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**IDENTIFICATION OF CONDITIONS SUPPORTING IN VITRO
MAINTENANCE OF BOVINE UNDIFFERENTIATED
SPERMATOGONIA**

A Thesis in
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by
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ABSTRACT

Proliferation and differentiation of the undifferentiated spermatogonial population provides the foundation for spermatogenesis, and spermatogonial stem cells (SSCs) represent a sub-population capable of infinite self-renewal. Long-term culture of SSCs in conditions supporting self-renewal and subsequent transplantation into testes of recipient males for reestablishment of spermatogenesis provides a means to preserve and expand male germlines. Moreover, these capabilities provide a platform for direct genetic modification of the male germline to produce transgenic animals. For cattle, these capabilities could provide a novel avenue for enhancing production efficiency and generation of genetically modified food animals. Studies with rodents revealed that culture of undifferentiated spermatogonia in serum-free media conditions promotes formation of germ cell clumps that contain SSCs. In addition, supplementation with the growth factor glial cell line-derived neurotrophic factor (GDNF) is essential for long-term expansion of germ cell clumps including self-renewal of SSCs, and fibroblast growth factor 2 (FGF2) enhanced these effects. To date, conditions supporting formation and expansion of bovine undifferentiated spermatogonial clumps that are similar in morphology to those in rodent cultures have not been reported. In this study, we examined several conditions that support rodent undifferentiated spermatogonia in vitro and identified conditions which supported formation of undifferentiated spermatogonial clumps from pre-pubertal bull testes that are identical in morphology to those in rodent cultures. These conditions included mitotically inactivated bovine embryonic fibroblast (BEF) feeder cells in DMEM/F12 medium containing StemPro supplement and addition of recombinant human forms of GDNF and FGF2. We determined these cells express the transcription factor Plzf, confirming an undifferentiated spermatogonial phenotype. Upon long-term culture we found that the number of

clumps declines in these conditions beginning with the first week and the cells could not be maintained for greater than 3 weeks indicating lack of key factors that promote their survival and proliferation. Thus, we conducted microarray analyses of isolated bovine undifferentiated spermatogonia to identify enriched expression of growth factor receptors whose corresponding ligands may enhance their maintenance in vitro. From these analyses we determined that expression of colony stimulating factor 1 receptor (*Csf-1r*) and chemokine (c-x-c motif) receptor 4 (*Cxcr4*) are highly enriched in the bovine undifferentiated spermatogonial fraction. Addition of recombinant human CSF-1, along with GDNF and FGF2, significantly enhanced the maintenance of undifferentiated spermatogonia during the first week of culture by greater than 2-fold compared to cultures receiving GDNF and FGF2 only. Supplementation of recombinant human SDF-1 in addition to the GDNF, FGF2, and CSF-1 increased the number of undifferentiated spermatogonia that were maintained by 1.75-fold compared with CSF-1, GDNF, and FGF2 alone. These results implicate CSF-1 and SDF-1 as additional extrinsic factors that promote survival and proliferation of bovine undifferentiated spermatogonia. Furthermore, a Sertoli-enriched feeder cell monolayer was tested and found to improve support of undifferentiated spermatogonia significantly when compared to the BEF feeders. Collectively, these studies have identified conditions that enhance the short-term maintenance of bovine undifferentiated spermatogonia in vitro and serve as an important step towards discovering conditions which promote long-term expansion of the cells including self-renewal of SSCs.

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CHAPTER 1: REVIEW OF THE LITERATURE

SIGNIFICANCE

The majority of genetic gain in livestock is made through the male germline. Therefore, any improvement in male reproductive efficiency can translate to greater improvements in production efficiency and profitability of the cattle industry. For the dairy industry, this equates to more pounds of milk produced per cow and, for the beef industry, greater gain to feed efficiency is desired. With the projected world population estimated at over 9.3 billion people by 2050, over 2 billion more than the current population, there is an ever-present need for increased production efficiency, particularly in the area of food agriculture (USCB, 2012). Although there are many different factors involved in animal production efficiency, an effective means for achieving greater efficiency is with genetic improvements through specialized breeding programs, and the male germline has been an effective avenue in which this is accomplished.

Spermatogenesis is the process by which mature male gametes or sperm are produced and is sustained throughout most of adulthood by a small population of stem cells within the testis termed spermatogonial stem cells (SSCs). SSCs differentiate to form sperm which transfer genetic information from father to offspring. Identifying methods to increase availability of gametes from superior sires for use in commercial cattle production can greatly affect the rate of genetic gain.

TESTIS

The two primary functions of the testis are to produce gametes through the process of spermatogenesis and to produce androgens which promote fetal development, male secondary sex traits, and libido, as well as maintenance of spermatogenesis. The anatomical structure of the testis is vital to these functions, in particular the development of sperm and is outlined in *Histological and Histopathological Evaluation of the Testis* by Russell et al. (1990). The testis develops in a retroperitoneal fashion and then moves into the peritoneal space, becoming encapsulated by peritoneal tissue layers. Within the testicular capsule lies the parenchymal tissue, which is composed of seminiferous tubules surrounded by interstitial cells. The seminiferous tubules, as well as the interstitium, are composed of specialized somatic cells which surround the germ cells, forming an environment capable of supporting spermatogenesis, termed the niche microenvironment. Contributors of the niche microenvironment include Sertoli cells within the seminiferous tubules as well as Leydig cells, myoid cells, and a blood supply in the interstitium. Collectively, these cells support spermatogenesis and one of the ways in which they do this is by maintaining SSCs in a balance between self-renewal and differentiation.

The Seminiferous Tubules

The seminiferous tubules are a series of tubes which house and promote the development of germ cells. Each tubule forms a convoluted loop with both ends connecting to the rete testis, a centrally located area within the testis. Sperm converge in this area before exiting the testes via the epididymis. Within the seminiferous tubules are two distinct regions: the outer, basal compartment and the inner, adluminal compartment, collectively called the seminiferous epithelium, which is composed of Sertoli cells and germ cells.

Sertoli Cell

The Sertoli cells, often called nurse cells, are crucial players in the developing niche due to their direct contact with the germ cells inside the seminiferous tubules. Without these cells, spermatogenesis is impossible (Mruk et al., 2004). Sertoli cells serve as a major structural component of the seminiferous tubules, surrounding the developing germ cells as they progress through differentiation to become elongated spermatids. Sertoli cells also interact with each other forming tight junctions creating what is referred to as the blood-testis barrier (BTB), which divides the basal compartment from the adluminal compartment of the seminiferous epithelium. All of the stages of spermatogonia before meiosis, including the SSCs, reside within the basal compartment of the seminiferous epithelium, while all haploid spermatocytes after completion of meiosis cross the BTB to the adluminal compartment. The precise function of this separation is not fully understood but it is thought that the BTB creates an immune-privileged area, possibly to prevent haploid germ cells from being recognized as foreign and being destroyed (Fiorini, 2004). In addition to these functions, Sertoli cells also secrete cytokines which aid in facilitating the fate decision of SSCs for either self-renew or differentiation. Glial cell line-derived neurotrophic factor (GDNF) is one of the more notable cytokines produced by Sertoli cells and is required for the self-renewal of SSCs (Tadokoro et al., 2002; Kubota et al., 2004). Furthermore, recent studies have demonstrated Sertoli cells to be the vital somatic cell determinant of the SSC niche, compared to other testis components (Oatley et al., 2011).

Germ Cells

Germ cells are the other cell type found within the seminiferous tubules. These cells originate from fetal primordial germ cells (PGCs) and ultimately differentiate into spermatozoa. The most undifferentiated germ cells reside along the basement membrane of the seminiferous tubules and move towards the lumen as they develop into spermatozoa. There are several different types of spermatogonia that can be identified within the testis and can be classified into one of two broad categories: undifferentiated spermatogonia and differentiating spermatogonia. The undifferentiated spermatogonia consist of A-single (A_s), A-paired (A_{pr}), and A-aligned (A_{al}) spermatogonia, with A_s cells being the least differentiated and considered to represent the SSCs (Oatley and Brinster, 2008). When SSCs differentiate, the A_s cells divide mitotically, forming two daughter cells termed A_{pr} spermatogonia. The mitotic cycle fails to complete telophase and these cells remain connected by an intercellular bridge. This bridge allows communication and content sharing between the cells, thereby keeping their development synchronized (Lee et al., 1995; Braun et al. 1989). A_{pr} cells undergo subsequent mitotic divisions becoming chains of 4 ($A_{al}(4)$), 8 ($A_{al}(8)$), and then 16 ($A_{al}(16)$) cells, all still connected by intercellular bridges. Although these cells have traditionally been considered differentiating cells, it was recently suggested that this process may, in some cases, be reversible by cells breaking off and reverting to the A_s stage (Nakagawa et al., 2010). Typically, however, when they reach the $A_{al}(16)$ stage, they differentiate further to become chains of A_1 spermatogonia. Cells in this stage are considered to be committed to terminal differentiation and are ready to undergo the process of spermatogenesis.

The Interstitium

The interstitium, although not as intimately associated as Sertoli cells, also plays a role in supporting germ cell development. The interstitial space, which lies between the convoluted seminiferous tubules, contains the Leydig cells, myoid cells, macrophages, and the vascular network. Each of these cell types have specialized functions within the testis and many studies have been conducted over the last decade to examine the possible contributions and role of the interstitium on the fate decision of SSCs (de Rooij, 2009).

Leydig cells

Leydig cells are the main cell type of the interstitial space. These cells are most noted for their luteinizing hormone-induced production of testosterone, the androgen responsible for secondary sex traits in males and required for the transformative phase of spermatogenesis, sometimes called spermiogenesis. Without adequate testosterone levels, mid-stage round spermatids and mature, elongated spermatozoa are lost (Russell and Clermont, 1977). Sertoli cells express testosterone receptors, indicating a possible indirect effect of Leydig cells on the stem cell niche through Sertoli cells. It was also shown that undifferentiated spermatogonia are most prevalent near areas where seminiferous tubules bordered interstitial space, as opposed to other tubules, indicating a possible affinity for Leydig cells (Chiarini-Garcia et al., 2001, 2003). Another possible effect is through the actions of colony stimulating factor 1(CSF-1), a cytokine secreted by Leydig cells that stimulates the proliferation of A_s and A_{pr} spermatogonia, thereby promoting self-renewal (Oatley et al., 2009).

Myoid cells

Peritubular myoid cells are the other main somatic cell type within the interstitial space. They are flat, mesenchymal-type cells that line the outside of each seminiferous tubule and aid in structural support (Hadley et al., 1985; Dym and Fawcett, 1970). In addition to structure support, Clermont (1958) demonstrated that myoid cells also have contractile properties which were shown to aid in the movement of spermatozoa through the seminiferous tubules (Clermont, 1958; Kormano and Suvanto, 1968). Oatley et al. (2009) showed that myoid cells, in addition to Leydig cells, express CSF-1 which may contribute to the niche microenvironment (Oatley et al., 2009).

Vasculature

Unlike the immune-privileged seminiferous tubules, the interstitial space is highly vascularized. As with any vascularized tissue, the blood vessels serve a number of purposes including gas exchange, nutrient delivery, and transport of other substances such as hormones and cytokines. It was shown by Yoshida et al. (2007) that undifferentiated spermatogonia tend to associate with areas of vascularization, specifically at branching points of capillaries. Therefore, Yoshida et al. (2007) proposed vasculature as a key director of where the germline niche becomes established. Rationale for this could be linked to the higher concentration of hormones and cytokines delivered to these areas through the blood supply (Yoshida et al., 2007). More recent work has raised questions regarding this proposal, however, and had redirected attention back to the Sertoli cell (Oatley et al., 2011).

SPERMATOGENESIS

Spermatogenesis is the term used to describe the formation of male gametes and is the process whereby diploid undifferentiated spermatogonia give rise to haploid spermatozoa. This is a highly regulated and productive process that results in approximately 100 million sperm each day in men and even greater numbers in most livestock species (Sharpe, 1994). Spermatogenesis is established in the bull by 32 weeks post partum but varies slightly by breed (Curtis and Amann, 1981). The ultimate goal of spermatogenesis is to transmit genetic information to future generations. In order for this to be successful, germ cells must undergo differentiation and specialization in a three-stage process including the proliferative phase, the meiotic phase, and the transformative phase (Oatley and Brinster, 2006). This process is maintained from puberty through the majority of adult life, under the regulated balance of SSC self-renewal and differentiation.

Proliferative phase

The first phase of spermatogenesis is termed the proliferative phase and is marked by multiple mitotic divisions which account largely for the immense number of spermatozoa that are produced each day. Cells recruited for spermatogenesis come from the stem cell pool, which resides on the basement membrane of the seminiferous tubule. SSCs, by signaling which is not fully understood, begin the process of differentiation and go through mitotic phases, forming a syncytium of 4, 8 and then 16 A_{al} cells, connected by intercellular bridges after each cell division. Without dividing, the 16 A_{al} cells differentiate into A_1 spermatogonia and regain c-kit expression (Yoshinaga et al., 1991). It is the A_1 spermatogonia which are first referred to as differentiated cells. These cells proliferate extensively through multiple mitotic divisions, with

16-cell A1 chains dividing to make 32 A2 cells which divide again, forming 64 A3 cells and yet again to create chains of 128 A4 cells. At this point, the A4 cells divide yet again to form an intermediate cell type which divide again to form type B spermatogonia. At this point, there are 512 type B spermatogonia for every A1 cell that entered mitosis. These cells will undergo one final cycle of mitosis resulting in 1024 round cells called primary spermatocytes. Consequently, for every chain of A1 (16) spermatogonia, a possible 1024 primary spermatocytes could be produced. Many of these cells will undergo apoptosis throughout this process, however, significantly reducing this number (Allan et al., 1987).

Meiotic phase

Following the highly proliferative, initial phase of spermatogenesis, primary spermatocytes then enter the meiotic phase. This is the second and longest phase of spermatogenesis and it is during this time that the cells undergo two meiotic divisions. Testosterone, in conjunction with cytokines such as tumor necrosis factor α (TNF α) and transforming growth factor- β 3 (TGF- β 3), remodel the Sertoli cell tight junctions of the BTB, allowing primary spermatocytes to move from the basement membrane to the immune-privileged, adluminal area of the seminiferous tubule without disrupting the immunological barrier (Li et al., 2009). Once inside the BTB, the primary spermatocytes immediately enter prophase I, which has five sub-phases designated preleptotene, leptotene, zygotene, pachytene, and diplotene (Bellve et al., 1977). During these phases, DNA is replicated, homologous chromosomes are paired, genetic recombination occurs via crossing over, and the chromosomes are separated to opposite poles of the cell for cell division, resulting in two secondary spermatocytes which rapidly enter a second meiotic division.

No DNA replication occurs during prophase II, therefore, meiosis II produces four small, haploid round spermatids that enter the next phase of spermatogenesis called the transformative phase.

Transformative phase

The transformative phase is the third and final stage of spermatogenesis. During this phase, the round spermatids will undergo a remarkable morphologic differentiation with functional sperm as the end product. This process of differentiation consists of the following four sub-phases: the Golgi phase, the cap phase, the acrosomal phase, and the maturation phase (Hess, 1999). The Golgi phase is characterized by the initial formation of the acrosomal vesicle through proliferation of membrane-bound vesicles by the Golgi apparatus (Nagano, 1962). Also during this phase, the mother-daughter centriole pair migrates from the base of the nucleus. The mother centriole forms an initiation site for the future flagellum and the daughter centriole forms the axoneme, which is the central segment of the flagellum (Nagano, 1962). During the cap phase, the acrosome spreads out over the anterior surface of the nucleus and continues to spread down the sides of the nucleus during the acrosomal phase, which is characterized by nuclear and cytoplasmic elongation. The final phase, maturation, involves the assembly of the 3-piece tail, which extends from the Sertoli cells out into the seminiferous tubule lumen (McIntosh and Porter, 1967). Finally, the elongated spermatid is ready to be released from the Sertoli cell into the tubule lumen, called spermiation. This process involves final modifications to the spermatid's nucleus and cytoplasm and removal of Sertoli-germ cell connections, called ectoplasmic specialization junctions (O'Donnell et al., 2011). Spermiation concludes with release of the spermatid, which is now called a spermatozoan, into the lumen. Spermatozoa then travel to the epididymis for final maturation and storage (Russell, 1993).

