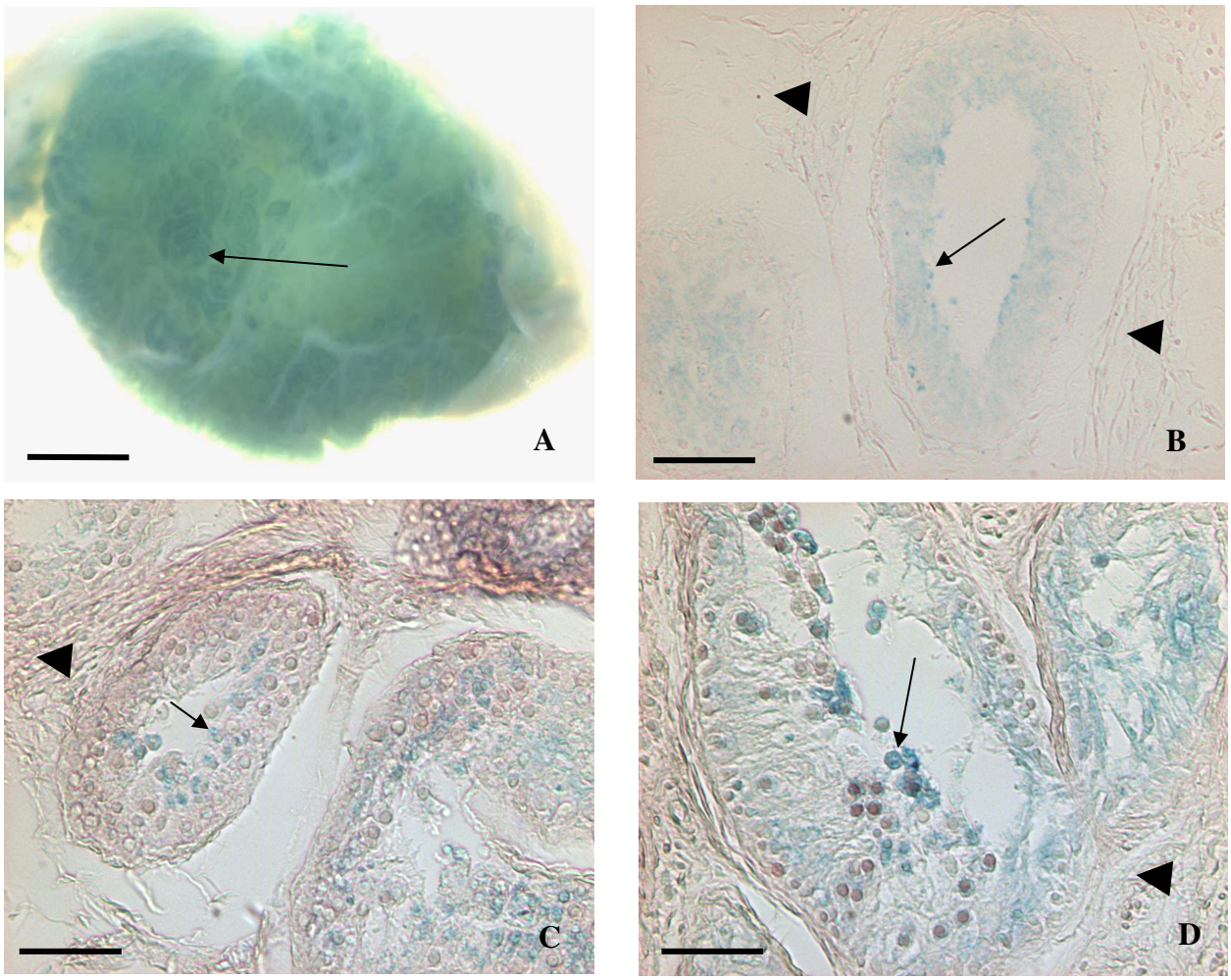


Figure 6. Images of electroporated neonatal bovine graft stained with X-Gal for β-galactosidase activity. **A:** Whole mount image of a 24wk electroporated graft, tubule specific staining was apparent (arrows). **B, C, D:** Micrographs of cross sections from 50mV grafts 24wk after electroporation, both germ cell (arrows) and Sertoli cell expression was seen. Note: Interstitial cell expression was not evident (arrow head). Bar = 2mm (A) or 100µm (B, C, D).

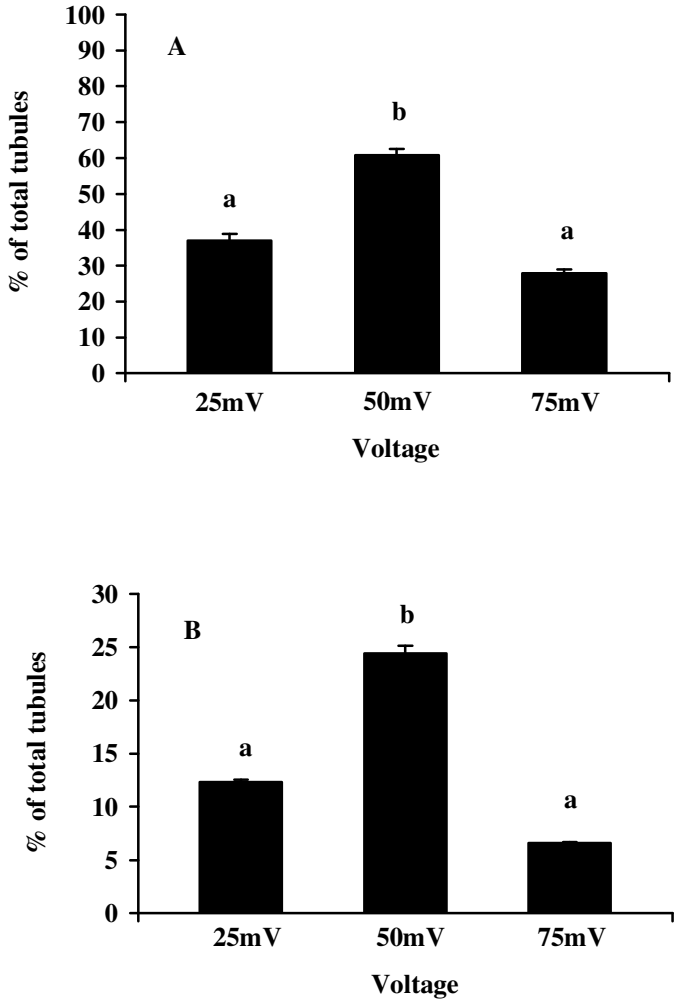


Figure 7. Average percentage of total seminiferous tubules (**A**) and seminiferous tubules that contained germ cell expression (**B**) of β-galactosidase from 24wk electroporated grafts. Data are presented as the mean±SEM.

CHAPTER FIVE

Establishment of spermatogenesis in neonatal bovine testicular tissue following ectopic xenografting varies with donor age*

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Abstract

Ectopic testicular xenografting can be used to investigate spermatogenesis and as an alternative means for generating transgenic spermatozoa in many species. Improving the efficiency of spermatogenesis in xenografted testicular tissue will aid in the utility of using this approach. The present study was conducted to evaluate age related differences in the establishment of spermatogenesis in grafted testicular tissue from bulls between 0 and 16wk of life. Testicular tissue was ectopically xenografted under the skin on the backs of castrated nude mice and subsequently evaluated for growth, testosterone production and establishment of spermatogenesis 24wk after grafting. The greatest weight increases occurred in donor tissue from calves of the ages 2, 4, and 8wk compared to the ages of 12 and 16wk. Recipient mouse serum testosterone concentration was at normal physiological levels 24wk after grafting and no significant differences were detected between recipients grafted with testicular tissue from bull calves of different ages. The development of germ cells to elongate spermatids could be detected in seminiferous tubules of grafts from donor calves of the ages 4, 8, 12, and 16wk but not observed in grafts from 2 wk donors which contained round spermatids as the most advanced germ cell stage. Grafts from 8wk donors contained a 10-fold significantly higher average percentage of seminiferous tubules with elongate spermatids than all other donor ages. These data demonstrate differences in the ability of testicular tissue from donor animals of different ages to establish spermatogenesis following ectopic testicular xenografting.

Introduction

Spermatogenesis is a highly organized yet complex process occurring in the testis that involves the coordinated interaction of both germ and somatic cell components. The germ cell contribution involves the proliferation and differentiation of many cell types beginning with diploid spermatogonial stem cells and ending with haploid spermatozoa. The somatic cell contribution involves steroid biosynthesis by Leydig cells and nurse function of Sertoli cells. Postnatal development of the testis encompasses a time period in which both the somatic and germ cells undergo proliferation and differentiation that will result in the first round of spermatogenesis and set framework for the future continuous sperm production.

The first round of postnatal spermatogenesis can occur in testicular tissue even when it has been removed and placed at locations distal from the testis [1-4]. Recently, postnatal spermatogenesis has been demonstrated in testicular tissue from several species xenografted onto the backs of recipient mice [1-4]. In mice, pigs, goats, and primates the first round of spermatogenesis in ectopically xenografted testicular tissue is accelerated [1-3]; however, in the bovine a comparable acceleration has not been reported [4]. In previous studies, successful establishment of spermatogenesis required neonatal donors as a source of testicular tissue [1-4]. This necessity has been attributed to donor germ and somatic cell populations that are immature, thus allowing the progression of the first round of spermatogenesis to occur following grafting.

During the time of prepubertal testis development both the somatic and germ cells undergo phases or stages of developmental change. In the early period of postnatal days 1-5 in the mouse the gonocytes undergo translocation to the basement membrane followed by conversion to spermatogonial stem cells eventually producing differentiating spermatogonia [5-7]. In bulls this period is much longer, lasting from day 0 to 16wk of life when differentiating

spermatogonia are first seen [8]. During this early stage of postnatal testicular development the somatic cell population consists of immature proliferative Sertoli and Leydig cells that become differentiated to function in their supporting roles of spermatogenesis. In the postnatal mouse testis, Sertoli cell proliferation occurs from day 0 to 10 at which time differentiation occurs [9]. Available data for the bovine indicates a comparable phase of proliferation lasting from day 0 to 20wk of life [8].

A major application of testicular grafting could be generation of transgenic spermatozoa. Production of stably transfected spermatozoa could be accomplished by transduction of spermatogonial stem cells prior to grafting. Use of a neonatal donor would be ideal since the germ cell population would be enriched for spermatogonial stem cells compared to other differentiated germ cell types. In previous experiments the percentage of seminiferous tubules in which differentiated germ cells were produced was low following ectopic xenografting of neonatal or prepubertal testicular tissue [3, 4]. Increasing the number of tubules containing haploid germ cells would be beneficial for enhancing the utility of this technique as a reproductive technology. In all species used to date, donors at a single neonatal age have been used as a source of testicular tissue. Different propensities for proliferation and differentiation of somatic and germ cells in testicular tissue during different periods of development in the postnatal mammalian testis exist and may correlate into different levels of sperm production following ectopic testicular xenografting. The objective of the present study was to evaluate age related differences in the establishment of spermatogenesis in grafted testicular tissue from bulls at developmental stages between 0 and 16wk of life. We hypothesized that testicular tissue from 12wk old bulls would result in the most effective establishment of spermatogenesis following

grafting since this is the age just before germ cell maturation into differentiating spermatogonia initiates.

Materials and Methods

Donor testicular tissue and ectopic grafting

All animal procedures were approved by the Washington State University Animal Care and Use Committee. Testicular tissue was collected from donor bulls at the postnatal ages of 2, 4, 8, 12, and 16wk (n=3 calves/age). Donor tissue was then diced into approximately 10mg pieces and maintained in Hanks balanced salt solution (HBSS) on ice until the time of grafting. Four pieces of donor tissue were ectopically grafted onto the backs of each castrated adult immunodeficient recipient nude mouse (n=3 mice/donor) (Taconic; Germantown, NY). Recipient mice were anesthetized with a combination of ketamine (0.1 mg/kg b.w) and xylazine (0.05 mg/kg b.w.) in sterile physiological saline. Mice were castrated before small incisions were made through the skin on the backs of the animal where the muscle was scored in individual areas, and four 10mg pieces of donor bull testis placed individually within the scored muscle areas of each recipient mouse. The incisions were closed with suture and the animals allowed to recover. Pieces of non grafted testicular tissue from each donor were also fixed in Boiun's solution for 4hr at 4^{o}C followed by dehydration and storage in 70% ethanol.

Histological analysis of donor grafts

Recipient mice were killed 24wk after grafting by CO_2 inhalation followed by cervical dislocation. Donor bovine grafts and recipient mouse seminal vesicles were removed, weighed, and fixed in Bouin's solution at 4^{o}C for 4hr followed by dehydration in 70% ethanol. All grafts

were subsequently blocked in paraffin, cross sectioned at 6 microns, and stained with hematoxylin. Sections were evaluated using light microscopy and digital images were captured with a Cool Snap *cf* digital camera (Media Cybernetics; Silver Spring, MD) at 100-200X magnification. The average percentage of tubules containing spermatogonia only, Sertoli cells only, pachytene spermatocytes, and elongating spermatocytes was calculated for each sample by dividing the number of round tubules containing each germ cell type/three microscopic fields by the total number of round tubules within the same three tubules.

Immunohistochemistry of Transition Protein-2

Representative cross sections of grafts from each aged donor 24wk after grafting were processed for detection of transition protein 2 (TP-2) by immunohistochemistry to identify late meiotic germ cells. Cross sections were deparrafinized and rehydrated followed by boiling in sodium citrate (pH 6.0). Endogenous peroxidase activity was then blocked by incubating the samples in 3% H_2O_2 in methanol for 10min followed by washing in PBS. Non specific antibody binding was then blocked by incubation of samples in 10% non immune rabbit serum for 15min at room temperature. Primary antibody (goat anti mouse TNP2; Santa Cruz Biotechnology, Santa Cruz, CA) diluted to 1:100 was added to samples and incubated at 4^{o}C overnight in a humidified chamber. The next day samples were washed in PBS three times for 2min each and biotinylated secondary antibody (rabbit anti goat IgG; Santa Cruz Biotechnology) was added and incubated at room temperature for 1hr followed by extensive washing in PBS. Streptavidin-HRP was then added and samples were incubated for 10min at room temperature followed by extensive washing in PBS. Samples were developed with aminoethyl carbazole (AEC) following manufacturer instructions (AEC substrate kit; Santa Cruz Biotechnology), counterstained with

hematoxylin and mounted with a coverslip. Stained sections were evaluated morphologically using light microscopy and digital images captured.

Recipient mouse analysis

At the time of sacrifice 24wk after grafting, recipient mice were bled by cardiac puncture. Serum was subsequently collected by centrifugation and subsequently analyzed for testosterone concentration by RIA using a commercial kit (DSL-400; Diagnostic Systems Laboratory Inc., Webster, TX).

Statistical analyses

Data were analyzed using the Proc GLM function of SAS systems software. Differences between means were determined for graft weight, % tubules with germ cell types, and recipient mouse testosterone concentration using Duncan's test for significance. Differences between means were considered significantly different at $P \leq 0.05$. Data are presented as the mean$\pm$SEM in all figures.

Results

Donor graft weight, recipient mouse serum testosterone concentration, and recipient mouse seminal vesicle weight

The average weight of individual grafts for each donor age was collected at removal to provide an evaluation of differences in growth of different aged donor bovine testicular tissue over the 24wk grafting period. Growth of donor testicular tissue under the skin on the backs of recipient mice could be visually identified over the 24wk grafting period. Analysis of testis graft

weights at removal indicated the growth potential varied depending on donor age. Average weights of grafts from 2wk old donor bull calves were significantly higher ($P \leq 0.05$) compared to both 12 and 16wk old donors, but not significantly different ($P \geq 0.05$) from 4 and 8wk donors (Figure 1A). The 4 and 8wk donor average graft weights were significantly higher than that of 16wk donors, but not different from each other or 12wk donor weights (Figure 1A). The average weight of 12 and 16wk donor graft weights were not significantly different from each other (Figure 1A). Overall, testis tissue from 2wk bull calves had the greatest growth potential.

Castrated recipient mouse serum testosterone concentration was measured to evaluate Leydig cell differentiation and function in grafted neonatal bovine testicular tissues. The average testosterone concentration in recipient mice was found to be at a physiological level of intact control mice (~3-5 ng/ml) regardless of the age of the donor testicular tissue (Figure 1B). No significant differences could be found between different donor ages.

Recipient mouse seminal vesicle weights were measured to further assess testosterone production by Leydig cells in grafted bovine testicular tissues. Only average seminal vesicle weights from 12 and 16wk donor mice were significantly different (Figure 1C). There was no detectable difference between the donor ages of 2, 4, 8, and 12wk. Overall, analysis of recipient seminal vesicle weights indicated testosterone production by grafted bovine testis tissue maintained seminal vesicle size.

Histological analysis

Histological examination of donor testicular tissue at the time of grafting was conducted to evaluate differences in germ cell populations between donor ages. The ability of germ cell differentiation to commence and extent of differentiation that occurs following grafting may be

influenced by the stage of germ cell development at the time of grafting. Evaluation of both 2 and 4wk old donors revealed seminiferous cords that contained only gonocytes (Figure 2A and B). Eight and 12wk old donor seminiferous tubules contained undifferentiated spermatogonia as the most advanced germ cell type (Figure 2C and D). Sixteen week old donor tissue contained differentiating spermatogonia and early meiotic germ cells at the preleptotene stage (Figure 2E).

Donor testicular tissue 24wk after grafting was investigated histologically for evaluation of establishment of spermatogenesis. Germ cell differentiation to the elongate spermatid stage has been demonstrated in neonatal testicular tissue from several species including bulls; however, the percentage of tubules with germ cell differentiation was low [1-4]. The extent of spermatogenesis in grafted bovine testicular tissue from different bull calf ages was determined. Evaluation of all grafts revealed the presence of intact seminiferous tubules and advanced stages of spermatogenesis in some tubules throughout the donor testicular tissue (Figure 3). Intact seminiferous tubules containing spermatogonia only (Figure 3A) and Sertoli cells only (Figure 3B) were identified. More advanced stages of spermatogenesis containing meiotic germ cells (Figure 3C) and elongate spermatids (Figure 3D) were also present within the grafts. The prevalence of different degrees of spermatogenesis in the donor testicular tissue varied with age of the donor bull.

Seminiferous tubules with Sertoli cells only could be observed in grafts from all the different donor ages. Upon comparison of donor ages for prevalence of Sertoli cell only tubules, grafts from 12 and 16wk donors were significantly different from each other (Figure 4A), the numerical values for these two donor ages were also the highest and lowest average percentage of all the donor ages at 29.8% and 17%, respectively. The range of average percentage of Sertoli cell only tubules in the 2, 4, and 8wk donor grafts was 19.1% to 26.9%.

The appearance of tubules containing spermatogonia only without any advancing germ cell types was seen in all grafts; however, the percentages of tubules with this phenotype varied by age of the donor (Figure 4B). Grafted testicular tissue from 2 and 4wk donors had a significantly higher percentage of tubules containing only spermatogonia than grafts from both 8 and 16wk donors. The percentage of tubules with spermatogonia only from 12wk old donor calves did not differ from any of the other donor ages. The highest percentage of spermatogonia only tubules was seen in 2 and 4wk donor grafts which comprised on average approximately 36% of the total tubules in each graft. The lowest percentage of tubules containing the spermatogonia only morphology was found in 8wk donor grafts comprising an average of 17% of the total tubules in each graft.

Maturation of germ cells to meiosis was seen in grafts from all donor ages. Grafts from 8 and 16wk old donor calves contained significantly higher percentages of tubules with meiotic germ cells than grafts from 2, 4, and 12wk donors (Figure 4C). The average percentage of tubules containing meiotic cells in 8 and 16wk grafts was approximately 60%; whereas, meiotic cells were present in an average range of 37% to 43% of the tubules in grafts from 2, 4, and 12wk donor testes.

