ABSTRACT

Title of Document: CHARACTERIZATION, ENRICHMENT, AND IN VITRO CULTURE OF SPERMATOGONIAL STEM CELLS IN THE DOMESTIC CAT: A MODEL FOR RARE AND ENDANGERED FELIDS

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Spermatogenesis is a highly prolific process in which millions of spermatozoa are produced daily. Spermatogonial stem cells (SSCs), the adult stem cell population of the testis, sustain this process by providing a constant source of new progenitor cells. The ability of this stem cell population to self-renew makes it a promising alternative to spermatozoa for genetic preservation of rare and endangered animals. While innovative advances in SSC technologies have been made in the mouse, there is a paucity of information concerning felid SSCs. Therefore, the overall objective of the dissertation was to develop SSC technology in the domestic cat (*Felis catus*) as a model for rare and endangered felids. In the first study, mRNA transcripts for six SSC marker genes (*THY1*, *GPR125*, *GFRalpha1*, *PLZF*, *UCHL1*, and *OCT4*) were identified in cat testes. Localization within the appropriate *in situ* niche was confirmed by immunohistochemistry for three of the markers (PLZF, UCHL1, and OCT4). The expression pattern of these markers was conserved in the cheetah (*Acinonyx jubatus*) and Amur leopard (*Panthera pardus orientalis*),

validating the cat as an appropriate felid model. In Study 2, we explored two techniques to enrich cat testis cells for SSCs. We found that the efficiency of enrichment depends on age of the donor and that prepubertal testes are the preferred source for differential plating. Magnetic-activated cell sorting did not achieve any level of enrichment for cat SSCs, likely due to unsuitability of the antibody. The final study modified the traditional mouse SSC culture system for use in the cat. A clear effect of feeder cell type was demonstrated, with mouse endothelial C166 cells supporting a significantly higher number of germ cell colonies as compared to STO cells or primary cat fetal fibroblasts. Identity of germ cell colonies was confirmed by co-expression of UCHL1, PLZF, and OCT4. During subculture, colonies maintained SSC marker co-expression and displayed alkaline phosphatase activity. At the time of writing, cells had been maintained for 78 days *in vitro*. Together, these studies provide the groundwork towards application of SSC technology in management of rare and endangered felid populations.

CHARACTERIZATION, ENRICHMENT, AND IN VITRO CULTURE OF SPERMATOGONIAL STEM CELLS IN THE DOMESTIC CAT: A MODEL FOR RARE AND ENDANGERED FELIDS

By

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Dedication

This dissertation is dedicated in loving memory to my mom, Bonnie Marie Vansandt.

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To my family, thank you for your love, support, and guidance. A special thanks to my husband Skyler who has had to endure my pursuit of not one, but two doctoral degrees. Thank you for understanding this crazy life I have chosen. I couldn't have accomplished any of this without you and I am deeply grateful for your love, support, and encouragement.

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AP	alkaline phosphatase
bFGF	basic fibroblast growth factor
BMP4	bone morphogenic protein 4
Cas9	clustered regularly interspaced short palindromic repeats-associated endonuclease 9
CD90	cluster of differentiation 90
cFF	cat fetal fibroblast
CRISPR	clustered regularly interspaced short palindromic repeats
CSF-1	colony-stimulating factor 1
DIV	days *in vitro*
DMEM	Dulbecco modified Eagle medium
DPBS(+)	Dulbecco phosphate-buffered saline with calcium and magnesium
DPBS(-)	Dulbecco phosphate-buffered saline without calcium and magnesium
EGF	epidermal growth factor
EpCAM	epithelial cellular adhesion molecule
ESC	embryonic stem cell
FACS	fluorescent-activated cell sorting
FBS	fetal bovine serum
Fc	fragment crystallizable region
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDNF	glial cell line–derived neurotrophic factor
GFRalpha1	glial cell line–derived neurotrophic factor family receptor alpha 1
GnRH	gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
GPI	glycosyl phosphatidylinositol
GPR125	G-protein coupled receptor 125
GS	germline stem
ICC	immunocytochemistry
ICSI	intracytoplasmic sperm injection
IMC	integrated modulation contrast
ITGA6	integrin alpha 6
IUCN	International Union for Conservation of Nature
IVF	*in vitro* fertilization
LIF	leukemia inhibiting factor
M	Gene expression stability value
MACS	magnetic-activated cell sorting
MEF	mouse embryonic fibroblast
MEMalpha	minimum essential medium alpha

mGSC	multipotent germline stem cell
NCBI	National Center for Biotechnology Information
OCT4	octamer-binding transcription factor 4
P1	passage 1
P2	passage 2
PGC	primordial germ cell
PGP 9.5	protein gene product 9.5
PLZF	promyelocytic leukemia zinc-finger
POU5FI	POU domain class transcription factor 1
Ret	Rearranged during Transfection
RPL17	ribosomal protein L17
RT-PCR	reverse transcription-polymerase chain reaction
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
SCF	stem cell factor
SCNT	somatic cell nuclear transfer
siRNA	short interfering ribonucleic acid
SSC	spermatogonial stem cell
SSEA-1	stage specific embryonic antigen-1
SSEA-4	stage specific embryonic antigen-4
STO	Sandos inbred mouse embryo-derived thioguanine and ouabain resistant
TALEN	transcription activator-like effector nuclease
TBST	tris-buffered saline containing 0.05% (v/v) Tween-20
Thy1	thymus cell antigen 1
UCHL1	ubiquitin carboxy-terminal hydrolase 1
VIM	vimentin
YWHAZ	tryptophan 5’-monooxygenase activation protein zeta isoform
ZBTB16	zinc finger and BTB domain-containing protein
ZFN	zinc finger nuclease

Chapter 1: Review of Literature

Background and Significance

Challenges Facing Family Felidae

There are 38 species in the family Felidae including the domestic cat (O'Brien and Koepfli, 2013). Twenty-five of those species are on the International Union for Conservation of Nature (IUCN) Red List of Endangered Species and nearly half of all the felid species are in the top three threatened categories (IUCN, 2010). A recent report estimates that 80 percent of cat species are experiencing population declines in the wild (Hunter et al., 2010). Conversely, the human population has more than doubled since 1950 and is projected to reach eight billion by 2025 (United Nations, 2009). To support this population growth, more and more wilderness areas are being developed for human use. Loss and fragmentation of habitat are cited as the primary threat to wild cat populations (Hunter et al., 2010; Nowell et al., 1996). As natural habitats dwindle, conservation efforts have focused on preserving genetic material in these species as a hedge against extinction.

Sperm cryopreservation is the main technique for genetic preservation currently in use because sperm are more amenable to freezing than either eggs or embryos. This procedure not only preserves existing genetic material but, when used with other assisted reproductive techniques, also allows for the introduction of new genetics into a captive population. Many captive cat populations were derived from a small founder population and hence, are subject to the deleterious effects of inbreeding (Nowell et al., 1996). Sperm collected from free-living felids can be used to introduce new genes into a captive population without removing additional animals from the wild (Howard et al., 1997).

Despite the advantages and widespread use of sperm cryopreservation, there are two key limitations to this method. First, the sperm cells collected are fully mature. They are terminally differentiated cells which cannot undergo replication. This severely limits the number of breedings that can be achieved with one collection. Multiple collections are needed if more insemination doses are desired. Increasing the number of collections enhances the risk to the animal, since non-domestic species usually require anesthesia; and to persons involved, as working with wildlife is inherently dangerous. The second limitation is that sperm cryopreservation is restricted to reproductively mature individuals. This is especially pertinent in felids as it is not uncommon for offspring to be abandoned or killed (Nowell et al., 1996). In populations with small founder size, the loss of genetic diversity, even from a single individual, can have a significant impact on the long-term viability of that population. Presently, few options are available to preserve genetic material from these individuals. Although testicular tissue xenografting in cats has been promoted as a last-ditch effort to rescue genetic material from immature animals, efficiency of mature sperm production remains extremely low (Kim et al., 2007). Hence, there is an urgent need to develop additional tools to recover valuable genetic material from individuals (Swanson et al., 2007).

The Domestic Cat as an Important Biomedical Research Model

The domestic cat (*Felis catus*) has proven to be an important model organism for biomedical research. Genetic analyses have demonstrated that the cat is phylogenetically closely related to human, and of the non-primate mammals examined, shows the highest level of syntenic conservation with humans (approximately 90 percent) (Menotti-Raymond et al., 1999; Murphy et al., 1999; Pontius et al., 2007). Indeed, cats possess approximately

250 naturally-occurring genetic disorders with analogous pathologies to human diseases (Griffin and Baker, 2002; Lenffer et al., 2006; O'Brien et al., 2002). The domestic cat serves as a model for a variety of human infectious diseases, including the human immunodeficiency virus (feline immunodeficiency virus) (Willett et al., 1997), avian influenza (Kuiken et al., 2004), and severe acute respiratory syndrome coronavirus (feline infectious peritonitis coronavirus) (Martina et al., 2003; Pearks Wilkerson et al., 2004). Finally, cat models have also led to important advances in the areas of ocular and neural physiology (Rah et al., 2005; Ryugo et al., 2005).

The domestic cat also serves as a model for the other 37 members of the family Felidae. Of particular relevance is the cat's contribution to the field of reproductive physiology. Virtually all assisted reproductive technologies employed for the management and conservation of wild felids, including artificial insemination (Roth et al., 1997; Swanson et al., 1996), *in vitro* fertilization and embryo transfer (Pope et al., 2006), and cryopreservation of sperm (Swanson et al., 2007) and embryos (Pope et al., 2006) were first developed in the domestic cat (Goodrowe et al., 1988; Howard et al., 1992).

Spermatogenesis

Testis Structure

Mammalian testes are paired organs that perform two main functions: male gamete production (spermatogenesis) and steroid synthesis. The functional subunit of the testis, the lobule, consists of one to four seminiferous tubules. Each tubule forms a hairpin loop and folds back on itself within the lobule (Figure 1.1). It is within the seminiferous tubules

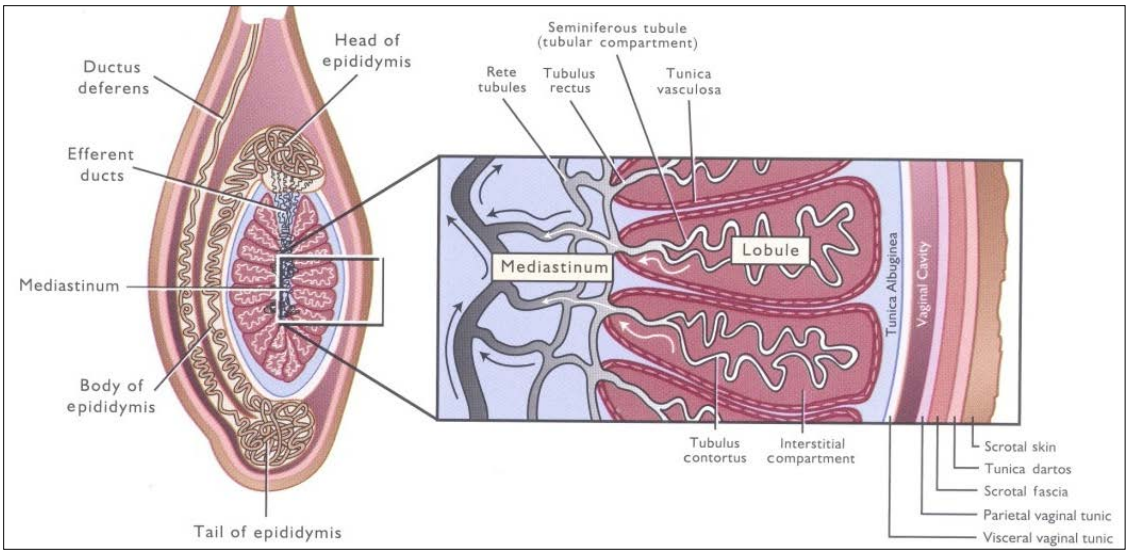

Figure 1.1 Mid-sagittal section of the mammalian testis. The seminiferous tubules are located within lobules of the testis. Each tubule forms a hairpin loop such that both ends empty into the rete testis, an interconnected series of collecting ducts that lead to the efferent ducts and ultimately, the epididymis and vas deferens. Modified from Senger (2005).

that spermatogenesis occurs. Leydig cells, also known as interstitial cells, are located in between the tubules and are responsible for androgen production (Hermo et al., 2010).

The epithelium of the seminiferous tubules consists of two intimately-associated cell types: Sertoli cells and spermatogenic cells. Sertoli cells are a specialized type of simple columnar epithelial cells that extend through the full height of the tubules and function to support and nourish the germ cells. Along with the outlying peritubular myoid cells, Sertoli cells contribute to the formation of the basement membrane that supports the seminiferous epithelium. Extensive apical and lateral processes from the Sertoli cells surround the adjacent spermatogenic cells.

The spermatogenic cells represent male germ cells in various stages of development. They are organized in poorly-defined layers of progressive development between the Sertoli cells (Ross et al., 2003). As the germ cells divide and develop, they

move from the basement membrane, through the tight junctional complexes of adjacent Sertoli cells, to the adluminal compartment (Figure 1.2). The Sertoli-Sertoli cell junctions form the blood-testis barrier, which helps to protect the developing germ cells from potentially harmful blood-borne substances, including cells of the immune system which would recognize the post-meiotic germ cells as non-self.

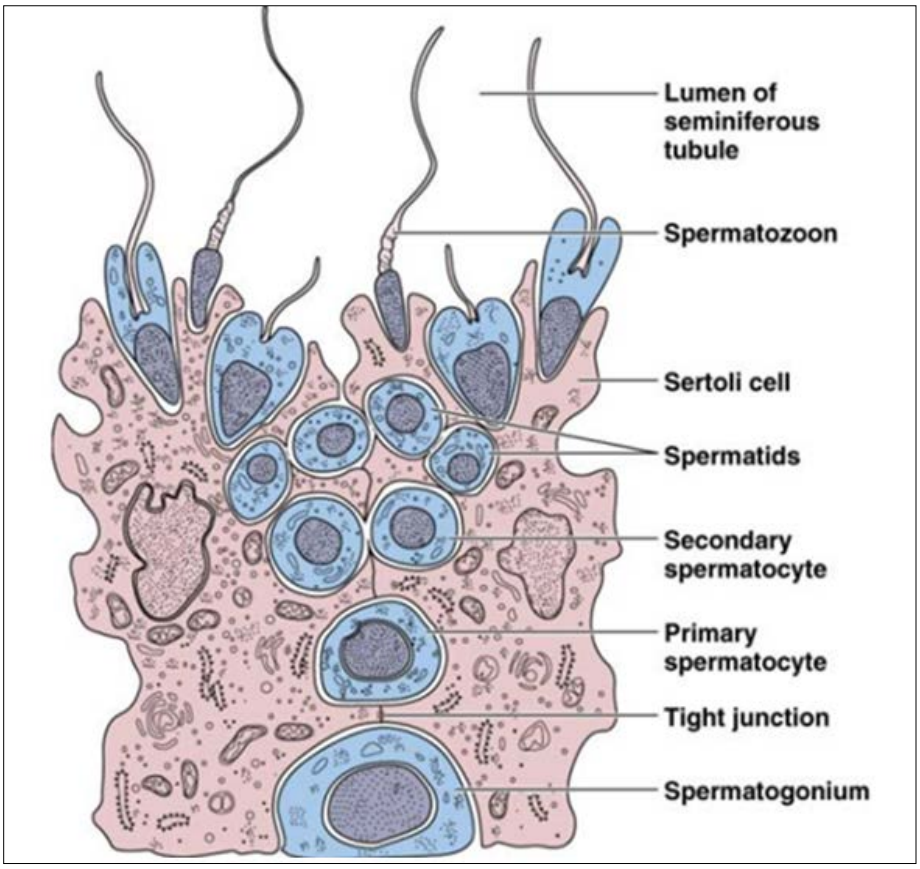

Figure 1.2 The cells of spermatogenesis. Spermatogonia and pre-leptotene primary spermatocytes are located in the basal compartment of the seminiferous epithelium, whose border is created by the basement membrane of the peritubular myoid cells and the tight junctions between adjacent Sertoli cells. During prophase I, primary spermatocytes cross the tight junctions to enter the adluminal compartment, where spermatids are also located. Adopted from Gill (2010).

Stages

Spermatogenesis is the process by which the immature spermatogonia develop, differentiate, and metamorphose into mature spermatozoa. It is a complex and orderly process that begins around puberty. The process of spermatogenesis starts with a series of mitotic divisions carried out by spermatogonia. The last mitotic division produces spermatocytes that pass through the lengthy prophase of the first meiotic division and subsequently complete two meiotic divisions to give rise to haploid spermatids. Initially, spermatids are round in shape but then elongate and become spermatozoa that leave the seminiferous tubules through the tubule lumen.

For descriptive purposes, spermatogenesis is divided into three phases. It is important to note, however, that spermatogenesis is a continuous process. The process of spermatogenesis begins with the proliferation phase. **Spermatogonia** are the most immature cell type and are located along the basement membrane of the seminiferous tubules. During the proliferative phase, they undergo a series of mitotic divisions. Classically, spermatogonia are placed in three categories based on the amount of heterochromatin present in the nucleus. As a general rule in biology, the more a cell has progressed in its lineage (and hence, the more differentiated it is), the more heterochromatin it displays (de Rooij and Russell, 2000). **Type A** spermatogonia, which display little to no heterochromatin in the nucleus, are considered the most primitive of the spermatogonia. Further cell divisions produce **Intermediate** and **Type B** spermatogonia, displaying an intermediate and high level of heterochromatin, respectively (Hermo et al., 2010).

When spermatogonia undergo mitosis, telophase is incomplete and an area of cytoplasmic continuity remains, called the cytoplasmic bridge. Spermatogonia without a cytoplasmic bridge are termed A-single (A_s). A_s spermatogonia give rise to pairs (A_{pr}) and then chains of 4 to 16 cells (A-aligned or A_{al}). The bridge allows gene products to be shared between cells, synchronizing cell division among the clones. In rodents, it is generally believed that SSCs make up a subpopulation of the A_s spermatogonia (although the two are morphologically indistinct), whereas A_{pr} and A_{al} represent the progenitor spermatogonial population (Seandel et al., 2008). A_s through A_{al} cells are collectively referred to as undifferentiated spermatogonia. This is a misnomer as most A_{pr} and A_{al} cells are irreversibly committed to develop into spermatocytes; however the term has persisted and is heavily used in the literature (de Rooij and Russell, 2000).

Each meiotic division is composed of prophase, metaphase, anaphase, and telophase. It is a process unique to germ cells that generates haploid cells and creates genomic diversity. Meiosis-I begins after Type B spermatogonia complete their last mitotic division to produce **primary spermatocytes**. The first meiotic division involves dramatic movements as the chromosomes undergo genetic exchange. Prophase-I is the longest stage in meiosis, lasting up to three weeks. Several subcategories of primary spermatocytes exist, named according to their stage in meiosis. DNA synthesis occurs in **preleptotene spermatocytes**. As prophase-I commences, chromosomes become more apparent in **leptotene spermatocytes**. In the **zygotene spermatocyte**, homologous chromosomes pair up to form the synaptonemal complex**.** Genetic recombination occurs through cross-over between paired chromosomes in **pachytene spermatocytes**. **Diplotene spermatocytes** separate the synaptonemal complexes and the chromosomes partially separate. During

diakinesis, the nuclear envelope disappears and the chromosomes condense (Hermo et al., 2010).

The remaining meiotic phases occur much more quickly that prophase-I. Diplotene cells undergo the first meiotic division to produce **secondary spermatocytes**. Secondary spermatocytes (2N) rapidly divide again by meiosis-II, producing haploid (1N) **round spermatids** (Hermo et al., 2010). Hence, a single primary spermatocyte produces four, genetically-distinct round spermatids.

Differentiation, the final phase of spermatogenesis, is commonly referred to as spermiogenesis. During this phase, round spermatids undergo dramatic morphological changes to become **elongate spermatids**, including nuclear elongation and chromatin condensation, formation of the tail, and modification of the Golgi apparatus to form the acrosome. Throughout spermatogenesis, the germ cells progressively move from the basement membrane towards the lumen of the seminiferous tubules. At the final stages, **spermatozoa** are released into the lumen. This step, termed spermiation, is analogous to ovulation in females except that it is occurring constantly throughout the testis. Spermatozoa then travel to the epididymis for the final steps of maturation, including the acquisition of motility (Senger, 2005).

Spermatogenesis in the Cat

Within a given cross-section of a seminiferous tubule, one can observe four to five concentric layers of germ cells. Because the germ cells move adluminally during development, each layer represents a different generation of cells, with the more mature cells located further from the basement membrane. Given that the length of the spermatogenic cycle is constant within a species, certain generations are generally found

together (Senger, 2005). The seminiferous epithelium cycle is a morphological description of the progression through a complete series of cellular stages at one location in the seminiferous tubules. In the domestic cat, eight stages are described. One cycle of seminiferous epithelium is defined as, within a given area of seminiferous tubules, the length of time from disappearance to reappearance of a stage. In the cat, the duration of one seminiferous cycle is 10.4 days and the total duration of spermatogenesis, based on 4.5 cycles, is 46.8 days (França and Godinho, 2003). Stages of the cat seminiferous cycle are shown in Figure 1.3. In all non-domestic felids investigated to date, the number of stages remained the same and germ cell morphology was very similar to that of domestic cats (Costa et al., 2008; Silva et al., 2010). Variation was seen in the frequency of each stage as well as the overall duration of spermatogenesis.

Spermatogenesis is one of the most productive cell-producing systems in adult animals, in which cells are continually being produced and lost. The constant rate of sperm production is heavily reliant on the regulation of the adult stem cell population of the testis, the spermatogonial stem cell (SSC).

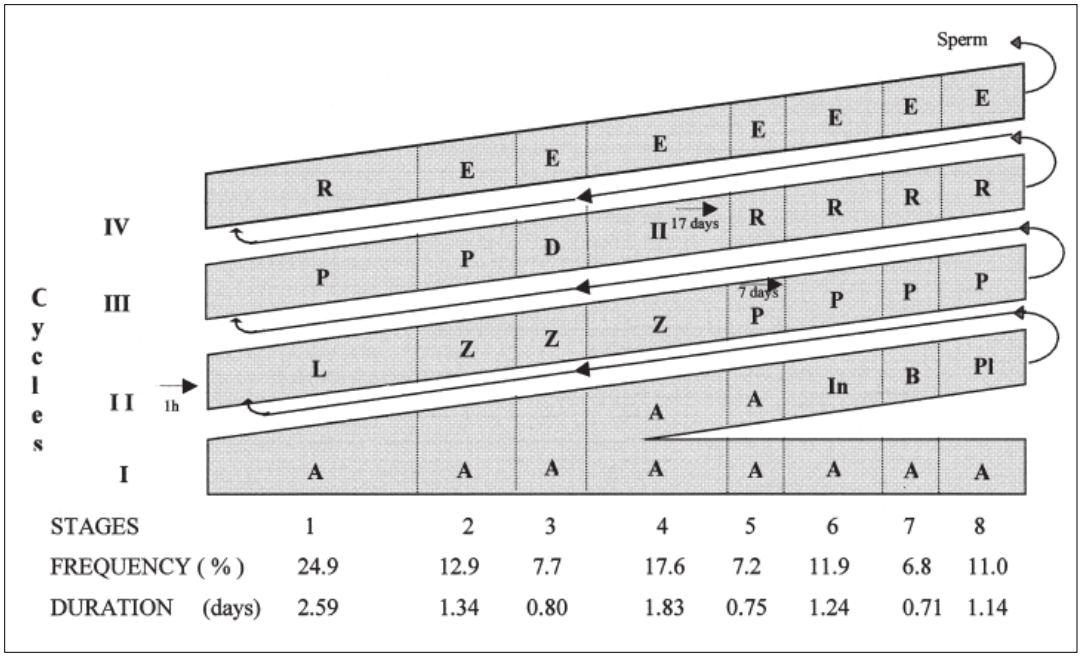

Figure 1.3 Seminiferous epithelium cycle in the domestic cat. This diagram illustrates the germ cell composition, frequency (%), and duration in days for each stage of the seminiferous epithelium cycle. Also depicted is the most advanced germ cell type labeled at the eight stages of the cycle at the different time periods (1 h, 7 days, and 17 days) following tritiated thymidine injections. The roman numerals indicate the spermatogenic cycle. The space given to each stage is proportional to its frequency and duration. The letters within each column indicate germ cell types present at each stage of the cycle. Type A spermatogonia (A); intermediate spermatogonia (In); type B spermatogonia (B); preleptotene spermatocytes (Pl); leptotene (L); zygotene (Z); pachytene (P); diplotene (D); secondary spermatocytes (II); round spermatids (R); elongate spermatids (E). Figure reprinted with permission from França et al. (2003).

