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THY1 IS A CONSERVED MARKER OF SPERMATOGONIAL STEM CELLS IN THE PRE-PUBERTAL BULL TESTIS

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by

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ABSTRACT

Spermatogonial stem cells (SSCs) are the precursors of sperm and their ability to self-renew and differentiate provides the foundation for sustaining spermatogenesis. Culture and transplantation of SSCs in bulls has the potential to enhance the efficiency of cattle production and provide a novel avenue for generation of transgenic animals. Because of their rarity, these techniques depend on the ability to isolate SSCs from the total testis cell population. However, this capability is currently unavailable for any livestock species. In both the mouse and nonhuman primate, SSCs express the cell surface molecule Thy1 and selection of Thy1+ cells from the total testis cell population results in SSC-enrichment. In this study, the hypothesis that Thy1 is a conserved marker of SSCs in the bull testis was tested. Flow cytometric analysis showed that the Thy1+ cell fraction comprises a rare subpopulation of cells in the testis of prepubertal bulls. Next, Thy1+ cells were isolated from prepubertal bull testes using magnetic activated cell sorting (MACS) and further examined for characteristics of the proliferating spermatogonial population. Expression of Plzf, a transcription factor expressed exclusively by proliferating spermatogonia, was highly enriched in isolated Thy1+ cells compared to the unselected total testis cell population. Further analysis revealed that the majority of cells in the isolated Thy1+ testis cell population are Plzf+. Lastly, xenogeneic transplantation of bull testis cells into seminiferous tubules of immunodeficient nude mice resulted in a greater than 6-fold increase of germ cell colonies from Thy1+ cells compared to the unselected total testis cell population. Collectively, these results demonstrate that Thy1 is a marker of SSCs in the prepubertal bull testis and isolation of Thy1+ cells produces an SSC-enriched fraction compared to the total testis cell population.

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CHAPTER 1 : REVIEW OF LITERATURE

Development of the Seminiferous Epithelium

Embryonic

Primordial germ cells (PGCs) are the first germ cells present during embryonic development (de Felici, 2000). During this time PGCs originate from the epiblast (McLaren, 1983). After arriving at the genital ridge Sertoli cells enclose the PGCs forming seminiferous cords which are the embryonic precursors of seminiferous tubules (de Rooij and Grootegoed, 1998). At the same time progenitor cells of somatic peritubular myoid and Leydig cell populations migrate to the embryonic gonad from the mesenephros and surround the seminiferous cords (Payne et al., 1996; McLaren, 2003). Differentiation of the pericordal, or future peritubular myoid cells occurs in accordance with differentiation of Sertoli cells (Fröjdman et al., 1992). Initial differentiation of Leydig cells also takes place at this time (Pelliniemi, 1985; Pelliniemi et al., 1993; Pelliniemi and Lauteala, 1981; Pelliniemi and Niemi, 1969). Tissue remaining outside the testicular cords will become interstitium (Fröjdman et al., 1992; Pelliniemi, 1975). When PGCs are enclosed in the seminiferous cords, they change morphologically and are then called gonocytes (de Rooij and Grootegoed, 1998). Many changes in cell junctions and contacts in fetal organization and re-organization of the seminiferous epithelium will also occur at this point in development (Byers et al., 1991).

Once the cords are formed there is a rapid increase of Sertoli cell numbers that continues until postnatal development, although the volume of Sertoli cells decreases (Rosen-Runge and Anderson, 1959; Russell and Griswold, 1993). During the time of

primary sexual differentiation of the gonads, factors from Sertoli and Leydig cells regulate the subsequent processes in sexual differentiation (Russell and Griswold, 1993). The key gene regulating sex determination in the male is testis determining factor (TDF) also called SRY, which is important in organizing testicular stroma into the male tunica albuguinea and interstitial tissue as well as essential for Sertoli cell development (Lovell-Badge and Robertson, 1990; Pelliniemi et al.,1993). Another substance important in male embryonic development is anti-Müllerian hormone, whose transcription starts in embryonic Sertoli cells (Münsterberg and Lovell-Badge, 1991). After sex determination, gonocytes have a burst of mitotic activity and then arrest in G_0 phase of the cell cycle where they will remain quiescent until after birth (McLaren, 1984; de Rooij and Russell, 2000).

Postnatal

Postnatally, development of gonocytes and other support cells are essential for spermatogenesis. In mice, Sertoli cell proliferation occurs in the fetal testis, continuing postnatally and is regulated by factors including the pituitary product follicle stimulating hormone (FSH) and contributions from Leydig cells (Orth, 1984; Orth, 1986). Sertoli cell proliferation continues until day 12 in the mouse , when meiotic cells first appear in the seminiferous epithelium (Kluin et al., 1984, Russell and Griswold, 1993), postnatal weeks 12-24 in bulls (Curtis and Amann, 1981, Wrobel, 2000) and postnatal years 3-3.5 in nonhuman primates (Kluin et al., 1983). After birth, quiescent gonocytes resume mitosis and migrate from the center of seminiferous tubules to the basement membrane at which point they transform into spermatogonia that will eventually give rise to spermatozoa (Kluin and de Rooij, 1981; McGuiness and Orth, 1989; de Rooij and

Russell, 2000). Once arriving at the basement membrane gonocytes have several fates including degeneration, differentiation into spermatogonial stem cells (SSCs), or give rise to differentiating A_2 spermatogonia (de Rooij, 1998). It is the gonocyte to A2 transition that initiates the first wave of spermatogenesis; whereas, all subsequent waves arise from SSCs from puberty until old age in males.

Spermatogenesis

Spermatogenesis is the process of male germ cell development from 2N spermatogonia into 1N spermatozoa. This process produces a continual supply of male gametes from puberty until death in all mammalian species and is one of the most highly specialized and productive tissue systems in the body, producing millions of spermatozoa every day. This process takes place within the seminiferous tubules in the testes and stability of this mechanism is dependent upon SSCs. Spermatogenesis is highly organized and can be broken into three distinct phases including proliferation, meiosis, and spermiogenesis (Sharpe, 1994).

Germ Cell Development

Several types of spermatogonia are present within the seminiferous epithelium and are broadly classified as two major subpopulations, proliferating (also referred to as undifferentiated) and differentiating spermatogonia. The proliferating spermatogonial population includes A single (A_s), A paired (A_{pr}) and A aligned (A_{al}) sub-types. A_s spermatogonia have traditionally been considered to comprise the SSC population which possess the capacity for self renewal and differentiation. SSC differentiation produces A_{pr} spermatogonia which remain connected by an intercellular bridge. These bridges maintain synchronous development within cohorts of spermatogonia and are

essential for normal germ cell development (Oatley and Brinster, 2006; Huckins, 1978). A_{pr} spermatogonia undergo further mitotic divisions becoming $A_{al}(4)$, $A_{al}(8)$ and $A_{al}(16)$ spermatogonia then transforming into $A_1(16)$ spermatogonia without a mitotic division. At the A_1 stage, spermatogonia are referred to as differentiating spermatogonia and additional series of divisions occur producing $A_2(32)$, $A_3(64)$ and $A_4(128)$ spermatogonia.

The next step in germ cell development involves type A_4 spermatogonial division and maturation into Intermediate (256) (In) and Type B (512) (B) spermatogonia. At this point the second phase of spermatogenesis, or the meiotic phase begins. Primary spermatocytes quickly enter the first phase of meiosis called prophase which consists of five sub-phases termed preleptotene, leptotene, zygotene, pachytene and diplotene. DNA is replicated and crossing over occurs during prophase of the first meiotic division at which point primary spermatocytes transform into secondary spermatocytes. The second meiotic division occurs quickly with secondary spermatocytes becoming haploid round spermatids which transform into spermatozoa through a specialized process termed spermiogenesis. During the beginning of spermiogenesis, round spermatids undergo a transformation in which the nucleus elongates and the acrosome forms the headcap (Nagano, 1962). At the same time the flagellum or axoneme is formed and specialized microtubules termed the manchette also form (McIntosh and Porter, 1967). At this point spermatids are embedded within Sertoli cells and their flagellum extend into the lumen of seminiferous tubules. Finally, spermatids enter the maturation phase where mitochondria assemble beneath the distal nucleus, around the flagellum, forming the middle piece. The fibrous sheath of the flagellum is formed and mature

spermatozoa are generated at which point spermatozoa are released into the lumen of seminiferous tubules, a process referred to as spermiation.

Somatic Support Cells of the Testis

Germ cell development from spermatogonia to spermatozoa relies on several different populations of somatic support cells including Sertoli, Leydig and peritubular myoid cells. Sertoli cells, also referred to as “nurse” cells, are located within seminiferous tubules and support germ cells throughout spermatogenesis. Additionally, Sertoli cells have been suggested as a major contributor of the spermatogonial stem cell (SSC) niche (de Rooij, 2009). Together Sertoli cells and germ cells comprise the seminiferous epithelium which is supported by a basement membrane formed by contributions of both Sertoli and peritubular myoid cells (Clermont, 1972). Sertoli cells are anchored to the basement membrane and surround developing germ cells. Adjacent Sertoli cells form tight junctions which separate the seminiferous epithelium into basal and adluminal compartments (Russell et al, 2000). Tight junctions also establish the blood-testis-barrier making the adluminal compartment an immune privileged site where blood borne substances such as cytotoxic agents have limited access, while the basal compartment is exposed to many blood and lymph borne substances (Russell, 1993). Sertoli cells have a cyclic function that depends on the stage of the cycle of the seminiferous epithelium (Russell and Griswold, 1993), supporting spermatogenesis by secreting a variety of biological substances including hormones, growth factors and other macromolecules needed to support germ cell development and maintain the normal endocrine pathways in the male. Sertoli cells express follicle stimulating hormone (FSH) receptors on their cell membrane and

respond to the concentration of FSH at a given time in the stages of spermatogenesis (Skinner, 2003).FSH action on Sertoli cells has an array of different functions, such as determining the number of Sertoli cells, thus the spermatogenic capability of the testis (Orth et al., 1988), and playing a key role in the final differentiation of Sertoli cells and in the first wave of spermatogenesis (Raj and Dym, 1976). Sertoli cells also possess phagocytic capabilities which eliminate left-over products of spermatogenesis such as residual cytoplasm left over from spermiogenesis.

Unlike Sertoli cells, Leydig cells reside in the interstium between seminiferous tubules where they play an essential role in regulating spermatogenesis and secondary sex characteristics by producing testosterone in response to pituitary secreted luteinizing hormone (LH; Payne et al., 1996). LH binds to its receptor expressed on the cell surface of Leydig cells maintaining the local and peripheral concentrations of androgens.