Differentiation of germ cells to the elongate spermatid stage was observed in grafts from 4, 8, 12, and 16wk old donors but not 2wk old donors. Comparison of all the donor ages revealed a significantly higher average percentage of tubules containing elongate spermatids in grafts from 8wk donors (Figure 4D). The average percentage of tubules containing elongate spermatids from 4, 8, 12, and 16wk donors was low at only approximately 0.5%, whereas, the average percentage of 8wk donors was approximately 10-fold higher at 5%.

Transition Protein 2(TP-2) Immunohistochemistry

Identification of late meiotic germ cells in grafted bovine testicular tissue were identified by immunohistochemical staining of TP-2. In grafts from all donor ages cells positive for TP-2 were detected within some tubules; however, the distribution and abundance of these cells varied by age of the donor. Grafts from 2, 4, 12, and 16wk donor bull calves contained tubules that had one or two positively stained cells. In comparison, grafts from 8wk donors had multiple positively stained cells with a distinct location towards the lumen of the tubules (Figure 3D). Based on morphological observations seminiferous tubules in 8wk donor testicular tissue contained the most advanced stages of spermatogenesis.

Discussion

The objective of this study was to evaluate differences in the establishment of spermatogenesis in ectopically xenografted testicular from donor bull calves at different ages of postnatal testicular development. During the neonatal phase of testicular development somatic Sertoli and Leydig cells undergo proliferation and differentiation. Changes in both the somatic and germ cells during the neonatal period could have an impact on the establishment of spermatogenesis in grafted testicular tissue. In order to increase the efficiency of sperm production in grafted testicular tissue it is important to gain an understanding of changes in testis cell differentiation throughout the early postnatal period of testicular development. This understanding may lead to pinpointing a certain developmental donor age that is most "susceptible" to establishing spermatogenesis following ectopic grafting. Moreover, the neonatal period of testicular development is poorly understood in many non rodent mammalian species and ectopic grafting of testicular tissue from donors at different stages of development could

provide a bioassay to investigate this critical period. Since the postnatal period of testicular development in the bull is several months long we used donor bull calves at the ages of 2, 4, 8, 12, and 16wk. Twenty four weeks after grafting recipient mice were sacrificed and the testicular grafts evaluated histologically. Graft weights were collected to provide an indication of somatic and germ cell proliferation and establishment of spermatogenesis. Recipient mouse serum testosterone concentration demonstrated Leydig cell maturation and function in grafted bovine testicular tissues. Histological analysis of donor bovine grafts was conducted to evaluate differences in establishment of spermatogenesis in the testicular tissue from bull calves of different ages.

Average weight of donor testicular tissue 24wk after grafting was higher with younger donor bull calves. The largest increase in weight occurred in grafted testicular tissues from 2wk old donors with a 6-fold increase from the weight of the starting tissue. The average final weight from 2wk donors was significantly higher than those of 12 and 16wk donors and numerically higher than 4 and 8wk donors. Both 4 and 8wk average donor graft weights increased approximately 4-fold compared to starting weight and were significantly higher than that of 16wk donors which did not increase in weight compared to the starting tissue. An increase in weight of grafted testicular tissue could be attributed to several things but was most likely a result of somatic cell proliferation and progression of spermatogenesis. In the bull, the time frame of postnatal Sertoli cell proliferation is not well established. There are available data to suggest that proliferation of immature Sertoli cells occurs between birth and 20wk of life [8]. At the cessation of proliferation, Sertoli cell differentiation begins at 20wk and is completed at 28wk when the maximal number of mature differentiated Sertoli cells are present [8]. These observations suggest that the Sertoli cell population during developmental periods between birth

and 20wk of postnatal life have different proliferation potential. Grafting of testicular tissue at different developmental stages, as was done in the present study, could have resulted in different abilities of Sertoli cells to undergo proliferation and differentiation following grafting. The different proliferative potentials of Sertoli cells from different aged donors could have contributed to differences in weight increases of testicular tissue from donors of the different ages. Bull testes from the younger donor ages of 2, 4, and 8wk may have contained a Sertoli cell population with higher proliferation potential at the time of grafting than older donors and would thus result in larger weight increases following grafting.

Use of castrated recipient mice for grafting provides an endocrine environment containing high FSH following grafting of the donor bovine testicular tissues [2]. In rodents, Sertoli cell proliferation has been shown to be regulated by FSH [10-14]. If the same principle holds true in the bovine then the high FSH environment that the Sertoli cells were exposed to following grafting would have supported proliferation. Differences in the proliferation potential of the Sertoli cells in the grafted bovine testicular tissues at different developmental ages, based on FSH receptor expression or other factors, would have responded to the high FSH in different ways. The lack of weight increase in 16wk donor tissues may have been a result of the inability of Sertoli cells to respond to recipient mouse FSH. Testis tissue from younger donor animals contained a more immature Sertoli cell population with greater potential for response to high FSH following grafting.

In order for a tissue to become established following grafting vascularization must occur to provide nutrients for survival and function. In order for Sertoli cells in grafted testicular tissue to respond to recipient mouse pituitary released FSH vacularization must occur. Differing degrees of vascularization in testicular tissue from donor bull calves may have contributed to

differences in the abilities of the Sertoli cells to respond to FSH for proliferation and thus increase in weight over the 24wk period.

Evaluation of recipient serum testosterone concentration revealed the ability of Leydig cells in the grafted testicular tissues to respond to recipient mouse LH for production of testosterone at physiological concentrations normally found in intact adult male mice. Differences between the testis tissue from different donor ages in the ability of Leydig cells to produce testosterone could not be detected. This observation suggests that Leydig cell maturation does not vary based on developmental stage of the testis. Like Sertoli cell responsiveness to FSH following grafting onto castrated recipients, Leydig cells in the bovine testicular tissue were exposed to high LH concentrations following grafting. In the intact bull, LH concentration in the serum does not become elevated until 18wk of age, at which time Leydig cell maturation occurs and testosterone production commences [15]. It appears that increasing LH concentration in the serum is a key component to Leydig cell maturation. In neonatal bovine testicular tissue grafted onto castrated recipients, the immature Leydig cells appear to undergo spontaneous maturation and begin to produce physiological concentrations of testosterone in response to a high LH environment. Unlike immature Sertoli cells at different developmental stages in the neonatal testis, Leydig cells do not appear to have different potentials for proliferation and differentiation following ectopic grafting onto castrated hosts. The lack of detectable differences in testosterone production by Leydig cells in grafted testicular tissues suggest that the degree of tissue vascularization does not vary by the age or developmental stage of the donor testis.

The appearance of seminiferous tubules containing a Sertoli cell only phenotype was observed in all testicular grafts with only marginal variation between donors of different developmental ages. The only significant difference in the average percentage of seminiferous

tubules containing Sertoli cells only was found between 12 and 16wk donors, which also constituted the highest and lowest percentage respectively. The overall average percentage of total tubules from all grafts was approximately 25%. Other studies have also reported the occurrence of seminiferous tubules containing this phenotype in ectopically grafted testicular tissue [1-4]. Like the incidence of Sertoli cell only syndromes in men, the causative reason for the formation of this phenotype remains elusive [16-18]. Formation of Sertoli cell only tubules could arise from defects in Sertoli cell proliferation and/or germ cell loss. Lack of a Sertoli cell differentiation for the attainment of proper supportive function to support germ cell maturation could result in germ cell apoptosis. The highest percentage of tubules with Sertoli cells only occurred in grafted testicular tissue from 12wk donor bull calves in this study. This age could represent a development stage in bulls in which the Sertoli cells are most susceptible to detrimental influences of endocrine disruption. The efficiency of spermatogenesis in grafted testicular tissues could be enhanced through reduction of the occurrence of Sertoli cell only tubules. Also, increasing the vascularization of grafted testicular tissues may increase the supply of nutrients to Sertoli cells resulting in lowered occurrences of Sertoli cell only tubules. Ectopic testicular grafting may also provide a valuable biological model to investigate factors leading to the formation of seminiferous tubules lacking germ cells.

The appearance of seminiferous tubules containing spermatogonia only was also evident in grafted tissues from all donor bulls. Testicular grafts from 2 and 4wk old donor bulls had significantly higher averages of seminiferous tubules with this phenotype than grafts from both 8 and 16wk donors. The causative reason for the establishment of these tubules is not clearly evident. Defects in proliferation and/or maturation of both Sertoli and germ cells may have been contributing factors. Lack of Sertoli cell maturation and attainment of normal differentiated

function would result in the inability for supporting germ cell maturation. Differences in the germ cell population at the developmental stages of testicular development in the different aged bulls used in this study may have had an impact on the incidence of spermatogonia only tubules. During neonatal testicular development gonocyte migration to the basement membrane, conversion to spermatogonial stem cells and establishment of spermatogonial stem cell niches must occur for normal spermatogenesis to commence. In mice this process occurs from postnatal days 3 to 4 [6, 7]. In bulls the time frame for this process has not been established, but if the same principles in rodents are applicable to bulls then the time frame would occur between the postnatal weeks of 0 to 16 when differentiating spermatogonia are first present [8]. Differences in the potential for spermatogonial stem cell conversion and establishment of niches by early undifferentiated spermatogonia during the first 16wk of postnatal life may have an impact on their ability to establish normal spermatogenesis following grafting. The data in this study suggests that testicular tissue at the developmental stages of 8wk and 16wk in the bovine testis contains a somatic and germ cell population with the best potential for establishing spermatogenesis in the highest percentage of seminiferous tubules following grafting.

Progression of germ cells to meiotic stages was evident in grafted testicular tissue from donor bulls of all ages used in this study. Like the occurrence of spermatogonial only tubules, both 8 and 16wk donor grafts resulted in significantly higher average percentages of total tubules containing meiotic germ cells. The lower average percentage of tubules containing meiotic cells in grafts from 2, 4 and 12wk donors was most likely a result of higher percentages of tubules containing Sertoli cell only and spermatogonia only phenotypes within the same grafts. Detection of late meiotic germ cells by immunohistochmeical staining for TP-2 showed the greatest advancement of germ cell maturation in seminiferous tubules from 8wk donors. These

data indicate that testicular tissue from bulls at the postnatal developmental stage of 8wk is most amenable to establishment of spermatogenesis following ectopic xenografting.

The presence of elongate spermatids in grafted tissues marked the differentiation of germ cells through meiosis and into spermiogenesis. The average percentage of total tubules containing this cell type was significantly higher in 8wk donor tissues with approximately a 10-fold increase compared to all other donor ages. Advancement of germ cells to the elongate spermatid stage could not be detected in testicular grafts from 2wk old donor bull calves. Prior studies have demonstrated an acceleration of the first round of postnatal spermatogenesis in neonatal testicular tissue of pigs, goats, mice, and primates ectopically xenografted onto castrated recipient mice [1-3]. Lack of observed elongate spermatids in 2wk old neonatal bovine testicular tissue 24wk after grafting suggests that a comparable acceleration does not occur with the bovine. In the intact bull, elongate spermatids can be first observed at 28wk of postnatal age [8]. Allowing testicular tissue from 2wk donors more time after grafting that is relative to the donor age (i.e. 26wk) could yield elongate spermatids. The lower average percentage of tubules containing elongate spermatids in 4wk donors was most likely a function of higher Sertoli and spermatogonia only tubules. Testicular tissue at the developmental ages of 12 and 16wk possibly contains a high percentage of seminiferous tubules with somatic and germ cell populations that are at a differentiated stage of development where the setup of the seminiferous epithelium is inhibited in the majority of tubules. Data from the current study again indicates that 8wk of postnatal age is a testicular developmental stage in the bovine testis where the somatic and germ cell population is most amenable for establishment of spermatogenesis in the highest percentage of tubules.

Ectopic testicular xenografting is a method that has potential use for investigating spermatogenesis in non rodent mammalian species where experimentation in the target species is difficult. As an applied technology, testicular xenografting could be utilized as an alternative means to generate stably modified transgenic spermatozoa through manipulation of spermatogonial stem cells prior to grafting. Use of neonatal donors is of benefit, since undifferentiated spermatogonia are the only germ cell type present and the population is enriched for spermatogonial stem cells. Understanding the establishment of spermatogenesis in grafted neonatal testicular tissue can lead to enhancement of sperm production. In the present study we demonstrate differences in the establishment of spermatogenesis in testicular tissue from bull calves at different neonatal stages of testicular differentiation ectopically xenografted onto recipient mice. The data indicates that 8wk of postnatal life is the developmental stage in the bovine testis when the Sertoli and germ cells are most amenable to establishing spermatogenesis in ectopically xenografted testicular tissue

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Figure 1. Growth and steroid biosynthesis of bovine testicular tissue from different aged bull calves ectopically xenografted onto castrated recipient nude mice. A: Average weight of bovine testicular grafts 24wk after grafting, B: Average serum testosterone concentration in castrated recipient mice 24wk after grafting with bovine testicular tissue, and C: Average castrated recipient mouse seminal vesicle weight 24wk after grafting with bovine testicular tissue. Data are presented as the mean$\pm$SEM.

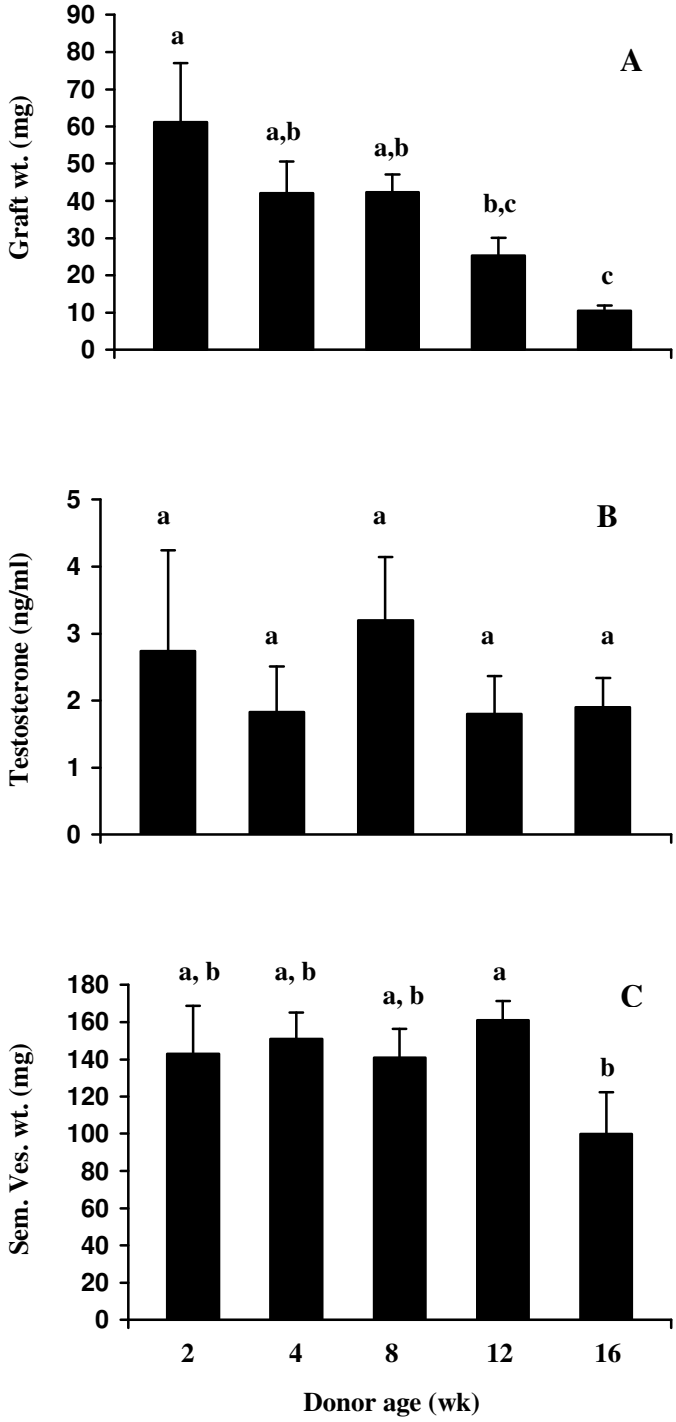
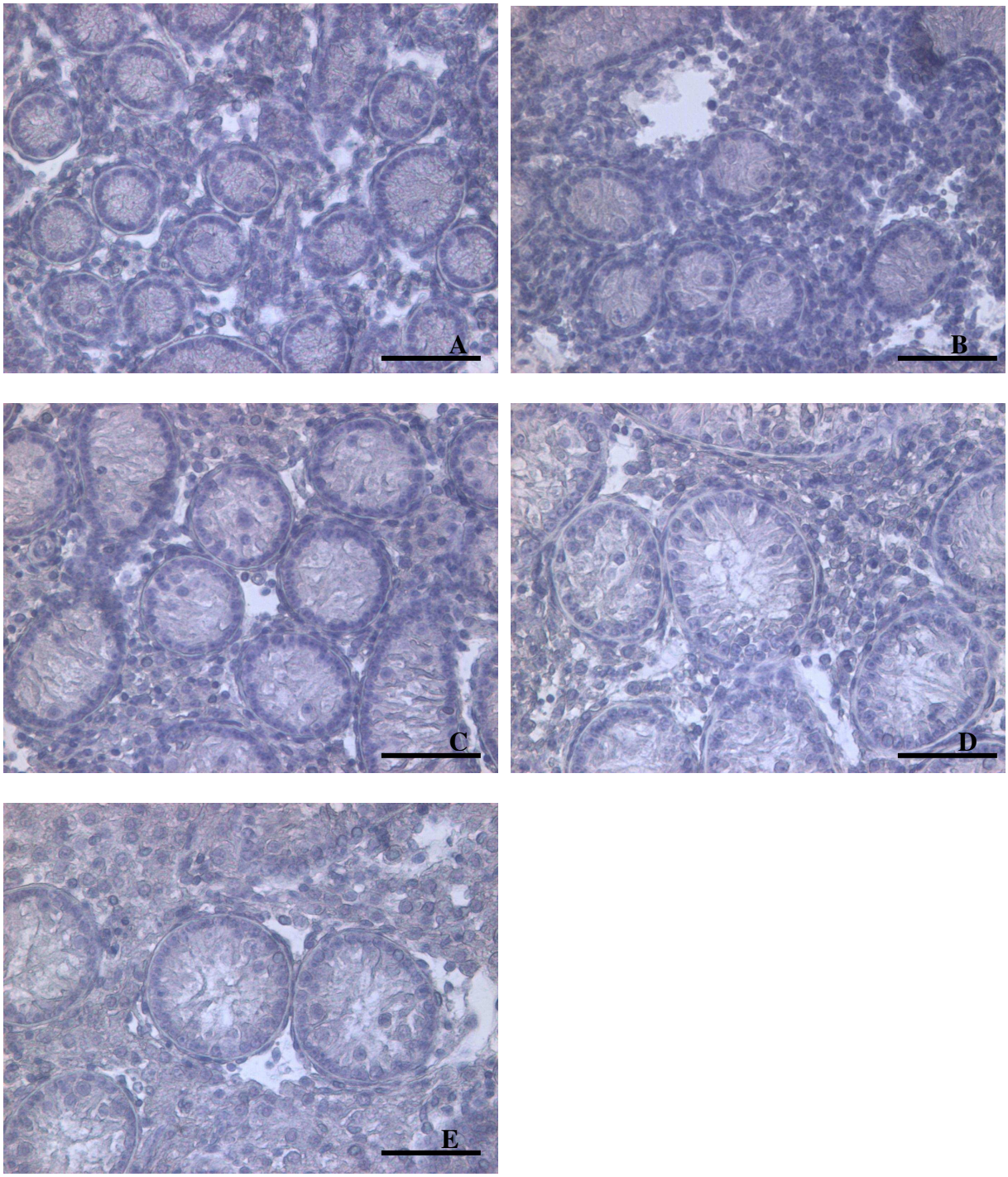

A
Graft wt. (mg)
90
80
70
60
50
40
30
20
10
0
a
a,b
a,b
b,c
c
B
Testosterone (ng/ml)
5
4
3
2
1
0
a
a
a
a
a
C
Sem. Ves. wt. (mg)
180
160
140
120
100
80
60
40
20
0
a, b
a, b
a, b
a
b
2
4
8
12
16
Donor age (wk)

Figure 2. Cross section micrographs of testicular tissue from donor bull calves of different developmental ages prior to ectopic xenografting. A: 2wk old donor bull calf, B: 4wk donor bull calf, C: 8wk donor bull calf, D: 12wk donor bull calf, and E: 16wk donor bull calf. Bar = 100µm for all panels.