Spermatogonial Stem Cell Biology

Stem Cell Classification

There are two broad classes of stem cells. Embryonic stem cells (ESCs) are derived from the inner cell mass of pre-implantation embryos. ESCs are pluripotent, meaning they have the ability to give rise to all of the cell types that make up the body (Evans and Kaufman, 1981; Martin, 1981) but typically cannot make the extra-embryonic tissues of the placenta (Beddington and Robertson, 1989), although it is important to note that ESCs

can be pushed towards a trophectoderm lineage under certain culture conditions (He et al., 2008).

Adult stem cells are undifferentiated cells found among differentiated cells in a tissue or organ. Adult stem cells are usually multipotent, able to develop into more than one cell type, but restricted within a particular tissue, organ, or physiological system. Typically, adult stem cells function to maintain and repair the tissue in which they are found (National Institutes of Health 2010; Weissman et al., 2001).

SSCs are unipotent adult stem cells, defined as the undifferentiated stem cells located along the basement membrane of the seminiferous tubules. SSCs are derived from gonocytes, which in turn, arise from primordial germ cells (PGCs). During embryogenesis, PGCs migrate from the yolk sac to the genital ridge. The arrival of PGCs stimulates the formation of the primitive sex cords. Once the seminiferous cords are fully formed, the PGCs are considered gonocytes (Senger, 2005). Gonocytes persist in the center of the seminiferous tubules until after birth. During the first wave of spermatogenesis, gonocytes resume mitosis, migrate to the basement membrane of the seminiferous tubules, and differentiate into SSCs. The first biologically active SSCs appear three to four days postpartum in the male mouse (McLean et al., 2003). The precise time period is undefined in the domestic cat. Because the average age of puberty in the male cat is nine months (Senger, 2005), the transition is estimated to occur over several months, similar to most livestock species.

Similar to other stem cells, SSCs have two fates. Cell division can give rise to either more undifferentiated SSCs (self-renewal) or to a differentiated cell population committed to enter meiosis and become mature spermatozoa (de Rooij and Russell, 2000). Currently,

there are two theories on the pattern of SSC cellular division (Figure 1.4). The symmetrical theory suggests that division would produce either two daughter SSCs or two committed differentiated daughter cells. Asymmetrical division would give rise to one new SSC and one committed daughter cell (Oatley and Brinster, 2008). It is a matter of debate whether mammalian testes utilize one or both of these pathways. Regardless, self-renewal provides a reservoir for future spermatogenic cycles while differentiation ensures an adequate number of spermatozoa are available for fertilization. The balance between the two fates is precisely regulated by the SSC niche (He et al., 2009).

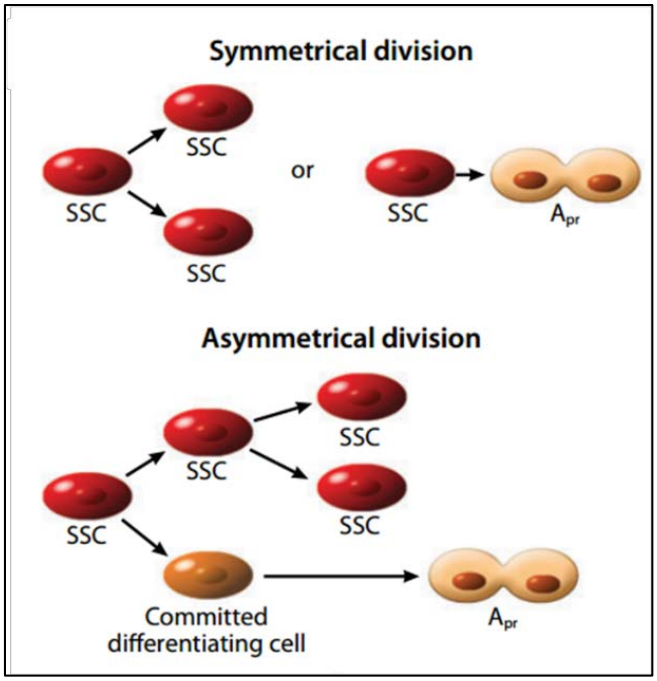

Figure 1.4 Possible division pathways of SSCs. The symmetrical division theory suggests that an SSC would divide to produce either two new SSCs (self-renewal) or two A_{pr} spermatogonia (differentiation) with an interconnecting cytoplasmic bridge. In asymmetrical division, an SSC division would produce two daughter cells: one new SSC (self-renewal) and one committed differentiating cell that produces A_{pr} spermatogonia upon its next division. It is unknown whether one or both of these pathways occur in mammalian testes. Adopted from Oatley et al. (2008).

SSCs are unique in many aspects of their stem cell biology. In contrast to other adult stem cells, SSCs are highly specialized cells whose sole function is to transmit genetic information to the next generation. SSCs are defined as unipotent because they differentiate into one end-product: spermatozoa. However, it has now been demonstrated that pluripotent cells can be derived from SSCs without the addition of genes. Through culture condition manipulation, ESC-like cells were established in mouse (Guan et al., 2006; Kanatsu-Shinohara et al., 2004a; Seandel et al., 2007b) and human (Golestaneh et al., 2009) that were able to differentiate into all three germ layers and organ lineages.

The SSC Niche Microenvironment

A stem cell niche is defined as a subset of cells and extracellular substrates that can indefinitely house one or more stem cells and control their self-renewal and progeny production *in vivo*. Niches provide extrinsic stimuli to regulate the balance between self-renewal and differentiation through architectural support and growth factor stimulation (Spradling et al., 2001). The SSCs reside on the basement membrane of the seminiferous tubules. Sertoli cell tight junctions form the apical border of the niche. All spermatogonia remain in this area for the duration of the mitotic divisions. As spermatogenesis proceeds, the cells lose contact with the basement membrane and, at the start of the first meiotic prophase, spermatocytes move through tight junctions to reside in the adluminal side (de Rooij and Grootegoed, 1998).

Using a specialized fixative and plastic embedding, Chiarini-Garcia et al. made testis sections in which the undifferentiated spermatogonia (A_s, A_{pr}, A_{al}) could be discerned from the more differentiated spermatogonia (A_1 to A_4) in the mouse (2001) and rat (2003).

As previously mentioned, the SSC niche exists between the basement membrane and tight junctions of the seminiferous epithelium. In both rodent species, it was discovered that the undifferentiated spermatogonia, including SSCs, are not evenly distributed along the basement membrane, but rather preferentially locate to areas bordering the interstitial tissue. This idea was expanded upon by Yoshida et al. (2007) who demonstrated that mouse A_s, A_{pr}, and A_{al} spermatogonia follow blood vessels in the interstitium, favoring locations where the vessels branch. Thus, it appears the presence of blood vessels and/or Leydig cell clusters helps determine the location of the niche.

Due to their intimate association with SSCs, Sertoli cells have been extensively studied for their role in the niche. Sertoli cells have been demonstrated to produce two growth factors that stimulate SSC self-renewal: glial cell line–derived neurotrophic factor (GDNF) (Kubota et al., 2004b; Meng et al., 2000; Tadokoro et al., 2002) and basic fibroblast growth factor (bFGF) (Goriely et al., 2005; Simon et al., 2007). The Growth Factors section contains a more complete description of these items.

While Sertoli cells are considered the major SSC niche contributor, it is important to note that Sertoli cells are influenced by other cell types. Leydig cells affect Sertoli cell function through the production of testosterone. Mice treated with leuprolide, a testosterone-reducing agent, showed an enhanced rate of colonization following SSC transplantation (Ogawa et al., 1998). However, these results must be interpreted with caution as leuprolide is a gonadotropin-releasing hormone (GnRH) agonist, which could have other effects as well. Studies suggest that gonadotropins have an important role in the SSC niche, but the specific effects vary depending on the developmental stage of the male (Kanatsu-Shinohara et al., 2004b; Oatley et al., 2005). Peritubular myoid cells have also

been shown to influence Sertoli cell function and spermatogenesis in several ways (Hoeben et al., 1999; Verhoeven et al., 2000).

In addition to their roles in Sertoli cell function, it appears Leydig and peritubular myoid cells may have a more direct role in SSC self-renewal. Colony-stimulating factor 1 (CSF-1) is produced by Leydig and peritubular myoid cells. Expression of the CSF-1 receptor is enhanced in SSC-enriched fractions of the mouse testis. Exposure to recombinant CSF-1 was subsequently demonstrated to enhance mouse SSC self-renewal *in vitro* (Oatley et al., 2009).

Interestingly, Sertoli cells also play a major role in the differentiation of SSCs. When added to cultures of mouse SSCs, Sertoli cell factors Activin A and bone morphogenic protein 4 (BMP4) reduce self-renewal (Nagano et al., 2003). BMP4 was also found to induce c-Kit expression in spermatogonia (Pellegrini et al., 2003). The c-Kit receptor is expressed in late A_{al} spermatogonia onward and is important for the transition into A_1 spermatogonia (Schrans-Stassen et al., 1999). Its ligand, stem cell factor (SCF), is also secreted by Sertoli cells and can induce differentiation of spermatogonia that express the c-Kit receptor.

Taken together, these studies clearly demonstrate that many factors control the behavior of SSCs. Precisely how the balance between self-renewal and differentiation is struck has yet to be elucidated. Additional factors such as permeability of the basement membrane and external cell-matrix attachment ability of the growth factors no doubt contribute to the regulation as well (de Rooij, 2009). Figure 1.5 depicts the various cell types that make up the SSC niche in the mammalian testis.

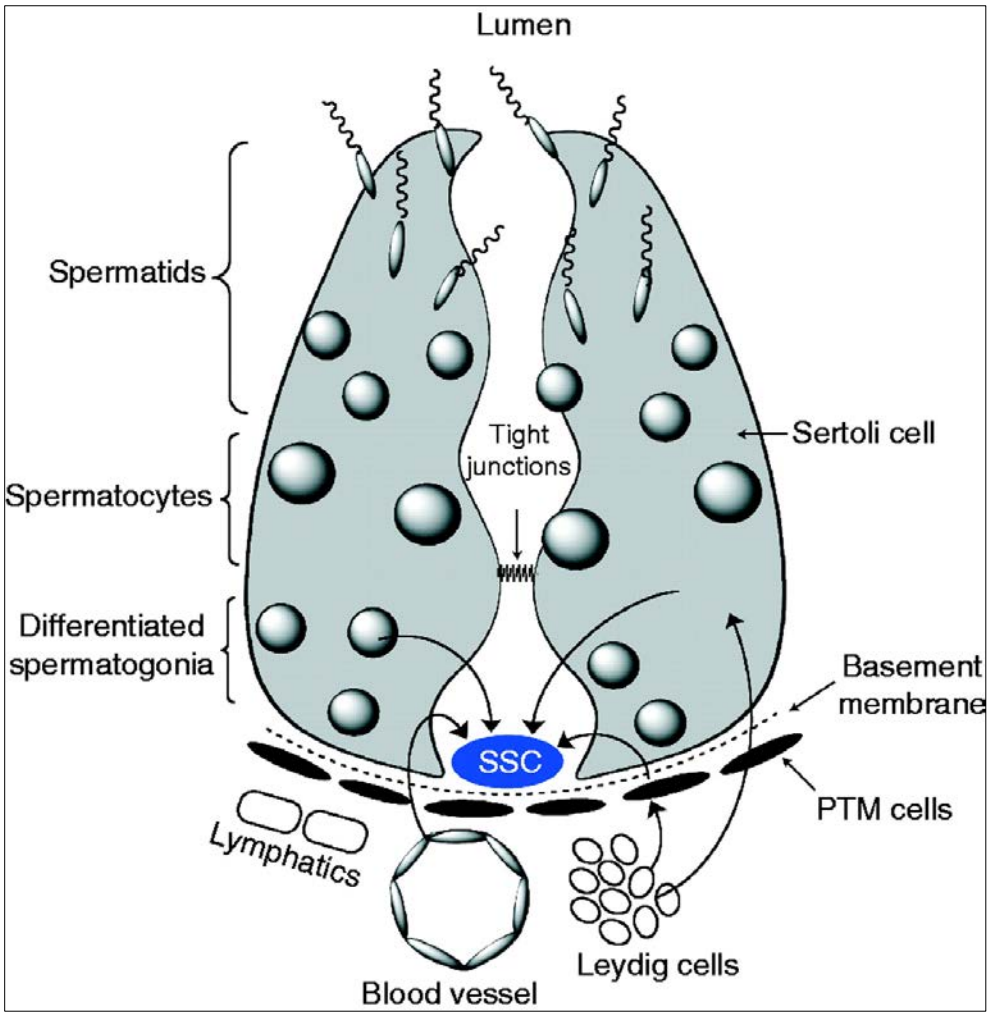

Figure 1.5 Spermatogonial stem cell niche in the mammalian testis. This model demonstrates the sources of potential autocrine, paracrine, and environmental cues that may influence spermatogonial stem cell fate in the mammalian testis. Adopted from Caires et al. (2010).

Molecular Markers of SSCs

Intracellular and cell surface markers are required to enrich stem cell populations, evaluate enrichment strategies, and monitor the success of *in vitro* culture systems. While several SSC markers have been described in a host of species, to date, none of the reported markers are expressed exclusively by the stem cells. The molecular markers mentioned

below are usually expressed by SSCs as well as other spermatogonia that are committed to differentiation and hence, have lost their stem cell activity. Some markers are expressed in somatic cells as well. For this reason, unequivocal identification of SSCs requires a functional transplant assay in which donor testicular cells are injected into the seminiferous tubules of recipient males whose endogenous germ cells are depleted (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). Only the SSCs are able to establish colonies and resume spermatogenesis in the host tubules. Each colony represents the clonal expansion of a single SSC (Dobrinski et al., 1999b). Utilizing the transplant assay, potential SSC markers have been evaluated based on their use in antigen-based cell sorting and the subsequent change in rate of seminiferous tubule colonization. Markers that significantly enhance the colonization rate are considered purported SSC markers.

Surface Markers

GFRalpha1

Glial cell line–derived neurotrophic factor (GDNF) is secreted by Sertoli cells and has been demonstrated to be critical for SSC self-renewal (Meng et al., 2000). GDNF family receptor alpha 1 (GFRalpha1), a glycosyl phosphatidylinositol (GPI)-linked cell surface protein, forms part of the GDNF receptor complex. GFRalpha1 is the cell surface receptor that binds GDNF, mediating the activation of the Rearranged during Transfection (Ret) receptor tyrosine kinase (Airaksinen and Saarma, 2002) (Figure 1.6).

In the mouse, GFRalpha1 is expressed in a subpopulation of spermatogonia that were able to proliferate and form colonies in culture. Furthermore, GFRalpha1 silencing through the use of short interfering RNAs (siRNAs) resulted in the differentiation of these

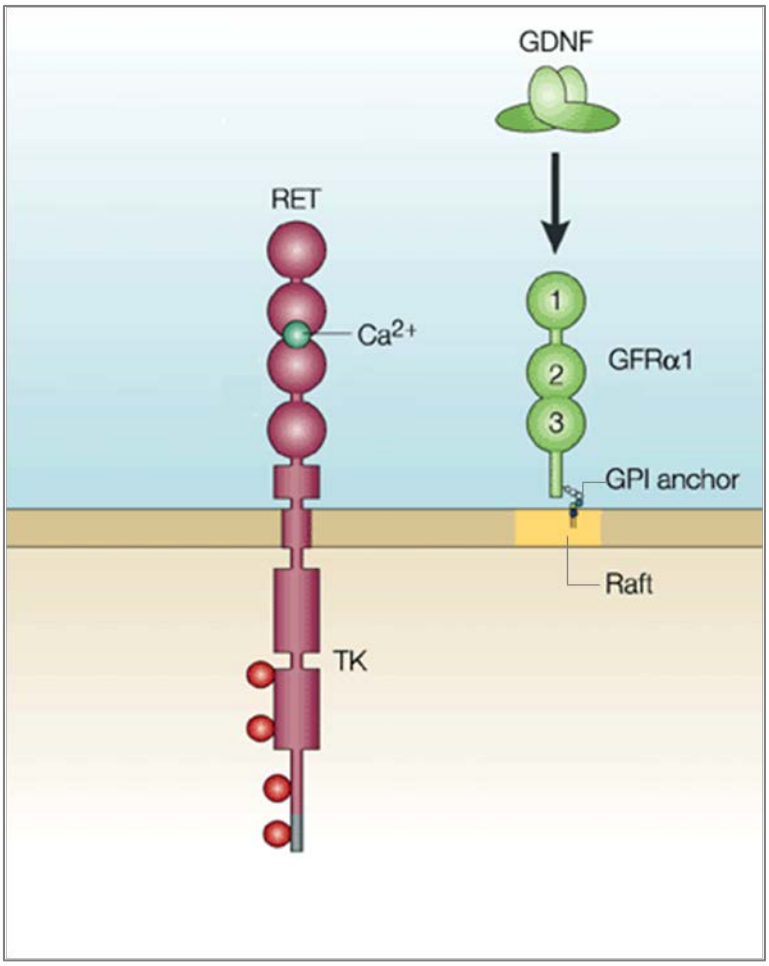

Figure 1.6 GDNF signals through a multi-component receptor complex. GFRalpha1 (GFRα1) is attached to the outer layer of the plasma membrane by a GPI anchor, and thus is localized to lipid rafts. The binding of GDNF to the GFRα1 recruits Ret to the rafts and triggers the formation of a signaling complex. Ret is a single pass transmembrane protein that contains four cadherin-like repeats in its extra-cellular domain and a typical intracellular tyrosine kinase domain. Activation of Ret induces autophosphorylation of the tyrosine residues, and intracellular relay of the signal. Modified from Airaksinen et al. (2002).

cells (He et al., 2007). GFRalpha1 expression has also been demonstrated in rat (Ryu et al., 2005), bull (Oatley et al., 2004), non-human primate (Hermann et al., 2007), human (He et al., 2010), and cat (Silva et al., 2012).

GPR125

GPR125 is a member of the adhesion family of G-protein coupled receptors (GPCRs). It is an orphan receptor, with no endogenous ligand yet identified. As with other adhesion-GPCRs, GPR125 has a long N-terminus that is believed to bind various proteins that promote cell-to-cell and cell-to-matrix interactions (Bjarnadóttir et al., 2004). Seandel et al. (2007b) first identified GPR125 as a potential SSC marker when evaluating a large set of mouse knockouts. Histological examination of the mouse testis revealed the expression pattern of GPR125 is restricted to the first layer of spermatogonia, adjacent to the basement membrane of the seminiferous tubules (Seandel et al., 2007b). When cultured, GPR125-positive cells displayed characteristic SSC morphology and expressed mouse germ-lineage markers. Cells retained GPR125 expression even after months of culture and were able to resume spermatogenesis upon transplantation into chemically-sterilized mice. The human testis has a similar expression pattern, with GPR125 localized to a subpopulation of spermatogonia that are adjacent to the seminiferous tubule basement membrane. Importantly, GPR125 displayed an extremely restricted expression pattern, with only one or two positive spermatogonia per cross-section (He et al., 2010). Magnetic-activated cell sorting (MACS)-isolated GPR125-positive cells were shown to co-express three mouse SSC/progenitor markers: integrin alpha 6 (ITGA6), Thy1 (thymus cell antigen 1), and GFRalpha1. He et al. (2010) were able to culture the cells for two weeks in an undifferentiated state while maintaining a high level of proliferation. However, no

xenotransplantation assay was performed so it is unknown whether these cells were truly SSCs.

THY1

Thymus cell antigen 1 (THY1), also known as cluster of differentiation 90 (CD90), is a GPI-anchored glycoprotein of the immunoglobulin superfamily. It is a marker for a variety of stem cells, including hematopoietic stem cells (Spangrude et al., 1988), mesenchymal stem cells (Pittenger et al., 1999), and embryonic stem cells (Henderson et al., 2002). Kubota et al. (2004a) demonstrated that MACS sorting with THY1 microbeads efficiently enriched SSCs in all age groups of mice tested: neonate, pup, adult wild-type, and adult cryptorchid. Furthermore, fractions obtained from adult testis experienced a greater than 30-fold enrichment of SSCs compared to the total testis cell population. It was subsequently revealed that all SSCs of the rat testis are THY1-positive (Ryu et al., 2004). Selection of THY1-positive cells in rhesus macaque testes has also demonstrated successful SSC enrichment (Hermann et al., 2009). Finally, THY1 MACS selection in the prepubertal bull resulted in a 3-fold enhancement of SSC activity, as demonstrated by a xenogeneic transplantation assay (Reding et al., 2010). An expression pattern restricted to a subset of spermatogonia has also been shown in the pig (Zheng et al., 2013a) and goat (Abbasi et al., 2013; Wu et al., 2013).

E-cadherin

Cadherins are a large family of transmembrane glycoproteins that mediate calcium-dependent homotypic cell adhesion (Wheelock and Johnson, 2003). In Drosophila, E-cadherin (also known as ovulomorin) functions to anchor the germ stem cells to their niche (Jin et al., 2008; Song et al., 2002). Two studies in the mouse reported that E-cadherin

expression is limited to a subset of undifferentiated spermatogonia that include SSCs, as demonstrated by the SSC transplant assay (Tokuda et al., 2007; Tolkunova et al., 2009). E-cadherin showed a similar expression pattern in the rat (Zhang et al., 2011) and buffalo (Yu et al., 2014), although a stem cell transplant assay was not performed with E-cadherin-selected cells. The exact role of E-cadherin in mammalian spermatogenesis is unknown. Give its role as an adhesion protein, it has been hypothesized that E-cadherin may help maintain the SSCs within their niche, similar to E-cadherin's role in Drosophila.

Intracellular Markers

In addition to surface markers, several intracytoplasmic and nuclear SSC markers have been identified. Although unsuitable for antigen-based cell sorting, these markers can be used to monitor the success of culture studies and may be more specific than the surface markers.

PLZF

Promyelocytic leukemia zinc-finger (PLZF), also known as Zinc finger and BTB domain-containing protein (ZBTB16), belongs to the POZ/zinc-finger family of transcription factors (Ching et al., 2010). PLZF is a spermatogonia-specific transcription factor in the testis that is required to regulate self-renewal and maintenance of the stem cell pool (Buaas et al., 2004; Costoya et al., 2004). It has been identified as the disrupted gene in the classic *luxoid* mutation in which mice exhibit a progressive loss of SSCs. PLZF stimulates SSC self-renewal through direct repression of c-Kit receptor transcription (Filipponi et al., 2007). As discussed above, c-Kit expression is associated with spermatogonial differentiation; specifically, the transition into A_1 spermatogonia (Schrans-Stassen et al., 1999). By blocking c-Kit expression in A_s, A_{pr}, and A_{al} spermatogonia, PLZF

appears to prevent their differentiation, thus promoting self-renewal. A similar expression pattern of PLZF has subsequently been demonstrated in a variety of species, including the bull (Reding et al., 2010), goat (Song et al., 2013), sheep (Borjigin et al., 2010), pig (Luo et al., 2006), horse (Costa et al., 2012), dog (Harkey et al., 2013), and non-human primates (Maki et al., 2009).

UCHL1

Ubiquitin-dependent proteolysis has been implicated in the control of mammalian gametogenesis and ubiquitin carboxy-terminal hydrolase 1 (UCHL1), also known as protein gene product 9.5 (PGP 9.5), is a deubiquinating enzyme that regenerates monoubiquitin from ubiquitin–protein complexes (Kwon et al., 2004; Sutovsky, 2003). In the mouse testis, UCHL1 is expressed in both spermatogonia and Sertoli cells (Kwon et al., 2004). This appears to be a rodent-specific phenomenon, as UCHL1 is expressed exclusively in spermatogonia for a variety of non-rodent species, including the pig (Luo et al., 2009; Luo et al., 2006), bull (Herrid et al., 2007; Reding et al., 2010; Wrobel, 2000), buffalo (Goel et al., 2010), goat (Heidari et al., 2012), dog (Harkey et al., 2013), non-human primate (Tokunaga et al., 1999), and human (He et al., 2010). Furthermore, UCHL1 expression was limited to a subset of the spermatogonia which were in contact with the basement membrane. This suggests that SSCs are included in the UCHL1-positive cells.

OCT4

OCT4 (octamer-binding transcription factor 4), also known as POU domain class 5 transcription factor 1 (POU5F1), is a homeodomain transcription factor of the POU family (Takeda et al., 1992b). OCT4 is critically involved in the regulation of pluripotency in ESCs; its expression level is closely linked to the balance between self-renewal and

differentiation (Boyer et al., 2005; Niwa et al., 2000). OCT4 is expressed in undifferentiated spermatogonia and, at least in the mouse, has a more restricted expression pattern than PLZF (Dann et al., 2008; Pesce et al., 1998). OCT4 also appears to play an important role in SSC self-renewal, through a separate pathway from PLZF (Dann et al., 2008). Similar expression patterns have been reported in the rat (Ryu et al., 2005) hamster (Kanatsu-Shinohara et al., 2008), bull (Fujihara et al., 2011), buffalo (Goel et al., 2010), and pig (Luo et al., 2006). The data on OCT4 expression in human testes is controversial; some investigators report a similar level of expression (Mirzapour et al., 2012), whereas others fail to detect it at all (He et al., 2010).