Like Leydig cells, the peritubular myoid cells reside in the interstitium. They aid in spermatogenesis through their contractile properties as well as contribute to the basement membrane. Androgen receptors are present on peritubular myoid cells, suggesting interactions of all somatic support cells in the testis (Skinner and Fritz, 1985). In vitro, peritubular myoid cells also closely interact with Sertoli cells forming basal lamina and seminiferous tubule-like structures as well as stimulating increased production of androgen binding protein by Sertoli cells (Skinner and Fritz, 1985).

The First Wave of Spermatogenesis

The first wave of spermatogenesis varies among all mammalian species and is completed at puberty. In the mouse, gonocytes are present at birth and transition into SSCs between 0 and 6 days postpartum (Huckins and Clermont, 1968). In 2003, McLean et al. showed that biologically active SSCs first appear at 3 to 4 days postpartum in the mouse testis. Evidence suggests the existence of two different populations of gonocytes in the neonatal mouse testis, one population that develops directly into differentiating spermatogonia, bypassing the stem cell stage and behaving like A_1 spermatogonia and eventually giving rise to A_2 spermatogonia, while the other population becomes SSCs with the ability to self-renew and differentiate, providing the foundation for future rounds of spermatogenesis (de Rooij, 1998, Yoshida et al., 2006). These findings suggest that the first wave of spermatogenesis begins from newly transformed differentiated spermatogonia and not SSCs; therefore, this round is shorter in duration compared to subsequent rounds which encompass 35 days in mice (Oakberg, 1956; Clermont and Trott, 1969). The first wave of spermatogenesis and the transformation of gonocytes to SSCs differ in nonrodent mammalian species. In the bull, gonocyte migration to the basement membrane does not occur until 4 weeks postpartum and type A spermatogonia are not present until 8 weeks postpartum (Curtis and Amann, 1981). The first wave of spermatogenesis is initiated between 16 and 24 weeks of age in the bull (Curtis and Amann, 1981) and subsequent rounds require 54 days for completion (Berndtson and Desjardins, 1974).

Following the first wave, all rounds of spermatogenesis are divided into different stages depending on the development of the seminiferous epithelium, and the number

of stages varies among species. In the bull and mouse, there are 12 stages (Oakberg, 1956; Berndtson and Desjardins, 1974), in contrast to humans, having only 6 stages of spermatogenesis (Clermont, 1963). At each stage of the cycle of the seminiferous epithelium, germ cells are at different phases of development. For example, Stage I consists of one generation type A spermatogonia, two generations of primary spermatocytes and one generation of immature spermatids, while in the middle of the cycle, stage IV, type A and Intermediate spermatogonia, one generation of primary and one generation of secondary spermatocytes, and one generation of immature spermatids are present. In stage VIII, type A and B spermatogonia, one generation of primary spermatocytes, and two generations of spermatids including a mature population that are about to be released into the lumen of the seminiferous tubule are present. In all mammals, these subsequent rounds of spermatogenesis are possible because the population of SSCs in the testis provide the foundation for continual spermatogenesis.

Spermatogonial Stem Cells

Spermatogonial stem cells (SSCs) are the adult tissue-specific stem cell population of the postnatal mammalian testes and provide the foundation for spermatogenesis, through the ability to both self-renew and differentiate (Oatley and Brinster, 2008). Similar to other adult stem cell populations these functions are regulated by extrinsic stimuli by a niche microenvironment and intrinsic expression of specific genes. Unlike other stem cell populations in the body, SSCs are the only adult stem cell population that has the ability to contribute genetic information to the next generation (Oatley and Brinster, 2006).

Development

Development of SSCs begins with primordial germ cells (PGCs), which eventually transition into gonocytes. After birth, a subset of gonocytes become SSCs between days 0 and 6 postpartum (Huckins and Clermont, 1968), with the first transition from gonocyte to SSC occurring around days 3 to 4 in the mouse (Mclean et al., 2003). In the bull, gonocyte migration to the basement membrane occurs around 4 weeks postpartum followed by transformation to spermatogonia at 8 weeks of age (Curtis and Amann, 1981).

Biological Actions

SSCs possess the capability for both self-renewal and differentiation. Currently, whether SSC division is a symmetric or an asymmetric process has not been defined. Regardless of the symmetry, SSC self-renewal is the process of infinite replication to maintain a stem cell pool in the testis, allowing continual spermatogenesis until old age in males (Oatley and Brinster, 2008). Differentiation initiates spermatogenesis by producing daughter progeny committed to eventual formation of spermatozoa (Oatley and Brinster, 2006). The traditional view of SSC functions has always been that a homogenous population of A_s spermatogonia exist that either self renew or differentiate (Oatley and Brinster, 2006). In a study by Nakagawa et al. (2007) a heterogenic SSC population in the mouse was proposed which includes a population of transiently amplifying SSCs and a second mitotically inactive SSC population. Further studies

proposed a model for these two populations (Yoshida et al., 2008). The actual stem cells were described as supplying differentiating cells maintaining their own population while potential stem cells function as transit amplifying cells and do not take part in the self-renewing pool, rather shift their mode from transit amplification to self-renewal and give rise to new actual stem cells in response to actual stem-cell loss or emptying of a stem-cell niche (Yoshida et al., 2008). Regardless of whether the SSC population is homogenous or heterogeneous, balance between self-renewal and differentiation must exist for normal spermatogenesis to occur.

Regulation

In general SSC functions are regulated by extrinsic signals from the niche microenvironment which activate intrinsic molecular pathways that ultimately affect their fate decisions. An extrinsic factor essential for SSC self-renewal is glial cell-line derived neurotrophic factor (GDNF), a member of the transforming growth factor-β (TGF-β) superfamily, initially described as a regulator of neuronal survival and differentiation in addition to being essential for ureteric branching in the embryonic kidney (Lin et al., 1993; Sariola and Saarma, 2003). In the testis, GDNF is produced and secreted by the Sertoli cells (Trupp et al., 1995), and its receptors, GDNF family receptor α1 (GFRα1) and c-Ret receptor tyrosine kinase, are expressed by spermatogonia (Meng et al., 2000, Yomogida et al., 2003). Meng et al. (2000) revealed that in the testes of mice with one GDNF null allele, spermatogenesis is disrupted due to depletion of differentiating spermatogonia resulting in seminiferous tubules adopting a Sertoli-cell-only phenotype, which is a condition where all spermatogonia are depleted from the seminiferous

tubules. In contrast, GDNF over-expression in the mouse testis causes inhibition of SSC differentiation resulting in an accumulation of proliferating spermatogonia within the seminiferous tubules eventually forming a seminoma or germ cell tumor (Meng et al., 2000; Yomogida et al., 2003). In vitro, exposure to GDNF is essential for the long-term expansion of rodent SSCs (Kubota et al., 2004; Kanatsu-Shinohara et al., 2005; Ryu et al., 2005; Kanatsu-Shinohara et al., 2008). Importantly, supplementation with GDNF enhances short-term proliferation of bovine SSCs in vitro (Oatley et al., 2004; Aponte et al., 2005). Collectively, these studies indicate that Gdnf plays a critical role in SSC fate decisions and spermatogenesis.

Molecular Markers

GDNFs influence on the expression of specific molecules within SSCs, controls self-renewal and differentiation. In cultured mouse and rat SSCs GDNF stimulation upregulates the expression of B cell CLL/lymphoma 6, member B (Bcl6b), Ets variant gene 5 (Etv5) and Lim homeobox protein 1 (Lhx1) (Oatley et al., 2007; Schmitt et al., 2008). Molecules implicated as regulators of SSC functions that have not currently been tied to signaling of specific niche factors include Pou domain protein 5 f1 (Pou5f1 or Oct4), promyelocytic leukemia zinc finger protein (Plzf) and TATA-box binding protein (TBP)-associated factor 4b (Taf4b).

Bcl6b

GDNF-regulated gene expression was investigated in cultured mouse SSCs using microarray analysis (Oatley et al., 2006). Those studies identified 79 genes that

dramatically decreased upon GDNF withdrawal and increased with GDNF replacement, one of which was the transcriptional repressor Bcl6b (Oatley et al., 2006). In vitro, apoptosis occurred in mouse SSCs when Bcl6b expression was reduced by siRNA treatment, suggesting that the transcriptional repressor plays a role in mouse SSC survival (Oatley et al., 2006). In vivo, targeted disruption of Bcl6b expression results in impaired spermatogenesis and a Sertoli-cell-only phenotype is observed in seminiferous tubules (Oatley et al., 2006). In Bcl6b null mice, sub-fertility at puberty is observed while fertility at an older age has yet to be examined. Studies by Oatley et al. (2007) localized Bcl6b expression to individual spermatogonia in pre-pubertal and adult mice, suggesting that Bcl6b plays an SSC specific role in male fertility.

Plzf

While GDNF regulates some intrinsic stimulators of SSC self-renewal and differentiation, there are molecular markers that do not require GDNF stimulation to be expressed. Similar to Bcl6b, Plzf is a transcriptional repressor expressed by spermatogonia in the mouse testes (Costoya et al., 2004). In contrast to Bcl6b, Plzf expression is not regulated by GDNF (Oatley et al., 2006; Oatley et al., 2007). Plzf is a BTB/POZ-domain family transcription factor and functions as a transcriptional repressor during chromatin remodeling (Hong et al., 1997; Payne and Braun, 2006). In the male mouse germline, Plzf expression is restricted to the proliferating spermatogonia population that consists of A_s, A_{al}, and A_{pr} spermatogonia (Buaas et al., 2004; Costoya et al., 2004). Disrupted Plzf expression in male mice causes infertility due to impaired spermatogenesis (Buaas et al., 2004; Costoya et al., 2004). Germ cells transplanted

from Plzf deficient mice cannot restore spermatogenesis in recipient testes suggesting impairment of SSC function (Costoya et al., 2004). Testes of Plzf deficient mice are smaller in size compared to mice that express Plzf, and lack developing germ cells within their seminiferous tubules (Buass et al., 2004; Costoya et al., 2004). The testes of males lacking Plzf expression start to degenerate, becoming more pronounced with older age. This causes an increase in apoptosis and subsequent loss of tubule structure, ultimately resulting in impairment of spermatogenesis (i.e. infertility).

Uchl1

Most of the molecular markers for SSCs have been investigated in rodents. Ubiquitin carboxyl-terminal esterase L1 (Uchl1) is an SSC molecular marker investigated in livestock species. In the adult boar testis, Uchl1 is exclusively expressed by A_s, A_{pr} and A_{al} spermatogonia (Frankenhuis et al., 1982). In the prepubertal bull, expression of Uchl1 is found in type A spermatogonia (Herrid et al., 2007). Recently, studies by Luo et al. (2009) found that Uchl1 in boar testes is highly expressed in Plzf(+) cells and weakly expressed in Plzf(-) spermatogonia, suggesting that Uchl1 is expressed by the undifferentiated spermatogonial population that contains SSCs. Currently, the role of Uchl1 in spermatogonial function has not been investigated.