For sperm production within the testis to be continuous, different areas along the length of the seminiferous tubules have to be in different stages of production at any given time. Each entire round of spermatogenesis in the bull can be divided into 12 stages and is referred to as the cycle of the seminiferous epithelium (Berndtson and Desjardins, 1974). Each stage is characterized by the different types of spermatogonia present within a given area of the seminiferous tubules. This continuous, cyclic nature of spermatogenesis is supported by the self-renewal and differentiation of SSCs.

SPERMATOGONIAL STEM CELL BIOLOGY

Spermatogonial stem cells (SSCs) are a small population of tissue-specific stem cells residing within the testis that provide the foundation for continual spermatogenesis throughout adulthood due to their ability to self-renew and give rise to spermatozoa (Oatley and Brinster, 2008). Tissue-specific stem cells are specialized, undifferentiated cells that are regulated by their niche microenvironment to maintain homeostasis of the specific tissue in which they reside, as well as facilitate the restoration of a tissue following physical damage or cytotoxic insult. Stem cells are present in most tissues throughout the body such as the crypt stem cells of the intestinal tract (van der Flier and Clevers, 2009), the hepatic stem cells of the liver (Schwartz and Verfaillie, 2010), the hematopoietic stem cells found in bone marrow (Ogawa, 1993), and many others including the SSCs in the testis (de Rooij, 1988). SSCs can be further defined as unipotent stem cells, meaning they only differentiate into one, specific cell type, for example sperm (de Rooij and Russell, 2000). This is in contrast to multipotent cells that can generate multiple cell types from a single lineage, such as crypt stem cells which differentiate into five different cell components of the intestinal lining (Hermiston et al., 1993). Unlike other tissue-

specific stem cell populations, however, SSCs are the only stem cells able to contribute genetic information to future generations, making them of great interest for the production of genetically modified animals (Oatley and Brinster, 2006).

Development

The precursors of SSCs are primordial germ cells (PGCs). These cells originate from the epiblast of the developing embryo and then, following a chemoattractant gradient, migrate to the genital ridge where the gonad begins to develop (Molyneaux, 2003). Late in embryogenesis or shortly after birth, PGCs transition to prospermatogonia, also called gonocytes (de Felici, 2000). Postpartum, a subpopulation of prospermatogonia migrate to the basement membrane of the seminiferous tubule and will transition into SSCs. This occurs in mice between 0 and 5 days post partum (dpp) and occurs around week 4 in bull calves (McClean, 2005; Curtis and Amann, 1981). Any prospermatogonia that do not migrate to the basement membrane will be lost by way of apoptosis. Those cells that migrated to the basement membrane will transition to SSCs and reside in special niche microenvironments. All sperm production, from puberty to adulthood, will result from differentiation of these cells. In the bull, transformation into A1 spermatogonia occurs around 8 weeks of age and spermatogenesis begins between 12 and 24 weeks of age (Curtis and Amman, 1981).

Functional activity

The main function of SSCs is the maintenance of spermatogenesis from puberty through most of adult life. This is possible due to their ability to maintain a pool of undifferentiated stem

cells, by the process of self-renewal, and then go through differentiation to produce a constant supply of gametes. Self-renewal is the continual replenishment of SSCs through undifferentiated cell divisions and, without this process, SSCs would be depleted through the course of differentiation, resulting in premature sterility of the male (Oatley and Brinster, 2008). Therefore, for continual spermatogenesis, a balance between these two pathways must be maintained (Oatley and Brinster, 2006)

When a SSC divides, it will form two new daughter cells. Whether they divide symmetrically, forming two new SSCs or two differentiated A_{pr} cells, or asymmetrically, producing one SSC and one cell that is committed to differentiate is currently debated (Oatley and Brinster, 2008). It is possible that both of these occur at different times within the testis depending on the signals from the surrounding environment. For example, it could be possible that asymmetric division is the normal mode for maintenance of SSCs and spermatogenesis and that, if the testis has just undergone cytotoxic insult or injury, the SSCs may undergo symmetric division to reestablish the SSC pool. Once the SSCs have been adequately expanded, they could transition back to asymmetrical division for tissue homeostasis. Whether or not this is the case, regulation of divisions is supported by the niche microenvironment, the specialized area in which the SSCs reside (Oatley and Brinster, 2012).

PHENOTYPE OF SPERMATOGONIAL STEM CELLS

SSCs are estimated to comprise 1 out of every 3000 cells in the mouse testis and their scarcity within the testis makes their isolation difficult (Tegelenbosch and de Rooij, 1993). This is further compounded by the lack of known cell surface markers specific to SSCs, making

isolation of pure populations of these cells virtually impossible. Isolation of SSCs is of great importance in order to study their regulatory mechanism, as well as use them in reproductive techniques. Therefore, many studies have examined ways in which to accomplish this, mainly by identification of potential SSC markers (Shinohara et al., 1999; Kubota et al. 2003, 2004a, 2004b). As a starting point, cell surface markers of other tissue-specific stem cells have been examined. Some of these markers have been valuable in obtaining a population enriched for SSCs, but each of them is also expressed by other types of undifferentiated or differentiating spermatogonia and, in some cases, even somatic cells (Oatley and Brinster, 2008). Recently, several cell surface markers expressed by undifferentiated spermatogonia have also been investigated to elucidate the SSC phenotype.

Cell Surface Markers

THY1

Thymus cell antigen 1 (THY1), also identified as CD90, is a glycosyl phosphatidylinositol- anchored glycoprotein present on the surface of many cells, including T lymphocytes, hematopoietic stem cells (HSCs), and embryonic stem cells (ESCs; Raff, 1971; Goldschneider et al., 1978; Ling and Neben, 1997). A sub-population of cells within the testis of mice and non-human primates express THY1 and it was shown, upon transplantation analysis, that THY1⁺ cells were enriched for SSCs (Kubota et al., 2003, 2004a; Hermann et al., 2007). Isolation of THY1⁺ cells, in conjunction with a negative selection marker, β 2M expressing somatic cells, yields a ~30-fold enrichment for SSCs, containing nearly 95% of the SSCs in this THY1⁺ MHC-I cell population (Kubota et al., 2004a). THY1, alone or in combination with other markers, continues to be an invaluable marker for SSC enrichment in rodents (Oatley and

Brinster, 2008). More recently, THY1 was demonstrated to be a conserved marker of SSCs in livestock species (Reading et al., 2010; Bahadorani et al., 2012). Reading et al. (2010) showed, via flow cytometric analysis, that a small sub-population of bull testis cells were THY1⁺ and THY1⁺ cells isolated by magnetic activated cell sorting (MACS) were positive for PLZF, a marker of undifferentiated spermatogonia. When transplanted into immunodeficient nude mice, THY1⁺ cells had 6-fold greater colony formation compared to the unselected total testis cell isolate (Reading et al., 2010).

CD9

Another marker expressed by certain stem cell populations that was thought to possibly be conserved in the SSCs was CD9, a tetrasparin transmembrane protein. CD9 is expressed in HSCs, neuronal stem cells, and mouse ESCs (Oritari, 1996; Klassen, 2001; Oka et al., 2002). After finding that a small population (~5%) of testis cells located at the basement membrane of the seminiferous tubules expressed CD9, Kanatsu-Shinohara et al. (2004) examined both mice and rats to investigate the possibility of this stem cell marker being conserved in SSCs. Their work showed that, indeed, CD9 was expressed in both mouse and rat SSCs, and there was a 6.7-fold and 5.1 –fold enrichment of SSCs, respectively, as estimated by transplantation analysis (Kanatsu-Shinohara et al., 2004).

GDNF receptor complex

GDNF, a growth factor produced by Sertoli cells, is known to influence SSC self-renewal in vivo and in vitro. It acts through a receptor complex consisting of the GDNF family receptor alpha 1 (GFR α 1) and the proto-oncogene tyrosine-protein kinase receptor (c-RET; Trupp et al., 1995; Kubota et al., 2004a, 2004b). Because SSCs are influenced by this growth factor, it is

reasonable to test whether this receptor complex would be present on SSCs. Therefore, GFR α 1⁺ and c-RET⁺ selected cells were investigated for SSC enrichment. MACS was conducted for these receptor components in neonatal, pre-pubertal, and adult mice testis cells. Following transplantation analysis, colony formation was not increased compared to the unselected testis cell population, demonstrating that the GDNF receptor complex is not a valuable marker for SSC enrichment (Ebata et al., 2005).

α 6-and β 1-integrin

Integrins are cell adhesion molecules for many extracellular matrix components and are involved in the regulation of proliferation, differentiation, survival and migration of cells (Miranti and Brugge, 2002). α 6-intergrin and β 1-integrin form a heterodimer which serves as a receptor for laminin, a component of seminiferous tubule basement membranes. SSCs are known to reside along the basement membrane and Shinohara et al. (1999) showed that SSCs preferentially bind to laminin, yielding a 3- to 4-fold greater colonization compared to control cells. For these reasons, α 6- and β 1-integrins were investigated as possible phenotypic marker for isolation of SSCs. It was determined that both adult and pup SSCs express α 6-intergrin with 6.7- and 7.7-fold enrichment in SSCs, respectively, compared with controls (Shinohara et al., 1999; Ebata et al., 2005). β 1-integrin selection resulted in a 3.8 fold increase in colonization compared to unselected testis cells and colonization increased to a 14.6-fold greater colonization when compared with β 1-integrin depleted cells (Shinohara et al, 1999). However, histological examination of testes transplanted with β 1-integrin depleted cells showed normal colonization and donor-derived spermatogenesis, indicating that β 1-integrin⁺ cells do not contain the total SSC population (Shinohara et al., 1999). Another study revealed that β 1-integrin was involved

in SSC homing to the basement membrane niche and that, when SSCs lack β 1-integrin, colony formation following transplantation is disrupted due to diminished homing efficiency (Kanatsu-Shinohara et al., 2008). Together, this information supports α 6- and β 1-integrins as cell surface markers of SSCs and that, in conjunctions with other phenotypic markers, these adhesion molecules should be effective in enriching for SSCs.

c-KIT

In addition to enriching SSCs by selecting for molecular marker expressed by SSCs, it is also possible to obtain an SSC-enriched cell isolate by eliminating cells with markers known to be expressed by non-SSC cells. One such marker that is used for this purpose is *c-Kit*, a proto-oncogene that encodes a tyrosine kinase receptor. *c-KIT* was originally investigated because of its known expression by gonocytes, HSCs, and ESCs (Yoshinaga et al., 1991; Snodgrass et al., 1992; Randall and Weissman, 1998). Gonocyte expression of the *c-KIT* receptor allowed these cells to respond to kit ligand production by cells in the hindgut causing their migration to the genital ridge early in development (Yoshinaga et al., 1991; Zhao and Garbers, 2002). Mice with *c-Kit* and kit ligand mutations are sterile due to disrupted PGC proliferation and migration to the genital ridge (Buehr et al., 1993). Shinohara et al. (1999) attempted to enrich mouse testis cell populations for SSC by selecting for *c-KIT* expression by fluorescent activated cell sorting (FACS) but, following transplantation analysis, there was no difference in colony formation between *KIT*⁺ cells and the unselected control (Shinohara et al., 1999). It was later determined that PGCs were *c-KIT* positive during migration, lost this phenotype during the transition to undifferentiated spermatogonial state, and then regained *c-KIT* expression upon transition into A1 differentiated spermatogonia (Manova et al., 1990; Yoshinaga et al., 1991; Shinohara et al.,

1999). With this knowledge, c-KIT expression has been used for removal of differentiating spermatogonia to achieve greater enrichment of SSCs (Randall and Weissman, 1998; Shinohara et al. 1999, 2000; Kubota et al., 2003).

Molecular Markers

PLZF

Promyelocytic leukemia zinc finger protein (PLZF), a BTB/POZ-domain family transcription factor, was the first protein identified to be required for mammalian SSC maintenance, and its expression is restricted to the A_s, A_{pr}, and A_{al} spermatogonia in mice (Buass et al., 2004; Costoya et al., 2004). PLZF is encoded by the gene zinc finger protein 145 (*Zfp145*) and luxiod mice, which possess a mutation in this gene, have a distinct phenotype including limb abnormalities and infertility (Green, 1955; Buass et al., 2004). Luxiod mice have smaller testes than their wild-type counterpart and, upon histological examination, it was determined that many seminiferous tubules lacked developing germ cells and continued to deteriorate with age, thus indicating a depletion of SSCs over time (Johnson and Hunt, 1971; Costoya et al., 2004).

Transplantation studies showed that spermatogenesis could not be restored with luxoid donor mouse cells, showing a direct dysregulation of the SSCs (Buass et al., 2004; Costoya et al., 2004).

PLZF is expressed by undifferentiated spermatogonia in rodents and non-human primates and recently studies have investigated the expression of PLZF in livestock species.

Immunohistochemical analysis of pre-pubertal bull testis cross-sections showed select spermatogonia within seminiferous tubules that were PLZF⁺ and, when SSC-enriched THY1⁺ cells were examined for PLZF expression, it was found that the majority of these cells were also

PLZF⁺ indicating that this transcription factor has conserved expression in pre-pubertal bull undifferentiated spermatogonia (Reding et al., 2010).

UCHL1

Ubiquitin carboxyl-terminal esterase L1 (UCHL1), formerly protein gene product 9.5 (PGP9.5), is a molecular marker of SSCs in livestock species including the bull and the boar (Frankenhuis et al., 1982; Herrid et al., 2007). UCHL1 expression is found within the A_s, A_{pr}, and A_{al} spermatogonia of both of these species, and a recent study showed enrichment of UCHL1⁺ cells in PLZF⁺ populations (Luo et al., 2009). Reding et al. (2010) further characterized this population in the bull, showing colocalization of PLZF expression in some, but not all, UCHL1⁺ Spermatogonia, suggesting that UCHL1 may be a more general marker of type A spermatogonia, with PLZF being more specific to A_s cells, however, not much is known about the functional role it plays in these, or other, cells (Reding et al., 2010).

BCL6B

B-cell CLL/lymphoma 6, member B (BCL6B), is a molecular marker of SSCs and its expression is up-regulated by GDNF (Oatley et al., 2006). When GDNF was withdrawn from mouse SSC cultures, a significant decline in *Bcl6b* expression was observed. This was reversed upon reintroduction of GDNF to the cultures. A direct effect of *Bcl6b* on SSCs was confirmed when mass apoptosis was observed following *Bcl6b* knockdown with siRNA (Oatley et al., 2006). BCL6B is localized to spermatogonia within the mouse testis and disruption of expression results in a Sertoli-cell only phenotype (Oatley et al., 2006, 2007). BCL6B expression is conserved in the rat and, more recently, Reding et al. (2010) showed that BCL6B

expression was present in the pre-pubertal bovine testis and further demonstrated that its expression was enriched in the THY1⁺ cell population (Schmidt et al., 2009; Reding et al., 2010).