Self-Renewal SSC Culture

Early studies of SSCs borrowed culture conditions from ESCs and utilized various concentrations of serum (Nagano et al., 1998; Nagano et al., 2003). While these studies demonstrated that SSCs could be maintained *in vitro*, neither study observed an expansion of stem cell numbers. Serum contains undefined materials and variation among batches can lead to inconsistent cell culture performance. Furthermore, serum is toxic for many cell types (Barnes and Sato, 1980; Enat et al., 1984).

In order to establish defined, experimentally modifiable conditions, cell culture systems should be serum-free (Barnes and Sato, 1980; Ham and McKeehan, 1979). For most cell types, serum-free conditions can be achieved through the addition of specific hormones and growth factors (Barnes and Sato, 1980; Hayashi and Sato, 1976). The first serum-free culture system for SSCs consisted of a commercially available medium (minimum essential medium alpha, MEMalpha), mitotically inactivated STO (Sandos inbred mouse embryo-derived thioguanine and ouabain resistant) cell feeders, and MACS-

sorted THY1-positive cells (Kubota et al., 2004a). The cells were kept at 37ºC in 5% CO_2. An enriched stem cell population was believed to be critical to the culture system as contaminating non-stem cells would likely influence the SSC behavior. Utilizing enriched stem cells and serum-free conditions, Kubota et al. (2004b) were the first to report the successful long-term culture of SSCs. The clumping germ cells expanded exponentially for over six months and retention of SSC activity was confirmed using a functional transplant assay. Rat and hamster SSCs have also been successfully cultured long-term (Kanatsu-Shinohara et al., 2008; Ryu et al., 2005).

Extension of SSC culture systems to non-rodent species has been limited. The only non-rodent species that has been successfully propagated long-term is the rabbit (Kubota et al., 2011). Short-term culture (typically less than two months) has been achieved in the bull (Aponte et al., 2008; Aponte et al., 2006; Fujihara et al., 2011; Nasiri et al., 2012; Oatley et al., 2004), buffalo (Goel et al., 2010; Kadam et al., 2013; Kala et al., 2012; Xie et al., 2010), goat (Bahadorani et al., 2012; Heidari et al., 2012; Zhu et al., 2013), pig (Goel et al., 2007; Kuijk et al., 2009; Luo et al., 2006; Zheng et al., 2013b), cat (Tiptanavattana et al., 2013), dog (Harkey et al., 2013), and human (Aponte et al., 2008; Sadri-Ardekani et al., 2009). In these species, the proliferation of putative SSCs appears to experience a gradual decline during subculture such that over time, differentiation and apoptosis leads to cessation of culture. The major factor limiting advancement is the failure to identify appropriate culture conditions (Feng et al., 2002). Human studies have not yet evaluated the effect of individual growth factors on SSC cultures (He et al., 2010; Sadri-Ardekani et al., 2009). Aponte et al. (2008) looked at the effects of individual growth factors on bovine SSC cultures. However, the authors did not obtain an enriched stem cell population

(cultures were initiated from a mixed population of cells that included thirty percent somatic cells) and used an undefined, serum-dependent culture system, both of which may contribute unknown factors to the cells. Similarly, the only report of *in vitro* propagated cat SSCs did not assess the purity of the starting cell population; used an undefined, serum-dependent culture system; and did not investigate the effects of individual growth factors (Tiptanavattana et al., 2013). In that study, only 2 of 30 presumptive SSC colonies survived the first passage. Colony proliferation was reduced by 30 days *in vitro* (DIV), and proliferation had completely ceased by 47 DIV.

Growth Factors for SSC Self-Renewal

GDNF

Glial cell line–derived neurotrophic factor is a member of the TGF-β superfamily of growth factors with demonstrated roles in kidney morphogenesis and neuronal progenitor cell function (Dressler, 2006; Sariola and Saarma, 2003). In regard to SSCs, it is a niche factor essential to self-renewal. The importance of GDNF was first demonstrated by Meng et al. (2000), who described disrupted spermatogenesis in mutant mice with one GDNF- null allele. The authors also observed accumulation of A_{pr} and A_{al} spermatogonia in male mice overexpressing GDNF. As described above, the GDNF receptor complex is made up of GFRalpha1 and Ret. Targeted disruption of any one of these elements results in impaired spermatogenesis in homozygous null male mice (Naughton et al., 2006). Inclusion of GDNF in culture media is essential for self-renewal in long-term culture of mouse (Kanatsu-Shinohara et al., 2005a; Kubota et al., 2004b), rat (Ryu et al., 2005), hamster (Kanatsu-Shinohara et al., 2008) and rabbit (Kubota et al., 2011) SSCs.

bFGF

Basic fibroblast growth factor (bFGF), also known as FGF2 or FGF-β, is a member of the fibroblast growth factor family. The first insight into its importance came when Resnick et al. (1992) reported that PGCs require bFGF supplementation to expand *in vitro*. Kubota et al. (2004b) discovered that when bFGF is added in combination with GDNF, mouse SSC expansion is enhanced. However, bFGF is unable to achieve a similar effect when added alone. Likewise, hamster SSCs require GDNF and bFGF to expand in culture (Kanatsu-Shinohara et al., 2008). On the contrary, it appears that bFGF alone is able to support rat SSCs during short-term culture (i.e., 1 week) (Ryu et al., 2005). However, more clump-forming cells were observed, and presumably more SSCs were present, when bFGF was supplemented with GDNF. In long-term culture studies of the rat, bFGF was not investigated alone but did display an additive effect when used in combination with GDNF (Ryu et al., 2005).

Soluble GFRalpha1

The fusion protein GFRalpha1-Fc is a soluble form of the GFRalpha1 receptor fused to the crystallizable region fragment (Fc) of an IgG antibody. First demonstrated in neuronal cells, soluble GFRalpha1 molecules are able to bind to GDNF with high affinity and trigger the activation of the Ret tyrosine kinase (Paratcha et al., 2001). When soluble GFRalpha1 was added to the culture media with GDNF and bFGF, mouse germ cell clumps expanded dramatically (Kubota et al., 2004b). Furthermore, cultured cells were transplanted at each time point in the study to confirm that the increase in clumps represented an increase in SSCs. Following 72 days of culture with the three growth factors, each stem cell was estimated to produce greater than 5,000 daughter cells. Taken

together, these results indicate that the combination of GDNF, bFGF, and GFRalpha1 support *in vitro* proliferation of mouse SSCs. Similar results were achieved with the rat (Ryu et al., 2005); however GFRalpha1 was not found to be required in the hamster (Kanatsu-Shinohara et al., 2008).

EGF

Epidermal growth factor (EGF) is the founding member of the EGF family of proteins. EGF has a wide variety of biological effects, the most notable being its ability to promote proliferation and differentiation. Similar to bFGF, EGF appears to have an additive effect on SSC self-renewal when supplemented with GDNF (Kanatsu-Shinohara et al., 2005a). However, that study was performed under feeder-free conditions with germline stem (GS) cells, which is a self-renewing germ cell population derived from the culture of gonocytes (i.e., prospermatogonia) collected from neonate mouse testes (Oatley and Brinster, 2008). These cells have varying degrees of stem cell potential and it is unclear how similar GS cells and SSCs behave in culture. Two other studies utilizing mouse SSCs were unable to demonstrate a significant effect using EGF (Kubota et al., 2004a, b). In the hamster, EGF does not potentiate the effects of GDNF (Kanatsu-Shinohara et al., 2008).

LIF

Leukemia inhibiting factor (LIF) is a pleiotropic cytokine of the interleukin-6 cytokine family. In addition to its classical roles in the immune response, inflammation, and hematopoiesis; downstream targets of LIF can affect cellular growth, differentiation, and survival (Hirano, 1998). LIF was investigated as a possible SSC self-renewal growth factor due to its demonstrated importance in ESC self-renewal (Williams et al., 1988) and

PGC proliferation (Smith, 2001). In mouse SSC cultures, the addition of LIF did not enhance the proliferation rate in serum-dependent (Kubota et al., 2004a; Nagano et al., 2003) or serum-free (Kubota et al., 2004b) conditions. In neonatal mouse GS cells, LIF has been shown to enhance the formation of germ cell colonies, but failed to affect self-renewal (Kanatsu-Shinohara et al., 2007). It was hypothesized that LIF facilitated the establishment of colonies by promoting the maturation of gonocytes into spermatogonia. Similarly, the addition of LIF to GDNF-containing culture media did not affect the proliferation of rat SSCs in short or long-term cultures (Ryu et al., 2005).

Applications of Felid SSC Technology

Domestic cats are the most popular household pet in the United States (AVMA, 2012). Currently, the cat receives a level of medical surveillance second only to humans and the domestic dog, and as such, feline health is a major focus of basic, translational, and clinical veterinary research (Menotti-Raymond and O'Brien, 2008). Due to its close phylogenetic relationship with humans (Menotti-Raymond et al., 1999; Murphy et al., 1999; Pontius et al., 2007), the cat also serves as an important biomedical model for a variety of hereditary (Griffin and Baker, 2002; Lenffer et al., 2006; O'Brien et al., 2002) and infectious (Kuiken et al., 2004; Martina et al., 2003; Pearks Wilkerson et al., 2004; Willett et al., 1997) human diseases. The domestic cat is also used as a model for rare and endangered felids because the reproductive physiology is well conserved in members of the Felidae family (Brown, 2006; Pelican et al., 2006). Thus, research related to cat SSCs has the potential to benefit human and veterinary medicine, as well as endangered felid species.

Assisted Reproduction in Conservation Biology

Historically, sperm cryopreservation has served as the primary means for preservation of the male genome. However, spermatozoa are fully mature cells which cannot undergo further replication. This severely limits the number of insemination doses that can be achieved with one collection. Furthermore, sperm collection is restricted to reproductively mature individuals, preventing the recovery of valuable genotypes from immature animals. This is especially pertinent in felids in which juvenile mortality is characteristically high (Nowell et al., 1996; Wielebnowski, 1996). For example, a study performed retrospectively on 11 felid species bred in North American zoos reported juvenile mortality rates ranging from 30 to 50 percent (Wielebnowski, 1996). Presently, few options are available to preserve genetic material from these young individuals. In populations with small founder sizes, the loss of genetic diversity, even from a single individual, can have a significant impact on the population's long-term viability (Pukazhenthi et al., 2006a; Swanson et al., 2007). Hence, there is an urgent need to develop additional tools to recover genetic material from these individuals.

SSCs are present in the testis from birth through adulthood (de Rooij and Russell, 2000). Therefore, in contrast to spermatozoa, SSCs can be collected from reproductively immature animals. Furthermore, because SSCs persist throughout life, these cells also can be collected from adults. As stem cells, SSCs maintain the ability to self-replicate, providing a potentially unlimited supply of cells and an alternate source for preservation of the male genome. Additionally, SSCs can be cryopreserved using a simple cryopreservation technique routinely used on cultured somatic cells (Robertson, 1987). First performed in the mouse (Avarbock et al., 1996), the same freezing procedure can be

used in a diverse group of species; including the rat (Clouthier et al., 1996), hamster (Ogawa et al., 1999), bull (Dobrinski et al., 2000; Izadyar et al., 2002a), pig (Dobrinski et al., 2000), horse (Dobrinski et al., 2000), rabbit (Dobrinski et al., 1999a), dog (Dobrinski et al., 1999a), baboon (Nagano et al., 2001), and human (Brook et al., 2001; Nagano et al., 2002). This is in sharp contrast to mature spermatozoa, in which cryopreservation protocols must be developed on a species by species basis. Marked differences can exist among individuals within the same species, or even between different ejaculates of the same male (Songsasen and Leibo, 1997; Songsasen et al., 2002). Such is the case with felids; the 38 members of this Family exhibit striking variability in the types and severity of sperm cryosensitivities (Baudi et al., 2008; Crosier et al., 2006; Gañán et al., 2009; Herrick et al., 2010; Pukazhenthi et al., 2006b; Thiangtum et al., 2006). Many of these species are notorious for being difficult to cryopreserve; a problem no doubt compounded by the high prevalence of teratospermia seen among felids (Pukazhenthi et al., 2000; Pukazhenthi et al., 1999; Pukazhenthi et al., 2002; Pukazhenthi et al., 2001). Thus, cryopreservation of SSCs could help alleviate these species-specific issues.

SSCs can be harvested from an animal post-mortem or collected from a live animal via a small testicular biopsy. This procedure is well-tolerated, and studies in both the dog (Hunt and Foote, 1997; James et al., 1979) and human (Ginsberg et al., 2010; Patel et al., 2005) have demonstrated that post-surgical complications such as inflammation and scarring remain localized to the biopsy site and do not reduce the spermatogenic potential of the individual. Development of a culture system would be particularly useful in this setting to greatly expand the number of SSCs prior to transplantation.

Studies in rodents, pigs, goats and dogs have demonstrated the ability to transplant cultured SSCs to a recipient animal testis within the same species for *in vivo* completion of spermatogenesis (Dobrinski, 2005). Cross-species spermatogonial transplantation has been successful within the rodent family. Transplants from rat to mouse (Clouthier et al., 1996), and mouse to rat (Zhang et al., 2003b) have led to the production of normal sperm in the host. SSCs transplanted to mouse testis from species outside the rodent family, including the cat, are able to colonize the host testes but are unable to successfully complete spermatogenesis (Dobrinski et al., 1999a, 2000; Kim et al., 2006; Nagano et al., 2001; Nagano et al., 2002). Thus, it appears that the taxonomic distance between recipient and donor strongly influences the success of SSC transplantation. It was recently demonstrated that domestic cat testes are capable of supporting spermatogenesis of transplanted ocelot SSCs (Silva et al., 2012). It is unknown whether the domestic cat will be a suitable host for the other members of the Felidae family. However, existing evidence suggests that the genome, as well as physiological mechanisms, is relatively well conserved across felids (Brown, 2006; Johnson et al., 2006; Pelican et al., 2006). Therefore, xenotransplantation of SSCs into more common species may help in the propagation of rare and endangered species.

Alternatively, *in vitro* spermatogenesis circumvents the need for a recipient host. Culture conditions are modulated to favor SSC differentiation. Haploid spermatids generated by *in vitro* spermatogenesis could be used in conjunction with intracytoplasmic sperm injection (ICSI). Resulting embryos could be transferred into recipients for further development *in vivo*. To date, sperm-like cells have been obtained in rodents (Kubota et al., 2004b; Lee et al., 2006b), bulls (Lee et al., 2001) and humans (Sousa et al., 2002;

Tesarik et al., 1998). However, efficiencies remain very low. It is encouraging to note that the supportive technologies including ICSI and embryo transfer have already been developed in the domestic cat as well as many non-domestic felids (Comizzoli et al., 2006; Gómez et al., 2000; Pope et al., 2006).

Transgenesis

Transgenics–the modification of the genome by human intervention, usually through the insertion of a DNA construct–has proven to be a particularly useful research tool for studying gene function. ESCs are frequently used to generate transgenic animals. ESC-based transgenesis allows for precise modification of the genome; including insertion, removal, or modification of DNA sequences. However, continuous culture of ESCs can lead to problems such as abnormal karyotypes, epigenetic instability, and loss of germline potential (Dean et al., 1998; Humpherys et al., 2001; Liu et al., 1997; Longo et al., 1997). Furthermore, this technology is only accessible to species with stable and fully characterized ESC lines, and hence is only currently feasible in the mouse (Evans and Kaufman, 1981; Martin, 1981) and rat (Buehr et al., 2008; Li et al., 2008). There are two reports of cat ESC-like cells that share many characteristics with ESCs, such as alkaline phosphatase activity and OCT4 expression (Gómez et al., 2010; Yu et al., 2008). Following 8 and 12 passages respectively, evidence of cell differentiation was observed, and neither study attempted to demonstrate germline competence.

Approaches to generate transgenic animals in species without established ESC lines include somatic cell nuclear transfer (SCNT) (Cibelli et al., 1998; Keefer et al., 2001; Lai et al., 2002; Park et al., 2002; Schnieke et al., 1997), pronuclear DNA injection (Hammer et al., 1985), sperm-mediated transfer (Lavitrano et al., 2002; Lazzereschi et al.,

2000), and more recently, using site specific nucleases such as zinc finger nucleases (ZFNs) (Hauschild et al., 2011; Whyte et al., 2011; Yang et al., 2011; Yu et al., 2011), transcription activator-like effector nucleases (TALENs) (Carlson et al., 2012), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated endonuclease 9 (Cas9) (Hai et al., 2014; Jinek et al., 2013; Mali et al., 2013). There are two reports of transgenic cat production using SCNT (Gómez et al., 2009; Yin et al., 2008). Animal cloning efficiency was low in both studies, with only two live transgenic kittens produced from 176 cloned embryos and one live transgenic kitten produced from 291 cloned embryos, respectively. More recently, transgenic cats were produced using gamete-targeted lentiviral transgenesis (Wongsrikeao et al., 2011). For this technique, the authors microinjected a lentiviral vector into the perivitelline space of *in vitro* matured cat oocytes before performing *in vitro* fertilization (IVF) with wild-type cat sperm. Following 22 embryo transfer procedures (30 to 50 embryos per transfer), three live transgenic kittens were produced. While overall efficiency remained low, it is encouraging to note that the transgenesis rate was quite high; of the eleven live and fetal offspring that resulted from the embryo transfers, ten were transgenic (embryos could not be preselected for transgene expression before transfer). Nevertheless, alternative approaches are needed for efficient transgenic production in non-rodent species that have proven useful as medical models (e.g., cats, dogs, and pigs).

SSCs are unique among adult stem cells because they already possess the ability to transmit genetic information to the next generation. This makes them an attractive tool for genetic manipulation, especially in species that lack germline competent embryonic stem cells. In contrast to ESCs that often develop genetic and epigenetic changes over time, a

study involving a two year continuous culture of mouse SSCs demonstrated that SSCs can maintain euploid karyotypes, normal methylation patterns, and germline competence during long-term culture (Kanatsu-Shinohara et al., 2005b).

Due to the relative rarity of SSCs, a culture system that supports SSC expansion is a prerequisite to utilizing this technology. However, once culture conditions have been established for the cat, cultured cat SSCs could be transfected with a vector containing the gene of interest and a selectable marker, such as drug resistance or expression of a fluorescent protein. Transgenic clones would then be selected and expanded *in vitro* before being transplanted into a recipient cat whose endogenous germ cells were already depleted, using either local irradiation (Kim et al., 2006) or a chemosterilant such as busulfan (Brinster and Zimmermann, 1994; Bucci and Meistrich, 1987). Once enough time has passed for the SSCs to colonize the recipient testis and resume spermatogenesis, spermatozoa carrying the transgene could be collected from the recipient cat with electroejaculation or an artificial vagina and manual stimulation (Platz et al., 1978). In theory, 50 percent of the resulting spermatozoa would carry the transgene. Recipient males can then be crossed with wild-type females, and a theoretical 50 percent of the offspring would be heterozygous for the transgene. Indeed, Kanatsu-Shinohara et al. (2005c) were able to demonstrate that 215 of the 571 F1 progeny (48.5 percent) carried the transgene when using this method with lipofected mouse SSCs. Subsequent studies have shown SSCs can also be used to create knockout animals (Kanatsu-Shinohara et al., 2006), with a homologous recombination frequency comparable to ESCs (Saitou et al., 2000). Application of gene editing tools such as ZFNs, TALENs, and CRISPRs should dramatically increase these efficiencies (Carlson et al., 2013).

It is also important to note that ES-like cells, designated as multipotent germline stem cells (mGSCs), have spontanously resulted from culture of neonatal mouse SSCs (Kanatsu-Shinohara et al., 2004a). These cells arise rarely, at a rate of 1 in 1.5×10^7 cells (the amount of cells in approximately 35 neonatal testes). Once established, mGSC colonies are selectively expanded when grown under standard ESC, but not SSC or embryonic germ cell, culture conditions. mGSCs could be differentiated into a variety of somatic cells, induce teratoma formation when injected into nude mice, and form germline chimeras following blastocyst injection (Kanatsu-Shinohara et al., 2004a). mGSCs have also been used to make knockout animals in a manner similar to ESC-based techniques (Takehashi et al., 2007).

Thus, SSC-based animal transgenesis provides an alternative to conventional methods, especially in species such as the cat in which germline competent embryonic stem cells have not been established. The unique biology of SSCs offers several advantages over ESC-based techniques. First, SSCs are stably committed to the germline, even following long-term culture. Second, due to the enormous capacity of spermatogenesis, founder males have the potential to generate a much larger pool of F1 offspring compared to founder females. Third, SSCs are collected from the postnatal testis, such that embryos do not need to be sacrificied to harvest the cells. Similarly, mGSCs could provide an alternative method to generate multipotent ES-like cells in the cat without the ethical issues associated with ESC-based technology.

Summary and Specific Objectives

In vitro propagation of SSCs will be a valuable tool for studying the mechanisms that regulate stem cell fate decisions. As the only cell in the adult body capable of both

mitotic division and germline transmission, SSCs represent a novel target for transgenesis, and could greatly expand the use of cats as a biomedical research model. SSC transplantation and *in vitro* spermatogenesis represent novel ways to generate male germ cells in the cat. Furthermore, SSC technology offers several advantages to conservation biology and can be used alone or in tandem with other assisted reproductive technologies already in use. SSCs can be preserved cryogenically for long-term storage in a genome resource bank or transported to another institution to infuse new genes into a population. Finally, development of SSC technology allows preservation and propagation of genetics from prepubertal animals, an advantage not available with current techniques.

While innovative advances in SSC technologies have been made in the mouse, there is a paucity of information available in the literature concerning felid SSCs. Therefore, the main objectives of this dissertation were to (1) identify markers of felid SSCs, using the domestic cat as a model, (2) optimize a technique to enrich SSCs from whole cat testis, and (3) develop a culture system to support cat SSC self-renewal *in vitro*. In the following chapters, the challenges and solutions that were employed to meet these objectives and ultimately develop a system for propagation of felid SSCs are described.

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Chapter 2: Molecular Markers of Spermatogonial Stem Cells in Felids

Portions of this chapter have been published in Reproduction in Domestic Animals (2012; Volume 47 Suppl 6: pages 256-260).

Abstract

Spermatogonial stem cells (SSCs) represent an exciting new avenue for assisted reproduction in endangered and genetically valuable species. Before this technology can be applied to wildlife, species-specific markers need to be identified so that SSC enrichment strategies and subsequent *in vitro* culture can be developed. This study was designed to evaluate in the domestic cat six conserved SSC markers (THY1, GPR125, GFRalpha1, PLZF, UCHL1, and OCT4) that had been previously identified in other species. Testes from prepubertal and adult cats were obtained following routine castrations and processed for mRNA extraction. Reverse transcription-polymerase chain reaction (RT-PCR) of whole testis and cell suspensions enriched for SSCs by differential plating confirmed that all six SSC markers were expressed in both the whole testis and SSC-enriched cell fractions. Western blot analysis of prepubertal, peripubertal, and adult cat testis were used to confirm protein expression for the intracellular markers PLZF, UCHL1, and OCT4. Furthermore, fluorescent immunohistochemical staining showed that these markers were detected in a subpopulation of testis cells adjacent to the basement membrane of the seminiferous tubules. Co-localization of the markers revealed that several cells within the subpopulation expressed all three markers. Western blot and fluorescent immunohistochemical studies performed on cheetah *(Acinonyx jubatus*) and Amur leopard

(*Panthera pardus orientalis*) testis exhibited a conserved expression pattern in protein molecular weights, relative abundance of these SSC markers, and localization of positive cells within the testis. The expression of six SSC marker genes and three SSC marker proteins in the domestic and wild cat testes confirms conservation of these markers, and perhaps SSC self-renewal mechanisms, in felids. These markers will facilitate further studies in cell enrichment and *in vitro* culture of felid SSCs. This study also validates the domestic cat as a model for rare and endangered felids.

Introduction

Historically, spermatozoa cryopreservation has been effective in preserving valuable genotypes and transferring genes between populations; however, semen collections can yield only a finite number of spermatozoa. Furthermore, prepubertal animals do not produce mature sperm and in the event of unexpected death, their genetic contributions are lost forever. This is especially pertinent in felids in which juvenile mortality is characteristically high (Nowell et al., 1996; Wielebnowski, 1996). For example, a study performed retrospectively on 11 felid species bred in North American zoos reported juvenile mortality rates ranging from 30 to 50 percent (Wielebnowski, 1996). Few options are available to preserve genetic material from immature individuals. Loss of genetic diversity, even from a single individual, can have a significant impact on long-term viability in small populations (Pukazhenthi et al., 2006a; Swanson et al., 2007).