SSC Phenotype

SSCs are a rare subpopulation within the proliferating spermatogonia in the testis. Isolation of SSCs is an essential tool to study the mechanisms regulating SSC function in all mammalian species. To date, there are no known specific phenotypic or

molecular markers to identify SSCs in any mammalian species; thus, isolating a pure population of SSCs from the testis is not possible. While specific SSC markers have not been identified, there are cell surface markers that are used for SSC enrichment from the total testis cell population. Because different stem cell populations share several characteristics, the expression of cell surface markers expressed by other stem cell populations, such as hematopoietic stem cells, have also been investigated in SSCs.

Cell Surface Markers

Thy1

Decades of research established that the phenotype of hematopoietic stem cells is c-kit(+), Thy1(+), Sca1(+) and Lin(-) (Randal and Weissman, 1998). In rodents (Kubota et al., 2003; Kubota et al., 2004) and nonhuman primates (Hermann et al., 2007; Hermann et al., 2009), SSCs also express the cell surface marker Thy1 (CD90), a glycosyl phosphatidylinositol (GPI) anchored glycoprotein molecule of the IgG superfamily. Studies by Kubota et al. (2003) showed that SSCs are enriched in the Thy1(+) cell fraction of mouse testes. Using transplantation analysis, SSC concentration was determined to be 1 in 15 cells, which in comparison to the total testis cell population, where 1 in 3,000 is an SSC (Tegelenbosch and de Rooij, 1993), represents a 200-fold enrichment. In nonhuman primates, the Thy1+ cell fraction is also enriched for SSCs (Hermann et al., 2009). Studies by Ryu et al. (2004) investigated the SSC enriched Ep-CAM+ cell fraction from the neonatal and pre-pubertal testis for Thy1 expression. Results demonstrated that 100% of neonatal and 90% of Ep-CAM+ rat

testis cells were also Thy1+, suggesting that rat SSCs also express Thy1 (Ryu et al., 2004). Currently, Thy1 has not been examined in any other mammalian species including livestock.

Ep-CAM

Epithelial cellular adhesion molecule (Ep-CAM) is a calcium-independent hemophilic adhesion molecule expressed by most epithelia and carcinoma cells (Litvinov et al., 1997). In rats, SSCs are enriched in the Ep-Cam+ testis cell fraction compared to the total testis cell population (Ryu et al., 2004). In addition to spermatogonia, embryonic and fetal germ cells express Ep-CAM and loss-of-function studies suggest a role in germ cell migration and homing (Anderson et al., 1999). Spermatogonial differentiation coincides with loss of Ep-CAM expression suggesting a role in compartmentalizing of germ cells within seminiferous epithelium (Anderson et al., 1999). Studies by Ryu et al. (2004) using fluorescent activated cell sorting (FACS) analysis showed that SSC activity is contained exclusively in the Ep-CAM+ expressing cell population within the neonatal and prepubertal rat testes. Selection of Ep-CAM+ cells from neonatal and prepubertal testes resulted in 70- and 11-fold enrichment of SSCs compared to unselected total testis cell population, respectively (Ryu et al., 2004).

α_6-integrin

Another cell membrane surface marker expressed in mouse SSCs is the cell adhesion molecule α_6-integrin. Together with β_1-integrin, α_6-integrin forms a

heterodimeric complex that binds the extracellular matrix laminin. Studies by Shinohara et al. (1999) investigated both α_6- and β_1-integrin as markers for SSCs. Isolated α_6-integrin expressing cell fractions of mouse testes results in increased colonization of recipient testes with an 8.4-fold enrichment of SSCs compared to unselected total testis cell population while β_1-integrin selection produced a 3.8-fold enrichment for SSCs. Studies by Kanatsu-Shinohara et al. (2008), also showed an α6- and β1-integrin phenotype of SSCs from the hamster testis. Collectively, these studies indicate that α6- and β1-integrin is a conserved phenotype of SSCs in the rodent testis; however, neither cell population is composed purely of SSCs.

CD9

Another surface marker of mouse and rat SSCs is the tetraspanin transmembrane protein, CD9 which is also expressed by hematopoietic stem cells, mouse embryonic stem cells and neural stem cells (Oritani et al., 1996; Oka et al., 2002; Klassen et al., 2001). Previous studies showed that CD9 is expressed by cells along the basement membrane of seminiferous tubules in human testes (Nakamura et al., 2001). Recent studies revealed that 4.7% of mouse testis cells express CD9 and its expression is localized to cells along the basement membrane including spermatogonia (Kanatsu-Shinohara et al., 2004). Using transplantation as an assay to study SSCs, the CD9(+) cell fraction was found to be enriched 6.9-fold and 5.1-fold for SSCs compared to the total testis cell population in adult mouse and rat testes, respectively (Kanatsu-Shinohara et al., 2004).

Most cell surface markers used to isolate SSCs have mainly been investigated in rodents and nonhuman primates, while limited research has been conducted in other mammalian species such as livestock.

Isolation of SSCs

SSCs are rare in the testis with an estimated concentration of 1 in every 3,000 cells which translates to about 0.03% of the entire testis cell population (Tegelenbosch and de Rooij, 1993). While studying the mechanisms controlling SSCs in the testis is of great importance, because of this rarity, in vivo examination is challenging. Also, while enrichment of SSCs is possible, isolating a pure population from the testis of any mammalian species has not been achieved. To date, a variety of different approaches for isolating an enriched population of SSCs from rodent testes have been devised. Three widely used methods are FACS analysis, magnetic activated cell sorting (MACS) and differential plating.

FACS isolation of testis cells based on expression of the surface markers Thy1 (Kubota et al., 2003; Hermann et al., 2009) and Ep-CAM (Ryu et al., 2004) yields varying degrees of SSC enrichment. FACS isolation based on the expression of Thy1 results in enhanced colonizing activity in nonhuman primates (Hermann et al., 2009) and a 200-fold enrichment of SSCs in mice (Kubota et al., 2003). In neonatal and prepubertal rats, FACs isolation of the Ep-CAM+ cell fraction results in 69.9- and 11-fold enrichment of SSCs compared to the unselected total testis cell population, respectively (Ryu et al., 2004).

Similar to FACS, MACS isolation utilizes magnetic columns for isolating single cell suspensions based on binding of an antibody that is conjugated to magnetic beads. When a single cell suspension is passed through the column, cells which have the magnetic antibody bound will adhere to the column and the remaining cells will flow through. MACS has been used widely to isolate SSC enriched fractions from testes of mice and rats based on expression of the cell surface markers Thy1, Ep-CAM, α6-integrin and CD9. This methodology is beneficial due to efficiency of isolation while maintaining the integrity and viability of cells for further analysis (von Schonfeldt et al., 1999).

An additional technique for isolation of SSC enriched fractions is differential plating, which employs coating culture plates with extracellular matrices (ECM) to which SSCs preferentially bind. Plating of a single cell suspension of testis cells on laminin, collagen type IV or fibronectin matrices have been used to isolate cell populations with limited enrichment for SSCs from testes of mice and rats (Shinohara et al., 1999). Laminin isolation resulted in 3-4 fold greater enrichment of SSCs compared to the unselected total testis cell population. In contrast, collagen type IV and fibronectin enrichment were not significantly different from the unselected total testis cell population.

SSC Applications in Livestock

Culture

Over the past 10 years culture systems have been developed that support expansion of rodent SSCs for long periods of time. Unfortunately, similar conditions for maintaining SSCs of any livestock species has not been devised. For many years, the belief was that germ cells could only survive in culture for short periods of time. Many studies have been conducted with testicular cells in vitro but whether SSCs could survive and remain viable had never been determined. Studies by Kierszenbaum (1994) revealed that testicular cells in culture retained some of the morphological characteristics of spermatogonia including a large nuclear to cytoplasmic ratio. Studies by Nagano et al. (1998) showed that mouse SSCs could be maintained in culture for four months and after transplantation some cells could generate colonies of spermatogenesis within seminiferous tubules of recipient mice. In those studies, testis cells isolated from a transgenic mouse strain carrying the E. coli LacZ gene, in the Rosa26 locus, were placed on either mitotically inactivated STO cells, an immortalized embryonic fibroblast feeder layer or without a feeder layer (Nagano et al., 1998). Cells carrying the E. coli LacZ gene produce β-galactosidase which stains blue when exposed to X-gal. After plating, LacZ expressing cells were examined and found to have a distinctly different appearance depending on whether they were cultured with or without STO feeders. The presence of STO cells supported the formation of a larger number of round putative germ cells compared to feeder-free cultures (Nagano et al., 1998). Transplantation analysis showed that some SSCs were present in the long-term

cultures of testicular cells but survival and proliferation was limited (Nagano et al., 1998).

Optimal in vitro conditions for long-term expansion of SSCs in the mouse also require a specific medium. Many cells maintained in vitro require the addition of fetal bovine serum (FBS) to help support growth. Yet, FBS is detrimental to rodent SSC survival (Kubota et al., 2004; Ryu et al., 2005); therefore, mouse SSC maintenance in vitro requires a serum-free culture condition supplemented with the growth factor GDNF (Kubota et al., 2004). Initially, GDNF was shown to enhance SSC maintenance during a short-term culture period of 7 days (Kubota et al., 2004). Further studies showed that long-term maintenance and expansion of SSC numbers in serum-free, chemically defined culture conditions required GDNF supplementation (Kubota et al, 2004a and b). Other growth factors added to culture medium that can enhance GDNF induced SSC self-renewal, but alone cannot support SSC expansion, are basic fibroblast growth factor (bFGF), insulin like growth factor (IGF1), epidermal growth factor (EGF) and leukemia inhibitory factor (LIF) (Kubota et al., 2004b; a; Kanatsu-Shinohara et al., 2005). Studies by Kubota et al. (2004b) reported that the addition of bFGF plays a complementary role to GDNF in SSC maintenance and expansion in vitro. The addition of IGF-1 to culture medium already supplemented with GDNF and bFGF also increases the number of SSCs in vitro (Kubota et al., 2004b). Kanatsu-Shinohara et al. (2005) reported that addition of LIF in combination with GDNF has a beneficial effect on establishment of long-term cultures of mouse SSC.

While long-term cultures for most mammalian systems have not been devised, major advances in culture of rat SSCs has been made over the last several years. A serum-free culture system has been established for rats that allows for the expansion of SSCs over 7 months similar to conditions that support mouse SSC maintenance in vitro (Ryu et al., 2005; Kubota et al., 2004). Hamra et al. (2005) also reported proliferation of rat SSCs in vitro when maintained in culture medium containing serum. Similar to mice, proliferation of rat SSCs in vitro requires supplementation of GDNF. More recently, Kanatsu-Shinohara et al. (2008) reported long-term culture of SSCs from hamster testes, also utilizing supplementation of GDNF into culture medium. These studies indicate a conserved mechanism for long-term maintenance of SSCs in rodents that requires GDNF stimulation.