ETV5

Another molecular marker which was shown to be up-regulated by GDNF is Ets variant gene 5 (*ETV5*; Oatley et al., 2006). Similar to *Bl6b*, knockdown of *Etv5* by siRNA treatment resulted in a decrease in THY1⁺ mouse cultures and infertility in *Etv5*-null mice was observed earlier than 3 months of age (Chen et al., 2005). Recent evidence showed a role for FGF2, in addition to GDNF, in the up-regulation of *Etv5* (Ishii et al., 2012). Combined, these data indicate that *Etv5* is needed for self-renewal of mouse SSC, a role which is conserved in rat SSCs (Schmidt et al., 2009).

REGULATION OF SPERMATOGONIAL STEM CELL FUNCTIONS

The regulation of SSCs is still relatively unknown and is an area of great interest because SSCs are essential for spermatogenesis and therefore maintenance of a species. Study of their regulation may also provide new insights into maintenance requirements of these cells in vitro. What is known currently is that extrinsic signals from the niche microenvironment regulate the fate decision of SSCs through up-regulation of several intrinsic factors. These extrinsic factors are secreted by the somatic cells within the testis, namely the Sertoli cells and the Leydig cells and, without these factors, in vitro maintenance is not possible, providing direct evidence for regulation of SSC fate decision. The main factors of interest are the cytokines glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2), and colony stimulating factor 1 (CSF-1).

GDNF

Sertoli cells, the only somatic cell population in direct contact with SSCs, are thought to be one of the key components of the niche microenvironment and the primary action of Sertoli cells on SSCs is through secretion of GDNF. GDNF is a member of the transforming growth factor- β (Tgf- β) superfamily that is crucial to the self-renewal process in vivo and in vitro (Trupp et al., 1995; Kubota et al., 2004a, 2004b). Kubota et al. (2004) showed that GDNF enhanced mouse SSC cultures over a 7-day period and, subsequently showed that GDNF was also required for the long-term maintenance and expansion of SSCs (Kubota et al., 2004a, 2004b). Transgenic mice with *Gdnf* over-expression exhibited an inhibition of spermatogonial differentiation resulting in accumulation of undifferentiated spermatogonia that, over time, formed seminomas (Meng et al., 2000, 2001; Yominga et al., 2003). Additionally, mice carrying a single *Gdnf*-null allele had a reduced production of GDNF and were agametic and therefore had disrupted spermatogenesis due to a lack of differentiating spermatogonia (Meng et al., 2000). Furthermore, the receptor complex for GDNF, GDNF family receptor alpha 1 (GFR α 1) and c-Ret receptor tyrosine kinase, is expressed by spermatogonia and, upon binding, up-regulate several downstream genes, such as *Bcl6b*, *Etv5*, and *Lhx1*, which serve as intrinsic regulators of self-renewal (Oatley et al., 2006, 2007). Collectively, these studies establish GDNF as a regulator of SSC self-renewal.

FGF2

Fibroblast growth factor 2 (FGF2) is a member of the fibroblast growth factor family and is secreted from various testis cell types including Sertoli cells, Leydig cells, and also

differentiating spermatogonia (Han et al., 1993). Resnick et al. (1992) showed that FGF2 stimulated proliferation of PGCs and addition of this factor stimulated in vitro proliferation of these cells. Because FGF2 influences the precursors of SSCs, research was conducted to determine if FGF2 had any effect on the regulation of SSCs. When added to mouse cultures, FGF2 enhanced GDNF-regulated long-term self-renewal expansion of SSCs, but could not maintain SSCs without GDNF supplementation (Kubota et al., 2004b). Kanatsu-Shinohara et al. (2008) also showed that FGF2 was required for culture of hamster SSCs. While the role GDNF has in self-renewal has been illuminated to a certain degree, the mechanisms of how FGF2 influences this process are unknown (Kubota et al., 2004a, 2004b; Oatley and Brinster, 2008). A recent study, however, proposed a mechanism whereby FGF2 mediated self-renewal of mouse SSCs through the activation of MAP2K1, up-regulating *Bcl6b* and *Etv5* (two genes thought to be involved in SSC self-renewal; Ishii et al., 2012). This latest information further suggests a role of FGF2 in SSC self-renewal regulation.

CSF-1

While Sertoli cells play a large regulatory role of SSCs through their production of GDNF and FGF2 and SSCs can be maintained in vivo for long periods of time under the influence of these two growth factors alone, the proliferative expansion of SSCs does not occur at a very rapid rate, thus indicating that additional growth factors may be beneficial to the self-renewal process. Oatley et al. (2009) conducted microarray transcript profiling of THY1⁺ cells from mouse testes to identify genes which were also enriched in this population and may be involved in SSC regulation. Of the over 200 genes with 10-fold or greater expression identified, colony stimulating factor 1 receptor (*Csf-1r*) showed the greatest enhancement (Oatley et al., 2009). CSF-1R is the receptor for colony-stimulating factor-1 (CSF-1), a cytokine ligand also

known as macrophage stimulating factor. The enrichment of this receptor in the SSC-enriched THY1⁺ fraction indicated that addition of CSF-1 to cultures might stimulate SSC self-renewal. Cultures of mouse SSCs which were supplemented with CSF-1, in addition to GDNF and FGF2, had enhanced self-renewal, indicating CSF-1 as an additional extrinsic regulator of SSC self-renewal (Oatley et al., 2009). Immunohistochemical analysis of mouse testis cross-sections localized CSF-1 expression to the Leydig and myoid cells of the interstitial space, suggesting that these cell populations may also contribute to regulation of the SSC through the secretion of this cytokine (Oatley et al., 2009). Further research must be conducted to determine the effects of CSF-1 on SSC regulation in other species.

SPERMATOGONIAL STEM CELL TRANSPLANTATION

Stem cells, by definition, must be able to regenerate the given cell lineage from their tissue of origin. SSCs, therefore, must be able to reestablish spermatogenesis when introduced into the testis. Consequently, availability of a functional assay which allows for the assessment of a given cell population's ability to generate differentiated tissue is needed. The technique of spermatogonial stem cell transplantation was first developed by Ralph Brinster at the University of Pennsylvania (Brinster and Averbock, 1994; Brinster and Zimmermann, 1994). Before this, there was no real way to confirm the existence of the SSC population (Oatley and Brinster, 2008). The SSC transplantation technique, therefore, has changed the way we are able to study SSCs. Transplantation was first developed in mice and involved the injection of testis cell isolates from a donor mouse into the seminiferous tubules of stem cell-depleted recipient mouse testis (Brinster and Averbock, 1994). If the donor cells contained SSCs, spermatogenesis was re-established in the recipient mouse testis. This process involved the following steps, which have

been outlined by Oatley and Brinster (2006). First, proper choice of a donor animal must be made. Donor cells must be able to be identified separately from the endogenous cells within the recipient mouse testis to ensure spermatogenesis is donor-derived. This is frequently accomplished using a transgene marker such as LacZ or green fluorescent protein (GFP). Thus, all cells from these animals will express the marker and be easily differentiated from recipient cells allowing donor-derived colonies to be visualized and counted. The number of colonies formed in the recipient testis is a direct indicator of the number of SSCs injected (Dobrinski et al., 1999). Because of this correlation, colony counting is done to determine the efficacy of different isolation methods or culture conditions. Increased colonization following addition of a new growth factor, for example, demonstrates the growth factor was beneficial to SSC maintenance and/or proliferation. Once the proper donor mouse has been selected, a recipient mouse must be prepared. Endogenous spermatogenesis must be eliminated for donor SSCs to successfully repopulate the testis. If this is not done effectively, SSCs will be largely blocked from reaching the basement membrane by endogenous spermatogenesis (Shinohara et al., 2002). Elimination of endogenous spermatogenesis can be accomplished using irradiation or with a number of chemotherapeutic drugs. One drug that was demonstrated to be very effective is busulfan, which is injected at a concentration of 30 mg/kg body weight intraperitoneally at least one month before transplantation (Bucci and Meistrich, 1987; Brinster and Averbach, 1994). Another mouse model that has been used in transplantation assays is the W/W^v mouse, which is sterile due to a mutation in the *c-kit* gene preventing proper PGC migration during embryogenesis (Brinster and Zimmermann, 1994). Injecting donor cells into the recipient mouse testes is the next step in the process of transplantation and can be done in a few different testis locations including the efferent ducts, the rete testis or directly into the seminiferous tubules

(Oatley and Brinster, 2006). Once injected into the testis, cells go through a three-phase migration (Nagano et al., 1999). During the first week, phase 1, cells are present throughout the seminiferous tubules, mostly in the lumen, with a few cells beginning to migrate to the basement membrane. Throughout phase two, which extends from week 2 until the end of the first month following transplantation, the cells continue to migrate through the BTB into the basal compartment and form a monolayer of cells along the basement membrane that give rise to colonies and differentiate, beginning spermatogenesis, in the third phase (Nagano et al., 1999). Analysis of colony formation and determination of spermatogenesis is the final step in the transplantation process. This is usually performed two months after initial introduction of the cells, which is sufficient time for completion of a round of spermatogenesis (Brinster and Averbock, 1994). If a transgene was used, such as LacZ, analysis is fairly simple. All donor derived cells will stain blue when exposed to the substrate X-gal and can be easily visualized (Brinster and Zimmermann, 1994). Early studies indicated that each colony is a result of a single SSC (Nagano et al., 1999; Dobrinski et al., 1999; Zhang et al., 2003). To substantiate this belief, Kanatsu-Shinohara et al. (2006) infected SSCs with an enhanced green fluorescence protein-expressing retrovirus and produced several transgenic mice from four different GFP-expressing colonies. The site of retroviral integration into the genome was unique to each SSC and, therefore, served as a marker to differentiate between cells which originated from different SSCs. Examination of the transgenic offspring revealed that all mice produced from a single colony carried the same retroviral integration site, demonstrating that the colony was clonally derived from a single SSC. Therefore, the number of donor-derived colonies is an indication of the number of SSCs originally transplanted (Kanatsu-Shinohara et al., 2006).

In addition to SSC transplantations conducted in mice, this technique has also been accomplished in rats (Jiang and Short, 1995). A year after this was achieved; Russell and Brinster (1996) were able to transplant donor cells from a rat into the testis of an immunodeficient mouse, resulting in rat spermatogenesis (Russell and Brinster, 1996). Subsequently, it was shown that mouse cells could be transplanted into rats, with similar results, thus demonstrating a conservation of signals for germ cell-Sertoli cell interactions between mice and rats (Russell and Brinster, 1996; Ogawa et al., 1999). These studies were the foundation for xenogenic transplantation of SSCs and laid the groundwork for transplantation in livestock species.

SPERMATOGONIAL STEM CELL TRANSPLANTATION AS A REPRODUCTIVE TOOL IN CATTLE PRODUCTION

The foundation for continual spermatogenesis is provided by the undifferentiated spermatogonial (und spg) population which includes SSCs and progenitor spermatogonia, and transplantation of these und spg between bulls may provide an efficient means for expanding the use of desirable genetics in cattle populations. Because genetic gain in livestock is primarily made through the male germline, preservation and increased availability of the male germline can greatly increase production efficiency and profitability. By greatly increasing the numbers of progeny to be produced by genetically desirable individuals, genetic progress can be made.

Currently, artificial insemination (AI) is the primary method by which genetic gain is made. Since its commercial availability in the 1940s, this technique has revolutionized the dairy industry and is used in many other species including swine, sheep, goats, turkeys, and horses. The beef industry, however, has not adopted this as a widely used practice, largely due to the way in which most beef cattle are managed. Whereas dairy cows are handled intensively each

day during the milking process, beef cattle are often maintained in range conditions with minimal human interaction. The amount of time and management involved in a successful AI program discourages most beef cattle operations from using this technique from a practical and economic standpoint. Approximately 7% of beef cattle are currently bred via AI, compared to the greater than 80% of calves resulting from AI in the dairy industry (USDA, 1993; Parish and Riley, 2011). Underutilization of AI limits beef cattle operations to the genetic lines within their local area, thus restricting the use of many superior sires. Improvements in production efficiency have therefore occurred at a much slower rate than the dairy industry, which has virtually any desired genetic line available for use (Blezinger, 2010). SSC transplantation would provide an alternative means of making superior genetics available to commercial beef cattle operations.

Transplantation in livestock, similar to the system already developed in rodents, would involve isolation of SSCs from the testis of a genetically desirable bull followed by a period of in vitro expansion and transplantation into recipient bulls (Brinster and Averbock, 1994; Brinster and Zimmermann, 2004). Donor-derived spermatogenesis would then occur within recipient testes and these bulls could breed by natural service to generate offspring containing donor genetics. Before this was attempted, however, Dobrinski et al. (2000) first endeavored to accomplish xenogenic transplantation in livestock species because a rodent transplant model was already shown to be effective (Brinster and Zimmermann, 1994; Brinster and Averbock, 1994). Cells from boars, bulls and stallions were collected and transplanted into immunodeficient mice immediately after isolated or following cryopreservation or 1 month in culture. Very few germ cells were identified following transplantation from stallions but cells from the pig resulted in chains of cell connected by intercellular bridges. Later stages of spermatogenesis, however, were not visible. The results from transplanting bull cells into mice were similar to that of the

boar but ultimately resulted in fibrotic tissue (Dobrinski et al., 2000). Another group showed that freshly collected germ cells from 1, 2, 3, and 4 months bulls were able to colonize a recipient nude mouse testis which had been treated with busulfan to remove endogenous spermatogenesis, thus developing a mouse bioassay model for bovine germ cell evaluation (Oatley et al., 2002). A polyclonal antibody was developed to recognize the bovine cells and, after two weeks, round bovine cells were still present within the mouse seminiferous tubules and appeared to have proliferated and begun colonization. Although spermatogenesis was not established, this still represented a first step in developing a transplant technique in bulls, which has great potential for preservation of male germlines in livestock in the future, helping to increase genetic gain in livestock herds and serving as an innovative means of generating transgenic animals (Oatley et al., 2002). This method was used to evaluate SSC content in the THY1⁺ cell population from the pre-pubertal bull testis, which showed a greater than 6-fold increase of germ cell colonies from THY1⁺ cells compared to the unselected total testis cell population (Reding et al., 2010). Although spermatogenesis did not result from these transplants, it was an important first step towards developing transplantations directly into livestock testes. There have been advances in utilizing this technique in other species as well. Transplantation of fluorescently labeled pig donor cells from 1- or 10-wk-old boar testes via ultrasound guided insertion of a catheter into the rete testis, representing the first attempt at direct transplantation in livestock (Honaramooz et al. 2002). Testes were evaluated for presence and localization of labeled cells for 4 weeks following transplantation and 10 of the 11 testes had fluorescently labeled cells within some of their seminiferous tubules (Honaramooz et al., 2002). The following year, this group published a similar study conducted in goats (Honaramooz et al., 2003a). Again, presence and localization of labeled donor cells were evaluated, this time for 12

weeks following transplantation. Labeled donor cells were found in 10-35% of the seminiferous tubules examined of all testes (Honaramooz et al., 2003a). Similar studies were conducted in rams and dogs (Rodriguez-Sosa et al., 2006; Kim et al., 2008) Transplantation in goats was reported to result in production of offspring with donor genetics which shows that transplantation of SSCs into livestock can reestablish spermatogenesis to a great enough extent to naturally sire offspring, however this process has not yet been reported in cattle (Honaramooz et al., 2003b). Development of methodologies for isolating and spg from donor bull testes and culture conditions that support long-term maintenance and expansion of these und spg are key to development of transplantation technology. If bovine und spg populations highly enriched with SSCs can be isolated and culture conditions which support their expansion are developed, transplantation will likely follow shortly after.