Spermatogonial stem cells (SSCs) are undifferentiated stem cells in the testes capable of self-renewal, thus providing a virtually unlimited supply of cells for genetic preservation. Furthermore, as this cell population is present throughout the life of the individual, age is not a limiting factor. While SSCs could serve as a valuable resource for

genetic banking, identification of this population in cats presents a challenge because markers used to identify SSCs in mice—the most studied species—may not be valid for felids.

Intracellular and cell surface markers are required to enrich stem cell populations, evaluate enrichment strategies, and monitor the success of *in vitro* culture systems. Several markers for SSCs have been described in other species. Thymus cell antigen 1 (THY1), is a glycosyl phosphatidylinositol (GPI)-anchored glycoprotein of the immunoglobulin superfamily. It is a marker for a variety of stem cells, including hematopoietic (Spangrude et al., 1988), mesenchymal (Pittenger et al., 1999), and embryonic (Henderson et al., 2002) stem cells. THY1 has been used as a SSC marker in several species, including the mouse (Kubota et al., 2004a), rhesus macaque (Hermann et al., 2009), and bull (Reding et al., 2010).

G-protein coupled receptor 125 (GPR125) is an orphan receptor of the adhesion family. The expression pattern of GPR125 in mouse testis is restricted to the first layer of spermatogonia, adjacent to the basement membrane of the peritubular myoid cells (Seandel et al., 2007a). In the human testis, GPR125 is localized to a subpopulation of spermatogonia that are adjacent to the seminiferous tubule basement membrane (He et al., 2010).

Glial cell line–derived neurotrophic factor (GDNF) is secreted by Sertoli cells and is required for SSC self-renewal (Meng et al., 2000). GDNF family receptor alpha 1 (GFRalpha1), along with Ret receptor tyrosine kinase, form the GDNF receptor complex. GFRalpha1 has been used as an SSC marker in a variety of species, including the mouse

(He et al., 2007), rhesus macaque (Hermann et al., 2007), human (He et al., 2010), and bull (Oatley et al., 2004).

Promyelocytic leukemia zinc-finger (PLZF) belongs to the zinc-finger family of transcription factors (Ching et al., 2010). PLZF is a spermatogonia-specific transcription factor that is required in the regulation of self-renewal and maintenance of the stem cell pool (Buaas et al., 2004; Costoya et al., 2004). It is the disrupted gene in the classic *luxoid* mutation in which mice exhibit a progressive loss of SSCs. PLZF stimulates SSC self-renewal through direct repression of c-Kit receptor transcription; expression of the c-Kit receptor is associated with spermatogonial differentiation (Filipponi et al., 2007). A similar expression pattern of PLZF has subsequently been demonstrated in a variety of species, including the bull (Reding et al., 2010), goat (Song et al., 2013), sheep (Borjigin et al., 2010), pig (Luo et al., 2006), horse (Costa et al., 2012), dog (Harkey et al., 2013), and non-human primates (Maki et al., 2009).

Ubiquitin-dependent proteolysis has been implicated in the control of mammalian gametogenesis and ubiquitin carboxy-terminal hydrolase 1 (UCHL1), also known as protein gene product 9.5 (PGP 9.5), is a deubiquinating enzyme that regenerates monoubiquitin from ubiquitin–protein complexes (Kwon et al., 2004; Sutovsky, 2003). In the mouse testis, UCHL1 is expressed in both spermatogonia and Sertoli cells (Kwon et al., 2004). This appears to be a rodent-specific phenomenon, as UCHL1 is expressed exclusively in spermatogonia for a variety of non-rodent species, including the pig (Luo et al., 2009; Luo et al., 2006), bull (Herrid et al., 2007; Reding et al., 2010; Wrobel, 2000), buffalo (Goel et al., 2010), goat (Heidari et al., 2012), dog (Harkey et al., 2013), non-human primate (Tokunaga et al., 1999), and human (He et al., 2010). Furthermore, UCHL1

expression is limited to a subset of the spermatogonia which are in contact with the basement membrane. This suggests that SSCs are included in the UCHL1-positive cells.

Octamer-binding transcription factor 4 (OCT4) also known as POU domain class 5 transcription factor 1(POU5F1), is a homeodomain transcription factor of the POU family involved in the regulation of pluripotency in embryonic stem cells (Niwa et al., 2000). OCT4 is expressed in undifferentiated spermatogonia and, at least in the mouse, has a more restricted expression pattern than PLZF. Similar expression patterns have been found in the rat (Ryu et al., 2005), hamster (Kanatsu-Shinohara et al., 2008), and pig (Luo et al., 2006). The data on OCT4 expression in human testes is controversial; some investigators report a similar level of expression (Mirzapour et al., 2012), whereas others fail to detect it at all (He et al., 2010). Discrepancies may in part be due to the various isoforms of OCT4 (Cauffman et al., 2006; Lee et al., 2006a) and the variability in detection by commercial antibodies (Warthemann et al., 2012).

Relatively little work has been done with felid SSCs. A few studies have explored the ability of transplanted felid SSCs to colonize seminiferous tubules in other species, but limited information has been reported on SSC marker specificity in cats (Powell et al., 2011; Silva et al., 2012). Cat SSCs are able to colonize mouse seminiferous tubules following germ cell transplantation, but are unable to successfully complete spermatogenesis (Kim et al., 2006). This failure is similar to that of other non-rodent SSC xenotransplantations, presumably due to the large phylogenetic distance between donor and host. It was recently demonstrated that domestic cat testes are capable of supporting spermatogenesis of transplanted ocelot SSCs (Silva et al., 2012). While the authors did detect the expression of GFRalpha1, no other markers were investigated. The objective of

this study was to determine whether SSC markers identified in rodents, would also be useful in the identification of felid SSCs. Three cell surface markers (THY1, GPR125, and GFRalpha1) and three intracellular markers (UCHL1, PLZF, and OCT4) were investigated.

Materials and Methods

Collection of Testes

Domestic cat testicles were obtained from local spay-neuter clinics. Immediately following surgery, tissue was aseptically wrapped in gauze pads moistened with 1X Dulbecco phosphate-buffered saline with calcium and magnesium (DPBS(+), Corning Cellgro) and stored at 4°C. Tissue was processed within 24 hours of surgery, and generally, within the first 6 hours. A portion of each testis was fixed in 4% formaldehyde and embedded in paraffin, as described below. Donor age was recorded if known or estimated by the attending veterinarian. Age group was then confirmed by histological analysis of hematoxylin and eosin-stained testis samples for progression through spermatogenesis.

Testis tissue samples from adult cheetah (*Acinonyx jubatus*) (n = 2) and Amur leopard (*Panthera pardus orientalis*) (n = 1) were obtained following euthanasia for health-related problems. Testes were collected post-mortem, wrapped in gauze-soaked saline, and transported on ice. Samples were donated by Smithsonian's National Zoological Park (cheetahs) and Bronx Zoo (Amur leopard). Testis tissue from one individual was processed within 6 hours of euthanasia, and within 24 hours for the other two donors.

Enzymatic Digestion of Testicular Tissue

A modified two-step enzymatic digestion (Honaramooz et al., 2002) was used as follows to obtain a single-cell suspension from the testicular tissue. Testicles were rinsed in 1X Dulbecco phosphate-buffered saline without calcium or magnesium (DPBS(-), Corning Cellgro) and mechanically dissected to remove the vaginal tunic, vas deferens, and epididymis. The testis was bisected and pointed forceps were used to remove the tunica albuginea. The tissue was minced into small pieces and grossly apparent connective tissue was removed. Tissue was incubated with 2 mg/ml collagenase (Sigma) for 30 minutes at 37°C in a shaking incubator at approximately 110 oscillations per minute. Tissue was washed twice with DPBS(-) and incubated with 1.25 mg/ml trypsin (Invitrogen) supplemented with 50 µg/ml deoxyribonuclease I (DNase I, Sigma) for 15 minutes at 37°C in a shaking incubator. Ten percent (v/v) fetal bovine serum (FBS, Hyclone) was added to inactivate trypsin before passing through a 70µm mesh filter (Fisher Scientific). Cell concentration was determined using a hemocytometer and cell viability assessed by trypan blue dye (BioWhittaker) exclusion (Strober, 2001).

Differential Plating

Differential plating utilizes the different adhesion properties of spermatogonia compared to somatic cells, with the somatic cells preferentially binding to the cell culture dishes (Herrid et al., 2009). To perform differential plating, cells were suspended in a basic cell culture medium of Dulbecco modified Eagle medium (DMEM, Invitrogen) supplemented with 10% (v/v) FBS, 1% (v/v) GlutaMAX™ (Gibco), 100 u/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco). The cells were plated at a concentration of 5×10^5 cells per ml on 100 mm untreated cell culture dishes (Corning). The cells were

incubated for 2 hours at 36ºC in 5% CO_2. Cells remaining in suspension at the end of the culture period were collected for further analysis. Cells at time zero (no enrichment) and after differential plating were stored in RLT lysis buffer (Qiagen) for reverse transcription-PCR (RT-PCR).

Endpoint RT-PCR Analysis

Total RNA was extracted from testicular cell lysates using the RNeasy Mini Kit, according to manufacturer's instructions (Qiagen). The expression of six candidate SSC genes (*THY1, GPR125, GFRalpha1, PLZF, UCHL1,* and *OCT4*) and the reference gene *Ribosomal Protein L17* were examined. *OCT4* and *RPL17* primers were designed as previously described (Gómez et al., 2010; Penning et al., 2007). At the time this work was performed, the remaining genes were not yet annotated in the National Center for Biotechnology Information (NCBI) cat genome database (http://www.ncbi.nlm.nih.gov/genome?term=felis%20catus). Therefore, primers for the remaining genes were designed by aligning the gene sequences from *Homo sapiens* and *Mus musculus*. Regions of high homology were blasted against the cat genome and cat-specific primers were generated by using Primer3 software (http://frodo.wi.mit.edu/primer3/). Primer pairs were designed to generate approximately 100 to 200 base pair PCR products and are outlined in Table 2.1.

One microgram of total RNA from each sample was used in the reverse transcription reactions. First strand cDNAs were synthesized using QuantiTect® Reverse Transcription (Qiagen) according to manufacturer's instructions. PCR amplification was performed for 30 cycles with GoTaq® Green Master Mix (Promega) using the specific

Gene	Primer	Product size (bp)
THY1	F: 5'-ACATTCTTGCTGGAGCTG GT-3' R: 5'-ACCCGTGAGAAGAAGAAGCA-3'	197
GPR125	F: 5'-CGACTCCTCGAGTGTCCTTC-3' R: 5'-AGCCGCCATCATAAAAACAG-3'	169
GFRalpha1	F: 5'-AGCATTCCGTAGCTGTGCTT-3' R: 5'-TGTAACCTCGACGACACCTG-3'	158
UCHL1	F: 5'-CGGAAAAGACATTCGTCCAT-3' R: 5'-ACAGAGAAGCTGTCCCCTGA-3'	155
PLZF	F: 5'-GCAAAGCTCGGTACCTCAAG-3' R: 5'-GTTCCCTGCAGGAGAGACTG-3'	208
OCT4	F: 5'-TGCAGCTCAGTTTCAAGAACA-3' R: 5'-ACAAGTGTCTCTGCTTTGCATA-3'	112
RPL17	F: 5'-CTCTGGTCATTGAGCACATCC-3' R: 5'-TCAATGTGGCAGGGAGAGC-3'	109

Table 2.1 Primer sequences used in RT-PCR. Figure reprinted with permission from Vansandt et al. (2012).

primers described above. The amplicons were analyzed on 1.3% agarose gels stained with ethidium bromide and visualized with UV light.

Preparation of Western Blots

Testes from prepubertal (n = 3), peripubertal (n = 3), and adult (n = 3) cats were used for Western blot analysis. The tunica albuginea was removed and small pieces of testis were flash frozen in liquid nitrogen and stored at -80°C. Protein was extracted using NP40 lysis buffer (50 mM Tris-HC1 pH 8.0, 150 mM NaC1, 1% (v/v) NP40) and protein concentrations were determined using the Micro BCA Protein Assay Kit (Thermoscientific). Forty micrograms of protein per sample were boiled at 98°C for 10 minutes in 6X SDS Sample Buffer (375 mM Tris-HCl pH 6.8, 12% (w/v) SDS, 60% (v/v) glycerol, 0.06% (w/v) bromophenol blue; 5% (v/v) 2-mercaptoethanol). Total protein lysates were subjected to SDS-PAGE on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Proteins transfer was confirmed by Ponceau S staining. Membranes were incubated in blocking solution (Tris-buffered saline (20 mM Tris-HCL pH 7.6, 137 mM NaCl) containing 0.05% (v/v) Tween-20 (TBST) and 5% (w/v) Sanalac nonfat dry milk) for 2 hours at room temperature and then incubated overnight at 4°C with primary antibody diluted in blocking solution. A different blocking solution (TBST containing 3% (w/v) bovine serum albumin (Sigma)) was used for membranes probed for PLZF but otherwise were processed as described for the other blots. See Table 2.2 for a complete list of antibodies and concentrations used. Membranes were washed in TBST and then incubated with a species- and isotype-specific horseradish peroxidase–conjugated secondary antibody (Table 2.2) in blocking solution for 1 hour at room temperature. Membranes were washed again in TBST, and antigen detection was performed using the Supersignal West Pico or Femto chemiluminescent substrates (Thermo Scientific) and visualized using a ChemiDoc XRS system (Bio-Rad

Laboratories). Immunoblots were stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) according to manufacturer's instructions and reprobed for β-actin as described above, except that the secondary antibody was only incubated for 45 minutes at room temperature. Immunoblot images were processed for figures using Photoshop CS6 (Adobe Systems). Intensities of immunoblot bands were quantified from unmodified immunoblot images using Image Lab software (Bio-Rad). Relative expression levels were calculated by normalizing band intensities to their corresponding β-actin band intensity. Comparisons were performed between each of the juvenile groups and the adult group using Dunnett's test from the MIXED procedure of SAS statistical software (SAS Institute). For purposes of the bar graphs, data were transformed by setting the prepubertal group to 1. However, statistics were performed on untransformed data. As a negative control,

Primary Ab	Isotype	Source	Dilution	Secondary Ab	Source	Dilution
OCT4	Mouse IgG_1	Millipore	1500	Anti-mouse IgG, HRP-linked	Cell Signaling Technology	1000
PLZF	Goat IgG	Santa Cruz	500	Anti-goat IgG, HRP-linked	Jackson Immunoresearch Laboratory, Inc.	2500
UCHL1	Rabbit IgG	Novus	4000	Anti-rabbit IgG, HRP-linked	Cell Signaling Technology	2500
β-actin	Mouse IgG_{2a}	Sigma	2500	Anti-mouse IgG, HRP-linked	Cell Signaling Technology	5000

Table 2.2 List of antibodies used in Western blot analysis.

additional immunoblots were performed for each antibody which included a known negative tissue or cell line (Supplementary Figure 1).

Fluorescent Immunohistochemistry

Testes were fixed in 4% methanol-free formaldehyde (Polysciences, Inc.) for 1 hour at room temperature or overnight at 4°C, dehydrated through a series of graded alcohols, and embedded in paraffin wax (Fisher Scientific) at 60°C. Five micron thick sections were cut from tissue blocks using a microtome and wet mounted onto Superfrost Plus™ microscope slides (Fisher Scientific). Sections were dewaxed using xylene and rehydrated through a series of graded alcohols. Samples were permeabilized with 0.2% (v/v) Triton-X 100 and 0.1% (v/v) Tween-20 diluted in DPBS(-) for 9 minutes at room temperature. Sections were blocked with MAXblock Blocking Medium (Active Motif) for 2 hours at room temperature. Primary antibodies were diluted in MAXbind Staining Medium (Active Motif) and incubated overnight at 4°C. See Tables 2.3–2.5 for a complete list of antibodies and concentrations used. Slides were washed in IHC washing buffer (DPBS(-) and 0.05% (v/v) Tween-20) and then incubated with a species- and isotype-specific fluorescent–conjugated secondary antibody (Tables 2.3-2.5) in MAXbind Staining Medium solution for 2 hours at room temperature. Hoescht 33342 (Sigma) was used to visualize cell nuclei and coverslips were mounted using Fluoromount G (Southern Biotech). Fluorescent imaging was performed using a Carl Zeiss AxioObserver Z1 inverted microscope, and images were acquired using the Zeiss AxioVision, release 4.6 software with a black and white Zeiss AxioCam MRm camera. Images were pseudocolored in AxioVision and exported to Photoshop CS6 for image processing. As a negative control, the primary antibody against the protein of interest was omitted (Supplementary Figures 2 and 3).

Primary Antibody				Secondary Antibody*				
Target	Isotype	Source	Dilution	Host	Reactivity	Isotype	Conjugate	Dilution
OCT4	Mouse IgG_1	Millipore	100	Goat	Mouse	IgG_1	647	400
PLZF	Goat IgG	Santa Cruz	50	Donkey	Goat	IgG	647	400
UCHL1	Rabbit IgG	Novus	200	Goat	Rabbit	IgG	647	400

*All secondary antibodies are Alexa Fluor dyes purchased from Molecular Probes

Table 2.3 List of antibodies used in single-label fluorescent IHC analysis.

Primary Antibody				Secondary Antibody*				
Target	Isotype	Source	Dilution	Host	Reactivity	Isotype	Conjugate	Dilution
UCHL1	Rabbit IgG	Novus	200	Goat	Rabbit	IgG	647	400
Vimentin	Mouse IgG_1	Abcam	1000	Goat	Mouse	IgG	488	400

*All secondary antibodies are Alexa Fluor dyes purchased from Molecular Probes

Table 2.4 List of antibodies used in double-label fluorescent IHC and ICC analysis

Primary Antibody				Secondary Antibody*				
Target	Isotype	Source	Dilution	Host	Reactivity	Isotype	Conjugate	Dilution
OCT4	Mouse IgG_1	Millipore	100	Goat	Mouse	IgG_1	647	200
PLZF	Goat IgG	Santa Cruz	50	Donkey	Goat	IgG	488	400
UCHL1	Rabbit IgG	Novus	200	Goat	Rabbit	IgG	594	400

*All secondary antibodies are Alexa Fluor dyes purchased from Molecular Probes

Table 2.5 List of antibodies used in triple-label fluorescent IHC analysis

Fluorescent Immunocytochemistry

Testes were enzymatically digested as described above, before being plated onto two-well glass chamber slides (Lab-Tek) pre-coated with 0.1% poly-L-lysine (Sigma). Cells were fixed with 4% methanol-free formaldehyde for 15 minutes at room temperature, gently washed 3 times with IHC wash buffer, and permeabilized with 0.2% (v/v) Triton-X 100 and 0.1% (v/v) Tween-20 diluted in DPBS(-) for 9 minutes at room temperature. Blocking, antibody incubation, mounting, and imaging were performed as described in the fluorescent immunohistochemistry section. See Table 2.4 for a complete list of antibodies and concentrations used. As a negative control, the primary antibody against the protein of interest was omitted (data not shown).

Results

Differential Plating Maintains Viability in Cat Testicular Cells

A two-step enzymatic digestion was used to obtain single-cell suspensions of testicular cells. The mean (±SEM) number of cells obtained from each prepubertal individual (n = 3) was 3.06 (±1.06) million with a mean of 13.07 (±0.27) million cells per gram of testis tissue. The mean (±SEM) number of cells obtained from each adult individual (n = 3) was 21.97 (±5.70) million with a mean 11.99 (±3.76) million cells per gram of testis tissue. The mean (±SEM) of the viability for all individuals (n = 6) processed was 93.3 (±2.4) percent. Differential plating was then used to enrich the cell population for SSCs. After 2 hours of incubation, the mean (±SEM) cell viability for the non-adherent (i.e., SSC-containing) fraction was 94.5 (±0.5) percent. The relative number of cells

recovered after differential plating was 20.1 (±2.6) percent and 23.8 (±2.4) percent for juvenile and adults, respectively.

Expression of Candidate SSC Marker Transcripts in Cat Testis

Single-cell suspensions derived from whole testis of juvenile (n = 3) and adult (n = 3) cats were analyzed by RT-PCR. Three cell surface markers (*THY1, GPR125*,and *GFRalpha1*) and three intracellular markers (*PLZF, UCHL1,* and *OCT4*) were examined. The reference gene *RPL17* was also amplified as a control (Penning, Vrieling et al. 2007). One band of the predicted base pair size was detected for all six putative SSC marker genes in both age groups (Figure 2.1). RT-PCR analysis was then repeated with enriched testicular fractions derived from differential plating. Messenger RNAs for the six candidate SSC markers were detected in the testis of juvenile (n = 1) and adult (n = 2) cats (Figure 2.2). PCR products were sequenced and gene identity was confirmed by BLAST with reference to the domestic cat and other mammalian genomes.

Confirmation of Protein Expression for Intracellular SSC Markers

To confirm translation of mRNA transcripts into proteins, Western blot analysis was performed on whole cat testis from three age groups: prepubertal juvenile cats (14 to 17 weeks, n = 3), peripubertal juvenile cats (6 months, n = 3), and adult cats (11 to 12 months, n = 3). While adult male cats are able to produce sperm throughout the year, it has been suggested that season can have an effect on the quality and rate of spermatogenesis (Axnér and Linde Forsberg, 2007; Blottner and Jewgenow, 2007; Tsutsui et al., 2009). Therefore, to reduce variability due to season, only samples collected between June and August 2013 were used.

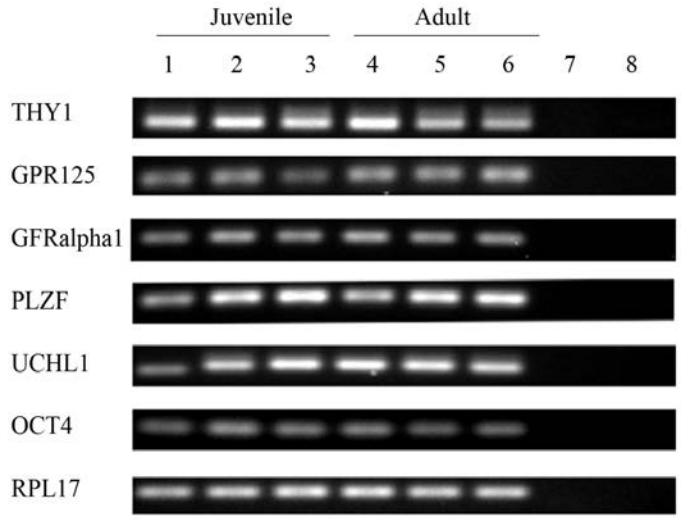


Figure 2.1 RT-PCR analysis of whole cat testis single-cell suspensions. Juvenile testis (lanes 1-3); adult testis (lanes 4–6); RT-minus control (lane 7); no template control (lane 8). The identities of the PCR products were confirmed by sequencing. Figure reprinted from Vansandt et al. (2012).