While culture systems have been devised for rodent stem cells, further studies need to be conducted to define in vitro conditions that will support long-term culture of bovine SSCs. Izadyar et al. (2002) evaluated the proliferation and differentiation of bovine type A spermatogonia in vitro and reported the formation of colonies of proliferating spermatogonia that eventually gave rise to differentiating spermatogonia. Unfortunately, the SSC content in these cultures was not thoroughly investigated. Oatley et al. (2004) reported that the culture of bovine testis tissue explants on pore membranes over a two week period supported the survival of SSCs. The same group reported that bovine embryonic fibroblast feeder cell layers, termed BEFs, could support short-term proliferation of bovine SSCs over a 7 day period (Oatley et al., 2004). 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., 2004). Aponte et al.

(2005) also investigated the effect of GDNF supplementation on bovine germ cells in vitro and showed that the number of spermatogonia was significantly greater in GDNF treated cultures at days 15 and 25. While short-term culture has been achieved, long-term culture conditions that support bovine SSC maintenance and proliferation have not been reported.

Cryopreservation

Cryopreservation of SSCs is a potential means to immortalize the male germline. Avarbock et al. (1996) demonstrated that mouse SSCs could survive long-term cryopreservation in simple conditions of Dulbecco modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) in a 1:31 ratio of dimethyl sulfoxide DMSO and retain the ability to reestablish spermatogenesis following transplantation into testes of recipient males. Cryopreservation of bovine SSCs has also been examined. Studies by Izadyar et al. (2002) reported a cryopreservation technique for type A spermatogonia in which different cryoprotectants and freezing protocols were examined. Cells frozen and thawed in medium containing 10% FBS and 10% DMSO had a greater survival rate than cells frozen and thawed in medium containing FBS only. Additional examination showed that addition of sucrose further increased the survival rate of germ cells (Izadyar et al., 2002). Also, a non-controlled-rate freezing protocol compared to a controlled-rate slow and controlled-rate fast freezing method produced the greatest efficiency of spermatogonial survival (Izadyar et al., 2002). Unfortunately, appropriate examination of SSCs was not conducted in these studies; thus, it is difficult to assess the efficacy of the different cryopreservation conditions on SSC survival. In 2004 a

study conducted by Oatley et al. investigated the proliferation of bovine SSCs in vitro after ten months of cryopreservation in DMEM containing 10% FBS, 30 mg/ml penicillin, 50 ng/ml streptomycin and 10% DMSO and after transplantation into recipient seminiferous tubules, colonies of bovine germ cells formed, demonstrating SSC survival. These studies indicate long-term cryopreservation of livestock SSCs is achievable in relatively simple conditions.

Transplantation

To date, specific phenotypic or molecular markers for SSCs have not been described for any mammalian species. The only unequivocal means to measure SSC content and activity is by functional transplantation into recipient testes upon which SSCs reestablish spermatogenesis fulfilling the defining criterion of a stem cell. The first report of SSC transplantation came from studies by Brinster and Avarbock (1994) and Brinster and Zimmerman (1994) using the mouse as a model. In these studies, germ cells from testes of donor mice at ages ranging from embryonic day 18 to postnatal day 28 were collected and microinjected into recipient mouse seminiferous tubules using two different protocols. In the first protocol, cells were collected from testes of C57BL/6 donor mice homozygous for a dominant mutation resulting in tan belly fur and transplanted into testes of heterozygous or homozygous mutant W mice that lack endogenous spermatogenesis. In the second protocol, cells were collected from testes of transgenic ZFlacZ donor mice that are heterozygous for the E. coli β-galactosidase (lacZ) transgene in which round spermatids stain blue when exposed to the substrate X-Gal. Donor germ cells were then transplanted into testes of non-

transgenic C57BL/6 x SJL F_1 mice treated with busulfan to deplete endogenous spermatogenesis. Recipient mice were then maintained for a minimum of 50 days after transplantation to allow for at least one round of spermatogenesis to occur. Overall, 245 testes were microinjected with donor germ cells and 173 of these testes harbored donor spermatogenesis upon analysis. Additionally, spermatozoa derived from transplanted donor cells were observed in ejaculates of recipient mice and some recipient males produced progeny carrying donor genetics upon breeding to wild-type female mice. Collectively, these results provide the first demonstration of a stem cell population in the mammalian testis and pioneered the establishment of a new field of reproductive biology. Over the last 15 years, this technique has been used to study SSCs and develop novel reproductive strategies in several mammalian species.

The SSC transplantation technique has also been developed for the rat (Jiang and Short, 1995; Russell and Brinster, 1996; Ogawa et al., 1999; Zhang et al., 2003). In studies by Russell and Brinster (1996) rat donor testis cells were transplanted into busulfan treated recipient mouse testes. Analysis showed that rat testis cells had the ability to reinitiate spermatogenesis in mouse seminiferous tubules. In a similar study, isolated mouse testis cells were transplanted into busulfan treated recipient rat testes resulting in the re-colonization of mouse germ cells in recipient rat seminiferous tubules (Ogawa et al., 1999). Another species in which the transplantation technique was utilized was the hamster. Ogawa et al. (1999) reported xenogeneic spermatogenesis from transplanted hamster SSCs within the seminiferous tubules of recipient mice; however, abnormalities in formation of elongated spermatids and in particular acrosomes were observed. Dobrinski et al. (2000) investigated whether the male germ

cell xenogeneic transplantation technique translated to livestock. Testis cells were collected from bulls at three different ages including 1 week, 6 months and adult and either immediately transplanted into recipient testes, cryopreserved and transplanted at a later date or cultured for 1 month and then transplanted. One month after transplantation, donor cells from the one week old bull calves were found in recipient seminiferous tubules, yet analysis at two months after transplantation resulted in fibrous tissue of bovine origin surrounding the recipient seminiferous tubules (Dobrinski et al., 2000). Oatley et al., (2002) conducted studies to develop a mouse bioassay model allowing for evaluation of transplantation efficiency of fresh and cultured bovine germ cells from calves with normal scrotal development and experimentally cryptorchid calves. Bovine germ cells were transplanted into immunodeficient mice to avoid immunological rejection, which were treated with busulfan to abolish endogenous spermatogenesis (Oatley et al., 2002). To identify donor cells in recipient testes, a polyclonal antibody against bovine testis cells was generated and applied to isolated recipient seminiferous tubules by whole-mount immunohistochemistry in which areas of round bovine cells were detected resembling germ cell proliferation indicating the possible formation of spermatogonial colonies (Oatley et al., 2002). The ability to xenogeneically transplant bovine cells into recipient mice testes is an important first step for eventual donor to recipient livestock transplantation techniques.

Nagano et al. (1999) used transplantation to gain insight into the biological activity of SSCs by examining recipient testes at increasing time intervals after transplantation to provide three-dimensional reconstruction of reestablished spermatogenesis from SSCs. On day 1 after transplantation, blue stained donor cells

were distributed throughout the recipient tubules, similar to 1 week after transplantation in which donor cells remained randomly dispersed, yet individual cells could be more easily visualized compared to day 1. At 1 month after transplantation, few individual cells or even short chains could be identified between colonies and at 2 months after transplantation, most colonies were well established and appeared as distinctive blue segments (Nagano et al., 1999). These results reiterate the importance of transplantation to study mammalian SSCs. In studies by Ogawa et al. (2000) transplantation of male germline stem cells was shown to restore fertility in two different infertile recipient mouse strains (i.e. Steel (SI) and white spotting (W)) and result in progeny with the genetic makeup of the infertile donor male. Results from this study are important to human health because a genetic defect in humans resulting in abnormal pigmentation is equivalent to the W mutation in mice; thus serving as preliminary studies for eventual application in a clinical setting.

The transplantation technique introduced by Brinster and colleagues has been utilized in other livestock species. In livestock species, an ultrasonographic guided cannulation of the centrally located rete testis technique was used for transplantation in the following studies (Dobrinski, 2005). Studies by Honaramooz et al. (2002) isolated donor testis cells from 10 week old boars, labeled with a red fluorescent cell linker dye and injected them into the testis of a recipient boar. After transplantation, fluorescent-labeled donor cells were identified in multiple seminiferous tubules in 10 of 11 recipient testes.

The following year, the same group isolated donor testis cells from transgenic goats carrying the transgene human alpha-1 antitrypsin and transplanted them into

immunocompetent recipient goats (Honaramooz et al., 2003). The transgene was detected in ejaculates from 2 of the 5 recipient goats. In 2003, Izadyar et al. isolated 5 week old donor testis cells from hemi-castrated bulls and then injected back into the testis of the same bull that had been subjected to irradiation to remove endogenous spermatogenesis. After 2.5 months, recipient bulls were castrated and testes were analyzed and about 60% of the tubules contained spermatocytes, 30% contained spermatids, and in about 15% of tubules spermatozoa were reported. More recently, Rodriguez-Sosa et al. (2006) reported using the male germ cell transplantation technique in rams. In seven of eight testes injected, donor cells were identified within the seminiferous epithelium for up to 2 weeks after transplantation. While these studies provide some evidence of successful donor to recipient germ cell transplantation in livestock, conclusions were inconclusive due to discrepancies in the studies.

Implications

Most genetic gain is made through the male germline in commercial livestock populations. While approximately 80-90% of the dairy industry has adopted artificial insemination (AI) for efficient utilization of superior genetics, approximately 5% of the beef cattle industry utilizes AI. This limited utilization is mostly due to impracticality for commercial beef cattle operations. Transplantation of SSCs would provide an alternative to AI in beef cattle. The ability to isolate SSCs from a genetically desirable donor bull and transplant them into a battery of recipient bulls would allow the industry to utilize the genetics of the superior bull without having to use AI. Currently, preservation of male germlines is achieved by cryopreservation of sperm which is a

nonrenewable resource. The ability to maintain bovine SSCs in culture would allow for the immortalization of germlines of genetically desirable sires even after death. Bovine SSC isolation, culture and transplantation would also allow for the production of transgenic livestock with favorable traits beneficial for human consumption in addition to creation of such disease resistant animals. Recently, a reproductive technology gaining popularity in the dairy industry is sexed semen. Isolation and culture of bovine SSCs would allow for the enhancement of sperm output and thus a greater amount of spermatozoa for use in sexing procedures that are inefficient and thus depend on the total number of spermatozoa available for sorting. While great advances have been made toward these goals, further investigation and knowledge of bovine SSCs is required.