A
B
C
D
E

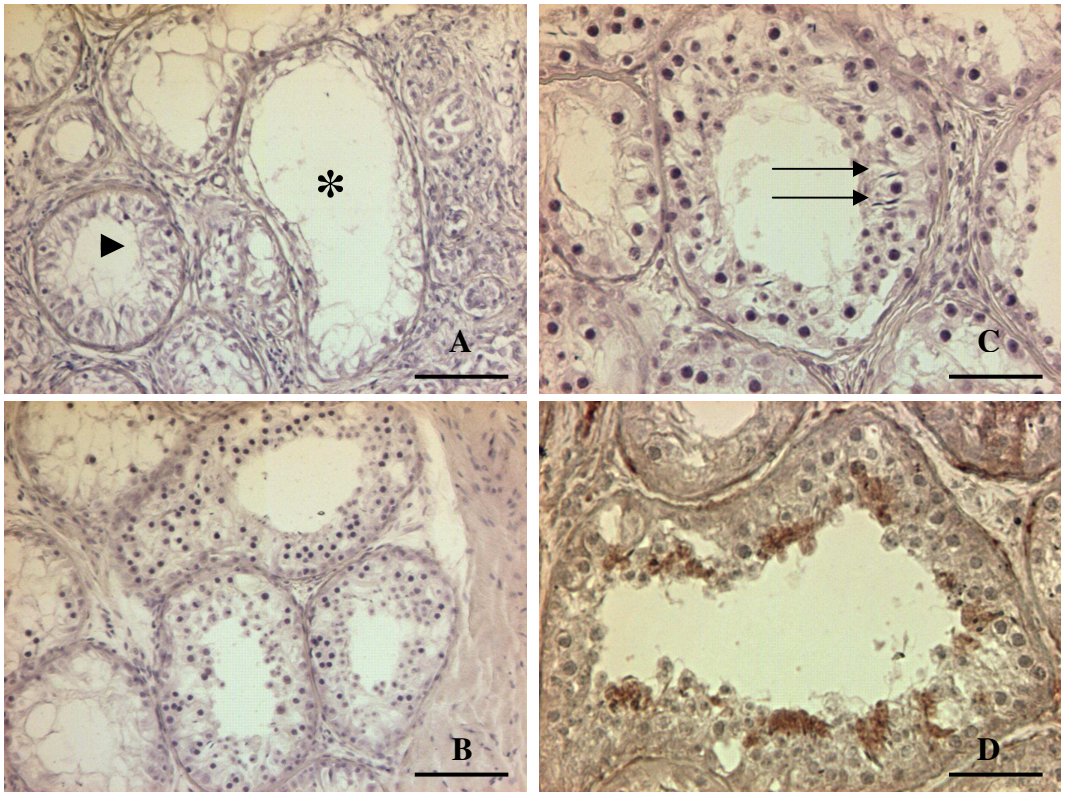
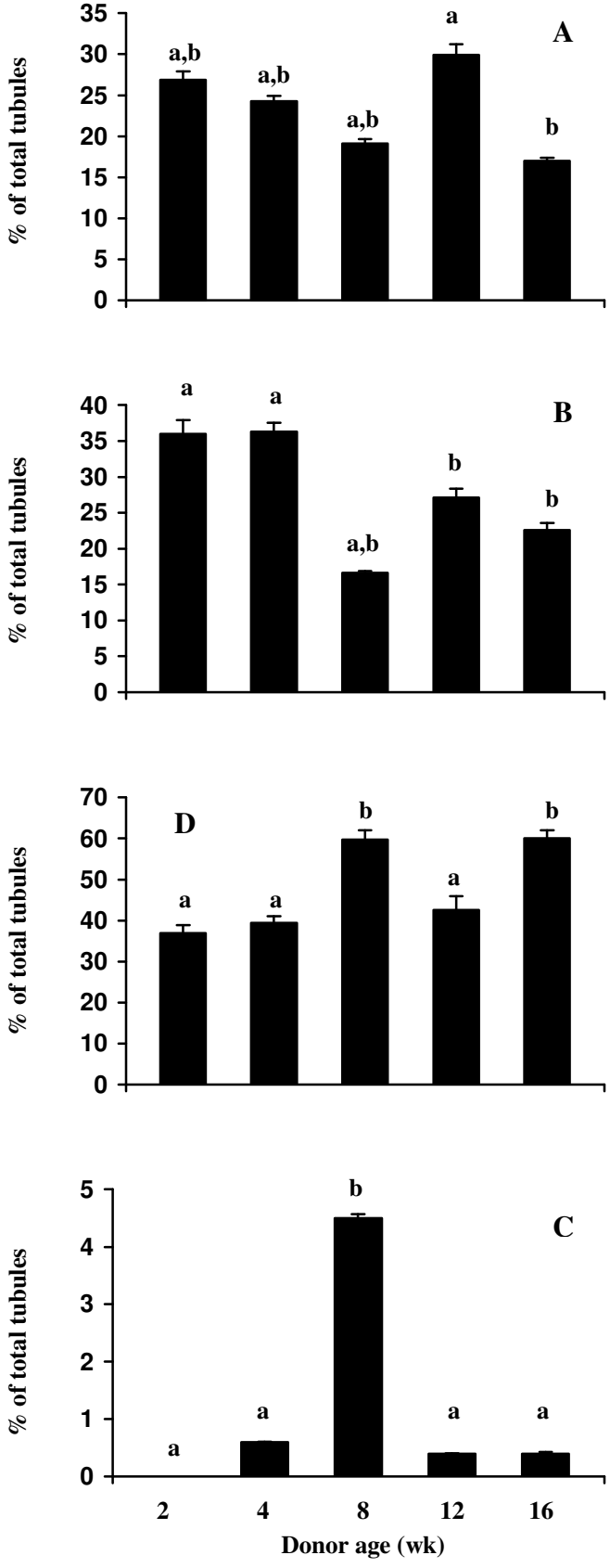


Figure 3. Cross section micrographs of donor bovine testicular grafts from 8wk donor bulls 24wk after ectopic xenografting onto castrated recipient nude mice. A: Sertoli cell only (star) and spermatogonia only seminiferous tubules (arrow head), B: Cluster of seminiferous tubules containing advanced spermatogenesis, C: maturation of germ cells to the elongate spermatid stage (arrows), and D: Immunohistochemical staining for TP2 to identify late meiotic germ cells (cells positive for TP-2 staining are brown). Bar = 100μm for all panels.

Figure 4. Average percentage of seminiferous tubules containing different phenotypes and germ cell types in bovine testicular tissue from bull calf donors of different ages following ectopic xenografting onto castrated recipient nude mice. A: Average percentage of tubules with a Sertoli cell only phenotype, B: Average percentage of tubules with a spermatogonia only phenotype, C: Average percentage of tubules with meiotic germ cells, and D: Average percentage of tubules containing elongate spermatids. Data are presented as the mean$\pm$SEM. Bars with different letters are significantly different at $P \leq 0.05$.


A
% of total tubules
35
30
25
20
15
10
5
0
a,b
a,b
a,b
a
b
B
% of total tubules
40
35
30
25
20
15
10
5
0
a
a
a,b
b
b
D
% of total tubules
70
60
50
40
30
20
10
0
a
a
b
a
b
C
% of total tubules
5
4
3
2
1
0
a
a
b
a
a
2
4
8
12
16
Donor age (wk)

CHAPTER SIX

Changes in spermatogenesis and endocrine function in the ram testis due to irradiation and active immunization against LHRH[6*]

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ABSTRACT: Spermatogonial stem cell transplantation is a technique that has potential in livestock to enhance genetic gain and generate transgenic offspring through the male germ line. A means for depletion of endogenous germ cells in a recipient's seminiferous tubules is necessary for this technology to be applied. The objectives of this study were to evaluate several different methods for depletion of endogenous germ cells in the testes of adult rams and evaluate ultrasound-guided injections into the rete testes as a means for infusing a suspension into the seminiferous tubules. Sixteen adult rams were randomly divided into 4 treatment groups (n=4/group). Treatments consisted of active immunization against LHRH (IMM), localized testicular irradiation (IR), LHRH immunization+irradiation (IMM+IR), and non-treated control. Serial bleeds were conducted pre-treatment and monthly after treatment for 4mo at which time all rams were castrated. Both IMM and IMM+IR rams received 3,000 IU of Perganol® weekly for 8wk prior to castration to bypass the immunization. All rams also received an ultrasound-guided injection of PBS containing 0.4% trypan blue into the rete testis of one testicle prior to castration. Rams receiving IMM and IMM+IR treatments had significantly ($P \leq 0.05$) higher average percentages of seminiferous tubules with depleted germ cells compared to controls. Serum testosterone was significantly reduced in IMM and IMM+IR rams 1mo after treatment and throughout the remainder of the study compared to controls and IR rams which were not different from each other. Serum inhibin concentration was unchanged in all rams following treatment indicating that Sertoli cell function was unaltered. A significantly greater average percentage of the total testicular area could be filled with the trypan blue solution by rete testis injection in IMM and IMM+IR rams. These data demonstrate the depletion of endogenous germ cells in adult ram testes without alteration of Sertoli cell viability and function that have potential as methods for preparing recipient animals for germ cell transplantation.

Introduction

Spermatogenesis is a complex and intricate process in which a spermatogonial stem cell undergoes phases of proliferation and differentiation to eventually become mature spermatozoa. Spermatogonial stem cell transplantation is a technique in which spermatogonial stem cells from the testis of a donor male are transferred into the seminiferous tubules of an azoospermic recipient male. In rodents and goats this technique has been shown to result in donor-derived spermatogenesis within recipient testes and generation of offspring with the donor genotype following natural breeding (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994; Honamarooz et al., 2003A). These advancements have given spermatogonial stem cell transplantation technology potential utility in livestock as an alternative means to enhance genetic gain in commercial herds and generate transgenic offspring.

In order for the technique of spermatogonial stem cell transplantation to be functional on an efficient level two main concepts must be developed. First, a means for preparing recipient animals for transplant would be beneficial. In goats, recipient animals were sexually immature and even though several were used only one transfer was successful in resulting in donor derived spermatogenesis. A system in which endogenous germ cell are destroyed or depleted in recipient testes prior to transfer of donor germ cells could allow for efficient colonization of donor stem cells and establishment of donor-derived spermatogenesis. In rodents this can be accomplished through use of chemotoxic drugs or whole testis irradiation (Ogawa et al., 1997). In large animals like bulls treatment with chemotoxic drugs is not practical and therefore more reasonable means must be developed.

Secondly, a means for infusing donor spermatogonial stem cells into recipient seminiferous tubules must be in place. Microinjection into the efferent ductiles, rete testis, or

seminiferous tubules themselves results in stem cell colonization and donor-derived spermatogenesis in rodents (Ogawa et al., 1997). Due to the testicular architecture of large domestic animals, injection into the efferent ductiles or seminiferous tubules is not plausible. Ultrasound guided injections into the rete testis of primates, pigs, and goats has shown to be a promising approach to overcome this obstacle (Honaramooz et al., 2002; Honoaramooz et al., 2003A; Honaramooz et al., 2003B). The objectives of this study were to evaluate several different methods for depletion of endogenous germ cells in the testes of adult rams and evaluate ultrasound-guided injections into the rete testes as a means for infusing a suspension into the seminiferous tubules.

Materials and Methods

Experimental rams and treatments

All animal procedures were approved by the Washington State University Animal Care and Use Committee. Sixteen adult rams ranging in breed from Suffolk, Colombia, and Karakul were randomly divided into four treatment groups (n = 4/group); (1) LHRH immunized, (2) irradiated, (3) LHRH immunized and irradiated, and (4) control. The timeline for experimentation is depicted in figure 1.

Eight rams were actively immunized against LHRH using a fusion protein cocktail (Quesnell et al., 2000). The treatment regimen consisted of a primary immunization using a modified Freund's complete adjuvant followed by two booster injections in Freund's incomplete adjuvant one month apart each. Approximately 12 months after the last booster, immunized rams began to receive a weekly treatment of 3,000 IU of Perganol® (Serono; Rockland, MA) in sterile PBS by subcutaneous injection to bypass the effects of the vaccine. Eight rams were subjected to

12 grays of irradiation localized to the testis. Four of the rams had been immunized against LHRH eight months prior and 4 rams received just irradiation. Four rams were also utilized as non treated controls.

Serial bleeds and serum analysis

In order to evaluate changes in the pituitary gonadal axis due to treatment, serial blood samples were collected one month prior to any treatment and monthly for 4 months after treatment. The bleeding regimen consisted of jugular venipuncture every 15min for 4hr. Serum was then collected by centrifugation at 3,000 xg for 20min. All serum samples were assayed for both luteinizing hormone (LH) and follicle stimulating (FSH) concentrations using double antibody RIA (Acosta et al., 1983). Serum was also evaluated for circulating concentrations of inhibin using a double antibody ELISA (Myer and Wheaton, 1996) and testosterone using a commercial RIA kit (DSL-400; Diagnostic Systems Laboratory Inc., Webster, TX). Due to the pulsatile nature of testosterone, four consecutive samples from each 4hr bleed were analyzed and the averages of these were used as a representation of each 4hr bleed. Since inhibin is not released in a pulsatile manner by Sertoli cells, its concentration was analyzed in one sample from each bleed.

Testicular diameter, castration, and histological analysis

Changes in testicular size were evaluated by measuring the testicular diameter of each ram using ultrasonography one month prior to treatment and monthly thereafter for 4 months. Approximately 4 months after treatment, which constituted two spermatogenic cycles, all rams were castrated using standard procedures and random section of testicular parenchyma were

collected in Bouin's solution from one testis of each animal. Samples were fixed for 4hr at 4°C followed by dehydration in ethanol and xylenes, and embedded in parrifin. Cross sections from each samples were cut at 8 microns and affixed to glass slides, followed by deparrifinization, rehydration, and staining with hematoxylin and eosin. Processed samples were evaluated using light microscopy at 100X magnification and digital images were captured with a *cf* Cool Snap digital camera (Media Cybernetics; Silver Spring, MD). Evaluation of histology samples included assessment of the average percentage of regressed seminiferous tubules and tubules containing active spermatogenesis. Average percentages were calculated for each sample by dividing the number of round tubules with depleted germ cells in three microscopic fields by the total number of round tubules in the same 3 fields.

Ultrasound-guided rete testis injection

Just prior to castration the testis not used for histology was infused with a 0.4% trypan blue/PBS solution using ultrasound-guided injection into the rete testis. Briefly, rams were positioned in dorsal recumbence in a surgery cradle and given a local anesthetic injection of lidocaine at the base of the scrotum. A small incision was then made through the scrotum. The testis was viewed by ultrasonography (Aloka 500-7.5 MHz) and a 20 gauge 3 inch anesthesia needle was guided into the rete testis. Three ml of trypan blue/PBS solution was then infused into the rete testis over a 5min period by positive pressure from a peristaltic pump (Harvard Apparatus, Holliston, MA). Immediately after injection the testis was removed split down the midline to expose the rete testis and surrounding testicular parenchyma, extensively washed with saline to remove trypan solution not infused into seminiferous tubules, and digital images were captured. Analysis of the percentage of area filled with trypan blue was conducted by evaluating the digital images with a software program (Scion image; Scion Corporation, Frederick, MD).

Statistical analyses

All data were analyzed using the Proc GLM function of SAS systems software. Differences between means for treatment values of serum hormone concentrations and testis diameters were compared to control values using repeated measures analysis with contrast transformation. This type of analysis utilized a multivariate ANOVA to determine differences between treatment and control means. Data for % regressed tubules and % area filled by rete testis injection were analyzed for differences between means using Duncan's test for significance. Means were considered significantly different at $P \leq 0.05$.

Results

Effect of treatment on testicular somatic cell endocrine function

Serial blood samples were collected from all rams to evaluate changes in the pituitary testis axis in response to treatment. Serum testosterone concentration was measured to assess Leydig cell functionality. Sertoli cell functionality was evaluated by measuring serum inhibin concentration. The average concentration of LH and FSH in all rams was not different ($P \geq 0.05$) prior to treatment (Figure 2). At 2 mo after treatment and throughout the remainder of the study serum LH concentration in immunized animals fell below the detection limit of the assay, but was not statistically different from control rams (Figure 2A). In contrast serum FSH concentrations in immunized rams 1 mo after treatment and continuing throughout the remainder of the study decreased by approximately 56% of pre-immunization levels and were significantly lower than controls (Figure 2B). In rams receiving irradiation serum LH concentration was

significantly higher than controls at 1 mo after treatment but not different at 2, 3 or 4 mo (Figure 2A).

Serum testosterone concentration in all rams was not different prior to treatment (Figure 3A). In control and irradiated rams serum testosterone concentration dramatically increased as the animals entered the breeding season at the end of the study. In both LHRH immunized groups serum testosterone concentration significantly decreased from that of the control group to below the detection limit of the assay 1 mo after the final booster and throughout the remainder of the study (Figure 3A). Serum testosterone concentration in the irradiated group and control group were not different throughout the entire treatment period (Figure 3A).

Serum inhibin concentration was significantly higher in the immunized and irradiated rams compared to controls prior to treatment (Figure 3B) but was not different between irradiated and immunized rams. The average concentration of serum inhibin actually rose in immunized rams that were exposed to testicular irradiation and remained significantly higher than controls throughout the treatment period. The average inhibin concentration in rams receiving LHRH immunization alone remained relatively constant throughout the treatment period and was not different than controls by the end of the study. In irradiated rams the average serum inhibin concentration rose throughout the treatment period and was significantly different than controls throughout but not different than rams receiving both LHRH immunization and irradiation.

Effects of treatment on testicular diameter

The testicular diameter of all rams was measured to provide an evaluation of changes in active spermatogenesis caused by treatment. Testis diameter has been shown to be correlated with sperm output in many livestock species (Courot and Ortavant, 1998; Berndtson et al., 1987).

One month prior to treatment mean testis diameter was not different between groups (Figure 4). One month after treatment rams immunized against LHRH and those subjected to testis irradiation had significantly lower testis diameters than controls. Rams receiving irradiation alone had a significantly smaller testis diameter than controls until 3 mo after treatment, but by the time of castration the average testis diameter of these rams had reached control sizes and were not different. All rams immunized against LHRH had significantly smaller testis diameters than controls throughout the 4 mo period after treatment and were not different than each other. Treatment of immunized rams with Perganol® had no effect on testis diameter.

Effects of treatment on spermatogenesis

Preparation of a recipient animal for germ cell transplantation requires that endogenous germ cells be depleted within the seminiferous tubules to allow donor spermatogonial stem cells to colonize. The effect of treatment on spermatogenesis in ram testes was evaluated histologically following two spermatogenic cycles. Seminiferous tubules without active spermatogenesis (Figure 5 B, C, and D) were considered regressed when endogenous germ cells were depleted. Visual evaluation revealed tubules with both active and regressed spermatogenesis (Figure 5) from all rams. Rams actively immunized against LHRH had a significantly higher average percentage of regressed tubules compared to rams receiving irradiation alone and control rams (Figure 6). Rams receiving testicular irradiation alone had a significantly higher average percentage of regressed tubules than control rams. Those animals receiving active immunization against LHRH+testicular irradiation had the highest average percentage of tubules with regressed spermatogenesis.

Ultrasound-guided rete testis injection

In order to evaluate differences in the ability to infuse a liquid solution into the seminiferous tubules of ram testes, an ultrasound-guided injection into the rete testis was performed on one testis from each ram. As an evaluation of the utility of any of these treatments for preparing recipient animals for germ cell transplantation the total filled area was measured as a percentage of the whole testis. After injection, dissection and extensive washing infused blue dye could be seen within the testicular parenchyma (Figure 7). The average area filled by the rete testis injection of the immunized and immunized+irradiated groups were significantly higher than those of the control and irradiated alone groups (Figure 8). Approximately 53% of the testicular area could be filled with the rete testis injection in immunized and immunized+irradiated groups.