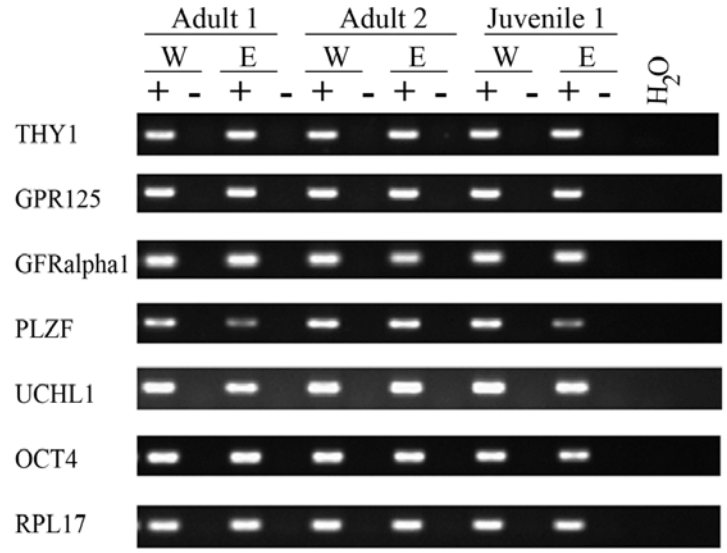
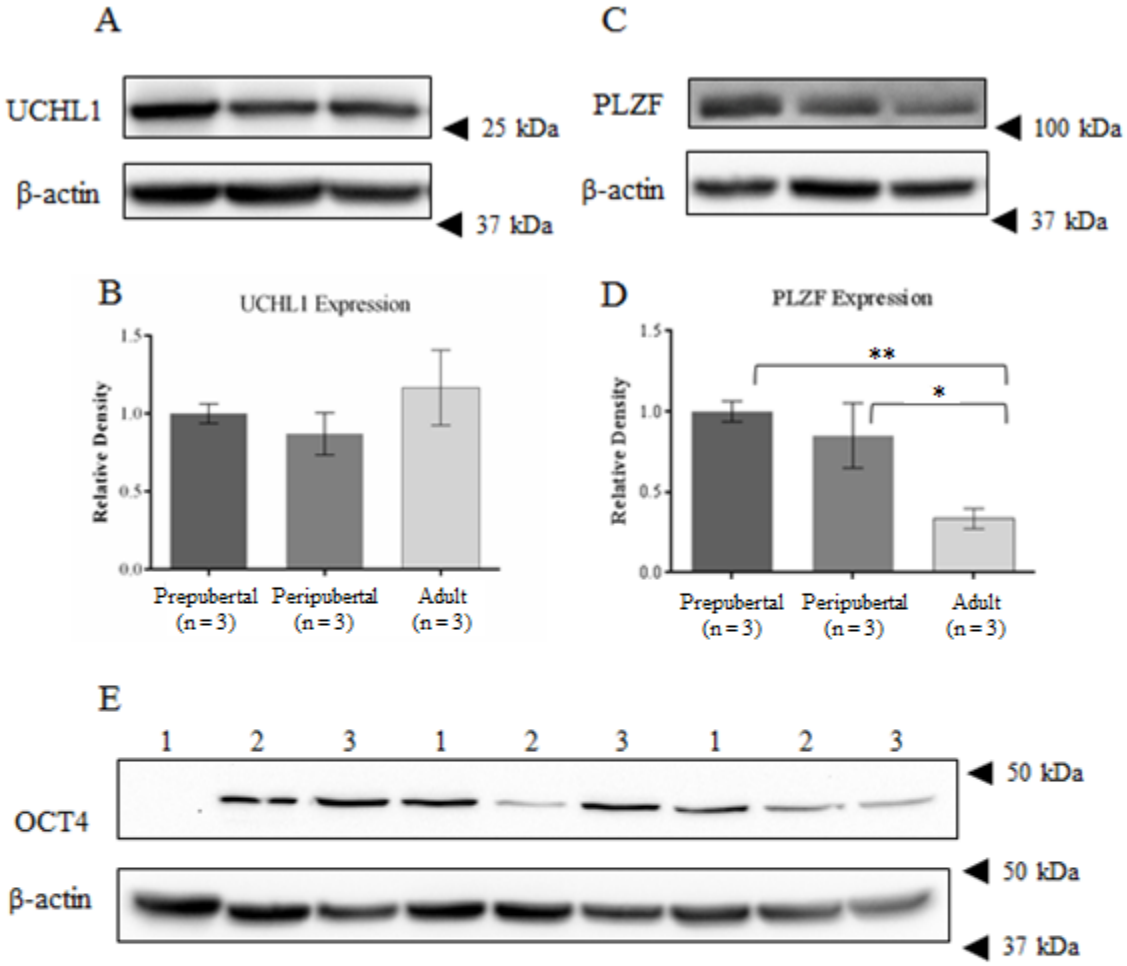


Figure 2.2 RT-PCR analysis of whole testis and enriched single-cell suspensions. W: whole testis sample; E: differential plating enriched sample. H_20: water control. +/- indicates presence (+) or absence (-) of reverse transcriptase enzyme. Figure reprinted from Vansandt et al. (2012).

A single band at approximately 27 kDa was detected for UCHL1 (Figure 2.3 A). No significant difference was detected between the prepubertal and adult cats ($p = 0.709$) or between the peripubertal and adult cats ($p = 0.392$) (Figure 2.3 B). PLZF displayed an immunoreactive band at approximately 115 kDa (Figure 2.3 C). Analysis of the band intensities revealed a significantly higher expression of PLZF for the prepubertal group as compared to adults ($p = 0.018$). Expression of PLZF was also higher ($p = 0.0502$) in peripubertal compared with adult donors (Figure 2.3 D). An approximately 44 kDa band was detected for OCT4 (Figure 2.3 E). The expression pattern was highly variable for this protein, both among and between age groups. As such, no significant difference of OCT4 expression levels were found between the two juvenile and adult groups (Figure 2.3 F). Interestingly, one individual in the prepubertal group (14 weeks old) failed to display an immunoreactive band for OCT4 (Figure 2.3 E, lane 1). To further investigate the expression of OCT4 in prepubertal cat testis, we assessed several individuals ranging from 8 to 18 weeks old ($n = 9$). To increase the sensitivity of the assay, Supersignal West Femto chemiluminescent substrate was used to visualize the bands. Immunoreactive bands were detected at 5 molecular weights: approximately 42, 44, 48, 60, and 75 kDa (Figure 2.4). A high level of individual variability was again observed, and there was no correlation between the age of the individual and number of bands, size of the bands, or band staining intensity.

SSC Markers are Expressed on a Subset of Cells Adjacent to the Basement Membrane

Our next aim was to characterize which cells of the testis were expressing the potential SSC markers. To this end, fluorescent immunohistochemistry was used to localize



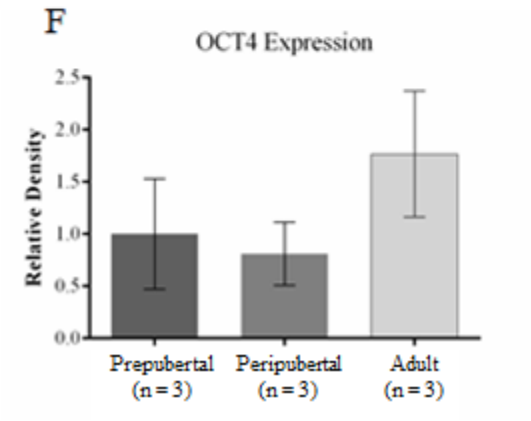


Figure 2.3 Quantitative analysis of SSC marker expression by Western blot. Representative Western blots of UCHL1 (**A**) and PLZF (**C**) protein expression in prepubertal (lane 1), peripubertal (lane 2), and adult (lane 3) domestic cat testis. (**E**) Western blot of OCT4 protein expression in domestic cat testis. Number denotes age group: prepubertal (1), peripubertal (2), and adult (3). Blots were visualized with Supersignal West Pico chemiluminescent substrate. Expression levels of UCHL1 (**B**), PLZF (**D**), and OCT4 (**F**) were quantified using Image Lab software. Values represent relative density normalized to β-actin.
*p = 0.0502; **p = 0.018;

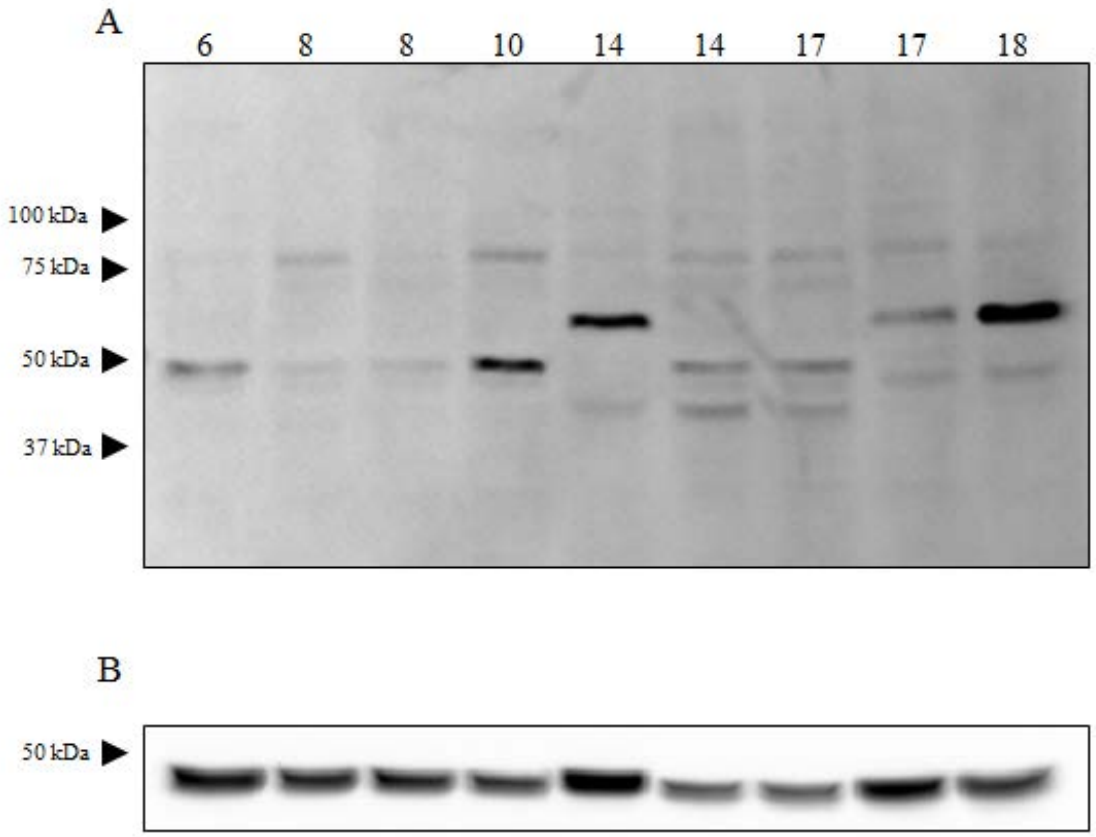


Figure 2.4 Western blot analysis of OCT4 expression in prepubertal cat testis. (**A**) Total proteins were isolated from prepubertal cat testis (n = 9). Age of donor cat is indicated at top of blot (in weeks). Eighty micrograms of total protein from each sample was assessed for OCT4 expression and bands were visualized using Supersignal West Femto chemiluminescent substrate. Multiple OCT4 immuno-reactive bands were observed with approximate molecular weights of 42, 44, 48, 60, and 75 kDa. (**B**) Blot was stripped and reprobed for β-actin as a loading control.

positive cells within prepubertal (8 to 14 weeks, n = 5) and adult cat testis (11 months to 4 years, n = 6). In the prepubertal testis, UCHL1 staining appeared specific to spermatogonia, as identified by their low cytoplasmic to nuclear ratio and location within seminiferous tubules (Figure 2.5 A-C). Single or paired UCHL1-positive cells were randomly distributed between the non-staining cells of the seminiferous tubule, adjacent to the basement membrane. In the adult testis, UCHL1 staining was also limited to spermatogonia (Figure 2.6 A-C). UCHL1-positive cells could be found as single, paired, or aligned spermatogonia in a single layer contiguous with the basement membrane of the seminiferous tubules. To investigate if UCHL1 expression is restricted to spermatogonia in the cat, double immunolabeling was performed with UCHL1 and vimentin, an intermediate filament specifically expressed by Sertoli cells (Steger et al., 1996). Double-labeled IHC sections demonstrate that Sertoli cells, as identified by vimentin expression, do not co-express UCHL1 (Figure 2.7 A-D). Because of the high number of cells in close proximity in the seminiferous tubules, it is possible that double-positive cells could escape detection. Therefore, immunocytochemistry was performed on dissociated testis cells so that individual cells could be evaluated (Figure 2.7 E-H). Of the approximately 1,000 testicular cells assessed per individual (n = 3), no cells expressed both markers, conforming that UCHL1 expression is specific to spermatogonia in the cat.

Single PLZF-positive cells were found interspersed among the non-staining cells of the seminiferous tubules in prepubertal testis (Figure 2.5 D-F). In adult testis, PLZF displayed an even more restricted expression pattern than UCHL1, with only a subset of spermatogonia staining positive (Figure 2.6 D-F). PLZF expression was localized to the

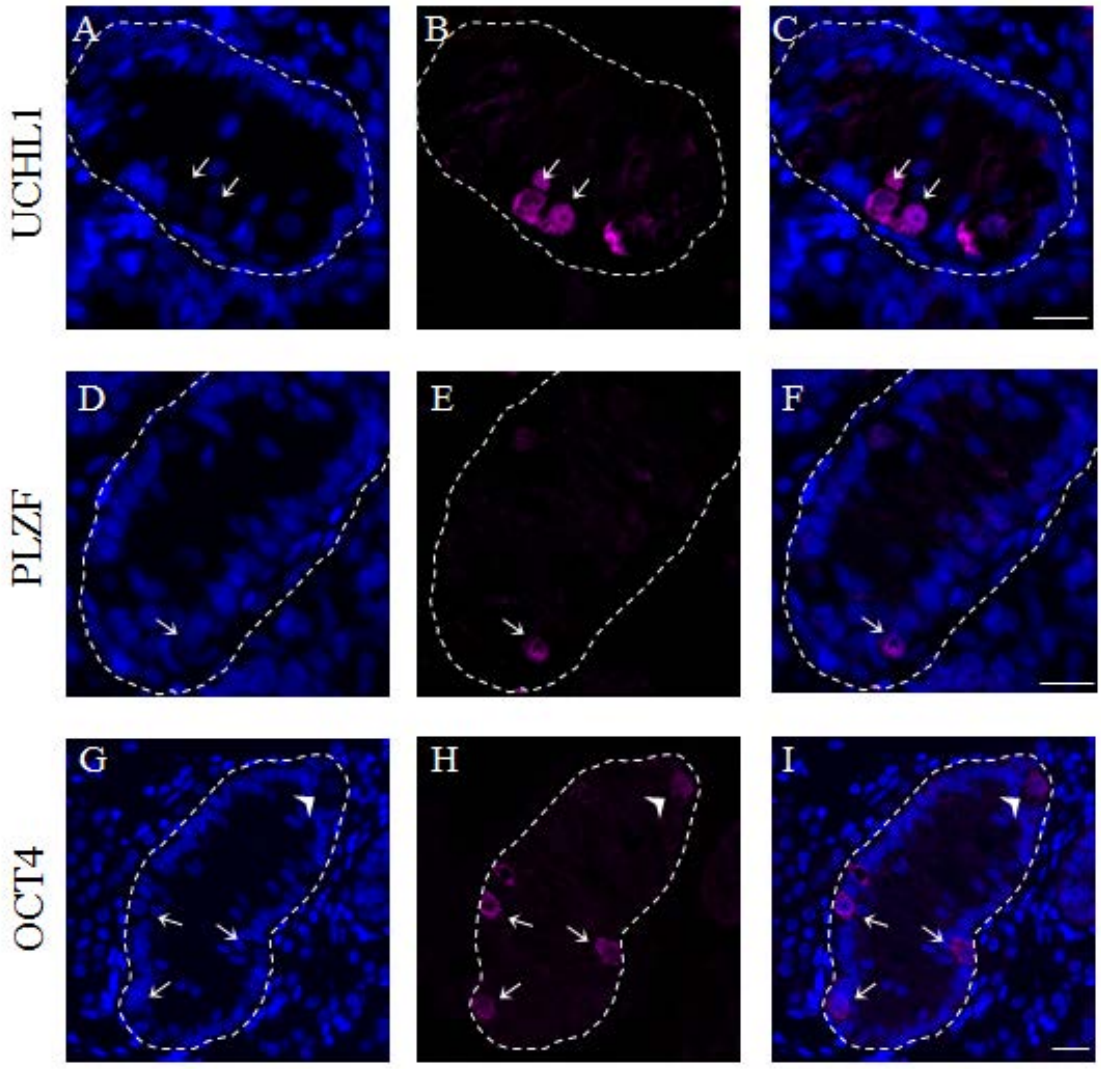


Figure 2.5 Distribution of SSC markers in prepubertal cat testis. Representative fluorescent IHC images of UCHL1 (**B**), PLZF (**E**), and OCT4 (**H**) protein in prepubertal cat testis. (**A**, **D**, **G**) Hoechst 33342 to mark cell nuclei. (**C**, **F**, **I**) Merged image. Arrows indicate positive cells. Arrowhead indicates nuclear localization of OCT4 protein. Seminiferous tubule borders are outlined in white. Scale bars; 20 µm.

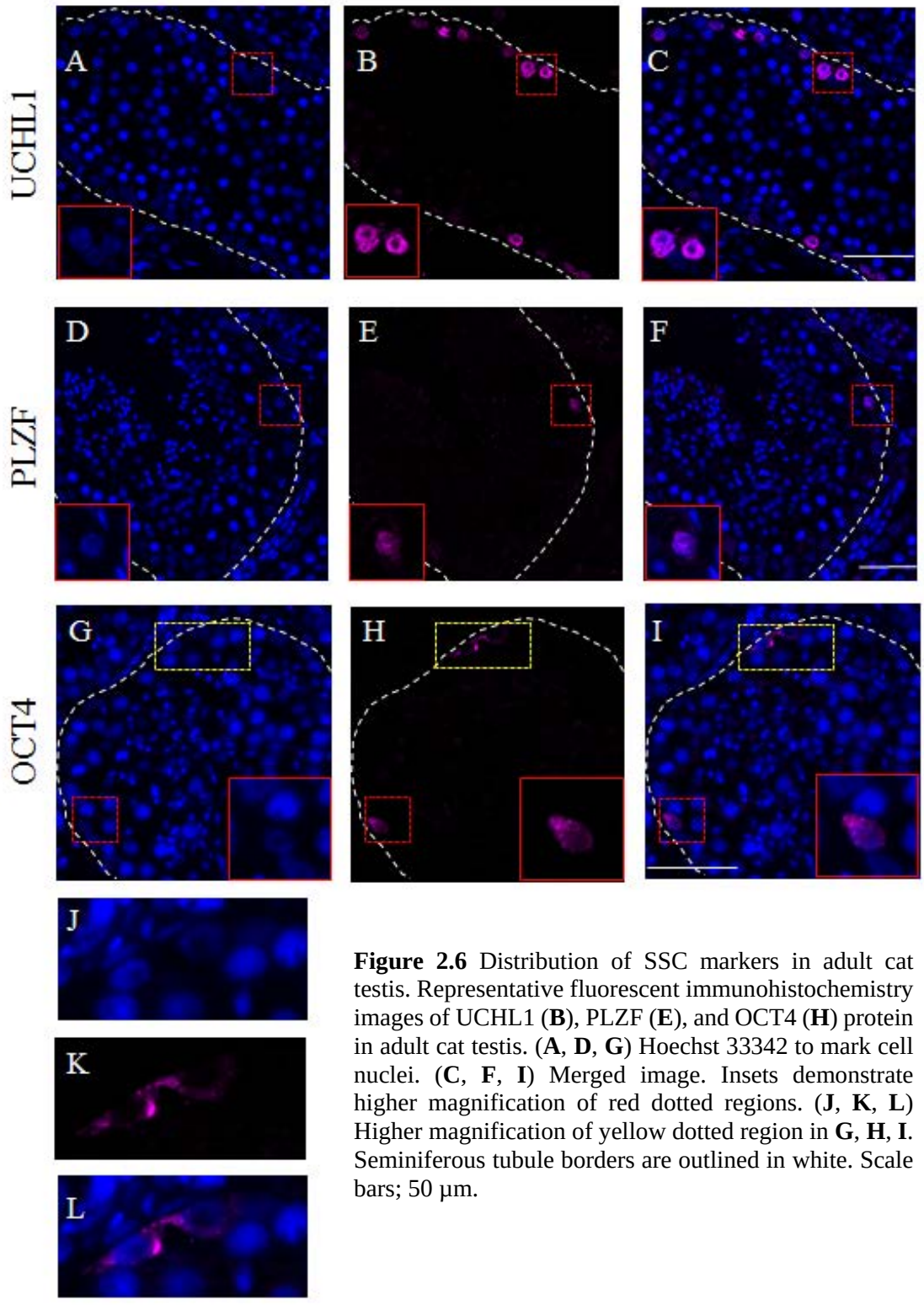


Figure 2.6 Distribution of SSC markers in adult cat testis. Representative fluorescent immunohistochemistry images of UCHL1 (**B**), PLZF (**E**), and OCT4 (**H**) protein in adult cat testis. (**A**, **D**, **G**) Hoechst 33342 to mark cell nuclei. (**C**, **F**, **I**) Merged image. Insets demonstrate higher magnification of red dotted regions. (**J**, **K**, **L**) Higher magnification of yellow dotted region in **G**, **H**, **I**. Seminiferous tubule borders are outlined in white. Scale bars; 50 µm.

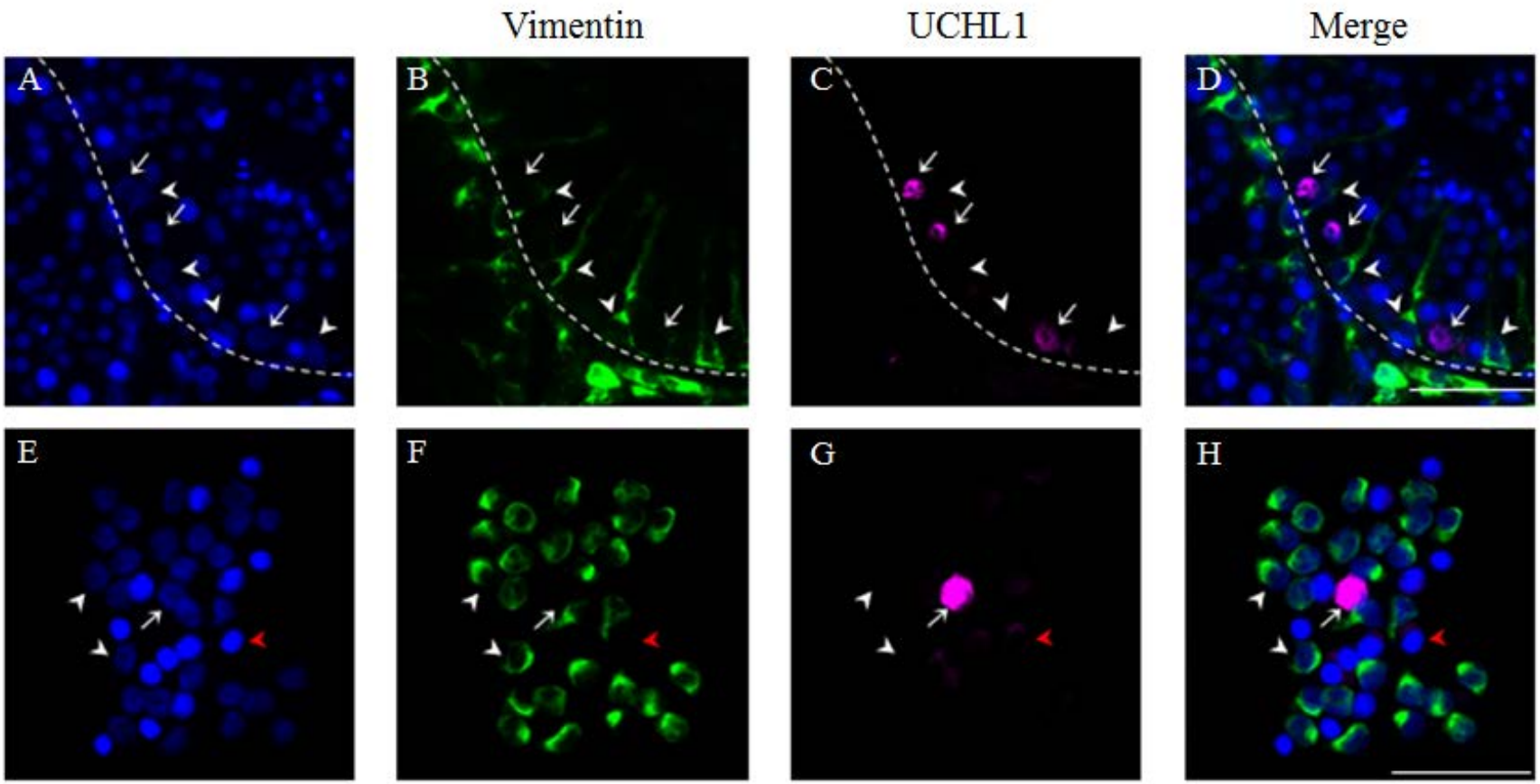


Figure 2.7 Co-localization of vimentin and UCHL1 in cat testis. Representative fluorescent immunohistochemistry images of vimentin (**B**), and UCHL1 (**C**) protein expression in different cells of adult cat testis. (**A**) Hoechst 33342 to mark cell nuclei. (**D**) Merge of previous images (**A**-**C**). (**E**-**H**) Fluorescent immunocytochemistry of dissociated adult cat testis cells showed a similar pattern of distribution for UCHL1 (**F**) and vimentin (**G**), with no cells co-expressing the two proteins. (**E**) Hoechst 33342 to mark cell nuclei. (**H**) Merge of images (**E**-**G**). Seminiferous tubule borders are outlined in white (**A**-**D**). Arrows indicate UCHL1 positive cells. White arrowheads indicate vimentin positive cells. Red arrowhead indicates cells negative for both vimentin and UCHL1. Scale bars; 50 µm.

nucleus, and positive cells were typically found as single spermatogonia located adjacent to the basement membrane.

In prepubertal cat testis, OCT4 was expressed in single or paired cells within the seminiferous tubules (Figure 2.5 G-I). Similar to PLZF, OCT4 showed a restricted expression pattern limited to a subset of spermatogonia along the basement membrane (Figure 2.6 G-L). Although the majority of OCT4-positive cells in the cat displayed a nuclear staining pattern, a subset of cells also expressed OCT4 in the cytoplasm.