CHAPTER 2

THY1 IS A CONSERVED MARKER OF SPERMATOGONIAL STEM CELLS IN THE BULL TESTIS

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Introduction

Spermatogenesis is a highly specialized and productive tissue system, producing millions of spermatozoa per day (Sharpe, 1994), and continual production depends on a rare germ cell population termed spermatogonial stem cells (SSCs) that possess the capacity for self-renewal and differentiation. The proliferating spermatogonial population includes type A single (A_s), A paired (A_{pr}) and A aligned (A_{al}) spermatogonia, of which A_s have traditionally been considered the SSC population (Oakberg, 1971; Huckins, 1971). Differentiation of SSCs produces A_{pr} followed by A_{al} spermatogonia, which give rise to differentiating spermatogonia that are committed to eventual production of spermatozoa. Molecular markers have been used to distinguish between the proliferating and differentiating spermatogonial subtypes. Promyelocytic leukemia zinc finger protein (Plzf) is expressed by the proliferating spermatogonial population in rodents and nonhuman primates (Buaas et al., 2004; Costoya et al., 2004; Hermann et al., 2007) but has yet to be examined in livestock species. Another molecular marker of spermatogonia previously studied in the bull (Herrid et al., 2007) and boar (Frankenhuis

et al., 1982; Luo et al., 2006) is ubiquitin carboxyl-terminal esterase L1 (Uchl1). In the adult boar testis, Uchl1 is expressed exclusively by A_s, A_{pr} and A_{al} spermatogonia (Frankenhuis et al., 1982; Luo et al., 2006). In the bull, expression of Uchl1 is found in type A spermatogonia of prepubertal testes (Herrid et al., 2007).

In livestock populations a majority of genetic gain is made through the male germline. Thus, the ability to isolate, culture, and transplant SSCs are tools that could enhance the efficiency of food animal production. Maintenance of bovine SSCs in culture would allow for immortalization of the germline from genetically superior bulls. Transplanting SSCs from a genetically superior donor bull into recipient males provides a novel means to efficiently expand the genetics of specific sires in commercial livestock populations. Additionally, culture and transplantation of bovine SSCs could provide a novel venue for generation of transgenic livestock.

Currently the ability to culture and transplant SSCs is only available for rodents (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994; Kubota et al., 2004b). The effectiveness of these procedures has been aided greatly by identification of surface markers expressed by SSCs, which has allowed for isolation of cell fractions enriched for SSCs compared to the total testis cell population. For rodents and nonhuman primates, selection of CD90 or Thy1 expressing testis cells results in an enrichment of SSCs (Kubota et al., 2004). Selection of Thy1+ cells from testes of adult mice produces an enrichment of greater than 30-fold for SSCs compared to the total testis cell population (Kubota et al., 2004). Studies by Ryu et al. (2004) revealed that all SSCs in the rat testis are Thy1+. Importantly, selection of Thy1+ cells from testes of

rhesus macaques also results in enrichment of SSCs compared to the unselected total testis cell population (Hermann et al., 2009). Collectively, these studies suggest a conserved phenotype of Thy1 expression in mammalian species. Expression of any SSC surface marker identified in rodents has not been reported for livestock species. The objective of the current study was to isolate an SSC-enriched fraction from the pre-pubertal bull testis. To achieve this we tested the hypothesis that Thy1 is a conserved marker of SSCs in the bull.

Materials and Methods

Cell Isolation

All animal procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee. Single cell suspensions of testicular cells were collected from 12 week old Holstein bull calves by two-step enzymatic digestion. Briefly, approximately 200-300 mg of testicular tissue was incubated in collagenase solution (1 mg/ml collagenase type 4; Worthington Biochemical Corporation, Lakewood, NJ and 7 mg/ml DNase Sigma-Aldrich Co., St. Louis, MO) at 37^{o}C for 30 minutes with agitation every 2 minutes. Seminiferous tubule fragments were then allowed to settle on ice and washed 3 times with Hank's balanced salt solution (HBSS) to remove interstitial cells. Single cell suspensions were then created by incubation in Trypsin-EDTA solution (0.25% Trypsin/2.21 mM EDTA; Cellgro Mediatech Inc., Manassas, VA) containing DNase (7 mg/ml) for 5 minutes at 37^{o}C. Cells were then passed through a 40 µm cell strainer (BD Biosciences, Durham, NC) to remove clumps and pelletted by

centrifugation at 600xg for 7 minutes. Cells were then resuspend in DPBS-S (PBS with 1% fetal bovine serum (FBS), 10 mM Hepes, $1x10^4$ U/ml penicillin, $1x10^4$ µg/ml streptomycin, 1 mM sodium pyruvate and 1 ng/ml glucose) and cell concentration was determined using a hemocytometer with trypan blue exclusion to determine live cells. Aliquots of $1x10^7$ 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 ^oC. Cells were then washed 2 times with DPBS-S by centrifugation for 7 minutes, followed by incubation with biotinylated goat anti-mouse antibody (1:100 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4 ^oC for 20 minutes. Cells were again washed 2 times in DPBS-S with centrifugation and then incubated with strepavidin 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 $2\text{-}4x10^5$ Thy1+ cells were isolated from 200-300 mg of testis tissue. For comparison, single cell suspensions not subjected to Thy1 MACS isolation (referred to as unselected total testis cell populations) were collected from identical tissue used for Thy1+ cell isolation.

Flow Cytometric Analysis of Thy1 Expression

The percentage of cells in the prepubertal bull testis that express potential SSC surface markers was evaluated using flow cytometric analysis. Single cell suspensions of bovine testicular tissue were collected as described above and incubated with mouse anti-human Thy1 (1:100 dilution; Abcam), primary antibody. Secondary detection involved incubation with Alexa 488 conjugated donkey anti-mouse IgG (1:1000 dilution;

R&D Systems, Emeryville, CA) antibody. The percentage of Thy1 expressing cells was determined using a Guava Easy Cyte Plus flow cytometer (Millipore Corporation, Billerica, MA). Cell suspensions from identical samples analyzed for Thy1 not incubated with antibody served as negative controls.

Immunofluorescence Analysis of Plzf and Uchl1 Expression

Expression of Plzf and Uchl1 was localized in cross-sections of pre-pubertal bull testes using immunofluorescence analyses. Testis samples were collected from 12 week old Holstein bull calves, fixed in Bouin's solution, dehydrated, embedded in paraffin and 5 µm cross-sections were adhered to glass slides. After deparaffinization and rehydration, antigen retrieval was achieved by boiling in citrate buffer for 20 minutes. Sections were then blocked for nonspecific antibody binding by incubation in 10% normal goat serum in PBS for 1 hour. Tissue was then incubated with goat anti-human Plzf (1:50 dilution; Santa Cruz Biotechnology) and/or rabbit anti-human/rat/cow Uchl1 (1:500 dilution; Abcam) primary antibodies at 4^{o}C overnight. Controls were testis cross-sections incubated in normal goat IgG (1:50 dilution; Santa Cruz Biotechnology) and/or normal rabbit IgG (1:500 dilution; Santa Cruz Biotechnology) as primary antibodies at 4^{o}C overnight. On the next day, sections were washed 3 times in PBS followed by incubation with Alexa 488 conjugated donkey anti-goat IgG secondary antibody (1:1000 dilution; Invitrogen Corporation, Eugene, OR) or Alexa 546 conjugated donkey anti-rabbit IgG (1:1000 dilution; Invitrogen Corporation) for 2 hours at room temperature. Sections were again washed 3 times in PBS and counterstained with DAPI to label cell nuclei. Sections were then examined using fluorescence microscopy

(Axioskop 2 Plus, Zeiss Inc., Thornwood, NY) and digital images were captured with an Olympus DP71 digital microscope camera (Olympus America, Inc., Center Valley, PA).

Quantitative RT-PCR Analysis of Plzf and Bcl6b Gene Expression

Expression of Plzf by isolated Thy1+ and unselected total testis cell populations were examined using qPCR. RNA was isolated from Thy1+ and unselected total testicular cell populations, using Trizol reagent (Sigma-Aldrich Co., St. Louis, MO) followed by treatment with DNase (DNA-free kit; Ambion, Austin, TX) to remove possible contaminating genomic DNA. RNA concentration in each sample was measured by spectrophotometry (Nano Drop-1000, Nano Drop Technologies Inc., Wilmington, DE) and 260/280 ratios were calculated to determine RNA purity. For each RNA sample 500ng of RNA was reverse transcribed to cDNA using oligo d(T) priming and M-MLV reverse transcriptase (Superscript III first strand synthesis kit; Invitrogen Corporation). Quality of resulting cDNAs were determined by PCR analysis for the expression of Gapdh. Primers for Plzf, Bcl6b and ribosomal protein S2 (RpS2) were designed using Primer Express 3 software (Applied Biosystems, Foster City, CA). The expression of Plzf and Bcl6b were then determined by qPCR analysis with SYBR green assays (Invitrogen Corporation) and a 7500 Fast Sequence Detection System (Applied Biosystems). To make comparisons between Thy1+ and unselected total testis cell populations, the expression of each gene-of-interest in each sample was normalized to that of the constitutively active gene RpS2 as described previously (Oatley et al., 2006; Oatley et al., 2007).

Western Blot Analysis of Plzf Protein Expression

Expression of Plzf protein in Thy1+ and unselected total testis cell populations was examined using western blot analysis. Protein lysates were collected using mammalian protein extraction reagent (MPER; Thermo Scientific, Logan, UT). Lysates from 293T cells (Santa Cruz Biotechnology) and sim 6-thioguanine resistant oubain resistant (STO) cells were used as positive and negative controls, respectively. For western blot analyses, samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Blots were blocked with 5% non-fat dry milk powder (Lab Scientific Inc., Livingston, NJ) in PBS containing 0.05% tween-20 (Sigma-Aldrich) for 2 hours. Blots were then incubated with rabbit anti-human Plzf antibody (1:2000 dilution; Santa Cruz Biotechnology Inc.) in PBS with 0.05%BSA and 0.1% Triton X-100 overnight at 4^{o}C. The next day, blots were washed 3 times with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) followed by incubation with goat anti-rabbit IgG polyclonal antibody conjugated to HRP (1:2500 dilution; Santa Cruz Biotechnology Inc.). Blots were again washed 3 times with TBS-T, developed with super signal west pico chemiluminescent substrate (Pierce, Rockford, IL), viewed using a ChemDoc imager (Universal Hood II, Biorad Laboratories, Hercules, CA), and digital images were captured. To make quantitative comparisons between Thy1+ and unselected total testis cell populations, blots were re-probed with a rabbit anti-human β-tubulin primary antibody (1:5000 dilution; Novus Biologicals, Inc., Littleton, CO). Secondary detection included incubation with HRP conjugated goat anti-rabbit IgG and development with chemiluminescent substrate. Plzf band density for each sample was normalized to

corresponding density of β-tubulin using Quantity One analysis software (Biorad Laboratories).