Discussion

In this study we evaluated three different treatment regimens for their individual abilities to deplete germ cells in the seminiferous tubules of adult ram testes without causing harmful effects on testicular somatic cell function or viability.

Adult rams receiving localized testicular irradiation had a significant decrease in average testis diameter 1 and 2 mo after treatment compared to controls. This observation suggests a depletion of germ cells and loss of active spermatogenesis within the testes. Studies in livestock have demonstrated the correlation of testis size to active spermatogenesis (Courot and Ortavant, 1998; Berndtson et al., 1987). Therefore, measuring testis diameter is an effective means for assessing loss of germ cells and active spermatogenesis following treatment. At 3 and 4 mo after treatment average diameter was not different from controls, suggesting that germ cell depletion occurred after irradiation but slowly came back after 3 mo, thus indicting that spermatogonial

stem cells were not destroyed by treatment. Spermatogonial stem cell viability following testis irradiation has also been demonstrated in rodents (Hacker-Klom UB et al., 2000; Meistrich et al., 1995).

Histological evaluation of testes revealed a significant 7.3-fold higher average percentage of regressed seminiferous tubules in irradiated rams compared to controls. Approximately 22% of seminiferous tubules in these rams were unable to reestablish spermatogenesis following two spermatogenic cycles after irradiation, suggesting that spermatogonial stem cells were destroyed in these tubules. Histological evaluation of the seminiferous epithelium in these rams occurred after their average testis diameter had regained comparable values to controls. Therefore, a higher percentage of tubules may have been regressed at earlier time points after treatment when testis diameter was significantly smaller than controls. A much higher percentage of germ cell deficient tubules would be ideal in animals intended as recipients for germ cell transplantation than that observed in irradiated rams 4 mo after treatment. Evaluation of regressed seminiferous tubules should be conducted at time points earlier after treatment (i.e. 1 and 2 mo).

Rams that were actively immunized against LHRH had a significant decrease in testes diameters compared to controls beginning 1 mo after the final booster and remaining until the conclusion of the treatment period. Localized testicular irradiation of rams that had been previously immunized against LHRH did not have an additive effect on testicular diameter. These observations alone suggest that active immunization against LHRH result in loss of active spermatogenesis and depletion of germ cells. Previous studies have also shown the inhibitive effects of immunoneutralizing LHRH on spermatogenesis (Carelli et al., 1982; Lad et al., 1989; Awoniyi et al., 1989). Treatment of immunized rams with Perganol® in this study was unable to bypass the immunization and stimulate an increase in testis diameter suggesting that some of the

spermatogonial stem cells were destroyed over the treatment period and could not reinitiate spermatogenesis in those seminiferous tubules.

Evaluation of the seminiferous epithelium in testes of rams immunized against LHRH showed significantly higher average percentages of tubules that were germ cell depleted. Rams receiving LHRH immunization had a 12.1-fold higher average percentage of regressed tubules compared to controls, whereas rams receiving LHRH immunization+irradiation had a 21.6-fold higher average percentage. Approximately 63% of the seminiferous tubules in rams receiving both immunization and irradiation were regressed. Those animals received localized testicular irradiation several months after the final booster. Therefore, mitotically active germ cells that were not depleted as a result of LHRH immunization were subjected to deleterious defects from irradiation and possibly eliminated resulting in a higher average percentage of regressed tubules than immunization alone. In both the immunized groups some seminiferous tubules contained active spermatogenesis; however, the percentage was so low that the animals were considered infertile upon electroejaculation and semen analysis (data not shown).

Evaluation of Leydig cell function in terms of testosterone production revealed no differences between rams subjected to irradiation and controls. However, beginning 1 mo after the final booster all rams immunized against LHRH had significantly lower concentrations of serum testosterone than controls. This observation suggests that active immunization against LHRH effectively neutralized LH output by the anterior pituitary. This observation is in accordance with previous studies demonstrated the effects of LHRH immunization on testicular steroidogenesis in bulls (Aissat et al., 2002). Even though the average serum LH concentration in the both groups of immunized rams was approximately 2-fold lower than controls they were not

statistically different at the 5% level. The low number of animals in each treatment group was most likely the reason for lack of statistical significance.

Treatment of the immunized rams with Perganol® was unable to reestablish testosterone concentrations to control values. In order for a treatment to be effective as a means for preparing recipient animals, detrimental influences on testicular somatic cell viability and function must be avoided. Testicular irradiation appears to not harm Leydig cell function in terms of ability to respond to LH and produce testosterone. The lack of response to exogenous gonadotropin in immunized rams suggests that Leydig cells in those animals were atrophied or unable to respond. A decline in Leydig cell number in the testes of adult rats following immunization against LHRH has been demonstrated (Duckett et al., 1997). Likewise, immunization of pigs against LHRH has been shown to result in reduced LH receptor in the testis and reduced ability of Leydig cells to produce testosterone upon exposure to exogenous LH (Awoniyi et al., 1988). Another explanation for lack of a response to exogenous gonadotropin in this study may have been to low of a dose administered. However, Oatley et al. (2001) was able to demonstrate a reestablishment of testosterone concentration in bulls given a similar dose of PMSG following active immunization against LHRH.

Even though active immunization against LHRH results in high percentages of tubules with germ cell depletion, Leydig cell function may be impaired. To serve as adequate recipient animals in germ cell transplantation these animals may need to be supplemented with exogenous testosterone. It is also important to note that spermatogonial stem cell colonization following transplantation in mice has been shown to be enhanced in a low testosterone environment (Ogawa et al., 1998; Dobrinski et al., 2001). Therefore, active immunization against LHRH may

be beneficial as a means to prepare recipient animals in terms of initial donor stem cell colonization.

An interesting observation in this study was the significantly elevated concentration of LH in rams receiving testicular irradiation compared to controls. A reduction in testosterone concentration would be thought to accompany a rise in LH however testosterone concentration slightly rose in these animals. These observations suggest that testicular irradiation caused a brief change in testicular somatic cells to create a positive feedback on the pituitary for LH production. This feedback may have resulted from Leydig cell aromatization of testosterone to estrogen that subsequently enhanced LH release from the pituitary.

Viability and functionality of Sertoli cells following treatment, evaluated as the ability to produce inhibin, revealed no deleterious differences in treated animals compared to controls. Both treatment groups receiving testicular irradiation had significantly higher serum inhibin concentrations compared to controls throughout the study. Rams only immunized against LHRH had significantly higher concentrations than controls initially but were not different by the end of the study. These data suggest that neither irradiation nor active immunization against LHRH has deleterious effects on Sertoli cell function.

In both of the LHRH immunized groups serum inhibin concentrations did not significantly decrease following immunization. Serum FSH concentrations in these animals was decreased by approximately 56% following immunization compared to their pre-immunization values. Since inhibin is regulated by pituitary FSH, these data suggest that the decrease in FSH concentration following active immunization against LHRH is not low enough to significantly inhibit Sertoli cell function. In these same animals testosterone concentration was significantly reduced compared to the pre-immunization levels suggesting that LHRH immunization inhibited

LH release from the pituitary. In fact LH concentration in the serum was below the detection limit of the assay. Other studies have also shown differential effects on FSH and LH release following neutralization of LHRH, in which LH was completely inhibited whereas FSH was not affected as severely (Culler and Negro-Vilar, 1986; Culler and Negro-Vilar, 1987; Aissat et al., 2001B).

Infusion of trypan blue into the rete testis of recipient rams demonstrated the ability to transfer a liquid solution into the testes of large domestic adult animals using ultrasound-guided injections. A significantly larger area was able to be filled with the solution in testes from rams immunized against LHRH than both control and irradiated rams. This observation suggests that treatment of rams by immunization against LHRH results in a testicular parenchyma mass with a higher percentage of seminiferous tubules devoid of germ cells that may allow for filling with a germ cell suspension. Previous studies have also shown the ability to inject a liquid solution into the seminiferous tubules of goats and pigs using ultrasound-guided injections into the rete testis (Honaramooz et al., 2002; Honaramooz et al., 2003); however, these studies did not evaluate the effectiveness of the procedures based on the percentage of area filled. These previous reports also used sexually immature animals as recipients and did not provide an evaluation of the depletion of endogenous germ cells.

Implications

Spermatogonial stem cell transplantation in livestock could provide a means by which to enhance genetic gain in domestic herds as well as generate transgenic offspring through the male germ cell line. In order for this technology to be applied a means for preparing recipient animals through depletion of endogenous germ cells within the seminiferous tubules that provides an

environment allowing donor stem cells to seed must be developed. This method must be pracitical and cost effective for the technology to be feasible. The treatment regimens evaluated in this study can be further refined and utilized in future germ cell transplantation experiments.

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Figure 1. Timelines of treatment regimens for experimental rams. Pre-treatment blood sampling was conducted 1 mo prior to any treatment. Rams receiving LHRH immunization were given a primary injection followed by two booster injections 1 mo apart each. Rams receiving localized testicular irradiation received that treatment 1 mo after the date of the final LHRH immunization booster. One month after the date of irradiation all rams were bled monthly for 4 mo, which constituted 2 spermatogenic cycles, followed by castration and analysis of germ cell depletion. Rams that had received immunization against LHRH were administered 3,000 IU of Perganol weekly for 2 mo prior to castration.

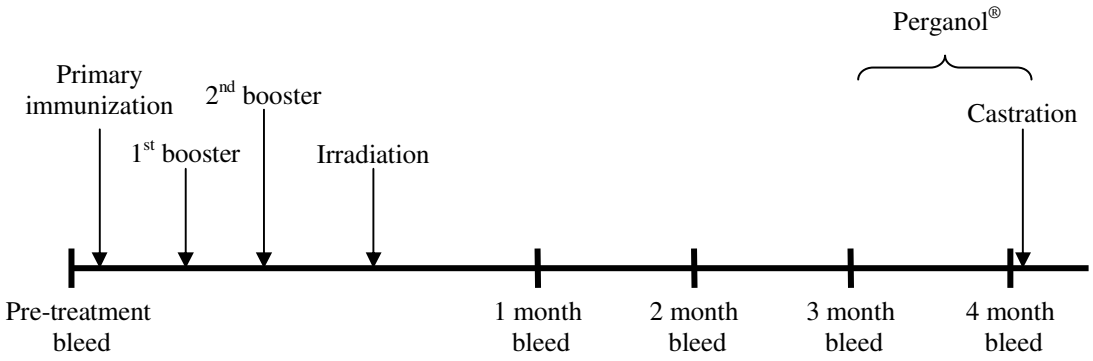
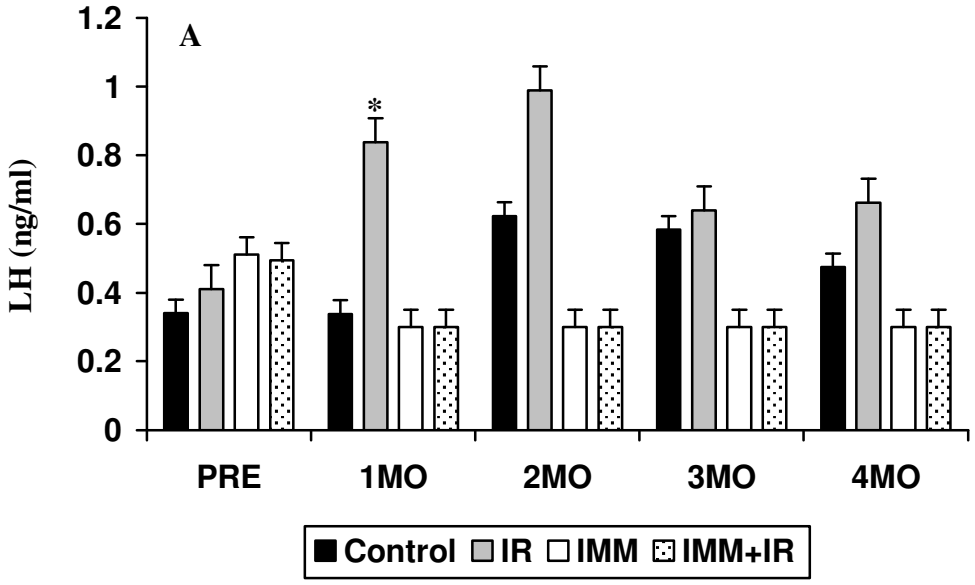

Perganol®
Primary immunization
2nd booster
Castration
1st booster
Irradiation
Pre-treatment bleed
1 month bleed
2 month bleed
3 month bleed
4 month bleed

Figure 2. Average serum concentrations of LH (A) and FSH (B) in rams subjected to different treatment regimens for depletion of endogenous germ cells. Hormone concentrations were measured to provide an evaluation of the effects of treatment on the hypothalamic-pituitary-gonadal axis. In both figures bars with an asterisk are significantly different than control at $P \leq 0.05$. Also, for both figures **Control** = non-treated animals, **IR** = animals receiving testicular irradiation, **IMM** = animals actively immunized against LHRH, and **IMM+IR** = animals receiving active immunization against LHRH and testicular irradiation. Perganol was given to all LHRH immunized rams between the 3 and 4 month bleeds. Data are presented as the mean$\pm$SEM.


A
LH (ng/ml)
1.2
1
0.8
0.6
0.4
0.2
0
*
PRE
1MO
2MO
3MO
4MO
Control
IR
IMM
IMM+IR

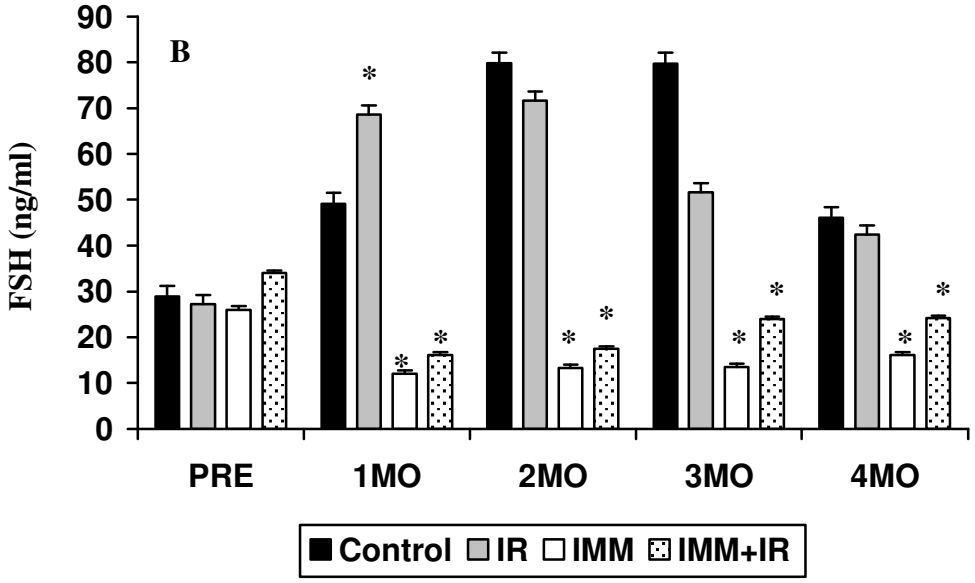
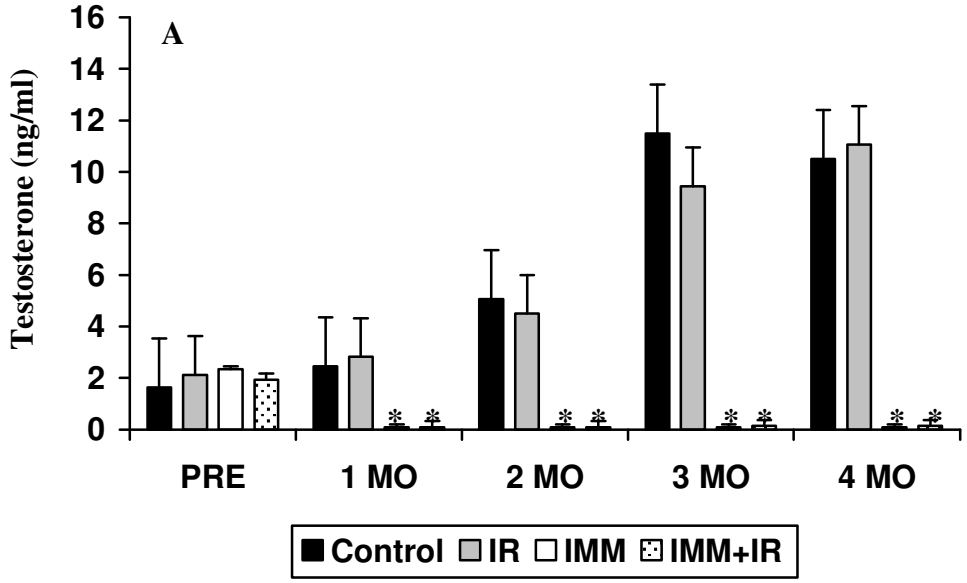

B
FSH (ng/ml)
90
80
70
60
50
40
30
20
10
0
*
PRE
1MO
2MO
3MO
4MO
Control
IR
IMM
IMM+IR

Figure 3. Average serum concentrations of testosterone (A) and inhibin (B) in rams subjected to different treatment regimens for depletion of endogenous germ cells. Testosterone concentration was analyzed for evaluation of Leydig cell function and viability. Inhibin concentration was determined for evaluation of Sertoli cell function and viability. In both figures bars with an asterisk are significantly different than control at $P \leq 0.05$. Also, for both figures **Control** = non-treated animals, **IR** = animals receiving testicular irradiation, **IMM** = animals actively immunized against LHRH, and **IMM+IR** = animals receiving active immunization against LHRH and testicular irradiation. Perganol was given to all LHRH immunized rams between the 3 and 4 month bleeds. Data are presented as the mean$\pm$SEM.