To determine whether the positive staining cells represented the same subpopulation, triple immunolabeling was performed. In the prepubertal testis, the same subset of cells expressed all three markers (Figure 2.8 A-D). In the adult testis, PLZF and OCT4-positive cells also expressed UCHL1 (Figure 2.8 E-H). However, several UCHL1-positive cells did not express PLZF or OCT4. The majority of cells expressing PLZF also expressed OCT4; however a subset of PLZF-positive, OCT4-negative cells were also identified. Taken together, these data demonstrate that UCHL1, PLZF, and OCT4 reliably identify a small subpopulation of undifferentiated spermatogonia, likely including SSCs, in the domestic cat.

SSC Markers are Conserved in Wild Felids

To investigate the utility of the domestic cat as a model for SSCs in rare and endangered felids, testis tissue from three wild cats were examined: one sample was obtained from an Amur leopard (*Panthera pardus orientalis*) and two from cheetahs (*Acinonyx jubatus*). Western blot analysis showed that SSC markers UCHL1, PLZF, and OCT4 are also expressed in wild felid testis, and immunoreactive bands are present at similar molecular weights to the domestic cat (Figure 2.9). Expression levels appeared

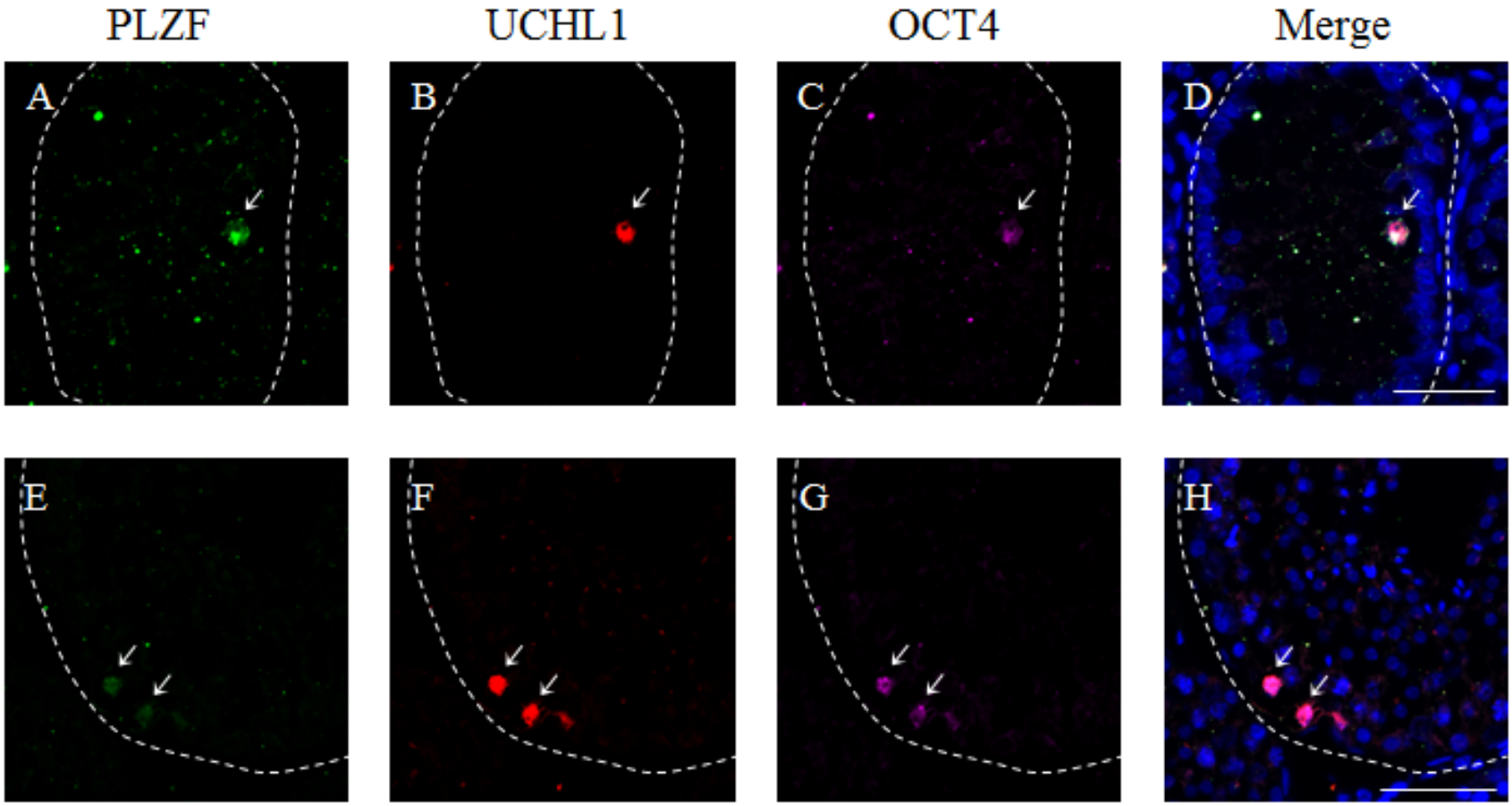


Figure 2.8 Co-localization of SSC markers in cat testis. Representative fluorescent immunohistochemistry images of PLZF (**A**, **E**), UCHL1 (**B**, **F**), and OCT4 (**C**, **G**) protein localizing to the same cells in prepubertal (**A-D**) and adult cat testis. (**E-H**) Merge of previous images and Hoechst 33342 (blue) to mark cell nuclei (**D, H**). Seminiferous tubule borders are outlined in white. Arrows indicate positive cells. Scale bars; 50 µm.

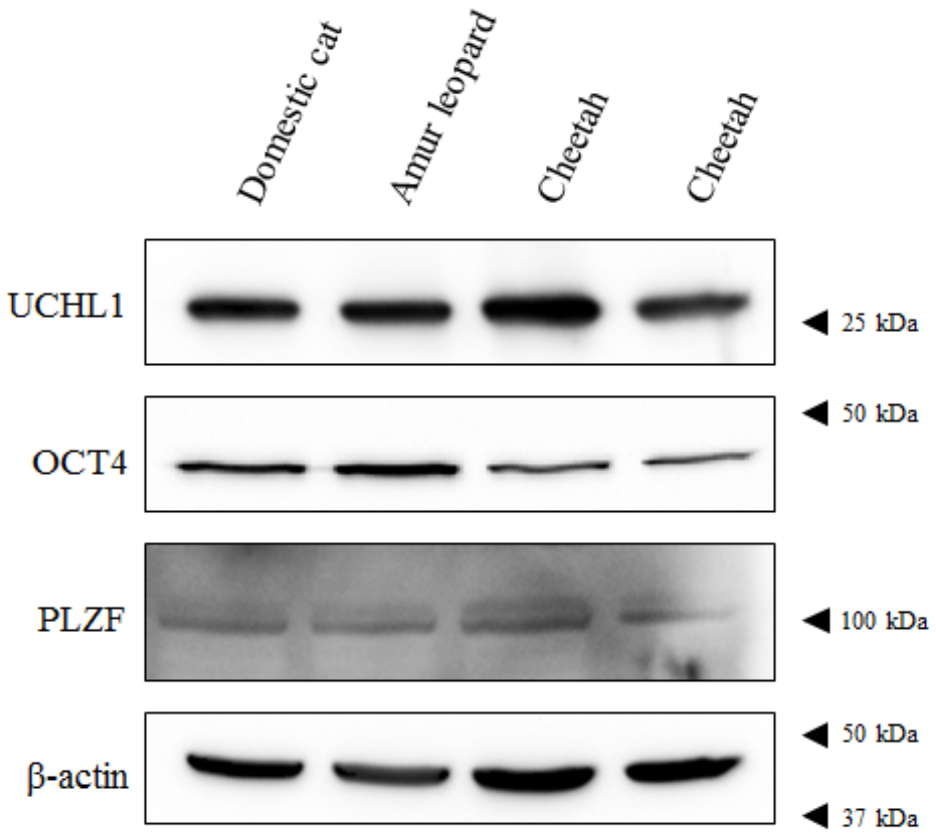


Figure 2.9 Western blot analysis of SSC marker expression in domestic cat and wild felids. Western blots of UCHL1, OCT4, and PLZF in the testis of Amur leopard (*Panthera pardus orientalis*) (lane 2) and cheetah (*Acinonyx jubatus*) (lanes 3-4). Adult domestic cat testis (age 1 year) was used as a positive control (lane 1). For a loading control, blots were stripped and reprobed for β-actin. Blots were visualized with Supersignal West Pico chemiluminescent substrate.

quite similar to the domestic cat: UCHL1 and OCT4 were strongly expressed, and PLZF expression was low (as was observed for the adult cat testis samples). Fluorescent IHC analysis also paralleled the domestic cat expression pattern. UCHL1 was the most abundant marker, with several positive basement membrane-associated spermatogonia per tubule cross-section (Figure 2.10 A-B, G-H). PLZF and OCT4 expression also appeared limited to spermatogonia bordering the basement membrane, and a more restricted expression pattern was seen (Figure 2.10 C-L). UCHL1 could be detected in both the nucleus and cytoplasm whereas PLZF and OCT4 generally remained localized to the nucleus. Overall, fewer positive cells were noted per tubule cross-section in the wild felid testes as compared to the domestic cat; likely due to the increased age of the wild cats (8 to 18 years old).

Discussion

In the present study, expression of six SSC markers was investigated in the domestic cat. We reasoned that markers that have been well-conserved across species would also be expressed in felids. Because it is often difficult to identify antibodies with appropriate homology to the cat, we first sought to examine the mRNA expression of these markers. This too can be problematic in non-traditional lab animals such as the cat, whose genome has been sequenced but not yet fully annotated. With the exception of *OCT4* (Accession: NM_001173441.1) and *RPL17* (Accession: NM_001128842.1), sequences for the genes of interest were not available on the National Center for Biotechnology Information genome browser. Therefore, primers for PCR amplification (Table 2.1) were designed by aligning gene sequences from other mammalian species for regions of high homology.

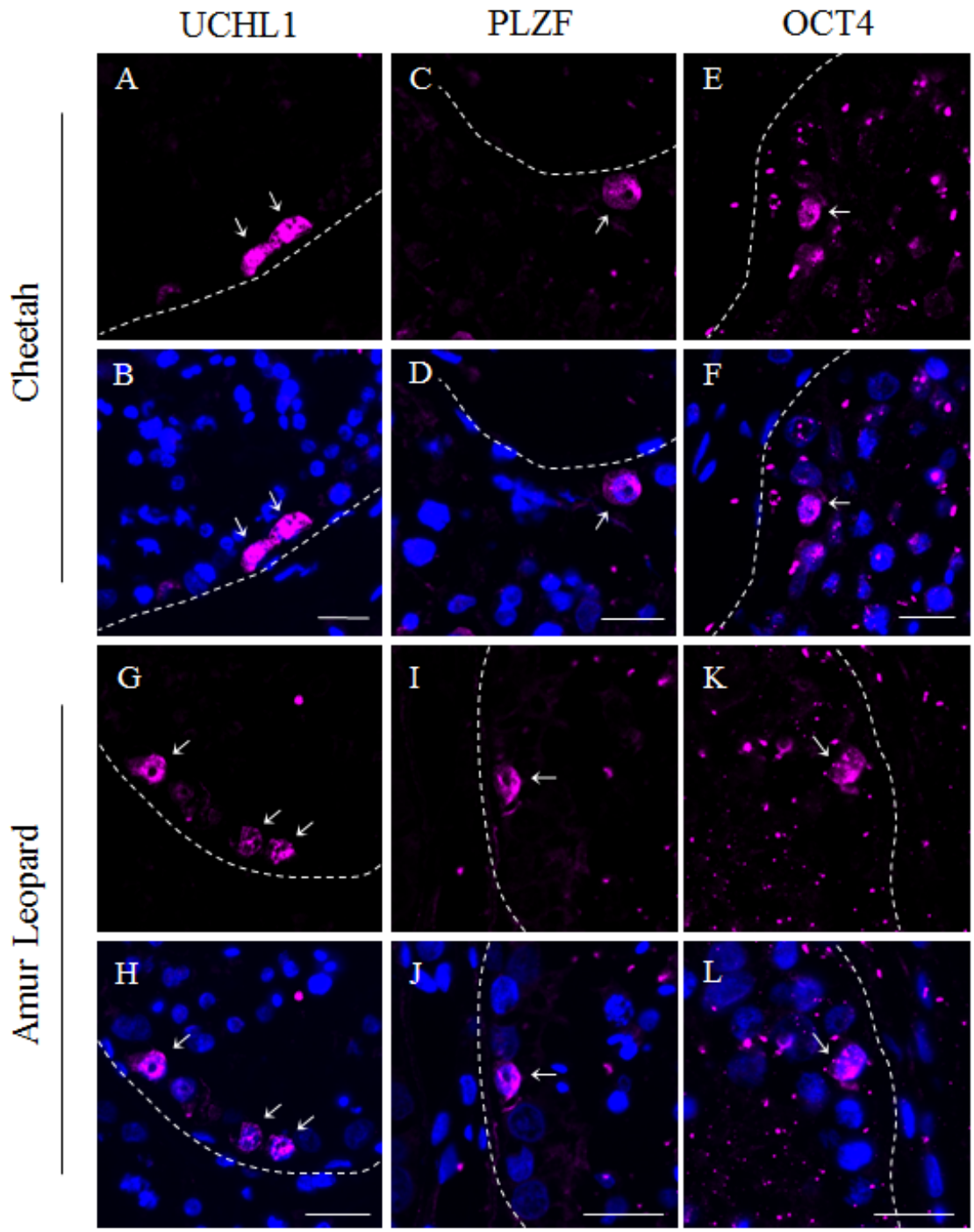


Figure 2.10 Distribution of SSC markers in wild felid testis. Representative fluorescent IHC images of UCHL1 (**B**, **H**), PLZF (**C**, **I**), and OCT4 (**E**, **K**) protein in wild felid testis. (**B**, **H**, **D**, **J**, **F**, **L**) Merge of previous image and Hoechst 33342 to mark cell nuclei. (**A-F**) Adult cheetah (*Acinonyx jubatus*) testis. (**G-L**) Adult Amur leopard (*Panthera pardus orientalis*) testis. Seminiferous tubule borders are outlined in white. Arrows indicate positive cells. Scale bars; 20 µm.

For the RT-PCR analysis, we separated the individuals into two age groups based on whether or not spermatogenesis had been initiated. The first biologically active SSCs appear three to four days post-partum in the male mouse (McLean, Friel et al. 2003) but the precise time period is undefined in the domestic cat. Because the average age of puberty in the male cat is nine months, the transition is estimated to occur over several months, similar to most livestock species. It is therefore reasonable to assume that at least some SSCs were present in the testis of the juveniles in our study. Indeed, transcripts for all six SSC marker genes were detected in the testis of both age groups (Figure 2.1).

Of course, germ cells are not the only cell type present in testis. Somatic cells such as Sertoli cells, Leydig cells, and peritubular myoid cells are also present in the whole testis cell suspensions. SSCs are estimated to make up one of every 4,000 cells in the testis (Tegelenbosch and de Rooij 1993). The rarity of the population makes it almost impossible to identify SSCs in unfractionated cell suspensions. Furthermore, differentiating germ cells are believed to secrete factors that inhibit the growth of SSCs (de Rooij, Lok et al. 1985). For these reasons, testicular cell suspensions must be enriched for SSCs prior to use in *in vitro* culture studies.

In our second RT-PCR study, differential plating was employed to enrich for spermatogonial cells by removing a portion of the contaminating somatic cells. As noted earlier, up to 80% of testicular cells were removed during the differential plating process. This technique does not provide a highly enriched population of SSCs, but it does achieve a gross reduction in the amount of contaminating cells that are present. Following differential plating, expression of all six marker genes was maintained in the SSC-enriched fraction (Figure 2.2).

We were unable to demonstrate protein expression for THY1, GPR125, or GFRalpha1 (data not shown). This is disappointing as these markers are commonly employed in rodent species for antigen-based enrichment, such as fluorescent- or magnetic-activated cell sorting. The lack of signal is likely due to the paucity of suitable antibodies available for the cat or the decreased sensitivity of immunohistochemistry for cell surface antigens (Brandtzaeg and Jahnsen, 2000) rather than a true absence of these SSC markers in the cat. Nonetheless, we confirmed expression of three key SSC markers: UCHL1, PLZF, and OCT4. Western blot analyses were critical to ascertain the specificity of our antibodies, as none had previously been verified in the cat (Figure 2.3 A, C, E). Indeed, all three antibodies detected specific bands consistent with previously reported sizes. Interestingly, PLZF expression was significantly reduced in the adult testis, as compared to either juvenile group (Figure 2.3 C-D). Due to the progressive ageing of the SSC niche (Ryu et al., 2006; Zhang et al., 2006) and dilution by meiotic and post-meiotic germ cells (Tegelenbosch and de Rooij, 1993), there were far less SSCs in the adult testis. PLZF is regarded as a more specific marker than UCHL1 (Buaas et al., 2004; Reding et al., 2010), and the reduced expression noted in the adult testis suggests that it is a more specific marker in the cat as well. Fluorescent immunohistochemical analyses demonstrated that all three markers label a subpopulation of spermatogonia located adjacent to the basement membrane (Figures 2.5 and 2.6), the ideal expression pattern for an SSC marker. PLZF and OCT4 appear to be more specific than UCHL1, consistent with reports in other species. Co-immunofluorescent studies confirmed that the same small subpopulation of undifferentiated spermatogonia express all three markers (Figure 2.8).

Unlike other members of the UCH family, UCHL1 expression is limited to neuronal tissue and gonads (Wilkinson et al., 1992; Wilkinson et al., 1989). In the mouse, both Sertoli cells and spermatogonia express UCHL1 (Kwon et al., 2004). However, in higher order mammals such as the bull (Herrid et al., 2007; Reding et al., 2010; Wrobel, 2000), pig (Luo et al., 2009; Luo et al., 2006), and monkey (Tokunaga et al., 1999), UCHL1 expression is exclusive to spermatogonia. Our fluorescent IHC studies revealed that UCHL1 expression is restricted to spermatogonia, as identified by their morphological appearance and location within the seminiferous tubules (Figures 2.5 A-C, 2.6 A-C). This finding was further confirmed by our co-immunofluorescent studies, which demonstrated that vimentin-positive Sertoli cells do not express UCHL1, both *in situ* (Figure 2.7 A-D) and when examining individual cells (Figure 2.7 E-H). Hence, we are confident concluding that UCHL1 is a spermatogonia-specific marker in the cat.

OCT4 expression appeared highly variable in the cat testis, with differences in expression levels noted among and between the three age groups in our study (Figure 2.3 E-F). In fact, one individual in the prepubertal group did not display an immunoreactive band for OCT4 (Figure 2.3 E, lane 1). However, a strong immunoreactive band was detected in lane 1 following stripping and reprobing of the blot for β–actin; thus it is unlikely that a loading error contributed to the lack of an OCT4-reactive band. More likely, this individual's OCT4 expression level was below the detectable threshold. Indeed, when probed with a more sensitive chemiluminescent substrate, a 60 kDa OCT4-immunoreactive band was detected in that individual (Figure 2.4 A, lane 5). In humans, the *OCT4* gene encodes three transcript variants and at least four protein isoforms (Atlasi et al., 2008; Takeda et al., 1992a) Similarly, the mouse *Oct4* gene can produce at least three distinct

transcripts, including the recently discovered *Oct4b* variant, which is translated into three protein isoforms (Guo et al., 2012). Currently, only one *OCT4* transcript has been characterized in the cat (Yu et al., 2009); however, other undiscovered isoforms are likely to exist. Additionally, post-translational modifications such as phosphorylation or sumoylation are known regulators of OCT4 function in other species (Saxe et al., 2009; Wei et al., 2007), and could also explain the difference in band sizes.

Equally intriguing is the cytoplasmic staining pattern noted in some spermatogonia (Figures 2.5 G-H, 2.6 G-L). OCT4 is a transcription factor and hence, is predicted to translocate to the nucleus to regulate gene expression via its DNA binding domain. OCT4 has a well-established pattern of nuclear localization in the mouse embryo (Palmieri et al., 1994). Following gastrulation, mouse OCT4 is confined to the germline, specifically undifferentiated germ cells (i.e., primordial germ cells, gonocytes, and spermatogonia in the male), again displaying a predominantly nuclear staining pattern (Ohbo et al., 2003). In contrast, a diffuse (i.e., cytoplasmic and nuclear) distribution of the OCT4 signal has been reported in pig (Kirchhof et al., 2000), bull (Kirchhof et al., 2000; van Eijk et al., 1999), and human (Cauffman et al., 2005) embryos, as well as bull (Fujihara et al., 2011) and buffalo (Goel et al., 2010; Mahla et al., 2012) germ cells. It has been suggested that localization of the OCT4 signal is determined by the protein isoform. Studies in human preimplantation embryos and embryonic stem cells demonstrated that the OCT4A isoform is nuclear localized and promotes self-renewal of embryonic stem cells, whereas OCT4B is expressed in the cytoplasm and does not appear to contribute to stem cell self-renewal (Cauffman et al., 2006; Lee et al., 2006a). However, a more recent study utilizing several human cell lines demonstrated a diffuse staining pattern for OCT4B, and its translocation

to the nucleus was up-regulated in response to cell stress (Wang et al., 2009). It is possible that the function of OCT4B depends on its localization and cell type. Certainly, the cytoplasmic localization of OCT4 suggests a biological function other than transcription activation. Additional experiments will be required to determine the functional significance of OCT4 localization in cat SSCs.

Of particular importance is the expression of these markers in wild felids. The Amur leopard and cheetah are two diverse members of the family Felidae, and are estimated to have phylogenetically diverged from the domestic cat 10.8 million and 7.2 million year ago, respectively (Johnson et al., 2006). The Amur leopard is a rare subspecies of leopard that has been listed as critically endangered since 1996. A 2007 census estimated that are only 14 to 20 adults are left in the wild (Jackson and Nowell, 2008). The cheetah has been listed as vulnerable since 1986, and the *in situ* adult population has experienced an estimated 30 percent population decline since 1975 (Durant et al., 2008). We were able to demonstrate that UCHL1, PLZF, and OCT4 display a similar expression pattern in the testis of these species (Figures 2.9 and 2.10). It is unfortunate that co-localization studies could not be performed with the wild felid samples. A high level of autofluorescence was noted when visualizing the wild felid slides with either the red or green filter. This was likely due to the testes being removed post-mortem and the increased time between euthanasia and tissue processing. As a result, IHC studies could only be performed using secondary antibodies in the far red spectrum (i.e., Alexafluor 647), precluding co-localization analysis. Regardless, conservation of SSC markers among these three species representing three distinct felid lineages is highly suggestive that these markers are conserved across all members of family Felidae.

SSC technology has the potential to impact the conservation of felids in several ways. It could provide an innovative means of generating spermatozoa both *in vitro* and *in vivo* following xenotransplantation for the propagation of valuable genotypes. Domestic cat testes are capable of supporting ocelot SSCs (Silva et al., 2012). It remains unknown whether the domestic cat will be a suitable host for the other members of the family Felidae. However, existing evidence suggests that the genome, as well as physiological mechanisms, are relatively well conserved across felids (Brown, 2006; Johnson et al., 2006; Pelican et al., 2006). SSCs can be collected from immature individuals thus providing an option for rescuing genotypes, especially from rare and endangered felids that might otherwise be lost due to premature death. Additionally, SSCs can be cryopreserved using a simple cryopreservation technique routinely used on cultured somatic cells (Robertson, 1987). This is in sharp contrast to mature spermatozoa, in which cryopreservation protocols must be developed on a species by species basis. Such is the case with felids; the 38 members of this Family exhibit striking variability in the types and severity of sperm cryosensitivities (Baudi et al., 2008; Crosier et al., 2006; Gañán et al., 2009; Herrick et al., 2010; Pukazhenthi et al., 2006b; Thiangtum et al., 2006). Many of these species are notorious for being difficult to cryopreserve; a problem no doubt compounded by the high prevalence of teratospermia seen among felids (Pukazhenthi et al., 2000; Pukazhenthi et al., 1999; Pukazhenthi et al., 2002; Pukazhenthi et al., 2001). Thus, cryopreservation of SSCs could help alleviate these species-specific issues.

The domestic cat serves as an important biomedical model in human research (Griffin and Baker, 2002; Ryugo et al., 2005). Transgenesis in the cat has been achieved through the use of somatic cell nuclear cloning (SCNT) (Gómez et al., 2009; Yin et al.,

2008). However, efficiencies remain low and several abnormalities resulting from faulty epigenetic reprogramming have been reported in cloned mammalian offspring (Yamanaka and Blau, 2010). Due to the unique ability of sperm to pass on genetic information to the next generation, SSC-based animal transgenesis offers an alternative to SCNT, especially in species such as the cat in which germline competent embryonic stem cells have not been established.

In conclusion, this study reports the conservation of three SSC markers in felids. These markers may be used to evaluate cell enrichment strategies and monitor the success of *in vitro* culture systems. Because many of these markers have specific roles in SSC self-renewal, conservation of the markers suggests that self-renewal mechanisms may be conserved as well.