Immunocytochemistry of Plzf Expressing Testis cells

Immunocytochemical staining was conducted to determine the percentage of Plzf+ spermatogonia in the Thy1+ cell fraction compared to the unselected total testis cell population. Isolated Thy1+ and unselected total testis cells were adhered to glass cover slips followed by fixation in 4% paraformaldehyde for 10 minutes at room temperature. Nonspecific antibody binding was blocked by incubating cells in 10% donkey serum for 1 hour at room temperature. Cells were then incubated with goat 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^{o}C overnight. On the next day, cells were washed 3 times with PBS and incubated with Alexa 488 conjugated donkey anti-goat secondary antibody (1:1000 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. Cover slips were then visualized by fluorescence microscopy at 20X magnification. For each replicate sample the total number of DAPI stained nuclei in 5 random fields of view were counted. The number of Plzf+ cells were also counted in each field and the average percentage of Plzf+ cells was determined by dividing the number of DAPI stained nuclei in each field by the number of Plzf+ cells.

Xenogeneic Transplantation of Bull Testis Cells and Analysis of Colonization

Xenogeneic transplantation assays were conducted to compare SSC content of the Thy1+ cell fraction to the unselected total testis cell population of prepubertal bull

testes as described previously (Oatley et al., 2004). Briefly, recipient NCr Swiss nude mice (Taconic, Germantown, NY) were treated with busulfan (33 mg/kg of body weight) 6 weeks prior to transplantation to deplete endogenous spermatogenesis. Following enzymatic digestion and MACS isolation, Thy1+ and unselected total testis cell populations were resuspended in PBS and fluorescently labeled with PKH26 red fluorescent cell linker dye following manufacturer instructions (Sigma-Aldrich). Cells were then washed 5 times in DPBS-S by centrifugation at 600xg for 7 minutes to remove residual dye. For each sample, cells were resuspended in DPBS-S at a concentration of 3 x 10^6 cells/ml and 3 to 7 μl of cell suspension (i.e. 3-7x10^4 cells) was microinjected into the seminiferous tubules of each recipient testis. Three recipient mice (6 testes) were transplanted with each replicate cell suspension and 3 replicate experiments were performed totaling 9 mice and 18 testes that were transplanted with Thy1+ or unselected total testis cell populations. Recipient mice were euthanized 4 weeks after transplantation and testes were recovered and incubated with collagenase (type 4, 1 mg/ml) and DNase (7 mg/ml) to separate seminiferous tubules followed by 3 washings in DPBS-S. Seminiferous tubules were then suspended in a small volume of DPBS-S, spread on 10 cm dishes and analyzed for colonization of bovine testicular cells using a fluorescence stereo-microscope equipped with a TRITC filter set (Olympus SZX10). The number of fluorescently labeled bovine colonies was counted for each recipient testis and digital images were captured with a DP71 digital microscope imaging system. To make quantitative comparisons between isolated Thy1+ cells and the unselected total testis cell populations, the number of colonies generated by each sample was normalized to 1X10^5 cells injected per recipient mouse testis.

Statistical Analysis

All data are presented as mean ± SEM for 3 replicated experiments. Data were analyzed using the General Linear Model (GLM) function of SPSS statistical software (Chicago, IL). Differences between means were determined using a one-way ANOVA in conjunction with Tukey-Kramer multiple comparisons test. Data were considered significantly different at $P \leq 0.05$.

Results

Thy1 is expressed by a rare subpopulation of spermatogonia in the prepubertal bull testis

In the mouse, SSCs are a rare subpopulation comprising approximately 0.03% of the total testis cell population (Tegelenbosch and de Rooij, 1993). Because of this rarity, isolation of SSCs facilitated by identification of specific cell surface markers. To date, isolation of a pure population of SSCs has not been achieved but cell surface markers for isolating an enriched population of SSCs have been described. One such marker is Thy1 which is expressed by rare subpopulations in the testes of rodents and non-human primates that are enriched for SSCs (Kubota et al., 2003; Hermann et al., 2009). Here we examined whether Thy1 is expressed by cells in the testes of prepubertal bulls. Using flow cytometric analysis, we determined that approximately 0.05±0.01% (n=3 replicate testis cell preparations from 3 different bulls) of the cells in the prepubertal bovine testis express Thy1 (Fig. 1). These results suggest that similar to rodents and nonhuman primates, Thy1 is expressed by a rare subpopulation in the testes of prepubertal bulls.

Expression of Plzf is localized to a subpopulation of proliferating spermatogonia within seminiferous tubules of prepubertal bulls

Previous studies revealed that expression of Uchl1 is a general marker of spermatogonia in the bull testis (Herrid et al., 2007). In rodents and nonhuman primates, expression of Plzf is specifically localized to proliferating spermatogonia including SSCs (Buaas et al. 2004; Costoya et al., 2004; Hermann et al., 2007); however, Plzf has not been evaluated as a marker of spermatogonia in the bull. Examination of Plzf expression in cross-sections of testes from prepubertal bulls using immunofluorescent staining revealed expression by select spermatogonia within seminiferous tubules (Fig. 2A). Interestingly, all spermatogonia did not stain for Plzf and further examination revealed that 0.9±0.15 Plzf+ cells (n=3 cross-sections from different bulls) are present per tubule (Fig. 2D). In comparison, expression of the general spermatogonial marker Uchl1 was observed at a frequency of 2.6±0.29 positive cells (n=3 cross-sections from different bulls) per tubule (Fig. 2B and D). These results suggested that Plzf expression is restricted to a subpopulation of spermatogonia in the prepubertal bull testis. To examine this possibility, co-immunoflurescent analyses were conducted for both Plzf andUchl1. In agreement with our prediction, only a portion of spermatogonia were found to express both Plzf and Uchl1 in the pre-pubertal bull testis (Fig. 2C) with all Plzf+ cells also being Uchl1+ (Fig. 2D). These observations demonstrate that Plzf expression is restricted to a sub-population of spermatogonia in the pre-pubertal bull testis which are possibly SSCs, whereas Uchl1 is a more indiscriminate marker being expressed by a greater number of spermatogonia and likely not restricted to SSCs.

The Thy1+ cell fraction of pre-pubertal bull testis is enriched for proliferating spermatogonia

In light of the above results we used Plzf expression as an indicator of enrichment for proliferating spermatogonia and possibly SSCs in further experiments. Next, we examined Plzf expression in the Thy1+ cell fraction isolated by MACS from prepubertal bull testes. First, qPCR and Western blot analyses were conducted to examine if Plzf was enriched in the Thy1+ cell population. Relative Plzf gene expression was significantly ($P = 0.036$) increased by 15.6±6.34-fold (n=3 cell populations from different bulls) in the Thy1+ cell fraction compared to the unselected total testis cell population (Fig. 3A). Additionally, the expression of another molecular marker of SSCs in rodents, Bcl6b (Oatley et al., 2006) was also significantly greater ($P \leq 0.05$) in Thy1+ cells compared to the unselected total testis cell population (Fig. 3B). Second, western blot analysis revealed that Plzf protein expression is also significantly ($P = 0.002$) greater by 13.6±10.4–fold (n=2 cell populations from different bulls) in the Thy1+ cell fraction compared to the unselected total testis cell population (Fig. 3C and D). These results suggested that the Thy1+ cell fraction from pre-pubertal bull testes is enriched for Plzf expressing spermatogonia. Third, immunocytochemical analysis of single cell suspensions revealed that the isolated Thy1+ cell fraction contains a significantly ($P = 0.001$) greater percentage of Plzf+ cells compared to the unselected testis cell population (Fig. 3E and F). On average 64.4±5.0% (n=3 cell populations from different bulls) of the cells in the Thy1+ population were Plzf+; whereas, only 24.7±3.5% (n=3) of cells in the unselected total testis cell population expressed Plzf. Thus, MACS isolation of the Thy1+ cells yielded a testis cell population enriched 2.6-fold (64.4/24.7)

for Plzf+ spermatogonia, confirming results from gene/protein expression analyses and indicating SSC enrichment compared to the total testis cell population.

The Thy1+ Cell Fraction in Pre-Pubertal Bull Testis is Enriched for SSCs

Stem cells are defined by a functional ability to colonize and reestablish homeostasis of a tissue system (Oatley and Brinster, 2006). The only means to unequivocally assess SSC content of an experimental cell population is by transplantation into recipient seminiferous tubules upon which SSCs will colonize and reestablish spermatogenesis (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994). Unfortunately, efficient and reproducible SSC transplantation between bulls has not been developed. However, a xenogeneic transplantation assay in which bovine germ cells are microinjected into seminiferous tubules of recipient immunodeficient mice is available (Dobrinski et al., 2000; Oatley et al., 2002; Oatley et al., 2004). In this assay, bovine germ cells colonize mouse seminiferous tubules but do not undergo complete spermatogenesis and these colonies are indicative of the SSC content of an injected cell population. To assay for SSC enrichment, Thy1+ cells isolated by MACS from pre-pubertal bull testes and corresponding unselected total testis cell populations were labeled with a fluorescent membrane linker dye and injected into seminiferous tubules of immunodeficient recipient mice. The number of fluorescently labeled bovine germ cell colonies was then evaluated in dispersed seminiferous tubules 4 weeks later. Regardless of cell suspensions, fluorescent colonies of bovine germ cells were observed in testes of recipient mice (Fig. 4A). Quantification of the colonies revealed that Thy1+ cells generated a significantly ($P = 0.009$) greater number of colonies compared to the unselected total testis cell population (Fig. 4B). Overall, the Thy1+

cells generated greater than 6-fold (94.0/14.4 colonies/10^5 cells transplanted; n=3 replicate experiments) more colonies than the unselected total testis cell population. These results demonstrate that the MACS isolated Thy1+ cell fraction from the pre-pubertal bull testis is enriched for SSCs compared to the unselected total testis cell population.

Discussion

Results of the present study demonstrate that selection of Thy1+ cells yields a cell fraction enriched for SSCs from pre-pubertal bull testes. Implementation of this finding will be a key component in future studies aimed at developing methodology for long-term culture and transplantation of bovine SSCs. Previous studies in rodents and nonhuman primates showed that Plzf is a molecular marker of the proliferating spermatogonial population that includes SSCs (Buaas et al. 2004; Costoya et al., 2004; Hermann et al., 2007), but has yet to be explored in bulls. The molecular marker Uchl1, also referred to as Pgp9.5, has been investigated and used as a marker for porcine and bovine spermatogonia (Frankenhuis et al., 1982; Luo et al., 2006; Herrid et al., 2007). In the current study, Plzf expressing cells were determined to be a subpopulation of spermatogonia within the more abundant Uchl1+ population of prepubertal bull testes. While all Plzf+ cells were Uchl1+, not all Uchl1+ cells were Plzf+ suggesting that Uchl1 expressing cells are more abundant in the testes. Based on this finding, Uchl1 seems to be expressed by all spermatogonia in a pre-pubertal bull in contrast to Plzf which is expressed by a sub-population of spermatogonia. Results of this study revealed that

the Thy1+ cell fraction of pre-pubertal bull testes is composed mostly (>60%) of Plzf+ spermatogonia. Surprisingly, the unselected total testis cell population in pre-pubertal bulls was determined to contain a higher than expected percentage of Plzf+ cells (i.e. ~20%) which was likely due to processing of the samples because initial collagenase digestion was conducted to reduce interstitial cells and enrich for cells within seminiferous tubules. Regardless, selection of Thy1+ cells resulted in nearly 3-fold enrichment of Plzf+ cells. In accordance with greater Plzf+ germ cell content, xenogeneic transplantation assays provided unequivocal evidence of SSC enrichment in the Thy1+ cell fraction compared to the unselected total testis cell population. Collectively, results of this study demonstrate that Plzf is a molecular marker of SSCs in the pre-pubertal bull testis and that Thy1 is a conserved marker of SSCs in the bull. Additionally, these results provide further evidence that Thy1 is a conserved marker of SSCs in several and possibly all mammalian species including other livestock.