ISOLATION OF SPERMATOGONIAL STEM CELLS

SSCs compose approximately 0.03% of the adult mouse testis, representing a very rare cell population within the testis (Tegelenbosch and de Rooij, 1993; Oatley and Brinster, 2008). Therefore, to be effectively studied and utilized, a method to isolate SSCs must be devised. However, because specific markers of SSCs have not been described, only enriched populations of SSCs can be isolated. Enrichment for rodent SSCs can be accomplished in a variety of ways including FACS, MACS, experimental cryptorchidism and differential plating.

The most common methods for obtaining an SSC-enriched cell population involve sorting cells by their surface markers. There are two main approaches to cell sorting, fluorescence-activated cell sorting (FACS) and magnetic activated cell sorting (MACS). FACS involves the sorting of cells according to whether or not they are tagged with a fluorescent

antibody against a specific cell surface marker, such as THY1. Kubota et al. (2003) attained a 200-fold enrichment for mouse SSCs using FACS for THY1 (Kubota et al., 2003). Another study using FACS identified epithelial cellular adhesion molecule (Ep-CAM) as a phenotypic marker of neonate and pup rat testis cells and obtained greater than 70- and 10-fold enrichment for SSCs, respectively (Ryu et al., 2004). Currently, FACS is the most effective method for enriching a population for SSCs, achieving the purest SSC populations to date (Kubota et al., 2003). MACS, however, is a more time and cost-efficient procedure and is therefore more often utilized (Ebata et al., 2005). MACS also isolates cells according to their cell surface phenotype but uses antibodies conjugated to magnetic beads in place of fluorescence proteins. Cells are passed through a magnetic column and the cells with the desired phenotypic marker adhere to the column, thus being isolated from the rest of the cell population. Similar to FACS, MACS is also able to isolate SSC enriched fractions from testes of mice and rats based on expression of the cell surface markers THY1 and Ep-CAM as well as other markers such as CD9, α 6-integrin, and β 1-integrin (Shinohara et al., 1999, Kubota et al., 2004a). Of these markers, THY1 has shown greatest enrichment for SSCs, consisting of 1 in every 15 THY1+ cells in mice (Kubota et al., 2004a).

Experimental cryptorchidism, another method for enriching for SSCs, is the process whereby one or both testes are surgically translocated from the scrotum back through the inguinal ring into the abdominal cavity, from where they originally descended. The temperature within the abdominal cavity is 2-3°C higher than that of the scrotum and this slight temperature increase is enough to be detrimental to spermatogenesis, preventing the differentiation of spermatogonia, without affecting the SSCs (Shinohara et al., 2000a, 2000b). Reducing the number of differentiating and differentiated spermatogonia concentrates the SSCs and Shinohara

et al. (2000) demonstrated a 25-fold increase in colony number in animals transplanted with cells from experimentally cryptorchid mice compared to their controls (Shinohara et al., 2000a, 2000b). Experimental cryptorchidism is frequently used in conjunction with other selection methods to obtain even greater SSC enrichment.

Placing testis cell populations on various extracellular matrix proteins, termed differential plating, has also shown some potential for SSC enrichment. Because SSCs are bound to the basement membrane of seminiferous tubules in vivo, one study looked at the effects of laminin, fibronectin, and collagen IV, three extracellular matrix molecules, to assess whether SSCs would preferentially bind to them. They reported that, of the three, SSCs showed the greatest affinity for laminin, obtaining a 3-4 fold increase in colonies following transplantation compared to the controls (Shinohara et al., 1999). Although enrichment was not as high as in previous methods discussed, this method is also often utilized in addition to other selection methods (Guan et al., 2009).

Methods similar to those used in rodents for enriching SSCs were also attempted on bovine testis cells with variable results. Izadyar et al. (2002) developed a method for enriching type A spermatogonia involving overnight differential plating followed by discontinuous Percoll density gradient centrifugation. This process yielded a population of cells containing $73 \pm 2.9\%$ type A spermatogonia although transplantation analysis was not conducted to assess SSC content (Izadyar et al., 2002). Utilizing a modified procedure for rodent THY1 MACS isolations, Reding et al. (2010) examined the possibility of THY1 being a conserved marker of SSCs in the bull testis. Flow cytometric analysis confirmed a small sub-population of approximately 0.05% THY1⁺ cells from the pre-pubertal bull testis. THY1 MACS isolation was conducted to further characterize these cells and it was discovered that there was a greater than 15-fold enrichment for

und spg in the THY1⁺ cells when compared to the unselected bovine testis cells. Subsequent transplantation of THY1⁺ cells into the testes of immunodeficient nude mice resulted in a 6-fold enrichment for SSCs compared to testes injected with the unselected control population, confirming that THY1 is a conserved marker of SSCs in cattle and could be useful for isolation of SSC for in vitro expansion (Reding et al., 2010).

CULTURE OF SPERMATOGONIAL STEM CELLS

For years it was thought that SSCs were not viable in culture outside of their special niche microenvironment in the testis and, until the development of transplantation by Brinster et al. (1994), there was no functional assay to determine whether this was accurate.

Transplantation provided a way to assess stem cell potential, making it possible to investigate methods for cultivating SSCs in vitro. Although early studies showed in vitro culture of testicular cells which maintained spermatogonial-type morphology, the first reported culture system for SSCs was by Nagano et al. (1998), who were able to maintain mouse SSCs in vivo for 4 months (Smith et al., 1992; Kierzenbaum et al., 1994; Nagano et al., 1998). Being able to culture SSCs has opened a whole new resource for studying regulatory mechanisms for SSC fate decision. Culture conditions for SSCs, however, are very specific and require defined media, co-culture feeder cells, and specific growth factors for proper maintenance and expansion (Nagano et al., 2003; Kubota et al., 2004a, 2004b).

Most cells require serum supplementation to survive and proliferate in vitro (Hayashi and Sato, 1976). Therefore, early developments of SSC cultures contained serum. The first medium used for culture of mouse SSCs was DMEM with 10% FBS along with supplementation of glutamine, penicillin, and streptomycin (Nagano et al., 1998). These conditions maintained cells

for over 3 months but little proliferation was observed and colonization of recipient testes following transplantation was limited (Nagano et al., 1998). Later studies demonstrated that serum was detrimental to SSCs and that they could be better maintained in serum-free conditions without significant loss of SSCs for over a week (Kubota et al., 2004a; Ryu et al., 2005). Serum-free culture systems have also been devised for the rat (Ryu et al., 2005; Hamra et al., 2005). Culture systems without serum must rely more heavily on the addition of growth factor for cell survival and use of serum-free, defined conditions has facilitated the identification of external factors needed for in vitro maintenance of SSCs (Kubota et al., 2004a). Many growth factors have been examined over the last 15 years of culturing rodent SSCs including GDNF, FGF2, leukemia inhibitory factor (LIF), epidermal growth factor (EGF), stem cell factor (SCF), and CSF-1. Kubota et al. (2004) showed that GDNF enhanced mouse SSC cultures for 7 days and this same group showed that GDNF was required for long-term maintenance and expansion of mouse SSCs (Kubota et al., 2004a, 2004b). GDNF was also shown to support rat SSC cultures through enhancement of self-renewal demonstrated by increased numbers of colonies following transplantation analysis after up to 5 months in culture with GDNF compared to transplants of fresh isolated cells (Ryu et al., 2005). Another growth factor that was shown to benefit culture of mouse SSCs is FGF2, which enhances GDNF-induced SSC self-renewal, but cannot maintain SSC expansion in the absence of GDNF (Kubota et al., 2004b). One additional growth factor that has shown great enhancement of mouse SSC maintenance and proliferation is CSF-1. In vivo, this cytokine is secreted by Leydig and myoid cells to regulate self-renewal of SSCs. Cells enriched for SSCs are also enriched for CSF-1R and when its ligand was added to the culture medium, along with GDNF and FGF2, self-renewal of mouse SSCs was enhanced to a greater extent (Oatley et al., 2009).

Optimal *in vitro* conditions for long-term expansion of SSCs in the mouse also require a feeder cell monolayer for co-culture and an early study examined testis cell isolates on either mitotically inactivated STO cells, an immortalized embryonic fibroblast feeder layer or without a feeder layer (Nagano et al., 1998). This study showed that cultures plated onto STO feeders were better supported, showing greater maintenance of SSCs than those without any feeder cells (Nagano et al., 1998). Collectively, optimal conditions for mouse SSC cultures were determined to include mitotically inactivated STO feeder co-culture with serum-free media supplemented with GDNF, FGF2, and CSF-1 (Oatley et al., 2009).

Although defined culture conditions have been determined for *in vitro* maintenance and expansion of rodent SSCs, a system has not yet been defined that can support long-term culture of bovine SSCs. Multiple attempts to culture bovine SSC have been made over the last decade, with limited success, and much of what has been attempted has been adapted from the rodent culture system. Proliferative type A spermatogonia cultures were developed by Izadyar et al. (2002) which eventually resulted in differentiating spermatogonia, however, SSC content was not assessed by transplantation analysis (Izadyar et al., 2002). Bovine testis tissue explants were maintained in culture for a two week period and, following transplantation into immunodeficient nude mice, colony formation was observed, demonstrating survival of SSCs in culture for a 2 week period (Oatley et al., 2004a). Short-term survival and proliferation of testis cell isolates was also achieved using a bovine embryonic fibroblast feeder cell layers, termed BEFs, for co-culture (Oatley et al., 2004b). In the same study, the effect of GDNF supplementation to culture medium was shown to enhance SSC maintenance after one week of culture (Oatley et al., 2004b). Aponte et al. (2005) also investigated the effect of GDNF supplementation on bovine germ cells *in vitro* and showed that, although numbers were initially lower than the controls, the

GDNF- treated cultures had greater numbers of spermatogonia after 15 days and maintained this difference out to day 25 in culture. A number of other studies have reported culture of SSCs but have either used inappropriate markers or have not validated their cultured cells through transplantation analysis (Aponte et al., 2005, 2008; Fujjihara et al, 2011; Nasiri et al., 2012). Consequently, while short-term culture has been achieved, long-term culture conditions that support bovine SSC maintenance and proliferation remain elusive. Clearly, however, conditions that support long-term maintenance and expansion of rodent SSCs in vitro will continue to serve as the foundation for developing long-term maintenance of bovine SSCs.

SUMMARY AND CONCLUSIONS

Artificial insemination has been the primary means of genetic gain in dairy cattle populations over the last 50+ years. Unfortunately, the management practices of most beef cattle operations are not conducive to the application of this technology and only an estimated 5-7% of beef cows are currently bred with this technology. Transplantation of SSCs from superior sires into a group of recipient bulls would provide a new method for increased utilization of these superior genetics, producing a greater number of offspring with the desired traits. Use of this technique on beef cattle operations should also be much more conducive to the management practices in place on many of these operations because the recipient bulls could simply be turned loose with the herd for natural service.

Long-term culture of SSCs in conditions supporting self-renewal expansion followed by subsequent transplantation into testes of recipient bulls for reestablishment of spermatogenesis would provide a means to preserve and expand the male germline. It would also be an instrument for generating transgenic cattle through manipulation of SSCs prior to transplantation,

a technique which has already been accomplished in rodents (Nagano et al., 2003). Transgenic cattle have the potential to increased production efficiency and development of disease-resistant livestock could also be a possibility. The ability to culture bovine SSCs would provide a means for induction of desired genetic modifications, and is an important first step in the development of these reproductive technologies. Although culture systems are already available in rodent species, long-term culture of bovine SSCs has not yet been accomplished. Developing methods for efficient isolation of SSC-enriched bull testis cell fractions and identifying conditions that support long-term maintenance and proliferation of bovine SSCs in vitro would advance development of transplantation as a commercially viable option for cattle industries.

CHAPTER 2: IDENTIFICATION OF CONDITIONS SUPPORTING IN VITRO MAINTENANCE OF BOVINE UNDIFFERENTIATED SPERMATOGONIA

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INTRODUCTION

Spermatogonial stem cells (SSCs) are a small pool of cells residing within the testis that support spermatogenesis throughout adulthood due to their ability to self-renew and differentiate into spermatozoa (Oatley and Brinster, 2006). There are two main types of germ cells within the testis: undifferentiated spermatogonia and differentiating spermatogonia. The nomenclature for undifferentiated spermatogonia (und spg) varies between different species. In rodents and livestock that have been studied, und spg consist of A-single (A_s), A-paired (A_{pr}), and A-aligned (A_{al}) spermatogonia, with A_s cells being considered the SSCs. Transformation of und spg into differentiating spermatogonia and further differentiation into spermatozoa, while still maintaining a pool of A_s cells, is the basis for continual spermatogenesis throughout adulthood in males.

Although there are techniques such as embryo transfer for female germline expansion, genetic gain in livestock species is primarily made through the male germline. This is possible due to the vastly greater number of male gametes produced, in comparison with the female, as well as the ability of a male to naturally sire multiple offspring in one breeding season.

Additionally, widespread implementation of artificial insemination (AI) as a reproductive tool has further enhanced genetic expansion from desirable sires. This technique requires intensive management strategies to be successful and has, therefore, been utilized primarily in the dairy industry where animals are already handled daily and the working facilities needed for AI are already in place. This is not the case throughout most of the beef cattle industry, however, resulting in a greatly reduced use of AI (USDA, 1993). The ability to isolate, culture, and transplant SSCs into bulls would provide an alternative means for expanding superior genetics in beef cattle. An isolation method for the enrichment of SSCs from pre-pubertal bull testes using the cell surface marker thymus cell antigen 1 (THY1) has recently been describe and several attempts to maintain bovine SSCs in culture have been minimally successful for a short time but, presently, long-term culture of bovine SSCs has not been reported (Reding et. al., 2010; Oatley et.al., 2004b; Aponte et.al., 2005, 2008).

Because SSCs are such a rare population within the testis, comprising only ~0.03% of the total testis population, expansion of these cells in vitro is necessary to achieve sufficient colonization following transplantation (Brinster and Zimmerman, 1994; Tegelenbosch and de Rooij, 1993; Nagano et.al., 1998). Methods for expanding rodent SSCs have been established through specific culture conditions including serum-free media, supplementation with glial cell-line derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2), and co-culture with mitotically inactive STO feeder cells (Nagano et.al., 1998; Meng et. al., 2000; Kubota et. al., 2004a, 2004b). Subsequent transplantation of these cells into recipient mice, previously treated with busulfan to eliminate endogenous germ cells, resulted in donor-derived spermatogenesis, unequivocally demonstrating that the cultured cells were SSCs (Kubota et. al., 2004b). In vitro expansion of SSCs from genetically desirable bulls followed by transplantation

into multiple recipient bulls which could then breed multiple cows via natural service would provide an alternative approach to AI for expanding utilization of superior beef cattle genetics. Additionally, introducing genetic modifications to SSCs prior to their transplantation would be a technique for producing genetically engineered cattle, a technique which has already been achieved in rodents (Nagano et al., 2001; Ryu et al., 2007). Genetic modifications in cattle could be used to increase the efficiency of milk and meat production and could also contribute to the creation of disease resistant animals. Before these technologies can be utilized in cattle however, in vitro support for long-term maintenance and expansion of bovine und spg must be achieved. The culture system developed to expand the number of mouse SSCs in vitro serves as a model for identifying conditions that would support maintenance and proliferation of bovine und spg in vitro. In the present experiment, therefore, multiple growth factors and feeder cells were tested in short-term culture to examine the potential effects of each condition on long-term maintenance and expansion of undifferentiated spermatogonia.