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Conflict of Interest

The authors declare no conflict of interest.

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Chapter 3: Enrichment of Spermatogonial Stem Cells in the Domestic Cat (*Felis catus*)

Abstract

Spermatogonial stem cells (SSCs) are an extremely rare population, estimated to represent only 1 out of every 4000 cells in the adult testis. Due to this rarity, testicular cell suspensions must be enriched for SSCs prior to genetic manipulations or *in vitro* culture studies. We explored two approaches for enrichment of domestic cat SSCs: differential plating and magnetic-activated cell sorting (MACS). Differential plating was performed on prepubertal (age 3 to 4.5 months, n = 4) and adult (age 9 months to 3 years, n = 3) cat testes. Differential plating resulted in recovery of 15.5 ± 4.6 and 26.3 ± 2.6 percent of total prepubertal and adult cat testicular cells, respectively. Cell fractions were assessed for SSC enrichment using reverse transcription-quantitative polymerase chain reaction. To allow for accurate normalization, we first validated three reference genes in cat testis: *GAPDH*, *RPL17*, and *YWHAZ*. Five genes (*UCHL1*, *PLZF*, *OCT4*, *CDH1*, and *THY1*) were used to assess the presence of SSCs and *VIM* was used to assess the presence of Sertoli cells. Following differential plating, *UCHL1*, *OCT4*, and *CDH1* (which encodes for E-cadherin) were all significantly increased in the prepubertal group, indicating a higher number of SSCs as compared to the unenriched fraction. The apparent increase in *UCHL1*, *OCT4*, and *CDH1* in the adult post-differential plating fraction was not statistically significant. This difference is likely due to the reduced proportion of SSCs present in the adult testis. Adult cat testicular cells (age 1 to 3 years, n = 3) were then subjected to differential plating and E-cadherin-selected MACS. Only 0.2 ± 0.1 percent of cells were recovered in the E-cadherin-selected fraction. Paradoxically, *UCHL1* and *OCT4* were significantly reduced

and *VIM* significantly increased in the E-cadherin-selected fraction. Cell viability did not differ between the fractions and remained above 90 percent for all techniques investigated. These data demonstrate that the efficiency of cat SSC enrichment depends on the age of the donor and that prepubertal testes are the preferred source for differential plating. Further studies are required to identify suitable cell surface SSC markers from felid testes.

Introduction

Spermatogenesis is one of the most proliferative systems in adult animals, in which cells are continually being produced and lost. The constant rate of sperm production is heavily reliant on the regulation of the adult stem cell population of the testis, the spermatogonial stem cell (SSC). SSCs are unique among adult stem cells because they possess the ability to transmit genetic information to the next generation. This makes them an attractive strategy for genetic manipulation, especially in species such as the cat in which germline competent embryonic stem cells have not been established.

Cells of the testis are a mixed population, consisting of Sertoli, Leydig, and peritubular myoid cells, as well as germ cells in various stages of spermatogenesis. Enzymatic digestion of testis tissue yields populations in which Sertoli cells and other somatic cells dominate. Spermatogonial stem cells are rare, estimated to make up only 1 of every 4000 cells in the adult testis, or 0.03 percent of the total cell population (Tegelenbosch and de Rooij, 1993). The rarity of SSCs makes it almost impossible to identify them in an unenriched cell suspension. Furthermore, differentiating germ cells are believed to secrete factors that inhibit the growth of SSCs (Meistrich and van Beek, 1993). Hence, it is also important to remove the majority of non-SSCs before initiating cell culture.

Several techniques have been employed to enrich SSCs. Differential plating utilizes the different adhesion properties of germ cells compared to somatic cells, with the somatic cells preferentially binding to cell culture dishes (Herrid et al., 2009). The relative simplicity and non-specific nature of differential plating has led to its widespread use in a variety of species including the mouse (Kanatsu-Shinohara et al., 2004a), bull (Herrid et al., 2009; Izadyar et al., 2002b), sheep (Borjigin et al., 2010; Rodriguez-Sosa et al., 2006), pig (Luo et al., 2006), and dog (Harkey et al., 2013). The level of enrichment varies widely, depending on the species, age of donor, plating density, and length of cell culture.

Two techniques using immunological separation, fluorescent-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS), represent the major approach for SSC enrichment in rodents. Kubota et al. (2004a) demonstrated that MACS sorting with thymus cell antigen 1 (THY1) microbeads efficiently enriched SSCs in all age groups of mice tested: neonate, pup, adult wild-type, and adult cryptorchid. The highest level of enrichment was noted in the adult wild-type testis (greater than a 30-fold enrichment of SSCs). Similarly in the rat, selection of epithelial cellular adhesion molecule (EpCAM)-positive cells led to a cell population highly enriched for SSCs (Ryu et al., 2004). Application of MACS to other species has been limited due to a failure to identify cell surface SSC markers and/or species-specific antibodies. It is encouraging to note that selection of THY1-positive cells has also demonstrated successful SSC enrichment in a subset of ruminants (Abbasi et al., 2013; Rafeeqi and Kaul, 2013; Reding et al., 2010) as well as in the carp (Panda et al., 2011) and rhesus macaque (Hermann et al., 2009).

Another cell surface marker of interest is E-cadherin. Two studies in the mouse report that E-cadherin expression is limited to a subset of undifferentiated spermatogonia

that includes SSCs, as confirmed by SSC transplant assays of E-cadherin-selected cells (Tokuda et al., 2007; Tolkunova et al., 2009). E-cadherin showed a similar expression pattern in the rat (Zhang et al., 2011) and buffalo (Yu et al., 2014), however neither study attempted to sort the E-cadherin-positive cells. In Drosophila, E-cadherin functions to anchor the germ stem cells to their niche (Jin et al., 2008; Song et al., 2002). The exact role of E-cadherin in mammalian spermatogenesis is unknown. Give its role as an adhesion protein, it has been hypothesized that E-cadherin may help maintain the SSCs within their niche as well.

With regard to the cat, there is one report of applying differential plating to adult cat testes prior to the initiation of *in vitro* culture studies (Tiptanavattana et al., 2013). No characterization was performed on the differentially-plated cell fraction, thus precluding enrichment analysis. Powell et al. (2013) attempted to enrich adult cat SSCs with FACS selection of stage specific embryonic antigen-1 (SSEA-1) and SSEA-4-positive cells. SSCs were present in these fractions, as confirmed by an SSC transplant assay. However, no attempt was made to quantify colonization rate as compared to unenriched cat testes. It remains unknown if any enrichment occurred, as the unenriched cell suspensions were also able to colonize host seminiferous tubules. Therefore, the objective of the present study was to assess two techniques to enrich SSCs in the domestic cat: differential plating and MACS.

Materials and Methods

Collection of Testes

Domestic cat testicles were obtained from local spay-neuter clinics. Immediately following surgery, tissue was aseptically wrapped in gauze pads moistened with 1X

Dulbecco phosphate-buffered saline with calcium and magnesium (DPBS(+), Corning Cellgro) and stored at 4°C. Tissue was processed within 24 hours of surgery, and generally, within the first 6 hours. A portion of each testis was fixed in 4% formaldehyde and embedded in paraffin. Donor age was recorded if known or estimated by the attending veterinarian. Age group was then confirmed by histological analysis of hematoxylin and eosin stained testis sections for progression through spermatogenesis.

Enzymatic Digestion of Testicular Tissue

Using a modified two-step enzymatic digestion (Honaramooz et al., 2002), testicular tissue was dispersed to obtain a single-cell suspension. Testicles were rinsed in Hanks Balanced Salt Solution without calcium or magnesium (HBSS, Gibco) and mechanically dissected to remove the vaginal tunic, vas deferens, and epididymis. The testis was bisected and pointed forceps were used to remove the tunica albuginea. The tissue was minced into small pieces and grossly apparent connective tissue was removed. Tissue was incubated with 2 mg/ml collagenase (Sigma) for up to 30 minutes at 37°C in a shaking incubator at approximately 110 oscillations per min. Tissue was washed twice with HBSS and incubated with 0.25% (w/v) trypsin (Invitrogen) supplemented with 7 mg/ml deoxyribonuclease I (DNase I, Sigma) at a 1:4 ratio for 5 to 10 minutes at 37°C in a shaking incubator. Ten percent (v/v) fetal bovine serum (FBS, Hyclone) was added to inactivate trypsin before passing through a 70 μm mesh filter (Fisher Scientific). Cell concentration was determined using a hemocytometer and cell viability assessed by trypan blue dye (BioWhittaker) exclusion (Strober, 2001).

Enrichment of Cat SSCs with Differential Plating

To perform differential plating, dissociated testis cells were suspended in a basic cell culture medium of Dulbecco modified Eagle medium (DMEM, Invitrogen) supplemented with 10% (v/v) FBS, 1% (v/v) GlutaMAX™ (Gibco), 100 u/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco). The cells were plated at a concentration of 5 x 10^5 cells/ml (10 ml per dish) on 100 mm untreated cell culture dishes (Corning). The cells were incubated for 2 hours at 37ºC in 5% CO_2. Cells remaining in suspension at the end of the culture period were collected for further analysis. Cells immediately following dissociation (no enrichment) and after differential plating were stored in RLT lysis buffer (Qiagen) for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

Magnetic-Activated Cell Sorting

MACS was performed using the quadroMACS magnet and LS columns (Miltenyi Biotech) according to manufacturer's instructions with minor modifications. Briefly, dissociated testis cells were washed once in PBS-S (PBS(+) containing 1% (v/v) FBS, 10 mM HEPES (Sigma), 1 mM sodium pyruvate (Gibco), 1 mg/ml D-(+)-glucose (Sigma), 50 u/ml penicillin (Gibco), and 50 µg/ml streptomycin (Gibco)). Approximately 1 to 4 x 10^7 cells were incubated with anti-E-cadherin antibody (rat anti-mouse IgG_1, Novus Biologicals), diluted 1:50 in PBS-S, for 20 minutes at 4°C. Cells were washed twice in excess PBS-S before incubation with goat anti-rat IgG magnetic microbeads (Miltenyi Biotech) for 15 minutes at 4°C. Cells were rinsed one more time with PBS-S before being passed through the LS separation column in the presence of the quadroMACS magnetic field. Following three PBS-S rinses, the column was removed from the magnetic field and

the E-cadherin-selected cells were eluted. Cells both immediately following dissociation (before enrichment) and after immunomagnetic separation were stored in RLT lysis buffer for RT-qPCR analysis.

RT-qPCR Analysis

The expression of five SSC genes (E-cadherin (*CDH1*), octamer-binding protein 4 (*OCT4*), promyelocytic leukemia zinc finger (*PLZF*), thymus cell antigen 1 (*THY1*), and ubiquitin carboxy-terminal hydrolase 1 (*UCHL1*)), one Sertoli cell gene (vimentin (*VIM*)), and three reference genes (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L17 (*RPL17*), and tryptophan 5'-monooxygenase activation protein zeta isoform (*YWHAZ*)) were measured with RT-qPCR. Total RNA was extracted using RNeasy Midi kits (Qiagen), according to manufacturer's instructions. RNA concentration was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). One microgram of total RNA from each sample was used in the reverse transcription reactions. For each sample, a second set of reverse transcription reactions were performed without reverse transcriptase enzyme, as a control for genomic DNA contamination. First strand cDNAs were synthesized according to manufacturer's instructions using the QuantiTect® Reverse Transcription kit (Qiagen), which utilizes an optimized blend of oligo-dT and random primers. The cDNA was diluted 1:20 for use in RT-qPCR reactions.

Primers (Integrated DNA Technologies) used in the RT-qPCR reactions were designed using the Primer3 software (http://bioinfo.ut.ee/primer3/) to have a melting temperature (T_m) of 58 to 60°C, GC content of 40 to 60%, length of 18 to 22 nucleotides, and amplicon length of 100 to 180 base pairs. Designs were based on transcript sequences in the National Center for Biotechnology Information cat genome database

(http://www.ncbi.nlm.nih.gov/genome?term=felis%20catus). Primers were designed to span an exon junction and/or primers pairs were separated by at least one intron whenever possible. Endpoint RT-PCR and gel electrophoresis were conducted to ensure that each primer pair produced a single amplicon of predicted size. PCR products for each set of primers were sequenced to confirm identity. Primer pair efficiencies were determined by performing a 5-fold serial dilution of cDNA (0.016 to 50 ng cDNA) pooled from prepubertal and adult testes, before and after differential plating. A linear standard curve was generated by plotting quantification cycle (Cq) against the log of template concentration. Primer pair efficiency was calculated with the equation $E = 10^{-1/slope}$ as described by Pfaffl (2001). Reference gene stability was determined using the geNorm software in qBase^{PLUS}(Vandesompele et al., 2002). Primer sequences and amplification efficiencies are listed in Table 3.1.

The PCR reaction consisted of 7.5 µl of iQ SYBR Green Supermix (Bio-Rad Laboratories), 300 nM forward and reverse primers, and 2 µl diluted cDNA (5 ng) in a total volume of 15 µl. PCR reactions were performed in triplicate, and RT-minus (pooled across each set of samples) and no template controls were performed in duplicate.

Cycling was performed in CFX96 Real-Time PCR Detection System (Bio-Rad) with the following conditions: initial denaturation at 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Following thermocycling, a dissociation protocol was performed to confirm that a single PCR product was amplified, and was absent in the RT-minus and no template controls.

Gene	Accession Number	Primer Sequence	Amplicon (bp)	Efficiency
Spermatogonial stem cells				
CDH1	XM_003998189.1	F: 5'-GCTGTGTCTTCAAATGGGCA-3'	178	97%
		R: 5'-CGTCGTCATCTGCATCTGTG-3'		
OCT4	NM_001173441.1	F: 5'-CTGGAGCAAAACACCGAGGAG-3'	160	113%
		R: 5'-TTTGGCTGAACACCTTCCCAAA-3'		
PLZF	XM_003992367.1	F: 5'-ATCCCTACGAGTGTGAGTTCTG-3'	169	96%
		R: 5'-TTCTCACCTGTATGCACCCTATAG-3'		
THY1	XM_003992443.2	F: 5'-CAAGGACGAGGGGATGTACA-3'		105%
		R: 5'-TATGCCCTCACACTTGACCA-3'		
UCHL1	XM_003985462.1	F: 5'-CCTGTGGTACCATCGGACTG-3'	168	98%
		R: 5'-CATCATGGGCTGCCTGAATG-3'		
Sertoli cells				
VIM	XM_003988131.1	F: 5'-GGGAGAAGTTGCAGGAGGAG-3' R: 5'-CAAGGTCAAGACGTGCCAAA-3'	105	101%
Reference Genes				
GAPDH	NM_001009307.1	F: 5'-AGAACATCATCCCTGCTTCTACT-3'	170	87%
		R: 5'-CTTCTTGATGTCATCGTATTTGGC-3'		
RPL17	NM_001128842.1	F: 5'-CCCGACAAAATCATGCAAATCAAG-3'	113	100%
		R: 5'-GATACTTGGTGGCTTTTCGGATAT-3'		
YWHAZ	XM_004000022.1	F: 5'-GCCCTTAACTTCTCTGTGTTCTAT-3'	142	98%
		R: 5'-TTATTAGCGTGCTGTCTTTGTAGG-3'		

Table 3.1 Primers used for reverse transcription-quantitative polymerase chain reaction.

Preparation of Western Blots

Testes from prepubertal (n = 3), transitional (n = 2), and adult (n = 2) cats were used for Western blot analysis. The tunica albuginea was removed and small pieces of testis were flash frozen in liquid nitrogen and stored at -80°C. Protein was extracted using NP40 lysis buffer (50 mM Tris-HC1 pH 8.0, 150 mM NaC1, 1% (v/v) NP40) and protein concentrations were determined using the Micro BCA Protein Assay Kit (Thermoscientific). Eighty micrograms of protein per sample were boiled at 98°C for 10 minutes in 6X SDS Sample Buffer (375 mM Tris-HCl pH 6.8, 12% (w/v) SDS, 60% (v/v) glycerol, 0.06% (w/v) bromophenol blue; 5% (v/v) 2-mercaptoethanol). Total protein lysates were subjected to SDS-PAGE on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Proteins transfer was confirmed by Ponceau S staining. Membranes were incubated in blocking solution (Tris-buffered saline (20 mM Tris-HCl pH 7.6, 137 mM NaCl) containing 0.05% (v/v) Tween-20 (TBST) and 5% (w/v) Sanalac nonfat dry milk) for 2 hours at room temperature and then incubated overnight at 4°C with anti-E-cadherin antibody (rat anti-mouse IgG_1, Novus Biologicals) diluted 1:1250 in blocking solution. Membranes were washed in TBST and then incubated with goat anti-rat IgG horseradish peroxidase–conjugated secondary antibody (Cell Signaling Technology) diluted 1:5000 in blocking solution for 1 hour at room temperature. Membranes were washed again in TBST, and antigen detection was performed using the Supersignal West Pico chemiluminescent substrates (Thermo Scientific) and visualized using a ChemiDoc XRS system (Bio-Rad Laboratories). Immunoblots were stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) according to manufacturer's instructions and reprobed for β-actin (1:2500, Mouse IgG_{2a}, Sigma) as described above,

except that the secondary antibody (1:5000, horse anti-mouse IgG horseradish peroxidase–conjugated, Cell Signaling Technology) was only incubated for 45 minutes at room temperature. Immunoblot images were processed for figures using Photoshop CS6 (Adobe Systems).

Statistical Analysis

Average Cq values from each triplicate were analyzed with the qBase^{Plus} version 2.5 software (Biogazelle), which allows for the use of multiple reference genes and incorporation of PCR efficiencies for each primer pair (Hellemans et al., 2007). Expression values were normalized to three reference genes previously validated in the cat (*GAPDH*, *RPL17*, and *YWHAZ*) (Filliers et al., 2012; Kessler et al., 2009; Penning et al., 2007) and primer efficiency values (Table 3.1) were taken into account for all relative expression calculations. All data were analyzed by analysis of variance using the MIXED models procedure of SAS statistical software (SAS Institute). Differences between before and after enrichment were determined using least square means. All data are presented as mean ± standard error.

Results

Validation of Reference Genes for RT-qPCR Analysis in Cat Testis Tissue

To allow for accurate normalization, we first focused on the selection of stable reference genes for cat testis tissue. Gene expression stability (M) is calculated based on the average pairwise variation between the reference genes. The highest M values correlates with the least stable expression and hence, genes with the lowest M value are the most stably expressed. The creators of this program proposed a cut-off value of 1.5; genes

with M values above which are considered too variable for accurate normalization. As shown in Figure 3.1, *RPL17* was identified as the most stable reference gene, followed by *YWHAZ* and *GAPDH* respectively. The M values for all three genes were well below the proposed cut-off value of 1.5, therefore all three were used for normalization in our RT-qPCR studies.

Differential Plating Enriches Prepubertal Cat Testis for SSCs

Differential plating was performed on freshly isolated cat testicular cells for two age groups: prepubertal (age 3 to 4.5 months, n = 4) and adult (age 9 months to 3 years, n = 3). In the prepubertal group, 15.5 ± 4.6 percent of total testicular cells were recovered following differential plating (Table 3.2). Cell viability of the enriched fraction (92.8 ± 1.4 percent) was comparable to the input cells (95.7 ± 0.4 percent), as determined by trypan blue dye exclusion.

RT-qPCR was performed to compare the gene expression profiles of the two fractions. Five genes (*UCHL1*, *PLZF*, *OCT4*, *CDH1*, and *THY1*) were used to assess the presence of SSCs and *VIM* was used to assess the presence of Sertoli cells (Steger et al., 1996). The results of the RT-qPCR study are shown in Figure 3.2. *UCHL1*, *OCT4*, and *CDH1* were all significantly increased in the post-differential plating fraction, indicating a higher number of SSCs as compared to the unenriched fraction. *PLZF* and *THY1* also trended upwards, but were not statistically significant. *VIM* expression appeared similar between the two fractions. The p-values for all prepubertal gene comparisons can be found in Supplemental Figure 4.

A high level of viability was also seen in the adult differential plating group fractions (94.1 ± 0.8 and 96.9 ± 1.6 percent viable) (Table 3.2). As compared to prepubertal

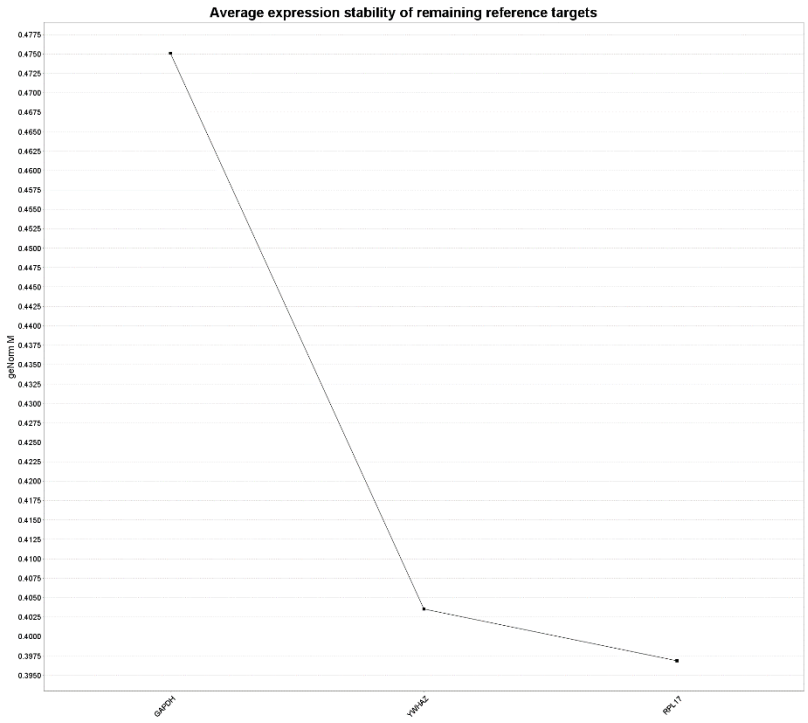

Figure 3.1 GeNorm analysis of candidate reference genes. Average transcript expression stability (M value) for the candidate reference genes in cat testis, plotted from the least stable (left) to the most stable (right).

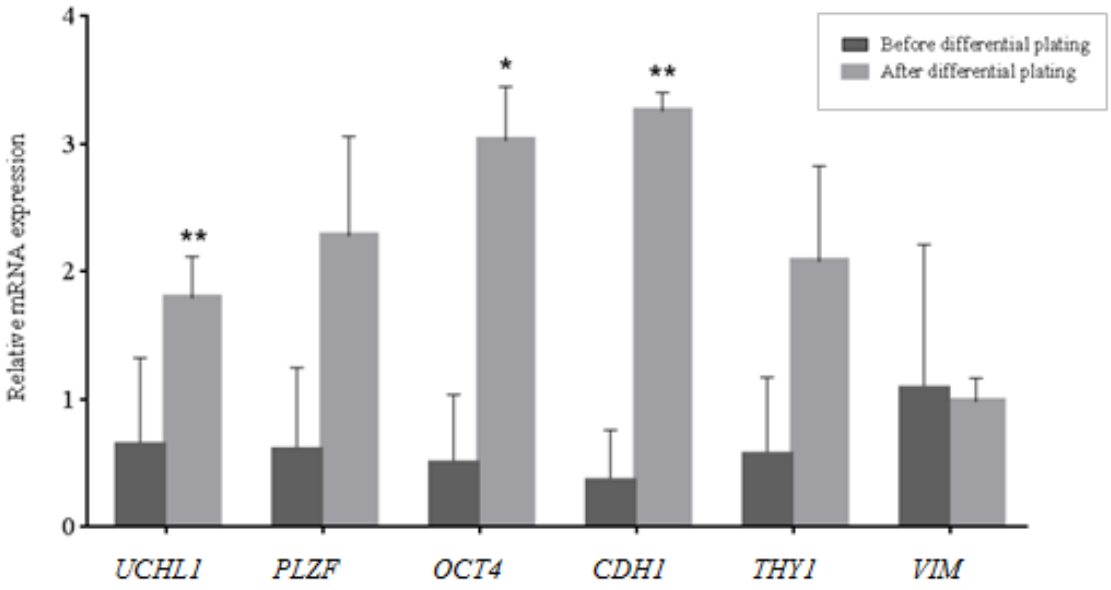

Figure 3.2 RT-qPCR analysis of SSC (*UCHL1*, *PLZF*, *OCT4*, *CDH1*, and *THY1*) and Sertoli cell (*VIM*) gene transcripts using mRNA isolated from prepubertal cat testis before and after differential plating. Four animals were used and analyses were performed in triplicate. Errors bars represent ± SE. Statistically significant data are represented by p-values < 0.05 (*) and < 0.001 (**).