Development of reproductive tools utilizing SSCs (i.e. long-term culture and transplantation) relies on isolation of these rare cells. Use of Thy1 as a marker for bull SSCs will aid in the progress of developing these tools in cattle. Maintaining bovine SSCs in culture could provide a means for immortalizing the germline of genetically superior sires. Transplantation of SSCs would provide an alternative assisted reproductive technique in the beef cattle industry where use of artificial insemination (AI) has been limited due to impracticality. Isolation of SSCs from a genetically desirable donor bull followed by transplantation into a battery of recipient bulls that subsequently produce donor sperm and are used for natural breeding could provide a novel means to expand the utilization of specific genetics within commercial cattle populations without

the technical limitations of AI. Both of these tools have the potential to increase efficiency of cattle production, thus, decreasing wastes, costs, and environmental impacts of producing food and fiber for human consumption. Additionally, these tools could provide a novel avenue for genetic modification of the male germline and subsequent generation of transgenic livestock with favorable traits such as disease resistance and animals producing food products beneficial for human consumption. The current study has provided the first step in achieving these goals by providing a means for isolating SSC enriched fractions from the bull testis which can be used in future studies to develop long-term culture conditions and efficient transplantation procedures.

Development of reproductive tools based on SSCs is at the forefront of development in livestock. In rodents, development of techniques for isolation, culture, and transplantation of SSCs spanned greater than a decade of research (Brinster, 2002). The first successful transplantation of SSCs was reported in 1994 by pioneering work of Brinster and Avarbock, and Brinster and Zimmerman. Cryopreservation of mouse SSCs was first reported 2 years later by Avarbock et al. (1996), and initial evidence that mouse SSCs could be maintained for long periods of time in vitro was reported 2 years after that by Nagano et al. (1998). By 1999, Shinohara et al. reported the first cell surface markers of mouse SSCs. In 2001, Nagano et al. reported the production of transgenic mice after transplantation of genetically modified SSCs into testes of infertile recipient mice. Two years later Kubota et al. (2003) investigated the cell surface marker Thy1 as a means to isolate an enriched population of SSCs from the mouse testis. Presently, Thy1 isolation continues to yield the greatest enrichment of SSCs from the

mouse testis (Oatley and Brinster, 2008). Also in 2003, Kantasu-Shinohara et al. reported the long-term culture of gonocytes from mouse testes. By 2004, Kubota et al. reported conditions for long-term maintenance of mouse SSCs in vitro, with similar methodology being identified for rats on year later (Ryu et al., 2005). Here we describe a means to enrich SSCs from the pre-pubertal bull testis based on Thy1 expression. This achievement is a crucial advancement for furthering the development of SSC applications in bulls. Based on the timeline of developments in rodents, major advancements in culturing and transplanting bovine SSCs should be made in the coming decade.

Fig. 1. Examination of the Thy1 expressing cell fraction in the prepubertal bull testis. Representative dot plots of flow cytometric analyses for the percentage of cells expressing Thy1 in the testis of prepubertal bulls. On average 0.05 ± 0.013% (n=3 different cell preparations from individual bulls) of cells in the prepubertal bull testis were Thy1+ (right panel) compared to unstained control cells (left panel).

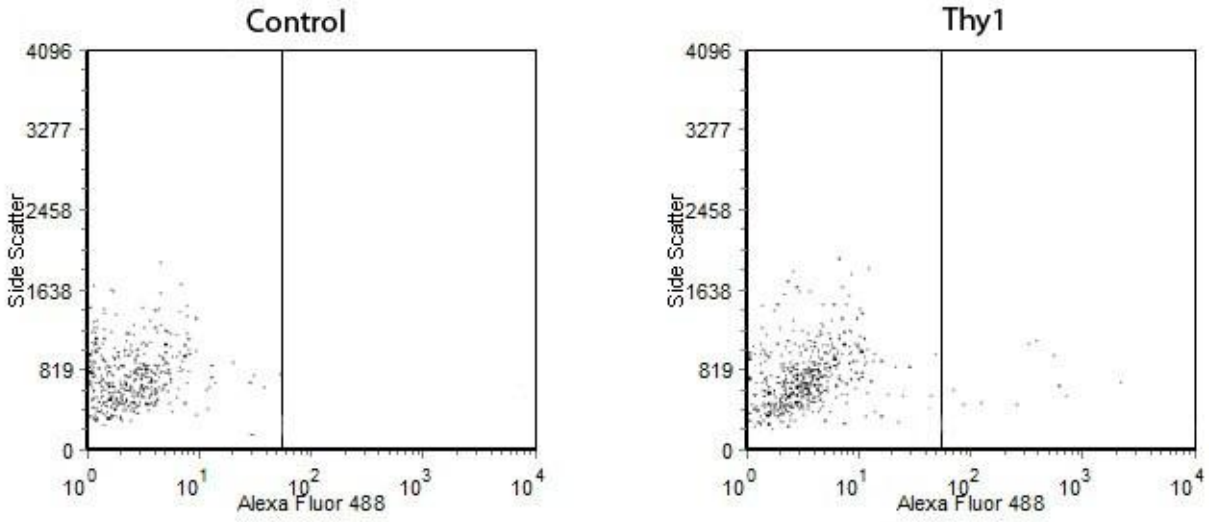


Fig. 2. Identification of Plzf+ spermatogonia in the prepubertal bull testis. A) Immunofluorescence examination of Plzf expressing cells within the seminiferous tubules of pre-pubertal bulls. Arrows indicate Plzf+ spermatogonia. B) Immunofluorescence analysis of Uchl1 expressing cells in the pre-pubertal bull testis. Arrows indicate Uchl1+ spermatogonia. C) Co-immunofluorescence analysis of Plzf (green) and Uchl1 (red) expressing cells in the pre-pubertal bull testis. All Plzf+ spermatogonia also expressed Uchl1 (arrows); whereas all Uchl1+ cells were not also Plzf+ (asterisks). Bars are 50µM. D) Average number of Uchl1+, Plzf+, Uchl+/Plzf+ and Uchl1+/Plzf- cells per seminiferous tubule. Data are mean ± SEM for 3 different replicate bulls.