A
Testosterone (ng/ml)
16
14
12
10
8
6
4
2
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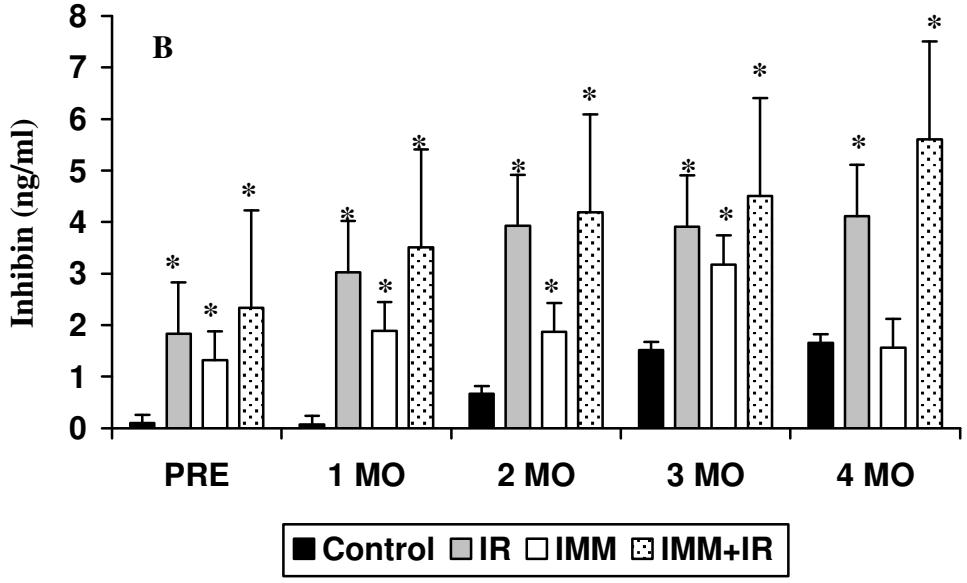
PRE
1 MO
2 MO
3 MO
4 MO
Control
IR
IMM
IMM+IR


B
Inhibin (ng/ml)
8
7
6
5
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3
2
1
0
PRE
1 MO
2 MO
3 MO
4 MO
Control
IR
IMM
IMM+IR

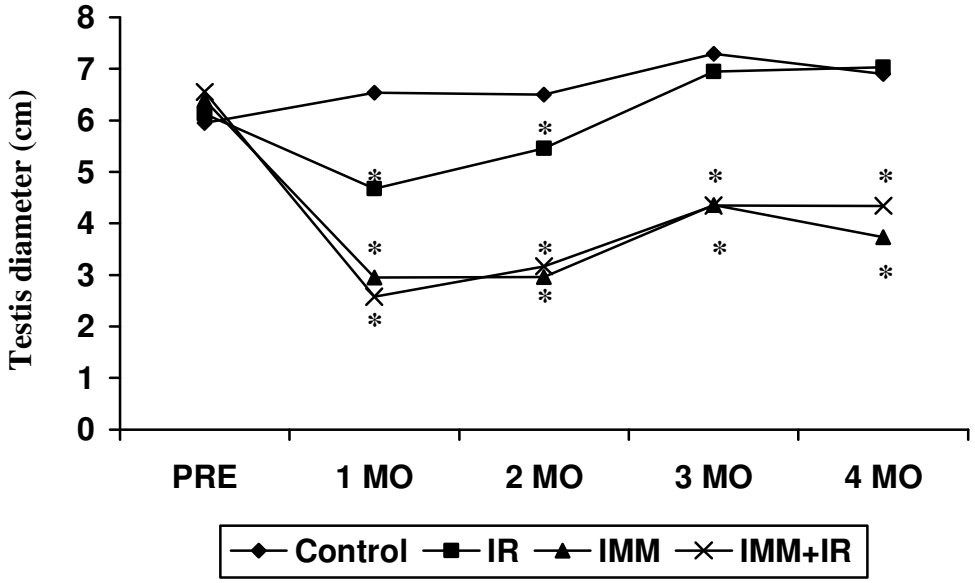
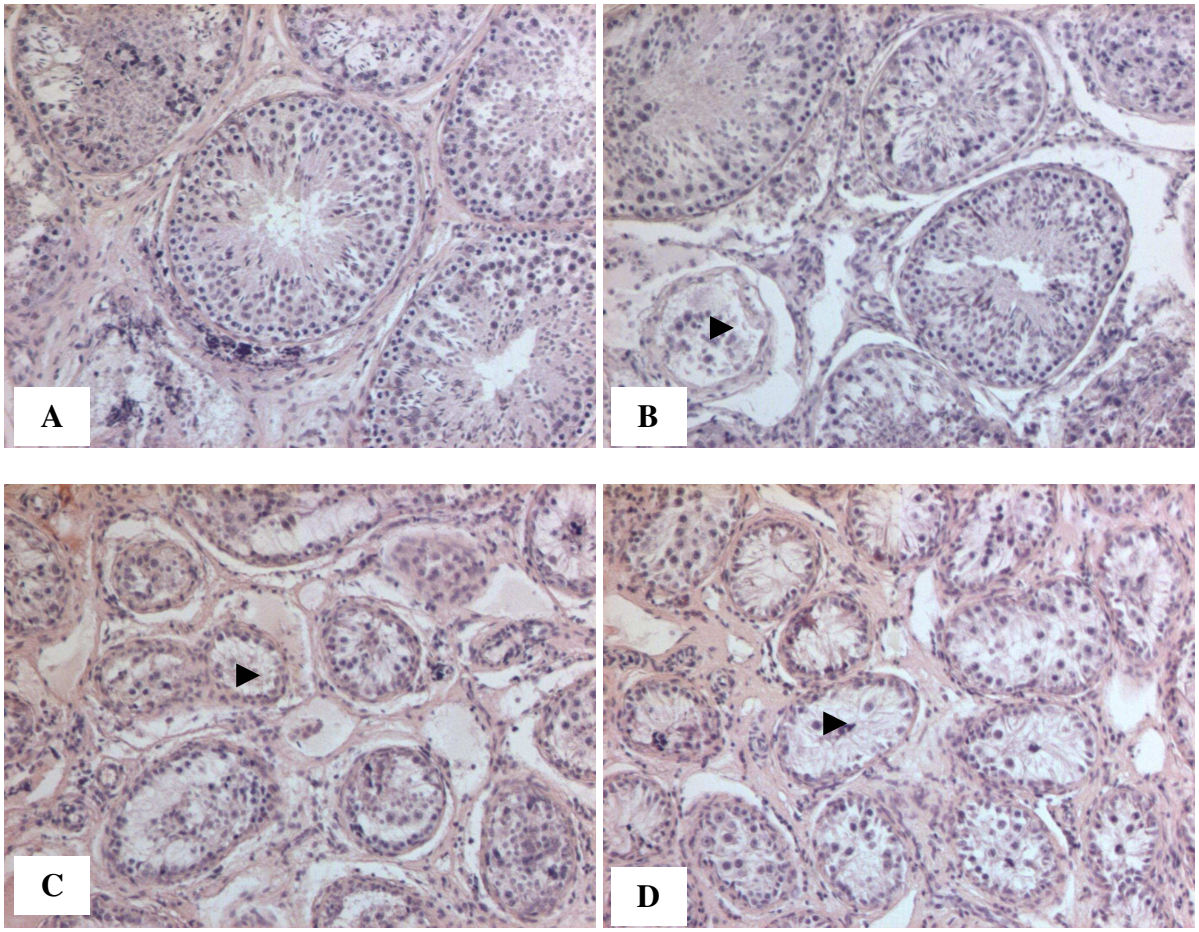


Figure 4. Average testicular diameter of rams receiving different treatments for depletion of endogenous germ cells. Testicular diameter was measured to assess depletion of active spermatogenesis. Data points with an asterisk are significantly different than control at $P \leq 0.05$. The data legend labels are **Control** = non-treated animals, **IR** = animals receiving testicular irradiation, **IMM** = animals actively immunized against LHRH, and **IMM+IR** = animals receiving active immunization against LHRH and testicular irradiation.

Figure 5. Photomicrographs of cross sections of seminiferous tubules from rams subjected to different treatment regimens to deplete endogenous germ cells. **A:** Control ram testis with normal spermatogenesis occurring, **B:** Irradiated ram testis with tubules containing normal seminiferous epithelium and regressed tubules (arrow head), **C:** LHRH immunized ram testis containing germ cell depleted seminiferous tubules (arrow head), and **D:** LHRH immunized+irradiated ram testis containing germ cell depleted seminiferous tubules (arrow head). Bar = 100 μm for each panel.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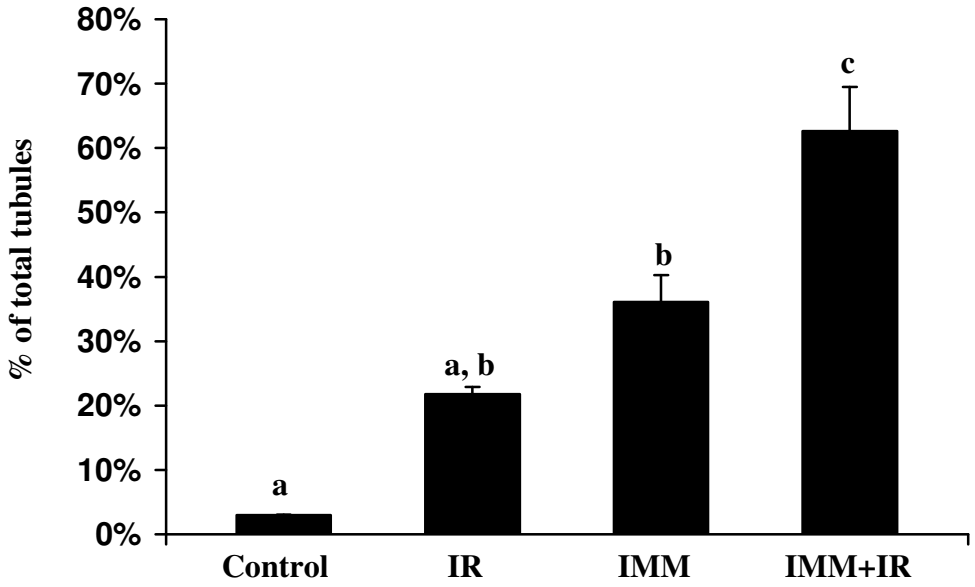
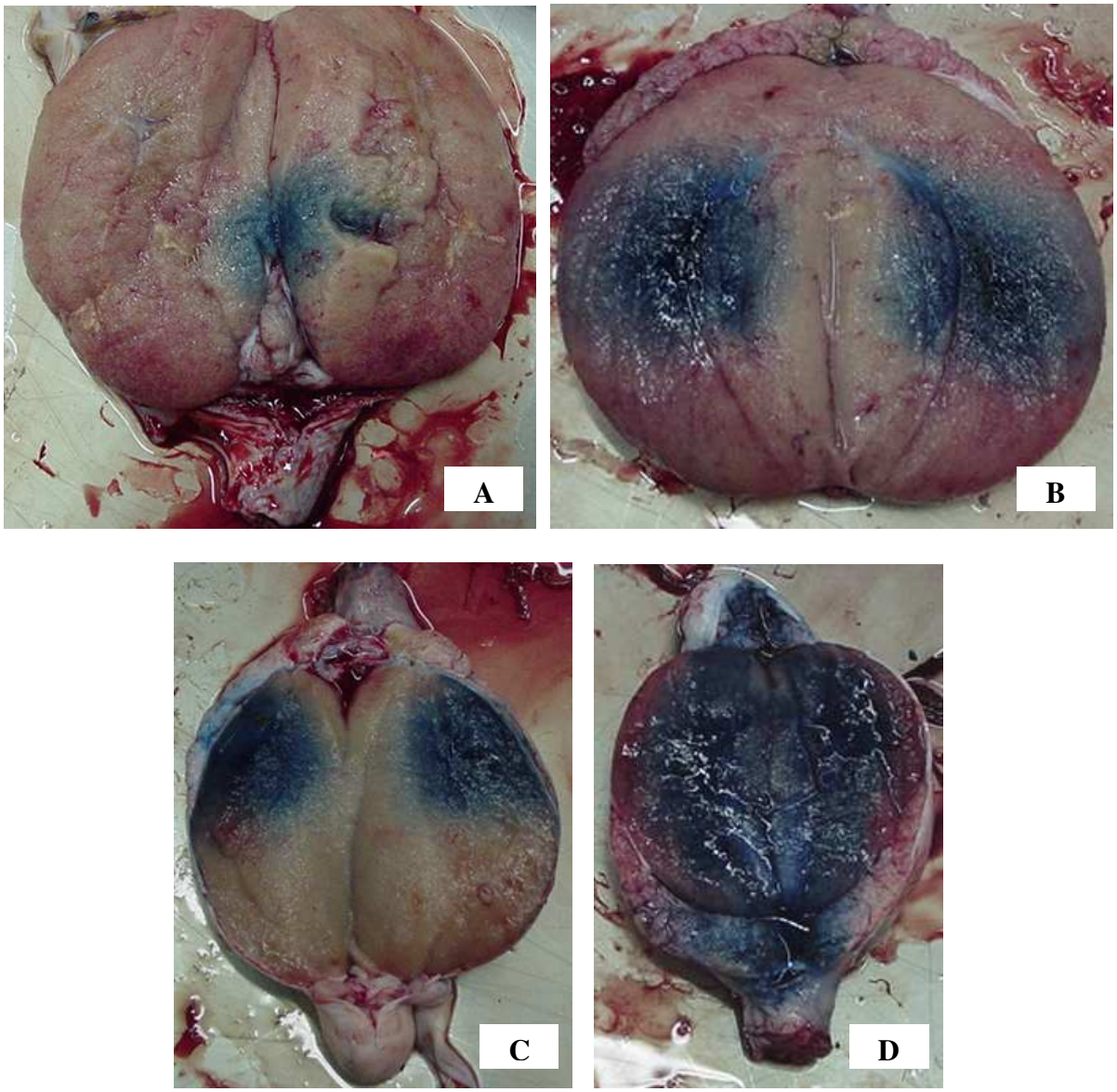


Figure 6. Average percentage of regressed germ cell depleted seminiferous tubules in rams subjected to different treatments for depletion of endogenous germ cells. **Control** = non-treated animals, **IR** = animals receiving testicular irradiation, **IMM** = animals actively immunized against LHRH, and **IMM+IR** = animals receiving active immunization against LHRH and testicular irradiation. Bars with different letters are significantly different at $P \leq 0.05$. Data are presented as the mean$\pm$SEM.

Figure 7. Photos of testes from rams subjected to different treatments to deplete endogenous germ cells following ultrasound-guided injections of trypan blue into the rete testis. **A:** Control ram testis, **B:** Irradiated rams testis, **C:** LHRH immunized ram testis, and **D:** LHRH+irradiated ram testis.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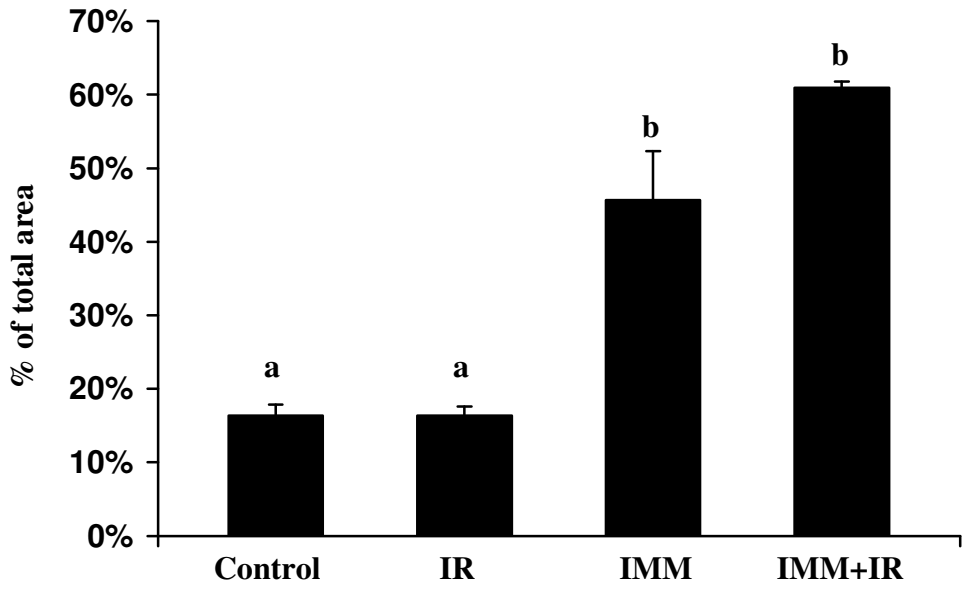

Figure 8. Average percentage of area filled by a ultrasound-guided injection of trypan blue into the rete testis of testes from rams subjected to different treatments for depletion of endogenous germ cells. **Control** = non-treated animals, **IR** = animals receiving testicular irradiation, **IMM** = animals actively immunized against LHRH, and **IMM+IR** = animals receiving active immunization against LHRH and testicular irradiation. Bars with different letters are significantly different at $P \leq 0.05$. Data are presented as the mean$\pm$SEM.

APPENDIX A

Gene expression in early postnatal testis development of the bull*

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Short Title: Gene expression in developing bovine testis

Key Words: Bovine, testis, gene

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* This work is part of an ongoing project that is scheduled to be submitted for publication in the fall of 2004.

Abstract

Postnatal spermatogenesis is a complex process of biological events in which both germ and somatic cells undergo proliferation and maturation resulting in production of spermatozoa at puberty. During this period the framework for future rounds of spermatogenesis are also established ensuring fertility. These biological events are driven by specific and coordinated expression of genes in both somatic and germ cells. The objective of this study was to evaluate the expression profiles of several key genes in the early postnatal testicular development period of 2 to 16 wk of age in the bull calf. Real time RT-PCR was used to evaluate the expression profiles of FSH receptor (FSHR), androgen receptor (AR), glial cell line-derived neurotrophic factor (GDNF) and GDNF family receptor α1 (GFRα1). Evaluation of FSHR expression showed significantly higher expression during the period of 2-12 wk of age with a peak expression at 8 wk. The expression profile for AR showed a steady increase at 2 and 4 wk of age with a peak at 8 wk followed by a significant decline at 12 wk and a sharp rise again at 16 wk of age. The age period of 2 to 8 wk of age showed a significantly elevated expression of GDNF, whereas the expression of its biological partner GFRα1 was significantly elevated during the age period of 2 to 12 wk. The FSHR and AR expression profiles suggest that Sertoli cell proliferation and differentiation occurs during the postnatal period of 2 to 12 wk in the bull calf. The profiles for GDNF and GFRα1 suggest that spermatogonial stem cell niche establishment occurs during the developmental period of 4 to 8 wk of age in the bull testis. Understanding the molecular events that control biological actions in the testis will help in management of bulls for optimal fertility and sperm production.

Introduction

Spermatogenesis is a complex process occurring within the testis which begins with the production of differentiated daughter progeny from diploid spermatogonial stem cells and ends with the maturation of haploid spermatozoa [1]. Postnatal testis development is a period in which the seminiferous epithelium becomes programmed for the first round of spermatogenesis. Several key cell biological events occur in the testis during postnatal development which constitutes the period between birth and puberty. These events are regulated by the expression of key genes by both somatic and germ cells. Postnatal testis development has been well characterized in the rodent and bovine from a cell biology standpoint, but the expression of genes that regulate these biological functions is poorly understood. Understanding the molecular aspects that regulate the biological progression of male germ and somatic cells during postnatal testicular development may one day help treat male infertility.

The first key event in postnatal testicular development involves the migration of gonocytes from the center of seminiferous cords to the basement membrane. Once migrated, gonocytes undergo a conversion to spermatogonial stem cells or differentiated type A spermatogonia [2]. Along with testicular somatic cells, spermatogonial stem cells niches form in which all future differentiated spermatogonia will arise. The differentiated type A spermatogonia then undergo several rounds of proliferation eventually differentiating into type B spermatogonia which are first appearant at 20wk of age in the bovine [3]. These type B spermatogonia then undergo two rounds of meiosis becoming primary and secondary spermatocytes which are appearent at 24 and 28wk of age in the bovine respectively [3]. Haploid spermatids are then formed and undergo the process of spermiogenesis to become mature spermatozoa which are first present at 32wk of age in the bovine [3]. At this point the first round of postnatal spermatogenesis has ended and a new wave of germ cell maturation has already started.

In coordination of germ cell maturation during the first wave of postnatal spermatogenesis, somatic cell proliferation and differentiation also occurs that aids in the initial setup of the seminiferous epithelium. Sertoli cell proliferation during testicular development is essential for postnatal spermatogenesis and establishment of the seminiferous epithelium. This process is dependent on pituitary release of follicle stimulating hormone (FSH) and expression of FSH receptor (FSHR) by Sertoli cells. Germ cell development is dependent on Sertoli cell influence through paracrine and autocrine factors. One factor is Sertoli cell produced glial cell line derived neurotrophic factor (GDNF) which has been implicated in playing an active role in spermatogonial stem cell proliferation and differentiation both in vivo and in vitro [4, 5]. In other physiological systems, GDNF has been demonstrated to act on progenitor cells through binding the receptor dimer of GFRα1 and Ret tyrosine receptor kinase [6-9]. Therefore, it has been theorized that spermatogonial stem cells may express GFRα1 and Sertoli cell GNDF production may play a role in stem cell development in the postnatal testis. Testicular testosterone has been shown to be essential for germ cell maturation both postnatally and in adulthood [10-12]. The main site of action of testosterone in the testis is the Sertoli cell through a nuclear androgen receptor (AR). The early postnatal period of spermatogenesis in the bovine, encompassing the first 16wk of life is poorly understood; however, any disruption in the events of germ cell or somatic cell maturation during this phase can lead to infertility at puberty. It is crucial to gain an understanding of the key events occurring during this period to better prevent and treat conditions of male infertility. The objective of this study was to examine the expression of several key genes in postnatal testicular development in the bovine testis at specific age or development stages from birth to 16wk of age.