MATERIALS AND METHODS

Cell Collection and Isolation

All animal procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee. Holstein bull calves between 12 and 16 weeks of age served as donors of testicular tissue for isolating spermatogonia. Animals were bilaterally castrated after washing the scrotum and surrounding area with betadine. Castration was achieved by removing the bottom inch of the scrotum with a sterile scalpel blade to expose the testes. Each testis was removed by manual stripping of the spermatic cord by hand, washed briefly in ethanol and then placed in HBSS on ice for transportation to the laboratory. Calves were monitored for several

minutes to ensure hemostasis. Using aseptic technique, a lateral incision was made into the testis capsule, exposing the parenchyma. Avoiding the rete testis, 4-5 pieces of tissue, each approximately weighing 50 mg, were removed with sterile scissors and forceps, mechanically disrupted with a sterile razor blade and then placed into collagenase solution (1 mg/mL collagenase type 4; Worthington Biochemical Corporation, Lakewood, NJ and 7 mg/mL DNase Sigma-Aldrich Co., St. Louis, MO) to begin the first of a two-step, enzymatic digestion. The tissue was incubated at 37°C for 30-40 minutes, with agitation every 5 minutes to facilitate the separation of the seminiferous tubules from the interstitial tissue. Once the seminiferous tubule fragments were visibly separated from the interstitial tissue, they were pelleted by brief centrifugation at 50 x g for 1 minute and the supernatant, containing interstitial cells, was discarded. The seminiferous tubule fragments were then washed with Hank's balanced salt solution (HBSS) and re-centrifuged three additional times to remove all remaining interstitial cells. The seminiferous tubules were then digested to obtain a single cell suspension of Sertoli cells and germ cells by incubating the tubules in a Trypsin-EDTA solution (0.25% Trypsin/2.21 mM EDTA; Cellgro Mediatech Inc., Manassas, VA) containing DNase (7 mg/mL) for 15 minutes at 37°C, with agitation by trituration every 5 minutes. Following digestion, cells were passed through a 40 µm cell strainer (BD Biosciences, Durham, NC) to remove clumps and centrifuged at 600 x g for 7 minutes to pellet cells. Cells were then re-suspend in DPBS-S (PBS with 1% fetal bovine serum (FBS), 10 mM HEPES, 1x10⁴ U/mL penicillin, 1x10⁴ µg/mL streptomycin, 1 mM sodium pyruvate and 1 ng/mL glucose) and cell concentration was determined using a hemocytometer. Cells prepared for THY1 selection were concentrated at 1x10⁷ cells/100µl and cells prepared for multi-parameter selection were concentrated at 1x10⁷ cells/5mL.

Cell Selection

Multiple procedures were utilized to enrich testis cell isolations for SSCs. The first method was selection via the cell surface marker, THY1. Aliquots of 1×10^7 cells were incubated with mouse anti-human THY1 antibody (1:10 dilution of a 0.11 mg/mL stock; Abcam, Cambridge, MA) for 20 minutes at 4°C. Cells were then washed 2 times with DPBS-S by centrifugation for 7 minutes at 600 x g, followed by incubation with a biotinylated goat anti-mouse antibody (1:100 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C for 20 minutes. Cells were again washed 2 times in DPBS-S with centrifugation and then incubated with streptavidin conjugated to magnetic microbeads (1:10 dilution; Miltenyi Biotec, Auburn, CA), for 20 minutes on ice. Cells were subjected to another 2 washes in DPBS-S using centrifugation and THY1⁺ cells were collected by magnetic activated cell sorting (MACS) (Miltenyi Biotec). Typical yields of $1-2 \times 10^5$ THY1⁺ cells were isolated from 200-300 mg of testis tissue.

The other method employed to enrich the testis cell isolate for SSCs was a multi-parameter technique involving gradient centrifugation and differential plating. Aliquots of 1×10^7 cells in 5 mL of DPBS-S were slowly layered over 2mL of a 30% Percoll gradient [phosphate-buffered saline (PBS) with 1% FBS, penicillin (50 U/mL), streptomycin (50 µg/mL), and 30% Percoll] in 15-mL conical tubes and then centrifuged at 600 x g for 8 minutes. The supernatant was removed and the pellets were washed, combined, and collected via centrifugation at 600 x g for 7 minutes. Cells were then placed on gelatin-coated wells at $1-2 \times 10^6$ cells/well of a 6-well plate and incubated at 37°C overnight. On the next day, the non-adherent germ cells were collected following gentle pipetting, washed, and resuspended in appropriate medium for analysis. Typical yields of $2-3 \times 10^6$ multi-parameter cells were isolated

from 200-300 mg of testis tissue. For comparison, single cell suspensions not subjected to THY1 MACS isolation or Percoll selection (referred to as unselected total testis cell populations) were collected from identical tissue used for THY1⁺ and Percoll cell isolation.

Cell Culture

Isolated cells were maintained on bovine embryonic fibroblast (BEF) or bovine somatic cell (BSC) monolayers that were mitotically inactivated by mitomycin-C treatment (Sigma-Aldrich). Cultures were maintained in StemPro media (Invitrogen) supplemented with different growth factor treatment conditions including recombinant human Glial cell line-derived neurotrophic factor (GDNF; 20 ng/mL of media; R&D Systems; Minneapolis, MN), recombinant human Fibroblast growth factor 2 (FGF2; 1 ng/mL of media; BD Biosciences; San Jose, CA), recombinant human Colony stimulating factor 1 (CSF-1; 10 ng/mL of media; R&D Systems; Minneapolis, MN), and Stromal derived factor 1 (SDF-1; 10 ng/mL of media; R&D Systems; Minneapolis, MN). Cells were cultured at 37°C in humidified incubators with an air atmosphere containing 5% CO₂, and media with growth factors was changed every other day

Creation of Feeder Cells

Two different feeder cell monolayers were developed for co-culture with the und spg. The BEF cell line was created from a Day 35 bovine embryo following previously described procedures for creation of embryonic fibroblast feeder cell lines (Flodby et. al., 2001; Oatley et. al., 2004b). Briefly, an E35 bovine embryo was collected into physiological saline and minced into small pieces with a sterile razor blade. The chopped tissue was then transferred to a conical tube containing a 10-mL solution of Hanks balanced salt solution (HBSS) with 0.05% trypsin

and 1 mM EDTA and incubated at 37°C for 15 minutes. Tissue was then allowed to settle on ice for 2 minutes followed by passing the suspension through a 14-gauge needle several times. Another 10 mL of enzyme solution was added and incubated again at 37°C for 15 minutes. The suspension was again allowed to settle on ice for 2 minutes, and the supernatant was removed for cell collection by centrifugation at 300 x g for 5 minutes. Collected cells were resuspended in culture medium (DMEM with 10% FBS, 30 mg/mL penicillin, and 50 mg/mL streptomycin), maintained at 37°C, and frozen stocks were created. Bovine somatic cell (BSC) lines were created using a modified procedure for multi-parameter testis cell selection. Following overnight differential plating on gelatin, the germ cells remained largely suspended in the media while the somatic cells adhered to the gelatin-coated well. After the germ cells were removed for further selection, the adherent cells were collected and it was these cells that were used as a Sertoli cell feeder.

Apoptosis Analysis of THY1 and Multi-parameter Selected Testis Cells

The percentage of cells undergoing apoptosis was assessed using the Guava Nexin kit and the Guava PCA system (Guava Technologies, Hayward, CA). The Guava Nexin assay utilizes two stains (Annexin V and 7-amino actinomycin D [7-AAD]) to quantify the percentage of apoptotic cells. Cells were selected using the THY1 and multi-parameter selection methods and incubated overnight at 37°C in StemPro media. The following day, cells were collected and diluted to 1×10^5 cells/well as per the manufacturer's recommendations. The Guava Nexin™ reagent was added to the cells in a 96-well plate and incubated on ice in the dark for 20 minutes prior to analysis on the Guava PCA system.

Immunohistochemical Analysis of PLZF, SOX9, CSF-1R, and CXCR4 Expression in the Bovine Testis

Bovine testis tissue was fixed in Bouin's solution overnight followed by 70% ethanol washes the following day. Tissue was then dehydrated, embedded in paraffin, and sectioned at a thickness of 7- μ m, which were then adhered to glass slides. Tissue was then deparaffinized in xylene and rehydrated with a descending series of gradient ethanol and water incubations. Antigen retrieval was accomplished by boiling the slides in citrate buffer for 20 minutes. For colorimetric staining, sections were blocked for endogenous peroxidase by incubating in 0.03% hydrogen peroxide and biotin activity using a commercial biotin blocking kit (Endogenous Biotin-Blocking Kit; Invitrogen). Slides were then incubated for 1 hour in 10% normal goat serum to block nonspecific antibody binding followed by overnight incubation with the primary antibody at 4°C. The following day, sections were washed in PBS and then incubated with the secondary antibody for 2 hours at room temperature. For colorimetric staining, sections were washed again in PBS, incubated with the Vectastain ABC Kit reagents (Vector Labs), developed with a horseradish peroxidase (HRP)-conjugated streptavidin kit (Vector Labs) and counterstained with hematoxylin. For immunofluorescence staining, the tissue was washed in PBS and then a glass coverslip was mounted on the tissue section using aqueous Dapi-containing medium (Invitrogen, Inc.). Staining for PLZF expression was accomplished using a rabbit anti-human PLZF polyclonal antibody (Santa Cruz Biotechnology) at 1:100 dilution followed by a goat anti-rabbit biotinylated secondary (1:250 dilution; Santa Cruz). Detection of SOX9 protein was achieved using a rabbit anti-human SOX9 polyclonal antibody at a concentration of 1:50 (Abcam) followed by a goat anti-rabbit biotinylated secondary (1:250 dilution; Santa Cruz). CSF-1R protein in cells was identified using a rabbit anti-human CSF-1R polyclonal antibody at

a concentration of 1:150 (Abcam) followed by a goat anti-rabbit biotinylated secondary (1:300 dilution; Santa Cruz). CXCR4 expression was identified by staining with a rabbit anti-human CXCR4 polyclonal antibody used at 1:10 dilution followed by a goat anti-rabbit IgG secondary antibody conjugated to Alexa 488 (1:300; Invitrogen, Inc).

Immunocytochemistry of PLZF and SOX9 Expressing Testis Cells

Immunocytochemical staining for PLZF was conducted to determine the enrichment of und spg following multi-parameter isolation and maintenance of und spg in various culture conditions. Multi-parameter selected cells were adhered to glass cover slips followed by fixation in 4% paraformaldehyde or fixed directly within the well of a 24-well plate for 10 minutes at room temperature followed by a 5-minute incubation in methanol at -20°C to permeabilize the cells. Nonspecific antibody binding was blocked by incubating cells in 10% goat serum for 1 hour at room temperature. Cells were then incubated with rabbit anti-human PLZF antibody (1:100 dilution; Santa Cruz Biotechnology Inc.) in PBS with 0.05% BSA and 0.1% Triton X-100 at 4°C overnight. On the next day, cells were washed 3 times with PBS and incubated with Alexa 488 conjugated goat anti-rabbit secondary antibody (1:200 dilution; Invitrogen Corporation) for 2 hours. Cells were again washed 3 times with PBS and incubated with Dapi for 3 minutes to stain cell nuclei. Cells were then visualized by fluorescence microscopy at 100X magnification. For each replicate sample, the total number of PLZF⁺ cells in 5 random low power fields of view was counted. The number of PLZF⁺ cells were counted for three separate experiments, averaged, and reported as PLZF⁺ cells per 10⁵ cells originally plated. To determine an average percentage of PLZF⁺ cells present immediately after multi-parameter isolation, total cell numbers were also counted in each field based on Dapi staining of all cell nuclei and the average percentage of

PLZF⁺ cells was determined by dividing the number of PLZF⁺ cells by the number of Dapi stained nuclei in each field. Immunocytochemical analysis of the BSC feeder cell monolayers was conducted to examine Sertoli cell content, using a rabbit anti-human SOX9 polyclonal antibody at a concentration of 1:50 (Abcam). The percentage of Sertoli cells was calculated by dividing the number of SOX9⁺ cells by the total Dapi stained nuclei.

Quantitative RT-PCR Analysis of *Plzf*, *Ret*, *Gfra1*, *Fgfr2*, *Csf-1r* and *Cxcr4* Gene Expression

Total cellular RNA was extracted from cells using TRIzol reagent (Invitrogen; Carlsbad, CA), DNase treated with a DNase-free kit (VWR) to remove any contaminating DNA and reverse transcribed with the SuperScript III First-Strand Synthesis System (Invitrogen) to get complementary DNA (cDNA). The cDNA was examined for relative abundance of *Plzf*, *Ret*, *Gfra1*, *Fgfr2*, *Csf-1r*, and *Cxcr4* by quantitative real time PCR (qRT-PCR) with SYBR Green (Platinum SYBR Green qRT-PCR SuperMix-UDG w/ROX; Invitrogen; Carlsbad, CA). Primers specific for *Plzf*, *Ret*, *Gfra1*, *Fgfr2*, *Csf-1r*, and *Cxcr4* were designed using Primer Express III software (Applied Biosystems) and Primer-BLAST (NCBI; Table 1).

To make quantitative comparisons between samples, the gene expression of *Plzf*, *Ret*, *Gfra1*, *Fgfr2*, *Csf-1r*, and *Cxcr4* were each normalized to expression of Ribosomal protein S2 (*Rps2*), a constitutively expressed transcript. Assays were conducted with the following cycle settings: 50°C for 2 minutes, 95°C for 5 minutes and 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds followed by a dissociation stage and data were collected on the 7500 Fast Real-Time PCR System (Applied Biosystems). Comparisons were made by normalizing each gene expression to that of *Rps2* using the formula: relative transcript abundance = $0.5^{(CT \text{ of gene of interest} - CT \text{ of } Rps2)}$ (Oatley et al., 2007).

Western Blot Analysis of PLZF Protein Expression

Western blot analysis was used to determine whether PLZF protein was enriched in the multi-parameter selected testis cells. Protein from the total cell lysate was obtained from multi-parameter selected and unselected cells using M-PER extraction reagent (VWR) immediately after isolation. Proteins (50 μ g) were separated using SDS-PAGE (Invitrogen) and then transferred to nitrocellulose membranes for downstream analysis. Membranes were blocked in TBS-T (10 mM Tris, pH 7.4, 100 mM NaCl, and 0.1% Tween-20) containing 5% nonfat dry milk for 2 hours and then incubated with gentle rocking overnight at 4°C with rabbit anti-human PLZF antibody (1:500 dilution; Santa Cruz Biotechnology Inc.) in TBS-T with 2% nonfat dry milk. The following day, membranes were washed in TBS-T and incubated with an HRP-conjugated goat anti-rabbit IgG polyclonal antibody (1:2000 dilution; Santa Cruz Biotechnology) for 1-2 hours at room temperature with gentle rocking followed by washing in TBS-T at room temperature. Proteins were detected by incubation with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and images acquired with a ChemiDoc XRS imager (Bio-Rad). Digital images were captured for further analyses and blots were stripped followed by incubation with rabbit anti-human tubulin-beta antibody (1:10,000 dilution; Novus Biologicals) to assess loading for each sample. Secondary detection included incubation with HRP conjugated goat anti-rabbit IgG and development with chemiluminescent substrate (Thermo Scientific). PLZF band density for each sample was normalized to corresponding density of β -tubulin using Quantity One analysis software (Biorad Laboratories).

Statistical Analyses

All numerical data are presented as the mean \pm standard error of the mean (SEM) for three replicate experiments. Differences between means were determined using the independent t-test function of SPSS version 17.0 statistical software package (IBM Corporation; Chicago, IL), and effect of treatment was determined using a general linear model with the one-way ANOVA. Multiple comparisons were conducted using the LSD post-hoc test for significance. A P-value of < 0.05 was considered significant.

RESULTS

Expression of PLZF is localized specifically to undifferentiated spermatogonia in testes of pre-pubertal bulls.