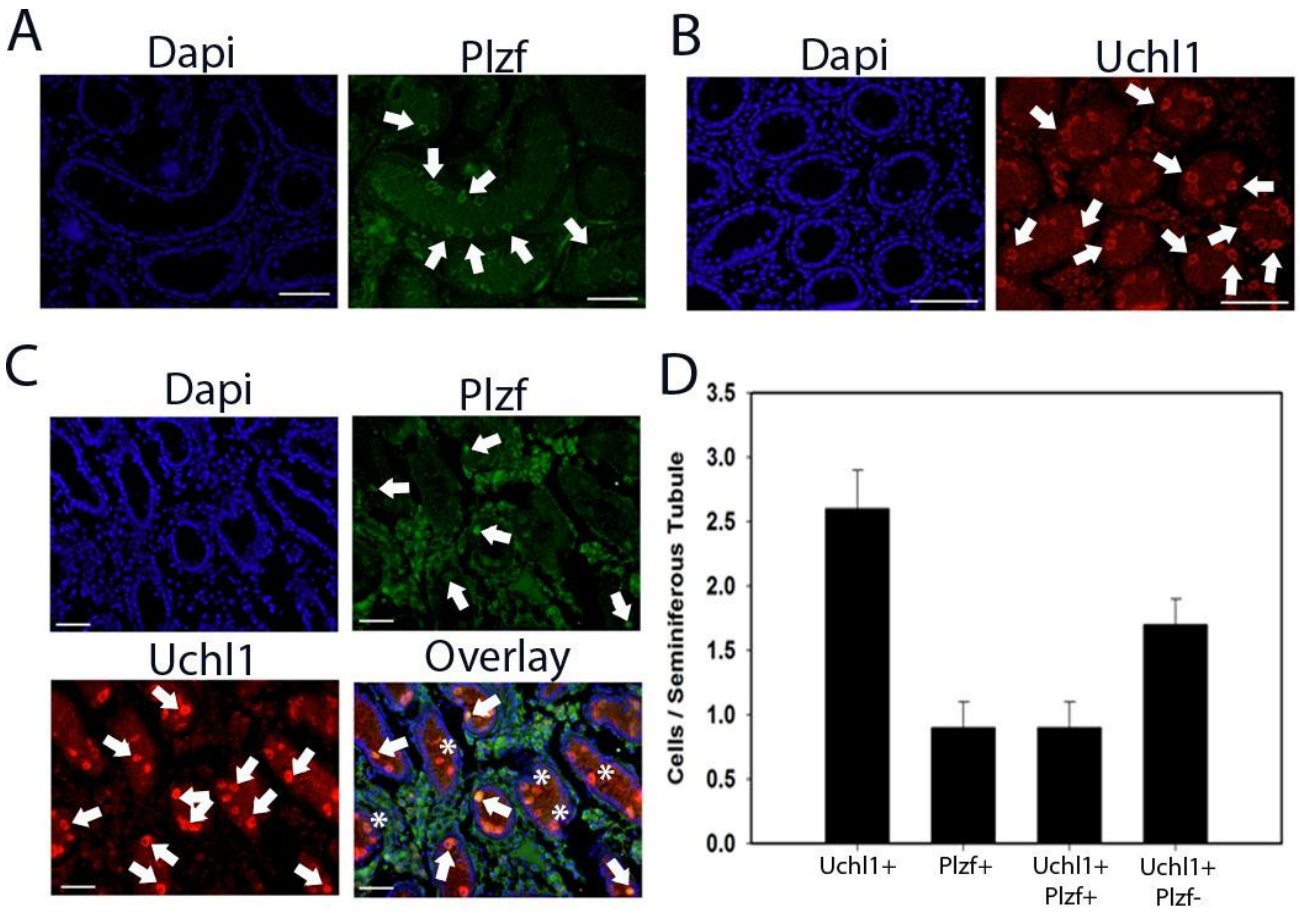


Fig. 3. Examination of Plzf expression in the Thy1+ cell fraction of prepubertal bull testes. Relative Plzf transcript and protein expressions were determined using quantitative PCR and Western blot analyses, respectively. A) Relative Plzf gene expression in the Thy1+ cell fraction compared to the unselected total testis cell population. Data are mean ± SEM for 3 different cell preparations and * denotes significant differences. Plzf expression in Thy1+ cells is 15.6 ± 6.3-fold greater in the isolated Thy1+ cell fraction compared to the unselected total testis cell population. B) Relative Bcl6b expression in the Thy1+ cell fraction compared to the total testis cell population. Data are ± SEM for 3 different cell preparations and * denotes significance. Comparison between means for both Plzf and Bcl6b were made by normalizing to the constitutively active gene Rps2. C) Representative image of Western blot analysis for Plzf protein expression in isolated Thy1+ fractions and unselected total testis cell populations. A distinct band of 37 kDA was observed for the bovine testis. D) Quantitative comparison for Western blot analyses for Plzf protein expression. Data are mean ± SEM for 3 different cell preparations and * denotes significant difference. Comparison of means was made by normalizing Plzf expression in each sample to that of β-tubulin. Plzf protein expression was determined to be 13.6 ± 10.4-fold greater in Thy1+ cells compared to the total testis cell population. E) Representative images of immunocytochemical analysis of Plzf+ cells in isolated Thy1+ cell fractions and unselected total testis cell populations. F) Quantitative comparisons of immunocytochemical analyses for the percent Plzf+ cells in isolated Thy1+ cell fractions and unselected total testis cell populations. Data are mean ± SEM for 3 different cell preparations and * denotes significantly different.

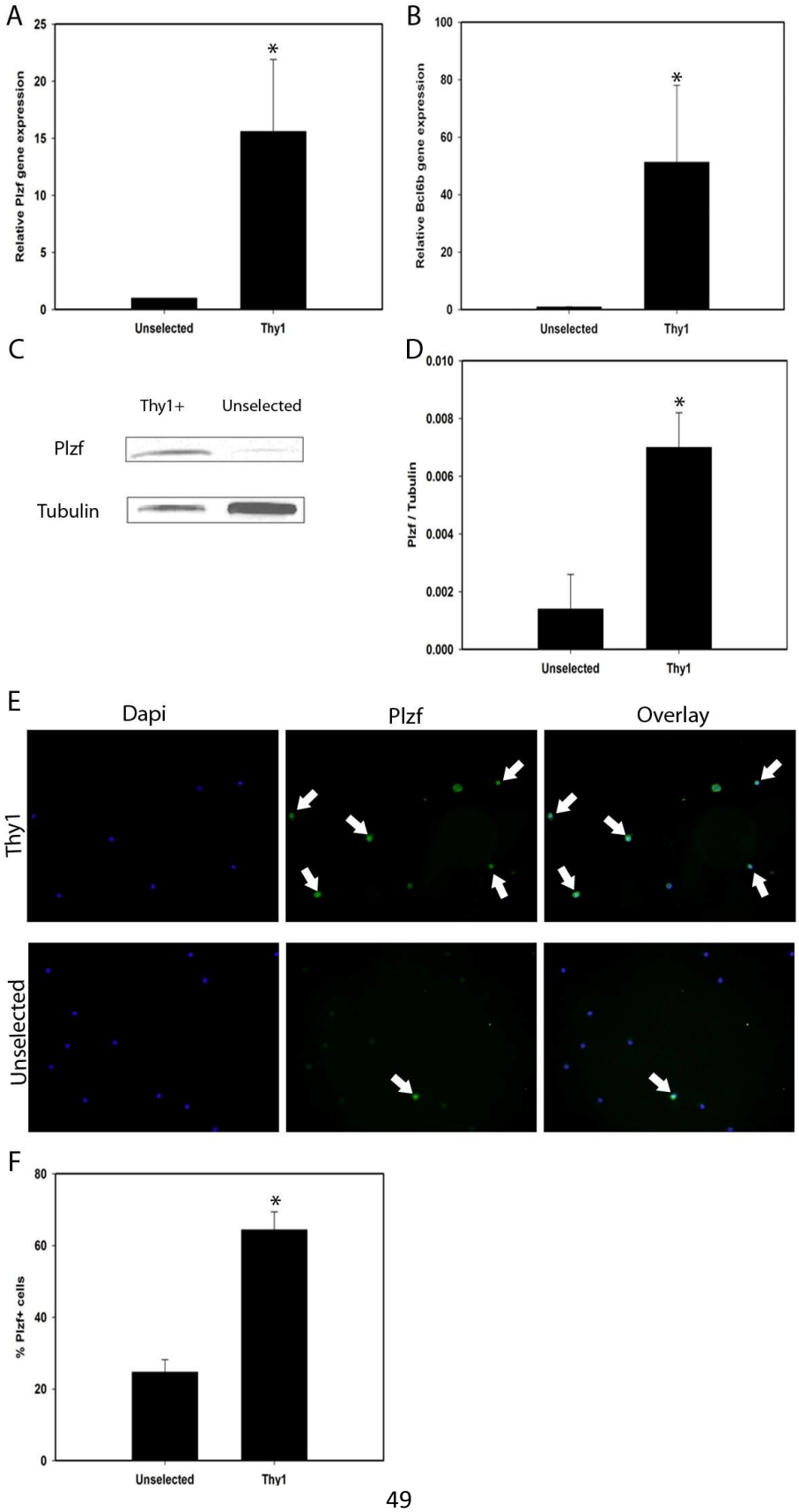
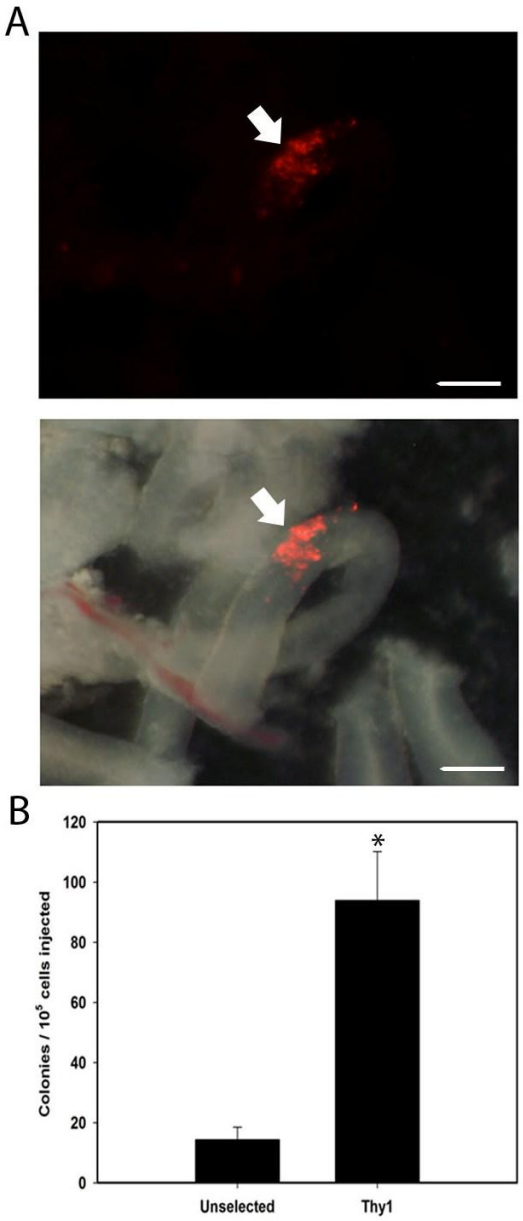

A
Relative Plzf gene expression
25
20
15
10
5
0
*
Unselected
Thy1
B
Relative Bcl6b gene expression
100
80
60
40
20
0
*
Unselected
Thy1
C
Thy1+
Unselected
Plzf
Tubulin
D
Plzf / Tubulin
0.010
0.008
0.006
0.004
0.002
0.000
*
Unselected
Thy1
E
Dapi
Plzf
Overlay
Thy1
Unselected
F
% Plzf+ cells
80
60
40
20
0
*
Unselected
Thy1

Fig. 4. Examination of SSC content in the Thy1+ cell fraction of pre-pubertal bull testes by xenogeneic transplantation analyses. Isolated Thy1+ bovine cells were labeled with a fluorescent dye and microinjected into the seminiferous tubules of recipient immunedeficient nude mice. A) Representative image of bovine germ cell colony (arrow) within recipient mouse seminiferous tubules transplanted with Thy1+ cells Top panel is fluorescent view and bottom panel is overlay of fluorescent view with the corresponding bright field image. Bars are 2 mm. B) Quantitative comparison of bovine germ cell colonies in recipient mouse testes generated by transplantation of Thy1+ or unselected total testis cell populations. Data are mean ± SEM for 3 independent experiments and * denotes significant difference.



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APPENDIX A

Castration Procedure

- Holstein bull calves 12-16 weeks of age were used for all experiments.
- Surgeries were performed in pens with clean shavings or in concrete floored rooms.
- Bull calves were physically restrained manually along with a halter and head catch.
- The scrotum and surrounding area were thoroughly washed with betadine solution.
- The lower 1/3 of the scrotum was removed with a sterile scalpel.
- The spermatic cord, containing the spermatic artery, was manually stripped by hand to remove the testis. The contralateral testis was then removed using the same procedure.
- The scrotal area was monitored for 1 minute for excessive bleeding and left open to properly drain.
- Banamine was administered following surgery and the animals were monitored for infection and/or pain 10 days after castration.

Sample Collection

- The tunica albuginea was removed using a scalpel.
- An incision was made in the testis to expose the parenchymal tissue.
- Sections of parenchymal tissue were cut into 200-300 mg pieces and put into 3 ml of HBSS.

APPENDIX B

Transplantation

- Overview: Bull spermatogonial stem cells (SSCs) will be collected freshly from testes and transplanted into the testes of recipient adult immunodeficient nude mice.

- Adult recipient male mice are anesthetized using an anesthetic and placed in dorsal recumbence.

- The abdominal fur is shaved and skin washed with an iodine scrub followed by alcohol and the animal will be placed on a surgical stage under microscopy.

- A midline incision approximately 1 cm in length is made through the skin and abdominal muscles to gain access to the peritoneal cavity.

- The testicular fat pad is pulled through the incision to expose the testis.

- A micropipette is threaded into the efferent duct running into the seminiferous tubules and a liquid suspension of SSCs (~10 ul total volume) is injected into the tubules of the testis.

- The testis is then placed back into the body cavity and the same procedure is performed on the contrallateral testis.

- The abdominal muscles and skin is then closed with absorbable suture.