Materials and Methods

Testsicular RNA Collection and Real Time RT-PCR analysis

All animal procedures were approved by the Washington State University Animal Care and Use Committee. In order to obtain a representation of the developmental changes in the bovine testis postnatally, 15 Angus bull calves were used at the ages of 2, 4, 8, 12, and 16 (n=3 calves/age). At each age, total cellular RNA was collected from testicular parenchyma using the Trizol method (Invitrogen, Carlsbad, CA). Complimentary DNA for each sample was synthesized by oligo(d)T primed reverse transcription using M-MLV reverse transcriptase (Invitrogen). All samples were then subjected to real time polymerase chain reaction for analysis of the relative gene expression levels of FSHR, AR, GDNF, and GFRα1. The gene expression levels for FSHR, AR, and GDNF were normalized to the Sertoli cell constitutively expressed GATA-4 gene in order to reduce germ cell contamination. Expression levels for GFRα1 were normalized to constitutively expressed ribosomal protein S2 (RPS2) levels. The expression levels for GATA-4 were also normalized to RPS2 to ensure that expression was even throughout all samples from donors of different ages. Primer sequences for all genes were designed from available GenBank sequences using Primer Express 3 (Applied Biosystems, Foster City, CA). Primer sequences were as follows; 5'-CTGCTGCTCATAGCCTCAGTT-3', 5'-AAAAAGCCGGCAGCATCA-3' (FSHR); 5'-AGACGCCCAAGGACATGAAA-3', 5'-AGAGAGGGAAAAAACAGGGTTGA-3' (AR); 5'-CCACCAGCATGTCCAATGAC-3', 5'-GCAGCCGAAACAATGTACGA-3', 5'-AAGGCGATGGGTCTGCAA-3'(GDNF); 5-'GAGCATCCCATAGCTGTGCTT-3' (GFRα1); 5'-GCTCTACGACGAGTACATGTT-3', 5'-ATCGTTGAACTCTCCCATCACA-3'(GATA4); and 5'-GGAGCATCCCTGAAGGATGA-3', 5'-TCCCCGATAGCAACAAACG-3' (RPS2). Reactions for real time analysis were setup at

25µl volumes containing 12.5µl of IQ™ SYBR® Green Supermix (BioRad, Hercules, CA), 1µl of each primer, 1.5µl of template cDNA diluted 1:5, and 9µl of H_2O. Reactions were run in a 96-well plate format on a BioRad I Cycler (BioRad).

Cloning of bovine GDNF

A GenBank submission for the mRNA sequence of bovine GDNF was unavailable. Therefore, in order to obtain a sequence that could be used to design real time primers we cloned a fragment of the mRNA sequence from adult bovine testis. Total cellular RNA was collected from adult bovine testicular parenchyma using the Trizol method (Invitrogen) and reverse transcription with oligo d(T) priming was used to obtain cDNAs. Sequencing primers were designed from a homologous region of the available rat, mouse, and human GDNF sequences. These primers were used in a PCR reaction with bovine cDNA as a template. The product was gel purified (Quiagen gel extraction kit, Quiagen, Valencia, CA) and ligated into pGEMT easy vector (Promega, Madison, WI) for sequencing. The resulting sequence was blasted to confirm homology to rat, mouse, and human GDNF sequences. Sequence data was then used to design real time primers for bovine GDNF.

Histology

Pieces of parenchyma from each donor testis were fixed in Bouin's solution for 4hr at 4°C. Tissues were subsequently dehydrated in 70% ethanol, embedded in paraffin, and sectioned at 6 microns. Sections were stained with hematoxylin to visualize cell nuclei and evaluated using inverted light microscopy. Digital images were captured with a CoolSnap *cf* digital camera (Media Cybernetics; Silver Spring, MD).

Results

Histological Analysis

We evaluated the different aged bovine testis samples histologically to assess the germ cell types present at the specific developmental stages. Postnatally at 2 and 4wk of age the bovine testis contains seminiferous cords with undifferentiated gonocytes as the only germ cell type (Figure 1A and B). At 8 and 12wk of postnatal age undifferentiated type A spermatogonia are present (Figure 1C and D). Primary spermatocytes are first detectable in the bovine testis at week 16 (Figures 1E). Seminiferous tubules diameter slowly increased throughout the age periods indicating Sertoli cell proliferation.

Bovine GDNF Cloning

In order to design real time primers for investigation of the expression of bovine GDNF we chose to clone the cDNA sequence from the adult bovine testis. A 363 base pair product was cloned and sequenced from adult bovine cDNA (Figure 2). The clone had 82% homology to GenBank available mouse (D88264) and rat (NM019139) GDNF sequences and 87% homology to the human (L19063) sequence (Figure 2).

Expression of specific genes during postnatal testicular development

The expression patterns of key genes in the establishment of the seminiferous epithelium were investigated in the developing bovine testis by real time RT-PCR. Specific age or developmental stage points from birth to 16wk of age in the bovine were investigated to provide an encompassing time period of early postnatal development. Each gene will be discussed

individually as to their relative expression profiles. The relative expression levels of GATA-4 from samples of donors from different ages were not significantly different from each other following normalization to RPS2 (data not shown).

FSHR

The expression of FSHR was investigated to provide an evaluation of Sertoli cell proliferation and maturation responsiveness to FSH during early postnatal testicular development. In the developing bovine testis FSHR expression was relatively level during the age periods of 2 and 4wk then peaked at 8wk and slowly declined at the age points of 12 and 16wk (Figure 3). The expression levels at 2, 4, 8, and 12wk of age were significantly higher ($P \leq 0.05$) than 16wk.

AR

Investigation of AR expression was conducted to provide an evaluation of somatic Sertoli cell responsiveness to testosterone in the developing testis. In the developmental period of 2 to 16wk of age in the bovine testis AR expression slowly increased during the first 8wk of age then declined at 12wk and rose again at 16wk of age (Figure 4). Expression significantly increased at 4wk of age and remained significantly elevated at 8wk before decreasing at 12wk.

GDNF & GFRα1

Recent data has indicated that spermatogonial stem cell proliferation and differentiation may be in part controlled by Sertoli cell secreted GDNF. This factor may act through a spermatogonial stem cell expressed GFRα1 receptor. Therefore, we chose to investigate possible

spermatogonial stem cell differentiation from undifferentiated gonocytes and their proliferation in the developing testis by investigating the expression of both GDNF and GFRα1. In the developing bovine testis GDNF expression was significantly higher during early development at 2, 4, and 8wk of age compared to the later developmental ages of 12, and 16wk (Figure 5). The expression of GFRα1 showed a different pattern than GNDF where expression significantly peaked at 4wk of age and slowly declined at the age period of 8-16wk of age (Figure 5B).

Discussion

In this study we investigated the expression profiles of several key genes known to be involved in regulation of spermatogenesis during the early testicular development period of 2-16wk of age in the bovine. Using real time RT-PCR we were able to evaluate relative expression profiles of the Sertoli cell genes FSHR, AR and GDNF. Normalizing the data for these gene expression levels to those of constitutively expressed GATA-4 allowed for evaluation of Sertoli cell specific expression patterns over time. Both FSHR and AR are key genes for Sertoli cell proliferation and differentiation in the developing mammalian testis. Neither of these events have been investigated in the bovine testis during development to any great extent. Investigating the expression profiles of these two genes during early testicular development will increase the understanding of Sertoli cell proliferation and differentiation. Recently, GDNF has been shown to have effects on spermatogonial stem cell biology in vivo [4] and in vitro [5] and has been suggested as a Sertoli cell produced paracrine factor that plays a role in regulation of spermatogonial stem cells [9]. Therefore, we chose to investigate the expression of this gene during early testicular development to provide better insights into spermatogonial stem cell niche development in the testis. In accordance, the expression pattern of GFRα1 was investigated to

provide insights into the putative GDNF spermatogonial stem cell axis during early establishment of spermatogonial stem cell niches in the developing bovine testis.

Evaluation of FSHR gene expression during the developmental period of 2-16wk of age in the bovine showed a slow increase from 2wk until peaking at 8wk of age followed by a slow decline in expression levels. This observation suggests that immature Sertoli cell responsiveness to FSH is highest at 8wk of age which marks a critical period of Sertoli cell establishment for the support of spermatogenesis. This age is also a developmental stage in which the germ cells have yet to undergo complete differentiation to committed spermatogonia. Sertoli cell proliferation and differentiation are essential events that must occur for normal male fertility to commence at puberty. The only cell type in the testis that expresses FSHR are the Sertoli cells, during the phase of proliferation FSHR expression is highest and then down regulated to a basal level upon differentiation. In the rodent, Sertoli cell proliferation is under the influence of FSH [13-16], whereas, the process of Sertoli cell differentiation is relatively unknown. Specific events that occur in the seminiferous epithelium that coincide with Sertoli cell maturation have been identified. One of these is the appearance of meiotic germ cells occurring at day10 in the mouse [1] at which time the Sertoli cells form tight junctions, a key indicator of differentiation. This event is not a dependent factor of Sertoli cell differentiation since tight junctions will form without the appearance of meiotic germ cells [17, 18]. In the bovine, Sertoli cell proliferation and differentiation is an understudied process and the little that is known is based on similar events in rodents. There is available data to suggest that Sertoli cell proliferation occurs from birth until 20wk of age [8]. The concentration of FSH in the serum remains relatively level from 4 to 28wk of age in the bull [19], suggesting that Sertoli cells regulate their differentiation autonomously. Understanding the expression profile of FSHR during early development in the

bovine testis aids in elucidate male infertility caused by disruption of this expression pattern by environmental or chemical inhibitors.

Expression of AR in the early developing bovine testis showed a relative increase from 2 to 8wk of age; however, at 12wk of age the expression level dropped only to come back up again at 16wk of age. The expression of AR in the testis is localized to Sertoli cells, Leydig cells, and peritubular cells [20]. Testosterone has been shown to be essential for spermatogenesis and male fertility [10-12]. In AR knockout animals germ cell maturation is suppressed [10], suggesting that androgen influence on Sertoli cells is essential for normal spermatogenesis. Evidence in *hpg* mutant mice suggests that testosterone plays an aiding role in Sertoli cell proliferation and differentiation in the developing testis [21]. The observation that peak FSHR expression occurs at 8wk of age suggest that Sertoli cell responsiveness to FSH for proliferation is maximal at this age. If this is the case then AR expression by Sertoli cells to aid in proliferation and maturation would also peak at 8wk of age, which was observed in this study. At 12wk of age AR expression dramatically decreased suggesting that Sertoli cell need for response to testosterone had passed. At 16wk of age expression increased again which could be a result of the need for Sertoli cell responsiveness to testosterone for support of germ cell development. This theory is also supported by the first appearance of differentiating spermatogonia between 12 and 16wk of age in the bovine [3].

Evaluation of the putative GDNF/GFRα1 axis in the early developing bovine testis revealed distinct expression patterns. The expression of GDNF remained at a steady level from 2 to 8wk of age and dramatically decreased at 12 through 16wk of age. The functional role of GDNF on spermatogonial stem cell maintenance remains debatable. Its expression by Sertoli cells in the seminiferous epithelium has been demonstrated [9] and the theory of a paracrine loop

between Sertoli and spermatogonial stem cells has begun to be proposed. Enhancement or stimulation of spermatogonial stem cell self-renewing proliferation could be one method of GDNF action. If spermatogonial stem cell self-renewing proliferation occurs autonomously then GDNF may exert its actions through inhibition of differentiation. Nagano et al. [5] proposed that GDNF inhibited spermatogonial stem cell differentiation in vitro thus increasing the overall number of surviving cells. Meng et al [4] demonstrated the accumulation of undifferentiated spermatogonia in transgenic mice over expressing recombinant GDNF. Whether this accumulation occurred due to stimulation of spermatogonial stem cell proliferation or inhibition of differentiation could not be elucidated. Establishment of the stem cell niche in the testis could require an increase in the expression of GDNF for stimulation of gonocyte to stem cell conversion and proliferation. It has been demonstrated that gonocyte to stem cell conversion and niche assembly occurs at days 3 or 4 in the mouse testis [22, 23], just prior to the appearance of differentiating spermatogonia at days 5 or 6. If the same theory holds true in the bovine then spermatogonial stem cell conversion and niche assembly would occur from birth until just prior to the appearance of differentiating spermatogonia at 12wk of age [8]. Data in this study leads to the hypothesis that elevated GDNF expression from 2 to 8wk of age in the bovine testis aids in spermatogonial stem cell conversion and niche formation which is complete by 12wk of age.

In this study GFRα1 expression peaked at 4wk of age in the bovine testis and slowly declined to 16wk of age. In neuronal systems GDNF has been shown to act on progenitor cells through binding the receptor dimer Ret tyrosine kinase and GFRα1 [6-8]. It has also been demonstrated that a subpopulation of undifferentiated spermatogonia in the mouse testis express Ret and GFRα1 [9]. If GDNF functions in the conversion of gonocytes to spermatogonial stem cells and establishment of niches then peak GFRα1 expression in the developing testis would be

expected to preclude or mimic that of GDNF. In this study that relationship was seen where GFRα1 expression was highest during the same period of peak GDNF expression at 2 to 8wk of age. It can be hypothesized that germ cell GFRα1 expression and Sertoli cell GDNF expression are necessary for conversion of gonocytes to stem cells and niche establishment in the early developing bovine testis that occurs from 2 to 8wk of postnatal life.

In this study the expression profiles of several genes known to be essential for spermatogenesis were investigated during early postnatal development in the bovine testis. A key event in testicular development and establishment of postnatal spermatogenesis is the proliferation and differentiation of Sertoli cells. Very little of this process is understood in the developing bovine testis. Here we show the profile of FSHR and AR expression during the phase of these events. Likewise, gonocyte to stem cell conversion and establishment of stem cell niches are essential for fertility at puberty. In this study we investigated the putative GDNF/GFRα1 axis existing between Sertoli cells and spermatogonial stem cells in terms of gene expression profiles during early testicular development. Investigating the molecular events in the testis that govern observed biological phenomenon can aid the understanding of factors necessary for fertility in males. In bulls this can lead to prevention of infertile or sub-fertile conditions at puberty and enhancement of fertility in genetically superior animals.

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Figure 1. Photomicrographs of cross sections of bovine seminiferous tubules at different stage of early postnatal testicular development. A: 2wk of age; B: 4wk of age; C: 8wk of age; D: 12wk of age; and E: 16wk of age. Bar = 100µm.

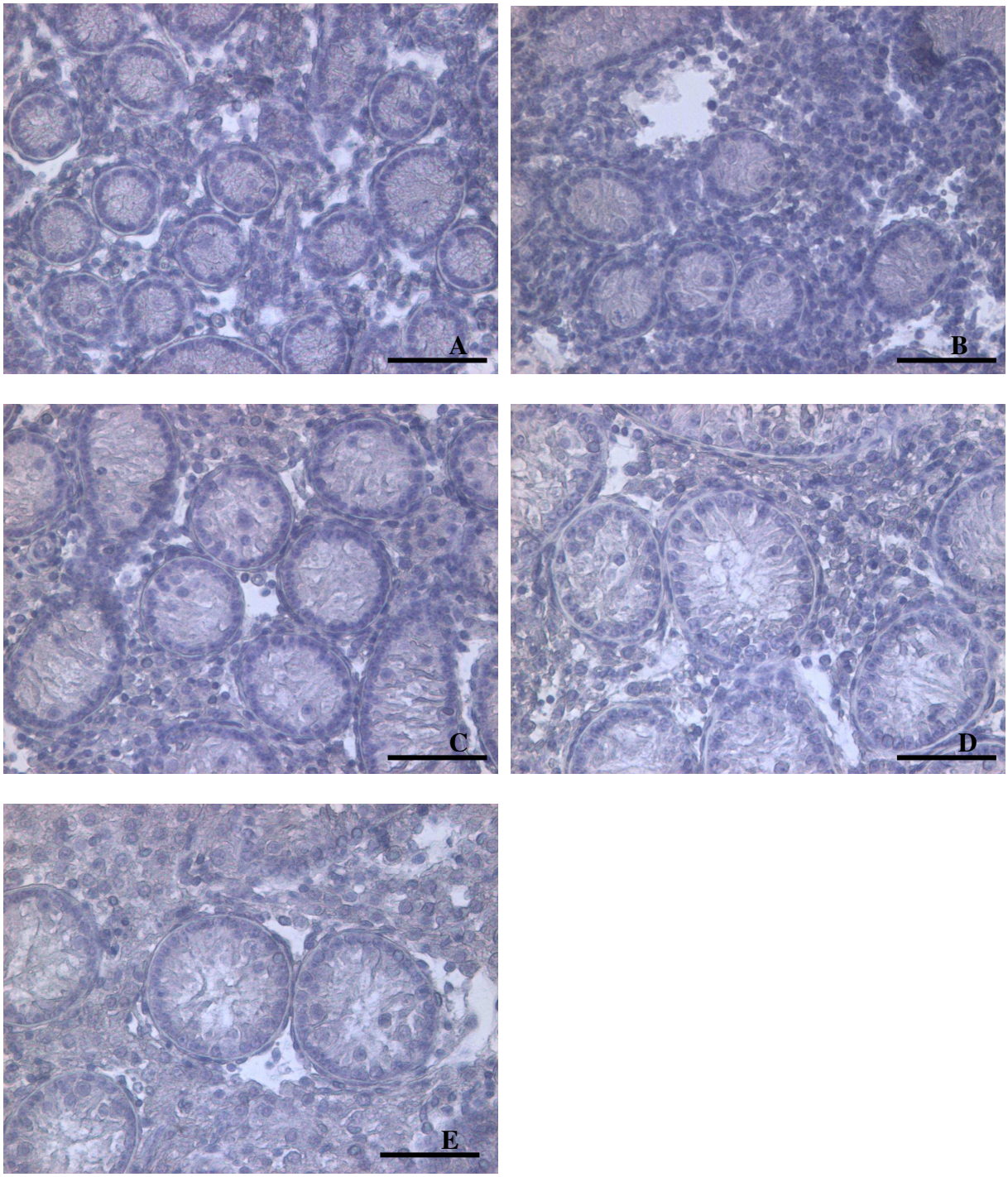
A
B
C
D
E

Figure 2. Base sequence and homology to other species of a cloned fragment of the bovine GDNF testis GDNF mRNA.