A marker was needed to assess enrichment and survival of und spg throughout this study. Immunohistochemical staining of PLZF in pre-pubertal bull testis cross-sections was evaluated to determine if its expression was restricted to the A_s , A_{pr} , and A_{al} spermatogonia in the bovine. The cross-sections showed staining exclusively in the large, round, und spg (Fig. 1A). The testis cross-sections which received normal goat IgG as a negative control showed no background staining, thus demonstrating that PLZF was a specific marker for und spg within the bovine testis (Fig. 1B).

Multi-parameter selection produces a cell fraction from testes of pre-pubertal bulls that is enriched for undifferentiated spermatogonia

In preliminary studies, we found that THY1⁺ testis cells isolated by MACS did not survive longer than 2 days in vitro (data not shown) and, upon further examination, it was determined that ~ 70% of the cells were dead or undergoing apoptosis 18 hours after isolation (Fig. 2A). Therefore, other methods for enrichment of und spg were investigated to find a procedure that would be less detrimental to the cells. A discontinuous Percoll density gradient centrifugation followed by differential plating, which had been used to isolate rodent SSCs and other types of stem cells previously, was examined for viability and enrichment of bovine und spg (Oatley and Brinster, 2008; Fong et. al, 2009; Kaul et. al., 2010). A protocol was developed using a 30% Percoll gradient followed by differential plating on gelatin overnight, which was referred to as a multi-parameter approach. Examination of multi-parameter cells by immunocytochemical analysis revealed that 47.1±8.53 % (Mean ± SEM, n= 3) of the cells stained for PLZF expression; whereas, only 7.3±5.6 % in the unselected total testis cell population was PLZF+ (Fig. 2B). Thus, the multi-parameter approach resulted in a 6.4-fold enrichment of und spg. In accordance, relative abundance of the *Plzf* transcript was found to be increased by 6.5-fold in the multi-parameter cells compared to the unselected population (P<0.05; Fig. 2C). Furthermore, Western blot analysis revealed that PLZF protein expression is also significantly enriched in the multi-parameter cell fraction compared to the unselected total testis cell population (Fig. 2D and E).

Bovine undifferentiated spermatogonia co-cultured with BEFs and supplemented with GDNF and FGF2 form clumps morphologically similar to mouse undifferentiated spermatogonia clumps

Our results indicate that multi-parameter isolated cells co-cultured with BEFs in StemPro media supplemented with GDNF and FGF2 form clumps that are morphology identical to cultures of mouse SSCs (Fig. 3A). Using RT-PCR and immunofluorescent staining, we confirmed that the clumps expressed *Plzf* mRNA (Fig. 3B) and PLZF protein (Fig. 3C) indicating they were indeed und spg. The bovine germ cell clumps could be maintained in vitro for several weeks but the number dramatically declined after 3 weeks (Fig 3D).

Sertoli-enriched somatic feeder cell monolayers from the bull testis enhance in vitro maintenance of undifferentiated spermatogonia

Our initial bovine und spg cultures used bovine embryonic fibroblast cell monolayers (BEF) and we observed clump formation early on, however, these declined over a 3 week period (Fig. 3A and Fig. 3D). In an attempt to maintain these clumps for a longer duration, a Sertoli cell line was developed to evaluate its potential benefit on SSC maintenance and proliferation. It was hypothesized that Sertoli cells might be more beneficial for co-culture with bovine und spg as they would provide germ cells with an environment that more closely resembled the in vivo surroundings. A marker specific for bovine Sertoli cells has not been reported in the literature but previous studies revealed SOX9 as a marker of Sertoli cells in mice and birds (Kent et. al., 1996; Morias da Silva et. al., 1996), but whether bovine Sertoli cells express SOX9 is not known. Here we used immunohistochemistry to characterize the expression pattern of SOX9 in

the pre-pubertal bull testis and found SOX9 expression to be restricted to the Sertoli cells, confirming SOX9 as a specific marker of Sertoli cells in the pre-pubertal bovine testis (Fig. 4A).

Seminiferous tubules contain two main cell types: Sertoli cells and germ cells. Cells isolated from seminiferous tubule, therefore, should only contain these two cell types, which can be separated using a modified procedure for multi-parameter testis cell selection. Following differential plating, the non-adherent germ cells were removed and the remaining adherent cells were collected. We used immunocytochemical staining for SOX9 expression to characterize the identity of the adherent cell population and demonstrated that SOX9⁺ cells represented a majority of the population, comprising $75.95 \pm 2.37\%$ (Mean \pm SEM, n= 3) of the adherent cells (Fig. 4B). Due to their heterogeneous identity, however, we referred to these cells as bovine somatic cells (BSCs). To evaluate the effect of BSCs on in vitro maintenance of und spg, we co-cultured equal numbers of multi-parameter isolated germ cells with BEFs or BSCs for 7 days followed by immunocytochemical staining for PLZF to evaluate the number of und spg maintained on BSCs compared to BEFs. There were 69.67 ± 31.39 PLZF⁺ cells (Mean \pm SEM, n=3) for cultures maintained on BSCs compared to 30.33 ± 10.48 und spg maintained on BEFs (Fig. 5). Statistical analysis showed that BSCs increase the number of und spg ($P < 0.03$) when compared to BEFs.

CSF-1R and CXCR4 are expressed by undifferentiated spermatogonia

We investigated whether bovine und spg cultures could be effectively maintained with the addition of GDNF and FGF2 and, although initial clump formation was evident, the cells slowly died over a 3 week period and long-term maintenance was not possible. Therefore, we conducted a microarray analysis of multi-parameter isolated and unselected total testis cells from pre-pubertal bulls to identify specific genes whose expressions was enriched in und spg. We

searched for enriched expression of growth factor receptors whose corresponding ligands could potentially support long-term maintenance of und spg in culture. The search revealed *Csf-1r* and *Cxcr4*, two receptors that had 39.4 and 40.1-fold transcript enrichment in the multi-parameter cells, respectively. CSF-1R, the receptor for CSF-1, was recently shown to be enriched in the THY1⁺ mouse cells and that addition of CSF-1 to mouse cultures significantly enhanced SSC self-renewal (Oatley et. al., 2009). To characterize the expression of CSF-1R in the bovine testis, immunohistochemical staining for CSF-1R expression was examined in pre-pubertal bull testis cross-sections. CSF-1R⁺ cells were detected within the seminiferous tubules, specifically in the und spg, as well as in the interstitial space, which was expected since macrophages located in the interstitial space are known to express CSF-1R (Cohen et. al., 1996; Fig. 6A).

The other growth factor receptor identified during the microarray scan, which had the greatest enrichment between the multi-parameter and unselected cells, was CXCR4, the receptor of stromal cell-derived growth factor 1 (SDF-1/Cxcl12). SDF-1 is a chemoattractant agent known to be involved in the migration of PGCs to the genital ridge during embryonic development and therefore PGCs express CXCR4 (Molyneaux et. al., 2003). Because PGCs are the precursors of SSCs, we examined whether bovine und spg also express CXCR4. Immunohistochemical analysis of CXCR4 expression in pre-pubertal bull testis cross-sections showed localization of the receptor to the und spg and absent in the control (Fig. 6B). Quantitative PCR also showed a higher ($P < 0.05$) relative abundance of *Cxcr4* gene expression in the multi-parameter isolated cells than the unselected total testis cell population (Fig. 6C).

Addition of growth factors CSF-1 and SDF-1 enhance survival of undifferentiated spermatogonia in vitro

To examine the effects of CSF-1 on und spg maintenance in vitro, we set up cultures of equal numbers of multi-parameter isolated cells supplemented with GDNF, GDNF + FGF2, and GDNF + FGF2 + CSF-1. After 7 days, immunocytochemical staining for PLZF expression was conducted to evaluate and compare the number of und spg maintained under the different culture conditions. Cultures supplemented with GDNF + FGF2 + CSF-1 had 60.33 ± 13.86 PLZF+ cells (Mean \pm SEM, n=3), a greater number ($P < 0.05$) compared to GDNF alone and GDNF + FGF2, which had 29.33 ± 3.48 and 29.33 ± 5.24 PLZF+ cells, respectively (Fig. 7A). Next, the effect of SDF-1 on maintenance of bovine und spg in culture was tested by comparing cells cultured with GDNF, FGF2 and CSF-1 to cultures also supplemented with SDF-1. Immunocytochemical staining for PLZF expression was conducted on the cells after a week in culture and we found that cultures which received SDF-1 had 145.33 ± 16.15 PLZF+ cells (Mean \pm SEM, n= 3), a greater number ($P < 0.05$) compared to cultures with only GDNF, FGF2, and CFS-1, which had 82.67 ± 8.67 PLZF+ cells (Fig. 7B). Additional cultures of the multi-parameter isolated cells receiving GDNF, FGF2, CSF-1 and SDF-1 were maintain for a second week and, although fewer number of PLZF + cells were observed at this time, there were still a few PLZF+ clumps present (Fig. 7C).

DISCUSSION

A long-term research goal of this lab is to utilize the unique characteristics of SSCs to enhance the production of spermatozoa from bulls for use in commercial cattle operations to

improve production efficiency. This study was focused on identifying conditions which would support long-term in vitro maintenance and expansion of und spg from the pre-pubertal bull testis. Long-term, in vitro maintenance of rodent SSCs was previously reported, and these culture conditions serve as a foundation for investigating conditions which would support bovine SSCs (Nagano et al., 1998). An in vitro system which supports survival and expansion of bovine SSCs will facilitate future study of the in vivo mechanisms of SSC self-renewal and differentiation and will also allow for utilization of these cells to enhance genetic progress in animal agriculture. For beef cattle operations, culture of SSCs could be employed as a means of increasing genetic gains in livestock as a more practical alternative to AI. Transplanting SSCs from superior bulls into multiple recipient bulls would facilitate distribution of these genetics to multiple offspring by natural service, providing an alternative to the labor-intensive process of AI.

Previous studies showed that bovine spermatogonia containing SSCs can be maintained for a short time in culture (Oatley et al., 2004b; Aponte et al., 2008; Fujihara et al., 2011); however, until now, maintenance of bovine SSC colonies which are morphologically similar to those present in rodent cultures have not been reported. In this study we showed that isolation of pre-pubertal bull testis cells using a multi-parameter approach, followed by culture in StemPro media supplemented with GDNF, FGF2, CSF-1, and SDF-1, and maintained in co-culture with BSCs, supports the formation of und spg clumps that are similar in morphology to rodent SSC cultures. Our approach was to conduct week-long studies with different culture parameters and then evaluate survival of und spg by PLZF immunocytochemical analysis on day 7. PLZF is a protein required for mammalian SSC self-renewal, and its expression is restricted to the A_s , A_{pr} , and A_{al} spermatogonia in mice (Buass et. al., 2004; Costoya et. al., 2004). PLZF is also

expressed exclusively by undifferentiated spermatogonia in non-human primates and was recently demonstrated to be expressed by a subpopulation of spermatogonia within seminiferous tubules of pre-pubertal bulls (Hermann et. al., 2007; Reding et. al., 2010). We showed that PLZF expression is localized specifically to the und spg in the pre-pubertal bull testis and, therefore, is an effective marker for identifying and evaluating enrichment for bovine und spg cells. We postulated that any conditions or factors which enhanced und spg cell maintenance over this short period would translate to conditions which would promote a long-term culture system.

Several conclusions were made during the preliminary trials for this study. First, although the most efficient method used to enrich for rodent SSCs is through MACS isolation with the cell surface marker THY1 (Oatley and Brinster, 2006) and our lab previously showed that THY1⁺ cells from the bovine testis are also enriched for und spg (Reding et al., 2010), the process by which these cells are isolated was detrimental to cell viability. A bovine-specific, anti-THY1 antibody conjugated to a magnetic microbead was not available for MACS. Therefore, we used Streptavidin conjugated magnetic microbeads to capture the THY1⁺ cells. This increased the amount of time and cell handling for SSC enrichment, compared to the rodent. Furthermore, the antibodies contained sodium benzoate, a preservative which is toxic to cells. Therefore, a different method for obtaining und spg enrichment had to be devised. Previous studies had reported a multi-parameter approach to isolate testis cells, involving differential plating and a discontinuous Percoll density gradient centrifugation (Izadyar et. al., 2002; Goel et al., 2010), for which a modified procedure was used. Examination by immunohistochemistry, qRT-PCR, and Western blot all showed enrichment for und spg. Collectively, these findings demonstrate that multi-parameter selection is an effective means for isolating a cell fraction

enriched for und spg from testes of pre-pubertal bulls and we utilized this isolation technique for all future cultures. Our preliminary studies further showed that of several different media tested, including mouse serum-free medium, rat serum-free medium, and StemPro, a commercial medium used for mouse embryonic stem cell cultures, StemPro was the only medium that supported maintenance of bovine und spg over a one week period (data not shown). Thus, all further experiments were conducted with StemPro.

Initial rodent SSC cultures were successfully established on STO cells, which are a mouse embryonic fibroblast cell line (Nagano et. al., 1998). Therefore, using the conditions of the rodent system as a foundation for developing culture condition which support bovine SSCs, an embryonic fibroblast cell line of bovine origin was created and utilized as co-culture cells for bovine SSC cultures (Oatley et. al., 2004b). Although BEFs were able to support formation of bovine clumps similar in morphology to the mouse SSC clumps, clump numbers were limited and diminished over a three-week period. In an attempt to improve and sustain these clumps, co-culture with testicular somatic cells containing mostly Sertoli cells was examined. We postulated that mimicking the in vivo environment of und spg as closely as possible, by culturing the und spg in close proximity to Sertoli cells, would provide the optimal microenvironment for und spg support. BSCs did enhance the maintenance of und spg, with higher numbers of PLZF+ cells after one week, but could not maintain the cells for any longer period of time than the BEFs.

In vivo, the stem cell population is influenced by a number of extrinsic and intrinsic regulators and growth factors which impact SSC fate decisions. Identifying and characterizing which of these factors are essential for self-renewal and differentiation of SSCs is vital to developing an in vitro system that effectively supports these cells. GDNF, a product of Sertoli

cells, plays a role in SSC self-renewal and is required for in vitro maintenance and self-renewal of SSCs (Meng et. al., 2000; Tadokoro et. al., 2002; Kubota et. al., 2004a, 2004b). Also, multiple studies have shown that supplementation of FGF2, in addition to GDNF, further enhances self-renewal proliferation of rodent SSCs (Kubota et. al., 2004b; Kanatsu-Shinohara et al., 2008). Because und spg were still not able to be maintained for greater than a few weeks with these two growth factors alone, we conducted microarray analysis to identify growth factors which may promote self-renewal of bovine SSCs and determine if addition of one or more of these growth factors would make long-term culture feasible. A similar approach was used successfully by Oatley et al. (2009). Identification of CSF-1R and CXCR4, two growth receptors with approximately 40-fold greater expression in the multi-parameter isolated cells, strongly indicated that addition of the growth factor ligands, CSF-1 and SDF-1, would enhance maintenance of bovine und spg. After determining, by immunohistochemistry, that CSF-1R and CXCR4 were expressed by und spg, we supplemented our cultures with CSF-1 and SDF-1 to determine their effect to long-term maintenance of bovine und spg in vitro. The results clearly indicated that both CSF-1 and SDF-1 enhanced the maintenance of und spg during the first week in culture and PLZF⁺ clumps were still present after a second week but, similar to what was seen in all previous experiments, these cells could not be maintained past three weeks.