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Mouse 388  TCCTCGAAGAGAGAGGAATCGGCAGGCTGCAGCTGCCAGCCCAGAGAATT
Rat   235  TCCTCGAAGAGAGAGGAACCGGCAAGCTGCAGCTGCCAGCCCAGAGAATT
Human 257  TCCTAGAAGAGAGCGGAATCGGCAGGCTGCAGCTGCCAACCCAGAGAATT
Bovine 1   TCCTCGGAGAGAGCGGCACCGGCAGGGGGCGGCCGCCAGCCCGGAGAGTG

Mouse      CCAGAGGGAAAGGTCGCAGAGGCCAGAGGGGCAAAAATCGGGGGTGCGTT
Rat        CCAGAGGGAAAGGTCGCAGAGGCCAGAGGGGCAAAAATCGGGGGTGCGTC
Human      CCAGAGGAAAAGGTCGGAGAGGCCAGAGGGGCAAAACCGGGGTTGTGTC
Bovine     CCAGAGGGAAAGGCCGGCGCGGCCAGAGGGGCCGAAATCGCGGCTGCGTC

Mouse      TTAACTGCCATACACTTAAATGTCACTGACTTGGGTTTGGGCTATGAAAC
Rat        TTAACTGCAATACACTTAAATGTCACTGACTTGGGTTTGGGCTACGAAAC
Huma       TTAACTGCAATACATTTAAATGTCACTGACTTGGGTCTGGGCTATGAAAC
Bovine     CTCACCGCCGTGCATCTAAACGTCACTGACTTGGGTTTGGGCTACGAAAC

Mouse      CAAGGAGGAACTGATCTTTCGATATTGCAGCGGTTCCTGTGAATCGGCCG
Rat        CAAGGAGGAACTGATCTTTCGATATTGTAGCGGTTCCTGTGAAGCGGCCG
Human      CAAGGAGGAACTGATTTTTAGGTACTGCAGCGGCTCTTGCGATGCAGCTG
Bovine     CAAGGAGGAACTCATTTTCAGGTACTGCAGCGGCTCCTGCGATGCAGCCG

Mouse      AGACAATGTATGACAAAATACTAAAAACCTGTCTCGGAGTAGAAGGCTA
Rat        AGACAATGTACGACAAAATACTAAAAATCTGTCTCGAAGTAGAAGGCTA
Human      AGACAACGTACGACAAAATATTGAAAACTTATCCAGAAATAGAAGGCTG
Bovine     AAACAATGTACGACAAAATATTAAAAACTTATCCAAAAGTAGAAGGCTG

Mouse      ACAAGTGACAAAGTAGGCCAGGCATGTTGCAGGCCGGTCGCCTTCGACGA
Rat        ACAAGTGACAAGGTAGGCCAGGCATGTTGCAGGCCGGTCGCCTTCGACGA
Human      GTGAGTGACAAAGTAGGGCAGGCATGTTGCAGACCCATCGCCTTTGATGA
Bovine     GTGAGTGACAAAGTCGGGCAGGCGTGTTGCAGACCCATCGCCTTTGATGA

Mouse      CGACCTGTCGTTTTTAGATGACAACCTGGTTTACCATATTCTAAGAAAGC
Rat        CGACCTGTCGTTTTTAGACGACAGCCTGGTTTACCATATCCTAAGAAAGC
Human      TGACCTGTCGTTTTTAGATGATAACCTGGTTTACCATATTCTAAGAAAGC
bovine     CGACCTGTCCTTTTTAGATGATAACCTGGTTTACCATATTCTAAGAAAGC

Mouse 737  ATTCCGCTAAACGG
Rat   584  ATTCCGCTAAACGG
Human 606  ATTCCGCTAAAAGG
Bovine 350 ATTCCGCTAAACGG
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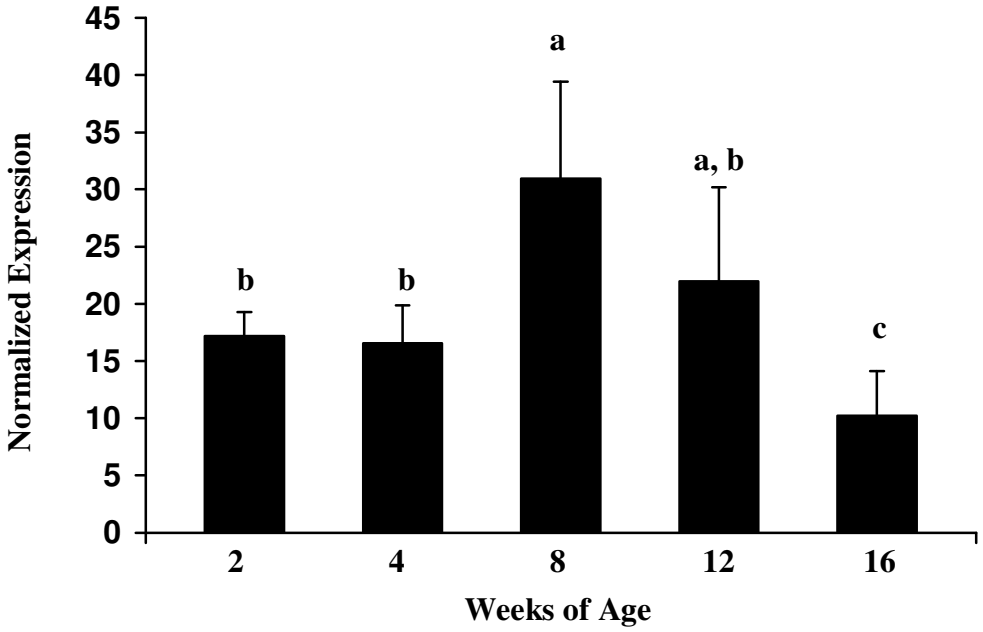


Figure 3. Expression of FSHR in the testis during the early postnatal period of 2 to 16wk of age in the bovine. Data were normalized to GATA-4 expression to evaluate Sertoli cell specific expression.

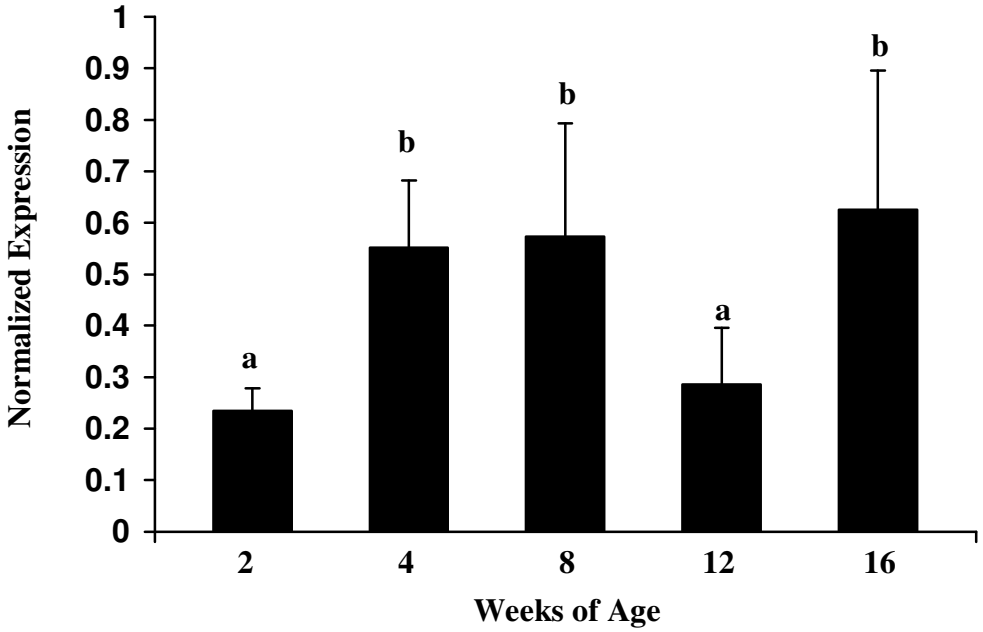
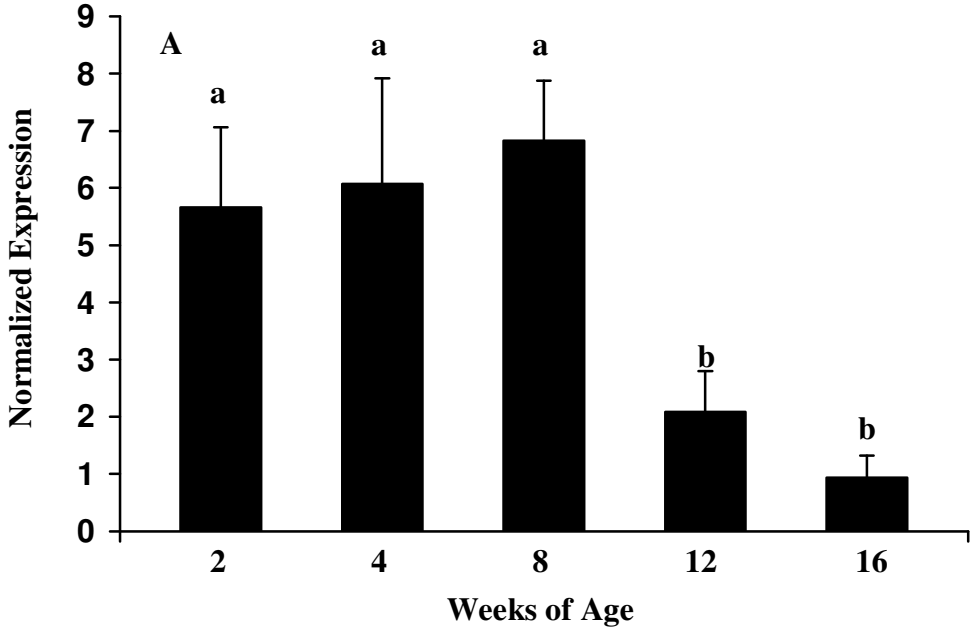
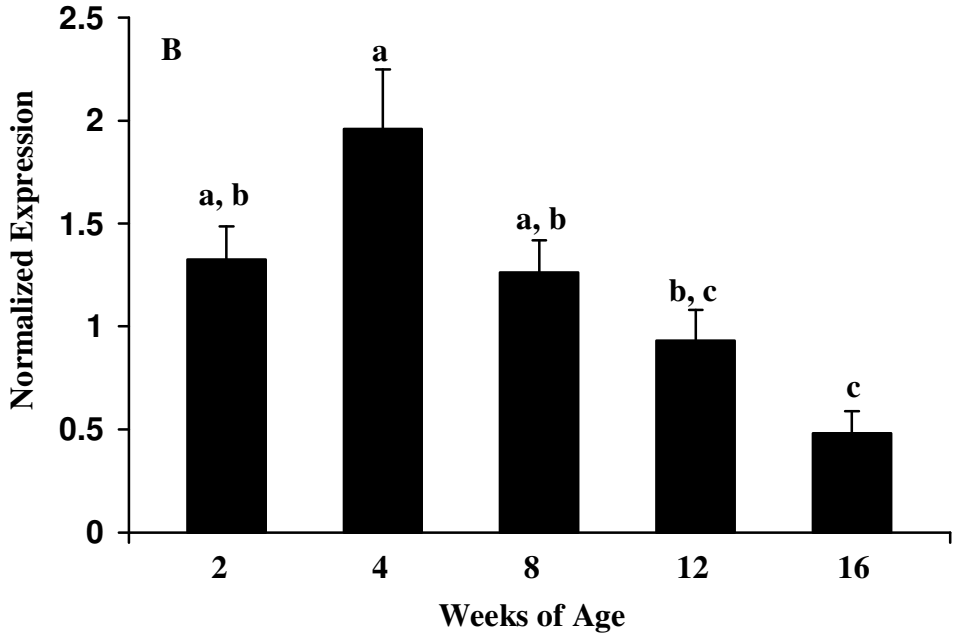


Figure 4. Expression of AR in the testis during the early postnatal period of 2 to 16wk of age in the bovine. Data were normalized to GATA-4 expression to evaluate Sertoli cell specific expression. Bars with different letters are significantly different at $P \leq 0.05$.

Figure 5. Expression of profile of mRNA for GDNF (A) and GFRα1 (B) in the testis during the early postnatal development period of 2 to 16wk of age in the bovine. Expression levels for GDNF were normalized to GATA-4 to evaluate Sertoli cell specific expression. Levels of expression for GFRa1 were normalized to ribosomal protein S2 in order to evaluate germ cell expression.


A
a
a
a
b
b
Normalized Expression
0
1
2
3
4
5
6
7
8
9
2
4
8
12
16
Weeks of Age


B
a, b
a
a, b
b, c
c
Normalized Expression
0
0.5
1
1.5
2
2.5
2
4
8
12
16
Weeks of Age

APPENDIX B

Active immunization against LHRH results in loss of spermatogonial stem cell biological activity in the murine testis*

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Short Title: Spermatogonial stem cell biological activity

Key Words: spermatogonial stem cell, LHRH, transplantation

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* This work is part of an ongoing project that will be presented as an abstract at the 2004 Society for the Study of Reproduction national meeting.

Abstract

Spermatogonial stem cells play an integral role in sustained sperm production through self-renewing proliferation and differentiation. Proper function of the hypophyseal-pituitary-gonadal (HPG) axis to supply gonadotropins to somatic cells of the testis is essential for spermatogenesis in mammals. The impact of this axis on spermatogonial stem cell biology is poorly understood. The objective of this study was to evaluate spermatogonial stem cell biological activity in mice with a disrupted HPG axis using active immunization against LHRH and spermatogonial stem cell transplantation. Donor Rosa26 mice were immunized against LHRH (n=7) using a fusion protein cocktail. The immunization regimen consisted of a primary immunization followed by two booster injections. One month after the last booster, germ cell populations were colleted by enzymatic digestion and transplanted into busulfan treated recipient mice (n=3 recipients/donor). Donor Rosa26 mice of similar age not immunized against LHRH were used as controls (n=3). Recipient mice were sacrificed six weeks after transplantation and the testes stained with X-Gal for detection of donor derived spermatogenesis to assess spermatogonial stem cell biology. Donor testis weight significantly decreased 2.3-fold following LHRH immunization compared to controls. Likewise, testosterone concentration in LHRH immunized donor serum decreased 12-fold compared to controls. Colonization of LHRH immunized donor spermatogonial stem cells could not be detected in recipient seminiferous tubules. On the other hand, control donor spermatogonial stem cell colonization was observed. These data support the conclusion that alteration of the HPG axis by active immunization against LHRH results in loss of spermatogonial stem cell biological activity in the adult testis.

Introduction

Spermatogenesis is the complex process by which a diploid spermatogonial stem cell produces differentiating spermatogonia that have the ability to undergo proliferation and differentiation to eventually become mature spermatozoa [1]. The key element to continuation of this process throughout the life time of the male is the dual actions of the spermatogonial stem cells. These cells have the unique abilities for both self-replicating mitosis and production of daughter progeny known as differentiating spermatogonia which are committed to the spermatogenic pathway [2]. It is essential that spermatogonial stem cells maintain these actions for fertility to be sustained in males. Despite their critical importance very little is known about the actual regulatory biology of spermatogonial stem cells. It is largely unknown whether spermatogonial stem cells regulate their own cell fate decision of self-replication or differentiation, or whether other factors such as the stem cell niche microenvironment have an influence.

A major contributor to the spermatogonial stem cell niche may be the Sertoli cell, the somatic cell of the seminiferous epithelium [3-5]. Sertoli cell function is regulated by pituitary released follicle stimulating hormone (FSH) and testosterone produced by Leydig cells under the influence of pituitary released luteinizing hormone (LH). The production and release of FSH and LH from the anterior pituitary is regulated by hypothalamic luteinizing hormone releasing hormone (LHRH). Thus, a functional hypophyseal-pituitary-gonadal (HPG) axis is essential for testis function and production of spermatozoa [6]. However, the effects of this axis on spermatogonial stem cell biology is unknown.

Spermatogonial stem cell transplantation is a technique in which donor germ cells are microinjected into the seminiferous tubules of a recipient mouse testis where they form colonies

of active spermatogenesis [7, 8]. This system is a functional assay that can be used to study the biology of spermatogonial stem cells. Using this assay, spermatogonial stem cell colonization has been shown to be enhanced in a low gonadotropin recipient environment [9, 10], indicating that gonadotropins or testosterone may have an impact on the spermatogonial stem cell niche microenvironment. One method for disruption of the HPG axis is through active immunization against LHRH [11, 12], thus blocking LHRH from reaching the pituitary and eliciting a release of FSH and LH.

Hypogonadotropic hypogonadism is a condition represented by dysfunction of the HPG axis resulting in low to undetectable concentrations of gonadotropins and testosterone causing severe to moderate oligospermia [13, 14]. Depending on the time point of development and how severely this condition represents to an individual, hormone replacement therapy may not remedy the disorder. Understanding the effects of a disrupted HPG axis on spermatogonial stem cell biological activity is important for understanding male fertility and designing treatment regimens for individuals with hypogonadotropic hypogonadism. The objective of this study was to evaluate the biological activity of spermatogonial stem cells in adult mice with a disrupted HPG axis using active immunization against LHRH and spermatogonial stem cell transplantation.

Materials and Methods

Donor Mice, active immunization against LHRH, and transplantation

Adult ROSA26 mice on a C57/B6 background (B6-Gtrosa26, Jackson Laboratory, Bar Harbor, ME) which express *LacZ* in all postnatal germ cell types were used as donors. All animal procedures were approved by the Washington State University Animal Care and Use Committee.

Donor ROSA26 mice (n=7) were actively immunized against LHRH with a fusion protein cocktail [12]. The immunization regimen consisted of a primary immunization in Freund's complete adjuvant followed by two booster injections in incomplete Freund's adjuvant one month apart. Adult donor ROSA26 (n=3) mice not receiving any immunization were used as controls. One month after the final booster donor mice were killed by CO_2 inhalation and blood was collected by cardiac puncture. With all donors both testes were removed and one testis was weighed and fixed in Bouin's solution for subsequent histological evaluation. Total germ cell populations were collected from the other testis by two-step enzymatic digestion as previously described [15] and suspended at a concentration of $1X10^6$ cells/ml in injection medium (DMEM with 0.004% trypan blue). Approximately 10µl of donor germ cell suspension was then microinjected into the seminiferous tubules of recipient C57 adult male mice (n=3 mice/donor) treated with busulfan 4wk prior to deplete endogenous germ cells as previously described [15]. With all recipients one testis was injected with donor germ cells while the contralateral testis was left as a non-injected control.

Donor mouse and recipient testis colonization analyses

Donor testes fixed in Boiun's solution were processed histologically for evaluation of spermatogenesis within seminiferous tubules using light microscopy. The average percentage of total regressed seminiferous tubules without active spermatogenesis was determined by dividing the number of regressed tubules in 3 microscopic fields by the total number of tubules within the same 3 fields. To assess effectiveness of the vaccine in inhibiting gonadotropin release, donor mouse serum was evaluated for testosterone concentration by RIA using a commercial kit (DSL-400; Diagnostic System Laboratories, Webster, TX).

Approximately six weeks after transplantation recipient mice were killed by CO_2 inhalation followed by cervical dislocation. The testes were removed, detunicated and fixed in 4% paraformaldehyde for 1hr at 4^{o}C. Testes were then washed twice in LacZ rinse buffer (0.2M sodium phosphate, 2mM magnesium chloride, 0.02% NP40, and 0.01% sodium deoxycholate) followed by incubation in LacZ staining solution (10mM potassium ferricyanide, 10mM potassium ferrocynaide, 2 mg/ml X-Gal, suspended in LacZ rinse buffer) overnight at 37^{o}C. The next day stained testes were destained twice with LacZ rinse buffer followed rinses in 50% and 70% ethanol and finally stored in 70% ethanol at room temperature. Testes were then evaluated for donor colonization using a dissecting microscope. The number of donor blue stained colonies were manually counted and digital images were captured with a CoolSnap *cf* digital camera (Media Cybernetics; Silver Spring, MD).

Statistical analysis

All data were analyzed using the SAS system software with the Proc GLM function. Differences between means were determined using Duncan's test for significance for average testis weight, serum testosterone concentration, and seminiferous tubules with regressed spermatogenesis. Data were considered significantly different at $P \leq 0.05$. In all figures data are presented as the mean$\pm$SEM.

Results

Donor testis weight and serum testosterone concentration

The testicular weight of each ROSA26 donor was collected to provide an evaluation of reduced spermatogenesis due to active immunization against LHRH. Donor mice immunized

against LHRH had significantly reduced testicular weight compared to control donors (Figure 1A and B). A 2.5-fold reduction in testicular weight was seen in immunized donors compared to controls.

Testosterone concentration in donor serum was measured to evaluate the degree of reduced gonadotropin production by the pituitary in response to immunization against LHRH. Immunized donor serum had significantly lower concentrations of testosterone than control donors (Figure 1C). An 11.9-fold reduction in testosterone concentration was seen in immunized donor mice compared to controls.

Donor testis histological analysis

To investigate the effects of active immunization against LHRH on spermatogenesis donor testes were processed for histological evaluation. The degree of regressed spermatogenesis was variable by seminiferous tubule and individual donor mouse, ranging from complete with a degenerative appearance (Figure 2A) to non-regressed with complete germ cell differentiation (Figure 2B). Control donor testes contained seminiferous tubules with full germ cell differentiation (Figure 2C). The percentage of total tubules with regressed spermatogenesis was variable by immunized donor but the average was significantly higher than controls which contained virtually no regressed seminiferous tubules (Figure 3).