Collectively, this study demonstrates a method for isolating viable bovine testis cells enriched for und spg and identifies a medium, a feeder cell for co-culture, and a cocktail of growth factors which significantly enhance survival of und spg over a one week period compared to previously reported conditions for cultures of these cells (Oatley et al., 2004b). Unfortunately, these cells diminish in culture over time and cannot be maintained for greater than three weeks, indicating that one or more essential components required for self-renewal of bovine SSCs is still

lacking in this current system. This may be an additional growth factor from the list of enriched growth factor receptors identified by our microarray analysis or from other factors, such as leukemia inhibitory factor and insulin like growth factor 1, which have previously been examined in mice (Kanatsu-Shinohara et al., 2007; Kubota et al., 2004b). Future studies must identify these missing factors if long-term culture for bovine und spg is to be accomplished. Although this study did not develop a complete formula for long-term culture of bovine und spg, it has provided greater knowledge of conditions which support maintenance and self-renewal of SSCs and should be a useful next step in moving forward with this research.

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APPENDIX

Table 1. Quantitative PCR primer nucleotide sequences

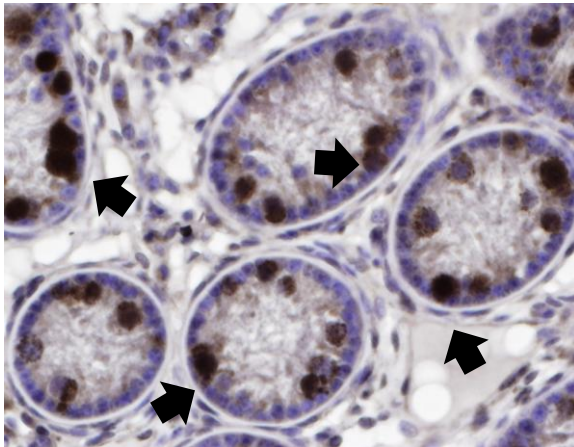
Gene	Primer	Sequence
<i>Plzf</i>		
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	R	CTGGAATGCTTCGAGATGAAGA
<i>Ret</i>		
	F	TTCCTCGGGGGCGTCGACAG
	R	GGCTGGCACCACGTCTGCAT
<i>Gfra1</i>		
	F	CTTCCAGCCACATAACCACAAA
	R	GCCAGCAGTGGGCTCAGA
<i>Fgfr2</i>		
	F	TGCGTGGTGGAGAACGATTAC
	R	GTGGTGATCGCTCAACAACGT
<i>Csf-1r</i>		
	F	ACTCTGCCCTCCGTCTTTTCTC
	R	GCCTCCTATAGATTCCCCCATT
<i>Cxcr4</i>		
	F	AGTGTGTTGGCTGTGGCAGCA
	R	CGGGGGCTACCGGCTACTCA

Fig. 1. Expression of PLZF is localized specifically to undifferentiated spermatogonia in testes of pre-pubertal bulls.

A) Representative image of immunohistochemical staining for PLZF in cross-sections from a 4 mo old bull calf (20X magnification). Staining is specifically localized to Und Spg (arrows). B) Representative image of negative control staining which included incubation with normal rabbit IgG (20X magnification).

A

PLZF



B

Control

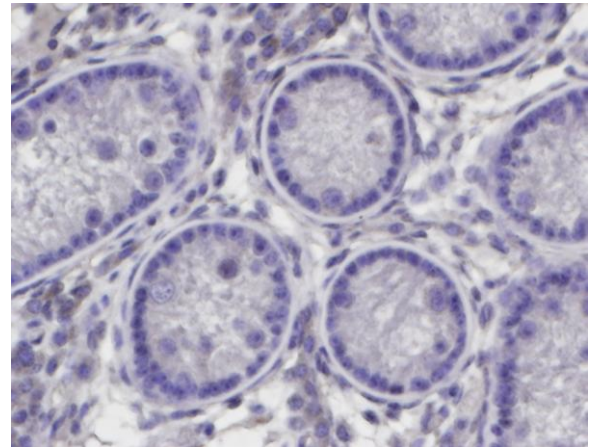


Fig. 2. Multi-parameter selected cells from the bull testis are highly enriched for undifferentiated spermatogonia.

A) Percentage of apoptotic cells in THY1 and multi-parameter (MP) testis cell populations. Data are reported as the mean \pm SEM for three separate cell isolations from three different bulls. * denotes significantly different at $P < 0.05$. B) Immunocytochemical analysis for the percent of PLZF+ cells in MP selected cells and the unselected total testis cell population. Data are reported as the mean \pm SEM for three separate cell isolations from three different bulls. * denotes significantly different at $P < 0.05$. C) qRT-PCR analysis for relative *Plzf* gene expression in multi-parameter selected and unselected total cell populations. Data are reported as the mean \pm SEM for three separate cell isolations from three different bulls. * denotes significantly different at $P < 0.05$. D) Representative image of a Western blot analysis for PLZF (top panel) and β -tubulin (bottom panel) protein expression in multi-parameter selected and unselected total testis cell populations. E) Quantitative comparison for Western blot analyses for PLZF protein expression. Data are reported as the mean \pm SEM for three separate cell isolations from three different bulls. * denotes significantly different at $P < 0.05$.

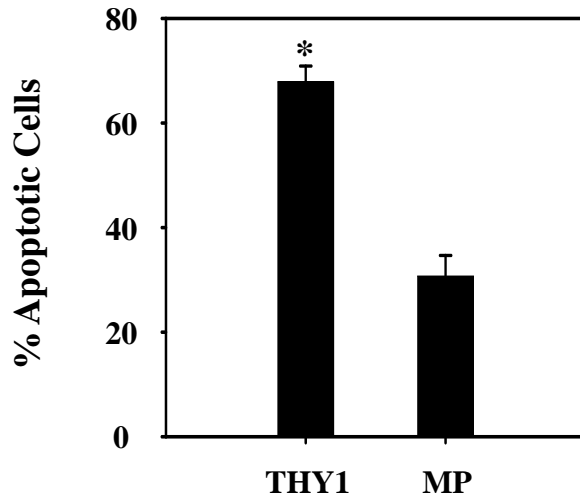
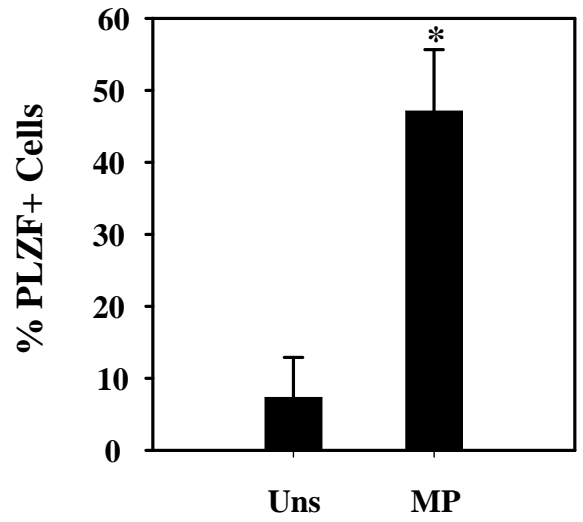
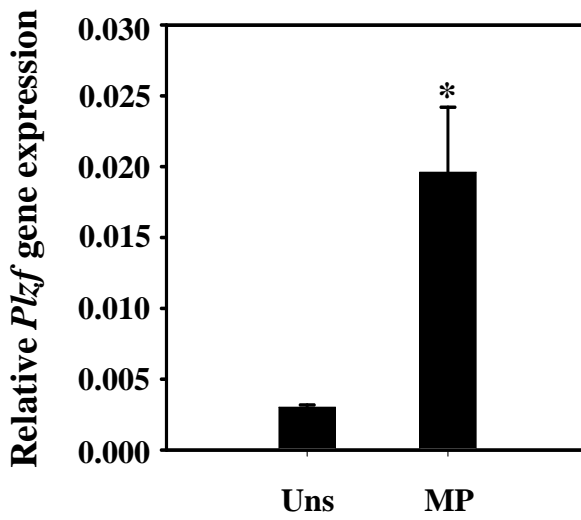
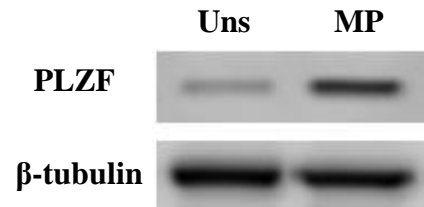
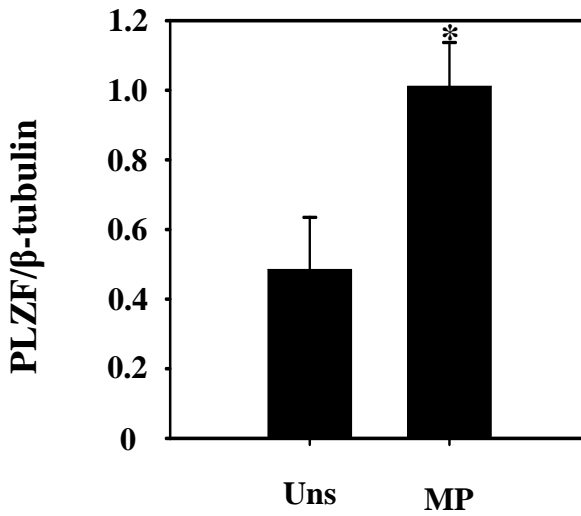
A**B****C****D****E**

Fig. 3. Multi-parameter selected cells supplemented with GDNF and FGF2 and co-cultured with BEFs support the formation of bovine undifferentiated spermatogonial clumps in vitro.

A) Representative images of bovine (left) and mouse (right) Und Spg clumps (arrows) that form during culture in serum-free medium supplemented with GDNF and FGF2. (10X magnification). B) RT-PCR analysis for expression of *Plzf* by cultured bovine Und Spg clumps. Lane 1 = 100 bp molecular weight (MW) marker, lane 2 = whole testis (positive control), lane 3 = Und Spg clumps, lane 4 = BEF feeders, lane 5 = whole testis – RT, lane 6 = Und Spg clumps –RT, lane 7 = BEF –RT. C) Representative image of immunofluorescence staining for PLZF expression in cultured bovine Und Spg clumps (arrows; 10X magnification). D) Immunocytochemical analysis of the number of PLZF+ cells isolated by multi-parameter selection, co-cultured with bovine embryonic fibroblast (BEF) feeders in DMEM/F12 StemPro media supplemented with GDNF and FGF2. PLZF+ cells were counted every 7 days over a 4 week period. Data are reported as the mean \pm SEM for three separate cell isolations from three different bulls.

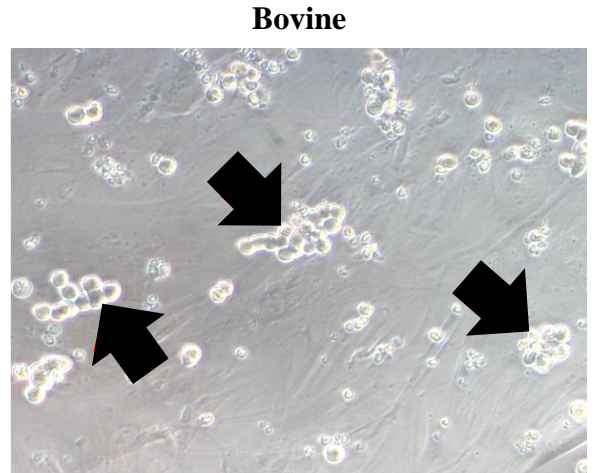
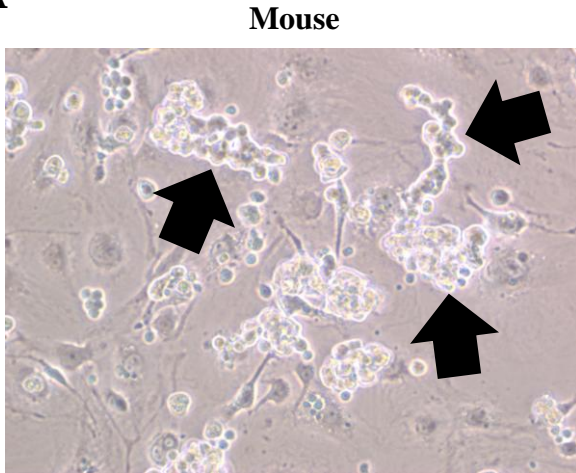
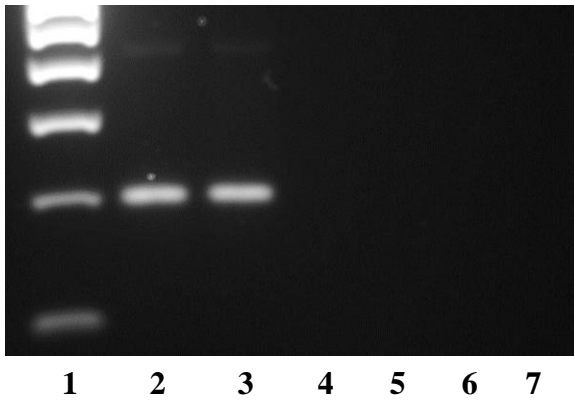
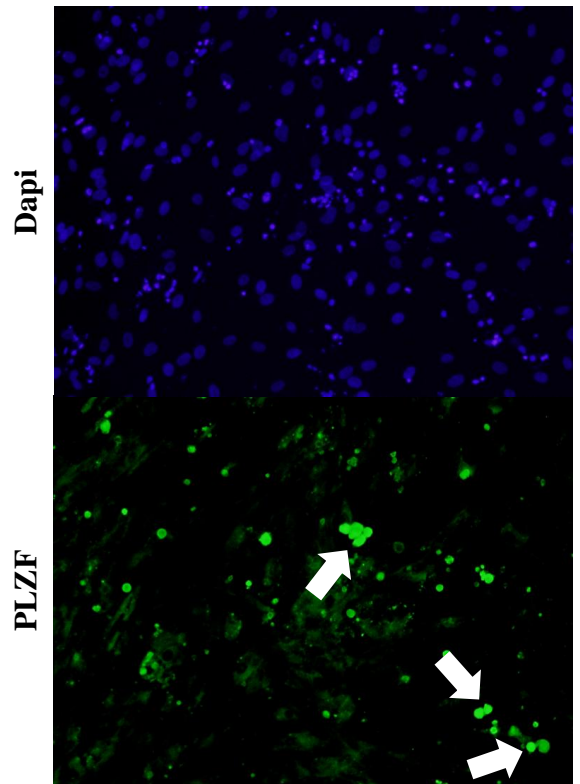
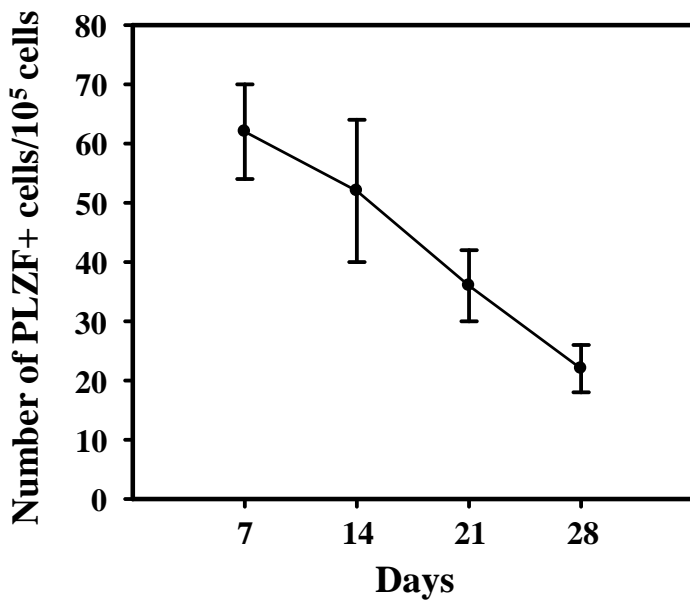
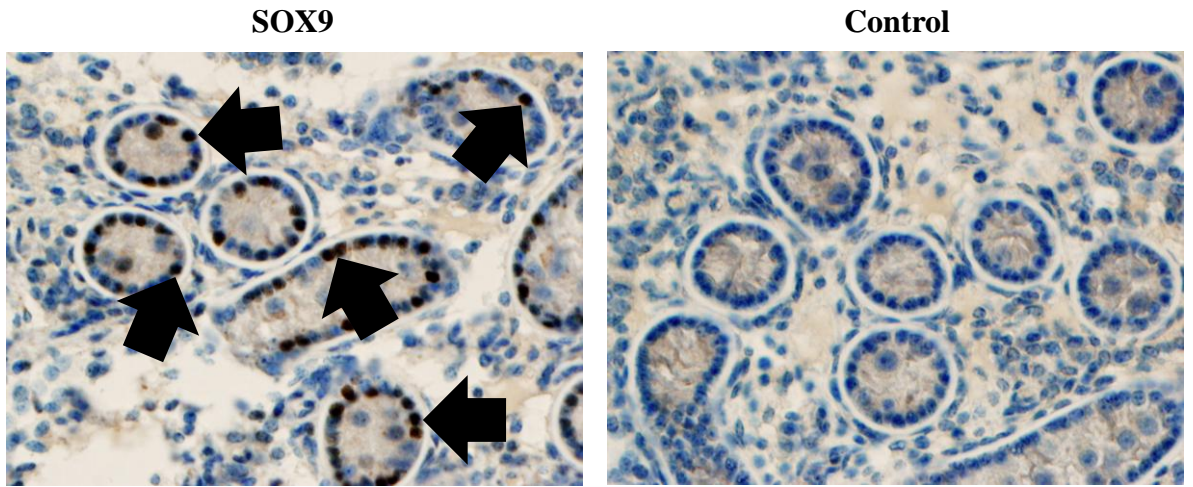
A**B****C****D**

Fig. 4. BSC feeder cells express SOX9, a conserved marker of Sertoli cells in the bovine testis.

A) Representative image of immunohistochemical staining for SOX9 expression in cross-sections of 4 mo old bull testes. Staining is specifically localized to Sertoli cells (arrows). Right panel is negative control staining which included incubation with normal rabbit IgG (20X magnification). B) representative image of immunocytochemical staining for SOX9 expression in the adherent cell population following overnight incubation on gelatin in the multi-parameter cell isolation protocol. Total cell numbers were determined by nuclei staining with Dapi and SOX9-specific staining was identified by FITC fluorescence. The lower panel is the negative control staining which included incubation with normal rabbit IgG (20X magnification).

A



B

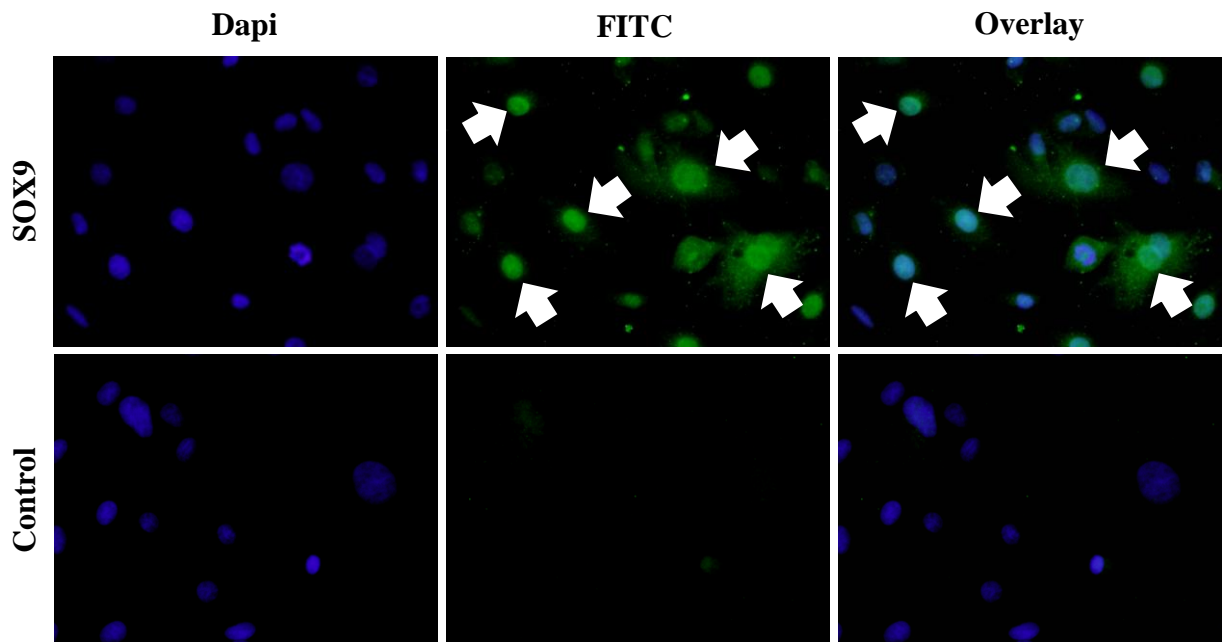


Fig. 5. Effects of feeder cell monolayers on maintenance of bovine undifferentiated spermatogonia during short-term culture.

Immunocytochemical analysis of the number of PLZF+ cells maintained in co-culture with BEF or BSC feeders for 1 week. Und Spg isolated by multi-parameter selection were co-cultured with bovine embryonic fibroblast (BEF) or bovine somatic cell (BSC) feeders in DMEM/F12 StemPro media supplemented with GDNF, FGF2, and CSF-1. Data are reported as the mean \pm SEM for three separate cell isolations from three different bulls. * denotes significantly different at $P < 0.05$.

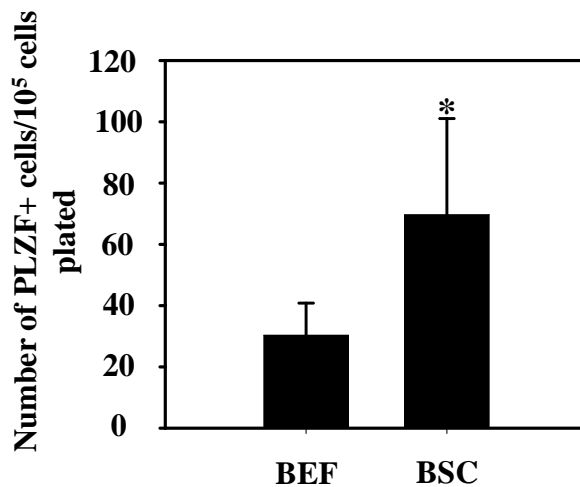


Fig. 6. CSF-1R and CXCR4 are expressed by the undifferentiated spermatogonia in the pre-pubertal bull testis and expression is enriched in multi-parameter selected cells.

A) Representative image of immunohistochemical staining for CSF-1R expression in testes of pre-pubertal bulls. Expression is localized specifically to Und Spg (arrows) within seminiferous tubules. Right panel is negative control staining which included incubation with normal rabbit IgG (20X magnification). B) Representative image of immunocytochemical analysis for CXCR4 expression in testes of pre-pubertal bulls. Staining is localized in the Und Spg (arrows). Total cell numbers were determined by nuclei staining with Dapi and CXCR4-specific staining was identified by FITC fluorescence. The lower panel is the negative control staining which included incubation with normal rabbit IgG (20X magnification). C) qRT-PCR analysis for relative *Cxcr4* gene expression in multi-parameter selected and unselected total cell populations. Data are reported as the mean \pm SEM for three separate cell isolations from three different bulls. * denotes significantly different at $P < 0.05$.

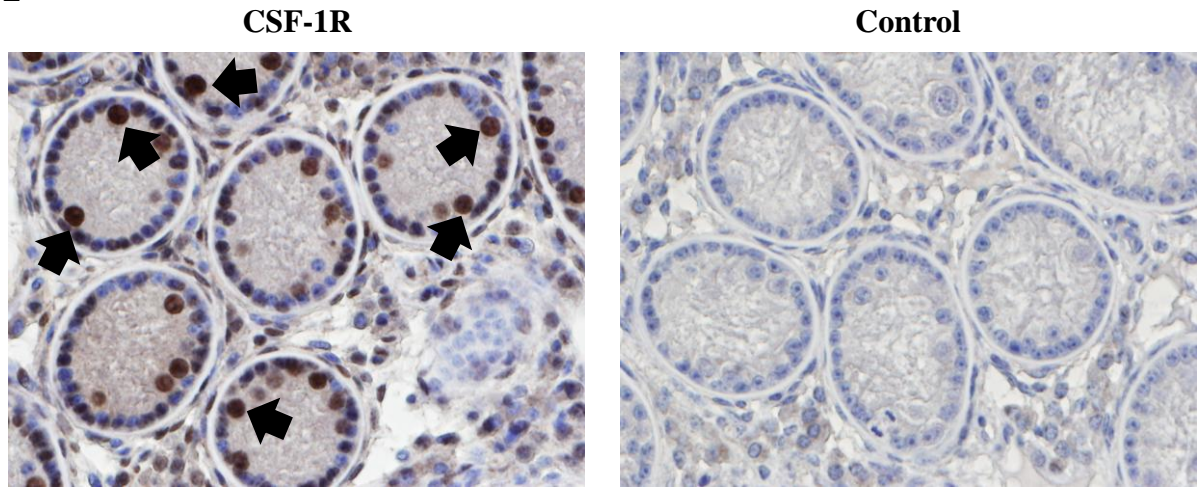
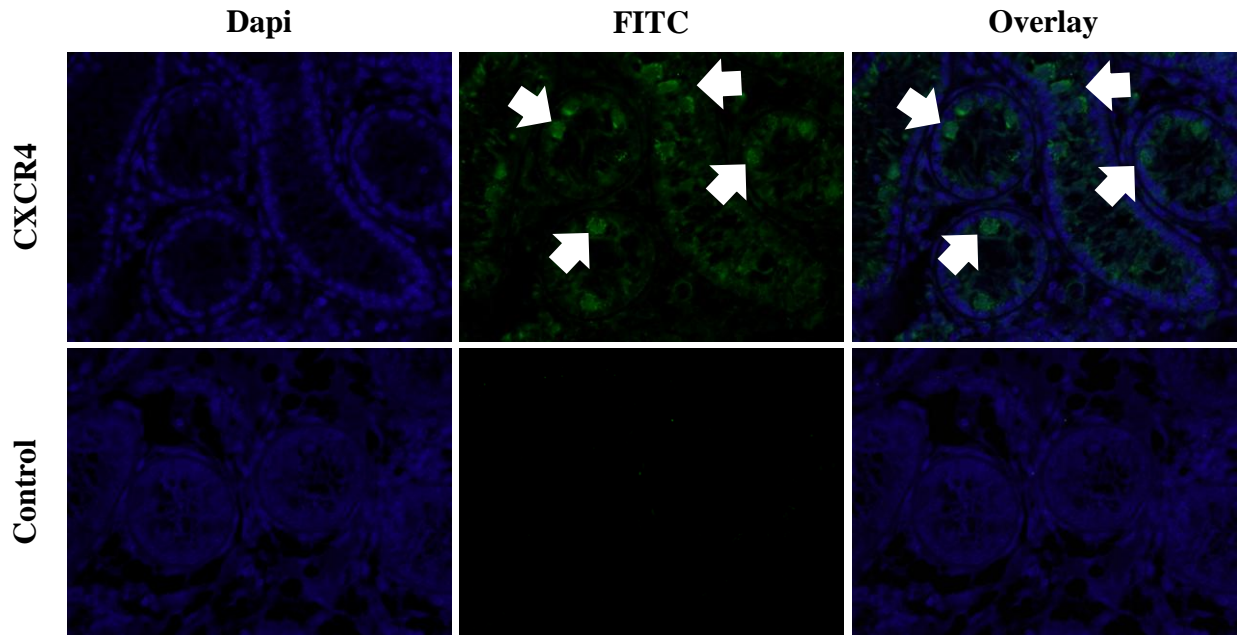
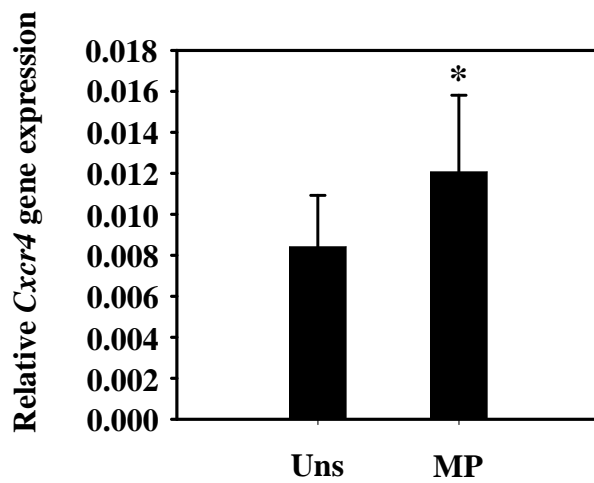
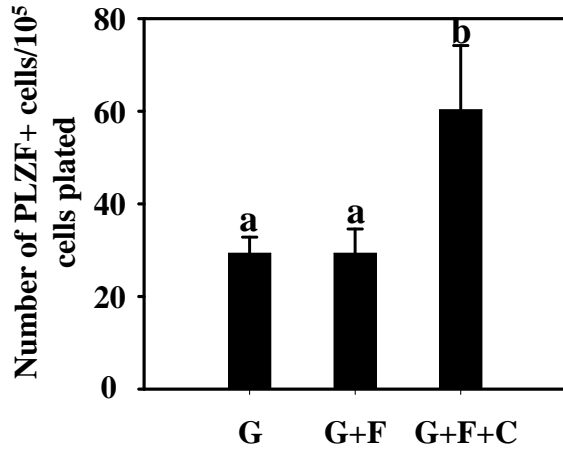
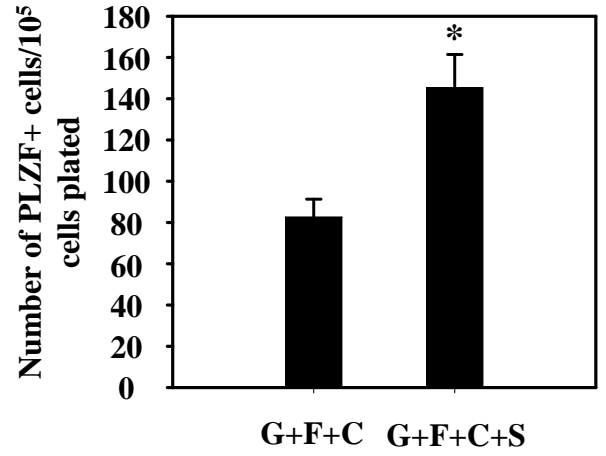
A**B****C**

Fig. 7. Supplementation with CSF-1 and SDF-1 enhances short-term maintenance of undifferentiated spermatogonia in vitro.

A) Immunocytochemical analysis of the number of PLZF+ cells maintained in co-culture with GDNF (G), GDNF and FGF2 (G + F) or GDNF, FGF2, and CSF-1 (G + F + C) for 1 week. Data are reported as the mean \pm SEM for three separate cell isolations from three different bulls. ^{a,b} Denote significant differences, $P < 0.05$. B) Immunocytochemical analysis of the number of PLZF+ cells maintained in co-culture with GDNF, FGF2, and CSF-1 (G + F + C) or GDNF, FGF2, CSF-1 and SDF-1 (G + F + C + S) for 1 week. Data are reported as the mean \pm SEM for three separate cell isolations from three different bulls. * denotes significantly different at $P < 0.05$. C) Immunocytochemical imaging of PLZF+ cell clumps observed in culture maintained with GDNF, FGF2, CSF-1 and SDF-1 for 2 weeks (20X magnification).

A**B****C**