Spermatogonial stem cell colonization analysis

In order to asses the biological activity of spermatogonial stem cells from the testes of immunized donor mice, germ cells were transplanted into the seminiferous tubules of busulfan treated recipient mice. Six weeks after transplantation recipient mice were sacrificed and the

testes analyzed for donor stem cell colonization by X-Gal staining. Recipient testes that were transplanted with control ROSA26 donor germ cells contained expanded donor colonies (Figure 3A), with an average of 2.6 colonies per testis. In contrast colonization of donor spermatogonial stem cells could not be detected in any seminiferous tubules of recipient testes transplanted with immunized donor germ cells (Figure 3B).

Discussion

Stem cells are defined solely by their functional abilities for regeneration. In the testis the spermatogonial stem cell is defined by its functional role of regenerating spermatogenesis. In order to study adult stem cells a regenerative assay must be utilized. Spermatogonial stem cell transplantation is one such assay that can be used to study male germ line stem cells.

A normal functioning HPG axis is essential for male fertility to provide gonadotropins to the somatic cells of the testis to support their functions in the sperm production process. Disruption of the HPG axis leads to a condition of hypogonadotropic hypogonadism with associated infertility. In many instances this condition can be treated with administration of exogenous hormones resulting in regained fertility; however, this treatment is not always 100% effective [13]. Investigating the influence of the HPG axis on spermatogonial stem cell biology can lead to a better understanding of the stem cell niche and regulatory factors of male fertility. In this study we evaluated the effects of disrupting the HPG axis in the adult animal on spermatogonial stem cell biology. Disruption of the HPG axis was achieved with active immunization against LHRH and germ cell transplantation was used as an assay to evaluate spermatogonial stem cell biology. Data collected in this study leads to the conclusion that loss of a functional HPG axis in the adult results in loss of stem cell biological activity.

Immunization of adult animals resulted in a 2.5-fold reduction in testicular weight compared to controls. This decrease in weight represents a loss in sperm production by the testis due to lack of gonadotropin influence for testosterone production and nurse function of Sertoli cells. Loss of gonadotropin influence for Leydig cell testosterone production was also evidenced by the 11.9-fold decrease in circulating testosterone concentration in immunized animals. In reported conditions of hypogonadotropism, loss of gonadotropin production by the pituitary results in decreased circulating testosterone concentration and infertility in pubertal patients which can usually be overcome with administration of exogenous hormones [13]. In cases of younger prepubertal males hypogonadotropism causes loss of Sertoli cell proliferation and establishment of a normal seminiferous epithelium [13]. Therefore, treatment with exogenous hormones does not usually result in complete reversal of the condition. Understanding the effects of gonadotropins on spermatogonial stem cell biology is essential for helping overcome these disorders. In this study a condition of infertility was created in adult animals in which over 50% of seminiferous tubules were regressed without active spermatogenesis occurring. This immunization against LHRH is functional as a hypogonadotropic model.

Regulation of spermatogonial stem cells in the testis is largely unknown and hard to investigate since self-renewal and production of differentiated daughter progeny occurs at the same time. Studies in invertebrates suggest the stem cell fate decision is strictly regulated by autonomous self-renewal by the stem cell and influence by it's microenvironment or niche [5, 16]. In mammals the Sertoli cell has been suggested as a key factor in the establishment and maintenance of the stem cell niche in the testis [3-5]. The exact influence that Sertoli cells have on spermatogonial stem cell biology is not understood. However, Sertoli cell action in the testis is regulated by both pituitary FSH and Leydig cell produced testosterone [17, 18]. Loss of the

production of both FSH and testosterone could have profound effects on the ability of Sertoli cells to maintain spermatogonial stem cell niches. Lack of spermatogonial stem cell colonization following transplantation of germ cells from immunized donor animals suggests that spermatogonial stem cell biology has been lost in these testes. Whether this observation was due to spermatogonial stem cell apoptosis cannot be undisputedly concluded from this study. Another possible cause of this observation is that the cell cycle kinetics of spermatogonial stem cells in immunized donor testes were pushed into a state of permanent quiescence and could not proliferate and establish spermatogenesis following transplantation. These observations suggest that gonadotropins have an important influence on spermatogonial stem cell maintenance in the adult testis. Also, long-term administration of anti-LHRH compounds proposed as male contraception's should be critically examined for reversibility.

Previous studies in which recipient animals were treated with LHRH analogs to reduce testosterone concentration prior to transplantation showed enhancement of spermatogonial stem cell colonization [9, 10]. These observations suggest that a low testosterone concentration enhances stem cell niche accessibility for donor spermatogonial stem cells following transplantation. This accessibility could be due to loss of spermatogonial stem cells in recipient testes due to apoptosis or alteration of spermatogonial stem cell proliferation kinetics. Observations from these studies support our conclusions from the current study that disruption of the HPG axis in adult males results in loss of spermatogonial stem cell biological activity. Understanding the regulatory functions of gonadotorpins on regulation of spermatogonial stem cell niche microenvironments could increase the understanding of spermatogenesis and male fertility.

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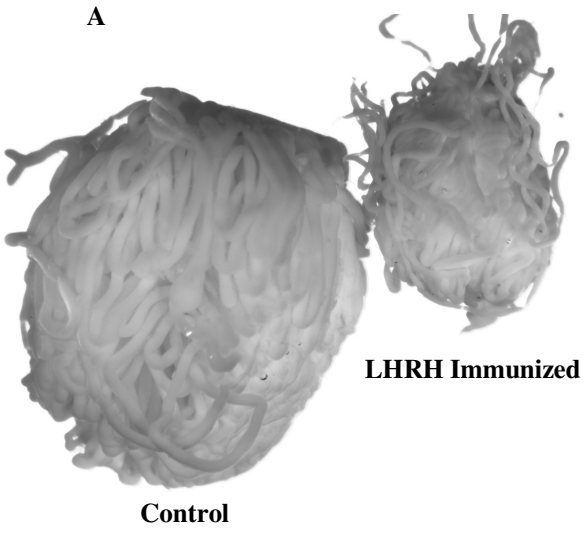
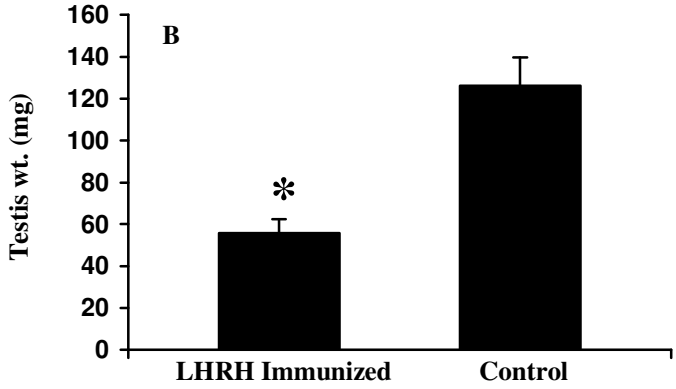
Figure 1. Testis size and testosterone concentration of Rosa26 mice immunized against LHRH. **A:** Photo of a testis from control mouse (left) and immunized mouse (right). **B:** Average testis weight (mg) of LHRH immunized and non-treated control mice. **C:** Average serum testosterone concentration in LHRH immunized and non-treated control mice. Data are presented as the mean$\pm$SEM. Bars with different letters are significantly different at $P \leq 0.05$.


A
LHRH Immunized
Control


B
Testis wt. (mg)
160
140
120
100
80
60
40
20
0
*
LHRH Immunized
Control

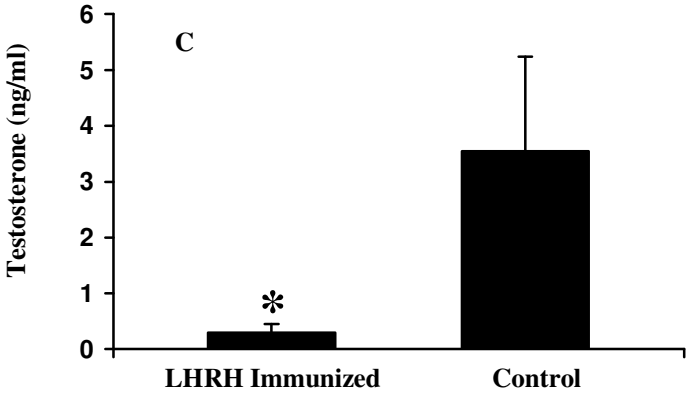
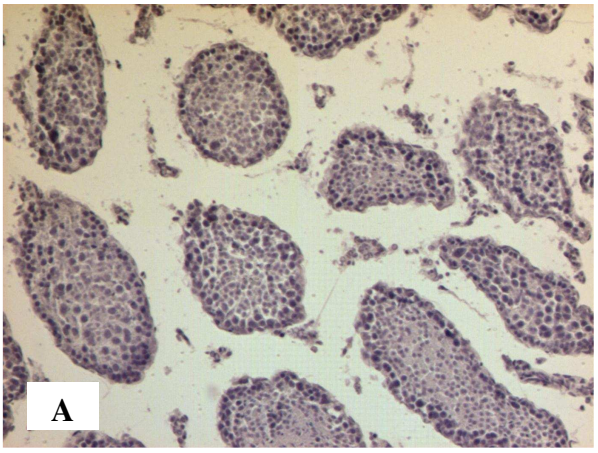
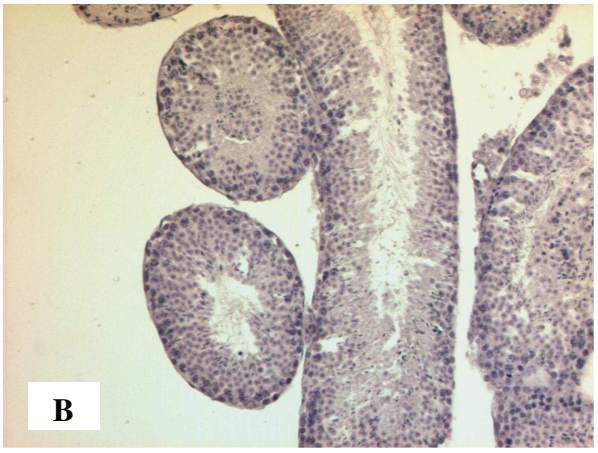

C
Testosterone (ng/ml)
6
5
4
3
2
1
0
*
LHRH Immunized
Control

Figure 2. Photomicrographs of seminiferous tubules from LHRH immunized (A and B) and non-treated control donor Rosa26 testes (C). Seminiferous tubules in LHRH immunized donor testes ranged from completely regressed (A) without any germ cell differentiation occurring to complete germ cell differentiation. Control donor testes contained seminiferous tubules with normal spermatogenesis occurring.


A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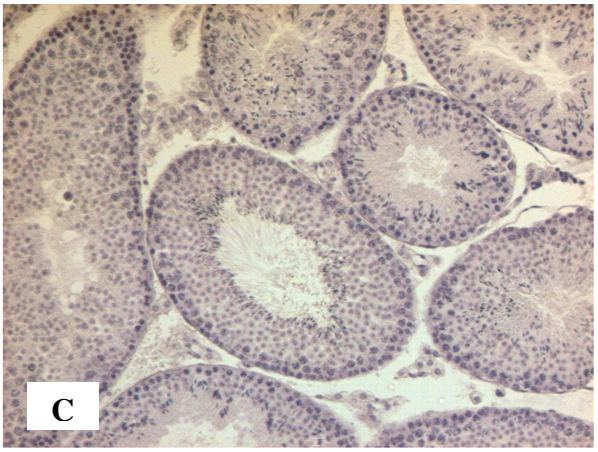
C

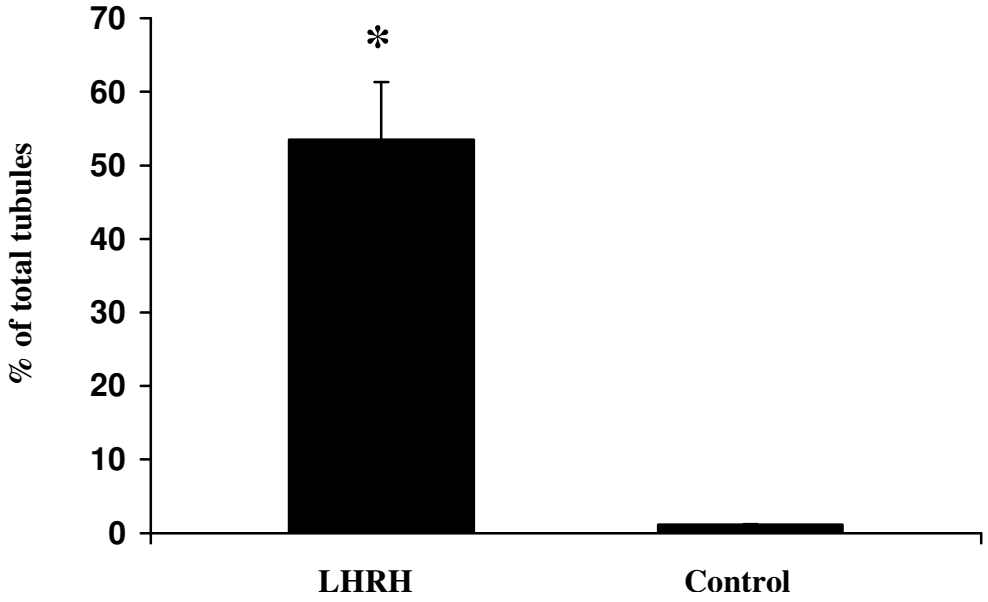

Figure 3**:** Average percentage of total seminiferous tubules with regressed spermatogenesis in LHRH immunized and non-treated control donor Rosa26 testes. Data are presented as the mean$\pm$SEM. Asterisk means significantly different than control at $P \leq 0.05$.

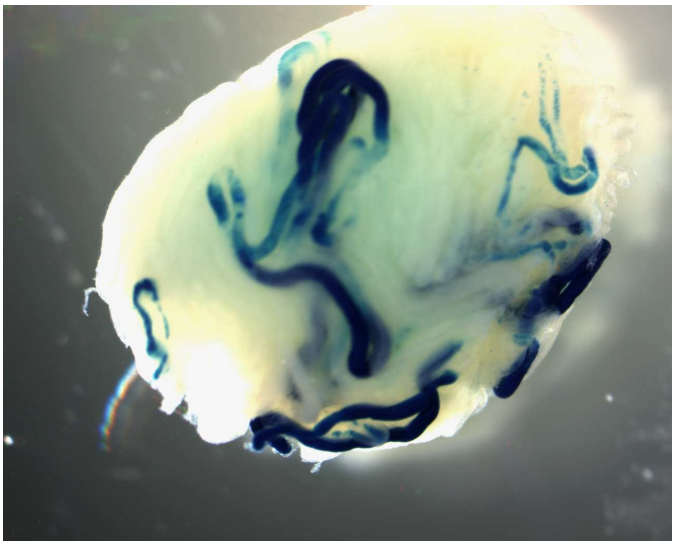

Figure 4. Photo of recipient mouse testes 6wk after transplantation of donor germ cells from control Rosa26 mouse. Donor derived spermatogenesis resulting from colonized spermatogonial stem cells are evidenced by blue colonies within recipient seminiferous tubules.

CONCLUSION

Spermatogonial stem cell transplantation, with the ability to maintain spermatogonial stem cells in culture being vital, and ectopic testicular xenografting are both methods with potential for efficient generation of transgenic animals through the male germ line. The global objective of the research in this dissertation is to develop model systems for generating transgenic cattle through the male germ line. In cattle, efficient generation of transgenic animals is animals that are resistant to particular pathogens that are harmful to humans of high importance for increasing food safety.

An important component to spermatogonial stem cell transplantation and genetic alteration of spermatogonial stem cells is generation of methods that support their survival and self-replicating proliferation in vitro. In the first two studies of this dissertation two different culture methods were used and colonization analysis from cross species transplantations demonstrated survival and proliferation of bovine spermatogonial stem cells over a 2wk period. Both of these culture systems can be refined to enhance spermatogonial stem cell maintenance and used to evaluate methods for genetically modifying bovine spermatogonial stem cells by transgene insertion. In study two, GDNF was shown to influence the biological activity of spermatogonial stem cells in vitro. Addition of GDNF as well as other growth factors to these culture systems may allow for optimization of spermatogonial stem cell maintenance leading to efficient genetic modification. Evaluation of other growth factors suspected as mitogens for spermatogonial stem cells can be conducted using these culture systems. When coupled with spermatogonial stem cell transplantation in bulls, genetic modification of bovine spermatogonial stem cells in vitro using the methods developed here may result in an efficient means for generating transgenic cattle through the male germ line by natural mating.

Ectopic testicular xenografting is another method that could be utilized to generate transgenic spermatozoa for in vitro fertilization to generate transgenic animals. Also, this system could be used to supply genetically altered spermatogonial stem cells for transplantation into recipient testes. In studies three and four establishment of spermatogenesis was demonstrated in neonatal bovine testicular following ectopic xenografting over a 24wk period. In study three incorporation of a β-galactosidase transgene in germ cells was demonstrated. These demonstrations support the hypothesis that ectopic testicular xenografting can be used as an alternative means for generating transgenic spermatozoa. Future studies can be conducted to evaluate the competency of these genetically altered germ cells for deriving transgenic embryos following fertilization.

Enhancing the percentage of seminiferous tubules that result in established spermatogenesis in xenografted bovine testicular tissue is essential for efficient application of this technique to derive genetically modified spermatozoa. In study four age related differences in the ability for testicular tissue to establish spermatogenesis was demonstrated in bull calves, with testicular tissue form 8wk old donor calves containing the highest percentage of tubules with germ cell differentiation. Thus, it appears possible that potential exist for optimization of grafting procedures to generate optimal numbers of spermatozoa. Future experimentation for generating transgenic spermatozoa in bovine testicular xenografts should utilize donor tissue from 8wk old bull calves.

Currently the ability to transfer spermatogonial stem cells from the testes of a donor bull into the seminiferous tubules of a recipient bull is not established. One major hurdle to accomplish this is the ability to deplete germ cells in recipient testes to allow for donor spermatogonial stem cells to efficiently colonize. In study five adult rams were used as an animal

model for evaluating three different treatment regimens for depletion of endogenous germ cells in large domestic animal testes. In order for a treatment to be effective at achieving the goal of preparing germ cell transplant recipients, somatic Sertoli cell biological activity must not be affected by treatment. Since the anatomy of the ram testis is nearly identical to the bull testis, any information garnered from studies with rams can be directly applied to bulls. In this study germ cell depletion without alteration of Sertoli cell viability or function was demonstrated using active immunization against LHRH and testicular irradiation. These treatment regimens can be used in future experiments to generate spermatogonial stem cell transplantation recipients and help further the development of this technology in bulls.

In the appendix portion two studies were conducted to increase the understanding of male fertility. Study one demonstrated the expression profiles of several key genes in postnatal testicular development in the bull calf. Specifically, Sertoli cell expression of FSHR and AR showed distinct patterns of expression suggesting the period of 2-12wk of age as the time when Sertoli cell proliferation and differentiation occurs in the bovine testis. The expression profiles for GDNF and GFRα1 showed coordinated interaction from 2-8wk of age in the bull calf suggesting this as the time period for establishment of spermatogonial stem cell niches. Increasing the understanding of establishment of sperm production in bulls can lead to critical information that can be directly applied to enhancing the efficiency of sperm production in xenografted testicular tissue.

In the second appendix study lack of spermatogonial stem cell colonization in recipient seminiferous tubules from donor mice actively immunized against LHRH was demonstrated. These data suggest that disruption of the *hpg* axis is detrimental to spermatogonial stem cell biological activity. Information gained from these experiments may lead to identification of

specific growth factors regulated by gonadotropins that are essential for spermatogonial stem cell biological activity. These factors could be supplemented in culture medium to mimic a defined environment that is essential for stem cell maintenance in vitro.

In cattle, development of model systems for efficiently generating transgenic animals is of high importance to increase food safety. Efficient generation of transgenic animals may lie through genetic alteration of spermatogonial stem cells. Both spermatogonial stem cell transplantation, with spermatogonial stem cell culture as an integral component, and ectopic testicular xenografting are valuable techniques that can be used to achieve this goal.