Group	Before Enrichment		After Enrichment		
	Number of cells (million)	Viability (percent)	Number of cells (million)	Viability (percent)	Cells Recovered (percent)
Prepubertal DP	22.3 ± 1.2	95.7 ± 0.4	2.1 ± 0.7	92.8 ± 1.4	15.5 ± 4.6
Adult DP	39.1 ± 0.9	94.1 ± 0.8	10.3 ± 2.7	96.9 ± 1.6	26.3 ± 2.6
Adult DP + MACS	182.0 ± 49.5	93.8 ± 1.6	0.4 ± 0.09	93.7 ± 2.3	0.2 ± 0.1

Table 3.2 Effect of differential plating and MACS on cat testicular cells. DP: differential plating.

samples, a larger percentage of cells (26.3 ± 2.6) were recovered from the adults. The three genes that were significantly higher in the prepubertal differential plating fraction (*UCHL1*, *OCT4*, and *CDH1*) also appeared to increase in adults (Figure 3.3). However, these differences were not statistically significant. *PLZF*, *THY1*, and *VIM* expression were not different between the two fractions. The p-values for all adult gene comparisons can be found in Supplemental Figure 5.

Enrichment using Immuno-based Separation

Due to the marginal enrichment of differential plating seen in the adult samples, we were interested in identifying an alternative strategy to enrich adult cat testicular cells. MACS, which separates various cell populations based on their surface antigens, is widely used to enrich SSCs in rodents (Kubota et al., 2004a; Ryu et al., 2005). A battery of antibodies directed against cell surface SSC markers which have been used in other species for selection purposes were investigated using Western blot analysis of whole cat testis lysates. Antibodies directed against G-protein coupled receptor 125 (GPR125), glial cell line–derived neurotrophic factor family receptor alpha 1 (GFRalpha1), and THY1 did not detect specific bands (data not shown). However, an antibody directed against mouse E-cadherin displayed two immunoreactive bands in cat testis, as shown in Figure 3.4 A. The larger 125 kDa band likely represents the full length protein, and the smaller 97 kDa band could be E-cadherin's extracellular domain following cleavage by a metalloprotease (Maretzky et al., 2005; Noë et al., 2001).

To test E-cadherin's utility in selection of cat SSCs, E-cadherin based MACS was performed on adult cat testicular cells (age 1 to 3 years, n = 3). Testes from three individuals were pooled for each replicate. In order to maximize the number of SSCs in the column,

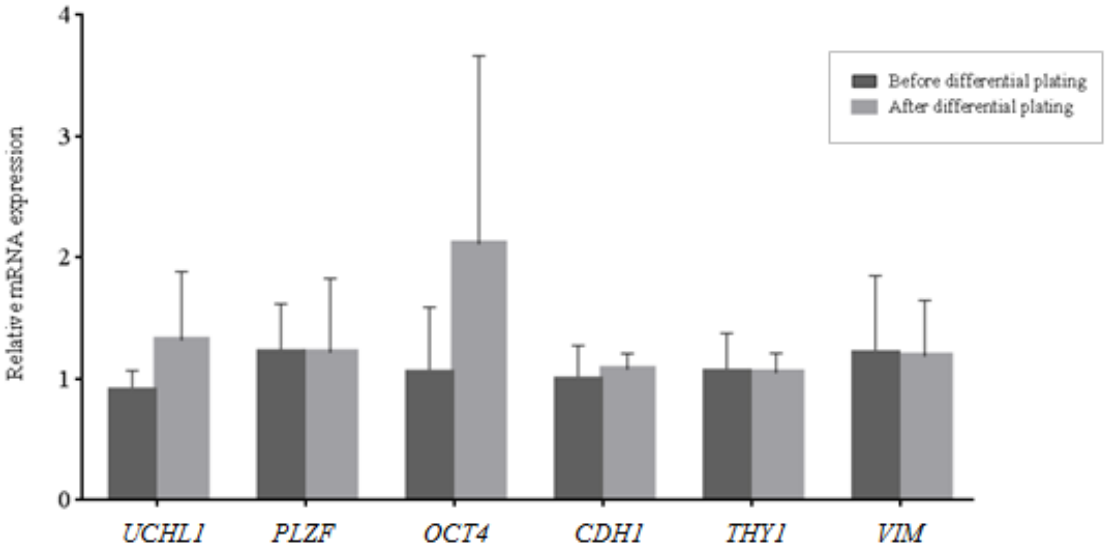

Figure 3.3 RT-qPCR analysis of SSC (*UCHL1*, *PLZF*, *OCT4*, *CDH1*, and *THY1*) and Sertoli cell (*VIM*) gene transcripts using mRNA isolated from adult cat testis before and after differential plating. Three animals were used and analyses were performed in triplicate. Errors bars represent ± SE. Statistically significant data are represented by p-values < 0.05 (*) and < 0.001 (**).

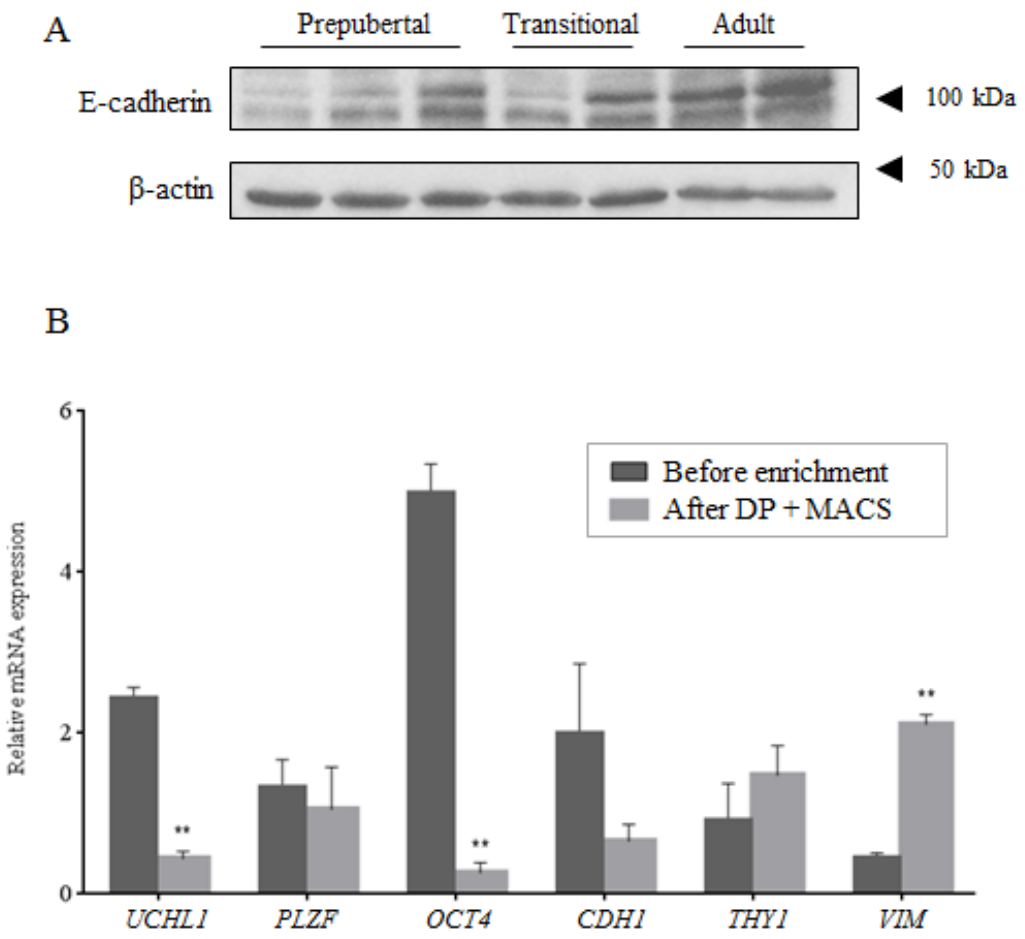

Figure 3.4 Analysis of E-cadherin-selected cat testis. **(A)** Western blot analysis of whole cat testis showing that prepubertal animals who had not initiated spermatogenesis (lanes 1–3), transitional animals who had started but not yet completed spermatogenesis (lanes 4–5), and adult animals with full completion of spermatogenesis (lanes 6-7) all displayed two immunoreactive bands for E-cadherin at approximately 97 and 125 kDa. Blot was stripped and reprobed for β-actin as a loading control. Ages were confirmed with histological analysis. **(B)** RT-qPCR analysis of SSC (*UCHL1*, *PLZF*, *OCT4*, *CDH1*, and *THY1*) and Sertoli cell (*VIM*) gene transcript expression using mRNA isolated from adult cat testis before and after differential plating and MACS. Testes from three adults were pooled for each replicate (n = 3), and analyses were performed in triplicate. Errors bars represent ± SE. Statistically significant data are represented by p-values < 0.05 (*) and < 0.001 (**). DP: differential plating.

cells were subjected to differential plating prior to E-cadherin sorting. Gene expression profiles of the freshly isolated cells and differentially-plated, E-cadherin-sorted cells were assessed with RT-qPCR.

Paradoxically, several of the SSC genes were reduced in the E-cadherin-selected cell fraction, including E-cadherin (*CDH1*) itself. (Figure 3.4 B). While only two of these genes were statistically significant (*UCHL1* and *OCT4*), four of the five SSC genes examined showed a downward trend, indicating that fewer SSCs were present in the E-cadherin-selected fraction. *VIM* was significantly increased in the E-cadherin-selected fraction, suggesting that our enrichment strategy had increased the number of Sertoli cells.

Discussion

In rodents, various strategies have been used to enrich for SSCs. Successful enrichment has been evaluated by both molecular and functional methods. Molecular methods include assessing enrichment of cells expressing SSC markers. Functional evaluations include transplantation of enriched cell fractions into the seminiferous tubules of recipient males whose endogenous germ cells are depleted. Only the SSCs are able to establish colonies and resume spermatogenesis in the host tubules (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). Donor cells are typically identified by a transgenic reporter gene. Owing to the fact that each colony represents the clonal expansion of a single SSC, the rate of recipient testis tubule colonization can be quantified and used as a measure of the relative abundance of stem cells in the input population (Dobrinski et al., 1999b).

This technique is far less practical in non-traditional lab animals such as the cat for several reasons. First, the paucity of available techniques to create transgenic cats make unequivocal identification of donor cells far more difficult. Second, the larger size of the

cat testis as compared to rodents prevents accurate quantitative analysis of colonization rate (Luo et al., 2006). Transplantation into a host mouse testis would ease identification of the donor cat cells; however this technique is less reliable because rodent testes can only support the initial steps of spermatogenesis in the cat (Kim et al., 2006). Finally, SSC transplantations are technically challenging and require a substantial investment of time and resources (Oatley et al., 2002).

Far more practical in the cat is the evaluation of SSC marker expression in each cell fraction. In our study, we used RT-qPCR to assess a panel of SSC markers well-conserved among other species. To date, no definitive marker has been identified that is solely expressed by the SSCs. All of the markers are expressed by more advanced spermatogonial cells, and some are expressed by testicular somatic cells as well. Simultaneously assessing several genes allowed for a more robust evaluation of our enrichment strategies.

Of fundamental importance was the selection of appropriate reference genes for accurate expression normalization. It has been well-documented that normalization with inappropriate reference genes can markedly alter the outcome of a study (Dheda et al., 2005; Fu et al., 2009; Tatsumi et al., 2008). There are a limited number of reports on feline reference genes (Filliers et al., 2012; Kessler et al., 2009; Penning et al., 2007). We chose three candidates with separate cellular functions (thus reducing the likelihood of co-regulation) that are stably expressed in other feline tissues. However, gene stability can vary greatly depending on the tissue tested and to our knowledge, no reference gene evaluation has been described for feline testis. Therefore, we assessed the stability of *GAPDH*, *RPL17*, and *YWHAZ* in prepubertal and adult domestic cat testis tissue. The geNorm analysis demonstrated that all three candidate reference genes were stable, with M

values well below the proposed cut-off of 1.5 (Figure 3.1). Conventional normalization strategies based on a single reference gene can lead to a large errors in normalization, up to 20-fold in some cases (Vandesompele et al., 2002). Therefore, all three genes were used to normalize our data sets. While it has been proposed as many as seven reference genes may be required for optimal normalization in feline tissue (Kessler et al., 2009), this would be difficult with the limited amount of RNA obtained from the enrichment studies. Furthermore, given the low M values and different functional classes of our three reference genes, we would anticipate only a marginal enhancement of reliability with additional reference genes.

We first investigated differential plating as a potential method to enrich cat SSCs because it is a simple technique that relies on the differential adhesive properties of somatic cells and SSCs and hence, does not require the use of an antibody. A variety of incubation times have been employed for differential plating. Two hours was chosen in our study to allow enough time for the somatic cells to firmly adhere to the culture dish, while maintaining a high level of viability and minimizing exposure to FBS, which has been shown to be detrimental to SSCs in other species (Kubota et al., 2004b). Indeed, cell viability remained high following differential plating for both prepubertal and adult cats (Table 3.2). Additionally, we plated the cells at a low density (1×10^6 cells per 100 mm dish; 1.8×10^4 cells/cm^2) to maximize the space available for the somatic cells to attach to the dishes.

Our gene expression data demonstrated that differentially plating achieved a much higher level of enrichment in the prepubertal group as compared to adults (Figures 3.2 and 3.3). These differences are also reflected in the percentage of cells recovered from

differential plating (Table 3.2). Approximately ten percent less cells were depleted in the adult group, suggesting a less stringent selection. This observation is consistent with reports from other species that enrichment of SSCs is more efficient in prepubertal testes (Borjigin et al., 2010; Herrid et al., 2007; Izadyar et al., 2002b; Luo et al., 2006). This is likely due to the cell population of each age group. Prepubertal seminiferous tubules contain only two cell types: gonocytes/SSCs and Sertoli cells. Following initiation of spermatogenesis, SSCs are diluted by the more advanced germ cells. Differential plating capitalizes on the higher affinity of somatic cells to adhere to plastic culture vessels (Chang et al., 2002); hence, differentiating germ cells would not be depleted. Ageing of the stem cell niche in adults leads to a further reduction of SSCs (Ryu et al., 2006). A longer incubation time and/or lower plating density may further enrich adult cat testes for SSCs. Further experiments would be required to determine an optimal differential plating protocol in adults.

In the mouse, identification of cell surface markers has greatly improved SSC enrichment. For example, MACS sorting of adult mouse testicular suspensions with THY1 microbeads efficiently enriched SSCs by more than 30-fold, as demonstrated by an SSC transplantation assay (Kubota et al., 2004a). Using Western blot analysis, we identified E-cadherin as a potential cell surface SSC marker in the cat (Figure 3.4A). Due to the fact that SSCs are present in the E-cadherin-selected fraction in the mouse (Tokuda et al., 2007; Tolkunova et al., 2009), we anticipated that cat SSCs could be also enriched through MACS selection of E-cadherin. Disappointingly, the E-cadherin-selected fraction contained less SSCs than the input fraction, as determined by the gene expression analysis (Figure 3.3B). Moreover, E-cadherin (*CDH1*) gene transcript levels were reduced in the

MACS fraction, suggesting a failure to select for E-cadherin expressing cells. One possibility is the antibody used in our study is not suitable for MACS. Success with a particular antibody in Western blots, (when the protein is denatured) does not guarantee success in other applications, such as immunosorting (when the protein is in its native state). Another potential explanation is loss of the E-cadherin antigen during enzymatic cell digestion. Mouse testes can be dispersed into a single cell suspension using a short (< 10 min) incubation with trypsin and vigorous pipetting (Kubota and Brinster, 2008). Cat testes contain more connective tissue and as such, dispersion requires the use of collagenase and/or hyaluronidase in addition to trypsin. In the dog, Harkey et al. (2013) were unable to achieve a single cell suspension of testis tissue without a considerable loss of cell viability. Our digestion protocol does maintain a high level of cell viability (Table 3.2). However, we cannot rule out the possibility that enzymatic treatment alters, damages, or removes cell surface proteins. Glycosyl phosphatidylinositol-anchored surface proteins, such as GFRalpha1 and THY1, lack a transmembrane domain and may be particularly susceptible to enzymatic treatment. In addition to providing a first level of enrichment, we had hoped a 2 hour differential plating treatment prior to MACS would give the cells enough time to recover from the enzymatic digestion (Farmer et al., 1978). Increased cell culture recovery times and/or alternative testis dispersion techniques warrant further investigation in carnivores.

In conclusion, this study explored two techniques to enrich cat testis cells for SSCs. Differential plating represents a simple and convenient way to enrich a large number of cells, while MACS can provide a highly enriched SSC population in some species. Cell viability remained well above 90 percent, regardless of the age group or enrichment

technique. Differential plating of prepubertal cat testis resulted in a significant increase in several SSC genes. A lower level of enrichment was noted when differential plating was applied to adult cats, likely reflecting the lower percentage of SSCs present in adult testes. MACS of E-cadherin labeled adult cat testis cells failed to achieve any level of SSC enrichment. It remains unclear whether this is due to a true lack of E-cadherin expression by cat SSCs *in vivo* or an artifact induced by *in vitro* manipulations. Further studies are required to identify a surface antigen that will facilitate production of a highly purified cat SSC population. Regardless, these results provide the basis for future studies aimed at improving SSC enrichment and establishing a long-term culture system for cat SSCs.

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Conflict of Interest

The authors declare no conflict of interest.

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Chapter 4: *In Vitro* Culture of Putative Spermatogonial Stem Cells in the Domestic Cat (*Felis catus*)

Abstract

Spermatogonial stem cells (SSCs) are unique adult stem cells that reside within the seminiferous tubules of the testis. As stem cells, SSCs maintain the ability to self-replicate, providing a potentially unlimited supply of cells and an alternate source for preservation of the male genome. While self-renewing, long-term SSC culture has been achieved in the mouse, there is virtually no information regarding the culture requirements of felid SSCs. Therefore, the objective of this study was to evaluate the ability of three different feeder cell lines to support germ cell colony establishment in the domestic cat: Sandos inbred mouse embryo-derived thioguanine and ouabain resistant (STO) cells, mouse derived C166 endothelial cells, and primary cat fetal fibroblasts (cFFs). When cultured on a STO feeder, cat germ cells were distributed primarily as single cells. Germ cell clusters (morphologically consistent with SSC colonies in other species) established on both C166 cells and cFFs. Immunocytochemistry with three SSC markers revealed that the single germ cells present on STO feeders were positive for UCHL1 and weakly expressed PLZF and OCT4. Cells within the germ cell clumps on C166 cells and cFFs co-expressed all three SSC markers. As demonstrated by the cluster forming activity assay, C166 cells supported a significantly higher number of germ cell clumps (77.4 ± 13.8 colonies per 10^5 cells input) as compared to STOs (3.5 ± 1.1 colonies, $p = 0.0003$) or cFFs (22.7 ± 1.0 colonies, $p = 0.0024$). Therefore, subsequent subculture experiments were performed exclusively with C166 feeder layers. Cultures from two individuals were passaged at 12 days in vitro (DIV),

and periodically as needed thereafter. Germ cell clumps consistently reestablished following each subculture. ICC analysis confirmed maintenance of all three SSC markers by the germ cell clumps. Cells were also positive for alkaline phosphatase activity. Taken together, these data strongly suggest that C166 cells can facilitate colony establishment and in vitro propagation of SSCs in the domestic cat.

Introduction

Spermatogonial stem cells (SSCs) are the adult stem cells of the testis that sustain spermatogenesis by constantly providing new progenitor cells. The ability of SSCs to self-renew and transmit genetic information to the next generation has important implications in human health, livestock management, wildlife conservation, and basic stem cell biology. The first self-renewing, long-term SSC culture system was reported in the mouse in 2004 (Kubota et al.). Also reported in this landmark paper was the required addition of glial cell line-derived neurotrophic (GDNF), GDNF family receptor alpha 1 (GFRalpha1), and basic fibroblast growth factor (bFGF) to the medium for long-term SSC self-renewal.

Since then, much research has focused on the development of SSC culture systems in other species. The mouse culture system has successfully been applied to the rat (Ryu et al., 2005). However, several key modifications (e.g., SSC selection marker, medium composition, oxygen concentration, and passaging method) had to be performed before rat SSCs could proliferate continually (Ryu et al., 2005). Similarly, changes were also required to translate the mouse system to the hamster. One notable difference being hamster SSCs proliferate better on laminin than on a mouse embryonic fibroblast (MEF) feeder layer (Kanatsu-Shinohara et al., 2008).

Investigations into optimal culture conditions for SSCs of non-rodents have been limited. To date, the only non-rodent species that has been successfully propagated long-term is the rabbit (Kubota et al., 2011). Short-term culture (i.e., typically less than two months) has been achieved in the bull (Aponte et al., 2008; Aponte et al., 2006; Fujihara et al., 2011; Nasiri et al., 2012; Oatley et al., 2004), buffalo (Goel et al., 2010; Kadam et al., 2013; Kala et al., 2012; Xie et al., 2010), goat (Bahadorani et al., 2012; Heidari et al., 2012; Zhu et al., 2013), pig (Goel et al., 2007; Kuijk et al., 2009; Luo et al., 2006; Zheng et al., 2013b), cat (Tiptanavattana et al., 2013), dog (Harkey et al., 2013), and human (Aponte et al., 2008; Sadri-Ardekani et al., 2009). In these species, the proliferation of putative SSCs appears to experience a gradual decline during subculture such that over time, differentiation and apoptosis leads to cessation of culture. The major factor limiting advancement is the failure to identify appropriate species-specific modifications (Feng et al., 2002).

A slow proliferation rate is characteristic of adult stem cells (Iscove et al., 1970; Lavker et al., 1993; Morrison and Weissman, 1994; Quesenberry and Levitt, 1979). Feeder cells can help promote proliferation by secretion of soluble factors and/or extracellular matrices as well as signals via direct contact (Puck and Marcus, 1955; Rheinwald and Green, 1975). A serum- and feeder-free culture system has been developed in the mouse (Kanatsu-Shinohara et al., 2005a). However, the number of SSCs was reduced, suggesting that feeder cells enhanced the SSC self-renewal rate. Thus, it seems likely that the derivation and maintenance of SSCs in non-rodent species will requires the presence of a feeder layer.

The Sandos inbred mouse embryo-derived thioguanine and ouabain resistant (STO) cell line was derived from MEFs and have been used to establish long-term SSC cultures in the mouse (Kubota et al., 2004b) and rat (Ryu et al., 2005). However, STO cells failed to support rabbit SSCs (Kubota et al., 2011). It is important to note that failure of STO cells to support SSCs was not due to media composition, as rabbit SSCs could be established and maintained on a C166 feeder layer. C166 cells are a yolk sac-derived cloned endothelial mouse cell line that have been used to support hematopoietic stem cells (Lu et al., 1996). As suggested by Kubota et al. (2011) the ability of C166 feeders to support SSC proliferation may be related to the endothelial origin of the C166 cells. *In situ*, SSCs are not evenly distributed along the basement membrane of the seminiferous tubules, but preferentially located to the areas bordering the interstitial tissue (Chiarini-Garcia et al., 2001; Chiarini-Garcia et al., 2003). This idea was expanded upon by Yoshida et al. (2007) who demonstrated that mouse single, paired, and aligned spermatogonia follow blood vessels in the interstitium and favor locations where the vessels branch. Thus, C166 cells may produce a SSC renewal factor similar to those secreted *in vivo* by endothelial cells of the testis.

Homologous feeder layers are commonly used as alternative to rodent-derived cell lines in higher-order mammals (Li et al., 2005; Richards et al., 2003; Sharma et al., 2012). Studies have utilized a variety of homologous and heterologous feeder layers for SSC propagation, including the pig (Kuijk et al., 2009; Lee et al., 2013), bull (Aponte et al., 2006; Nasiri et al., 2012; Oatley et al., 2004), buffalo (Yu et al., 2014), dog (Harkey et al., 2013) and human (Mirzapour et al., 2012). However, feeder layer effects vary widely depending on the species and cell type used. Differences in how the testis cells and feeder

cells were derived, as well as variables in other aspects of the culture system preclude a direct comparison of these studies. In the cat, Gómez et al. (2010) demonstrated a beneficial effect of using a homologous feeder layer to derive embryonic stem cell-like cells. No similar study has been performed for cat SSCs. In fact, there is only one report on SSC culture in the cat. Tiptanavattana et al. (2013) established colonies on a MEF feeder layer, but at passage 2 (P2) began using homologous Sertoli cells. No comparison between the two feeder types was performed. Therefore the objective of our study was to evaluate the ability of feeder layers produced using three different cell lines (STO, C166, and cat fetal fibroblasts (cFFs)) to support *in vitro* propagation of cat SSCs.

Materials and Methods

Preparation of Feeder Cells

STO cells (CRL-1503, American Type Culture Collection) and C166 cells (CRL-2581, American Type Culture Collection) were obtained commercially. STO cells were cultured in Dulbecco Modified Eagles Medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone), 1% (v/v) GlutaMAX™ (Gibco), 100 u/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco). C166 cells were cultured in DMEM supplemented with 10% (v/v) FBS, 1mM sodium pyruvate (Gibco), 100 u/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco). To inhibit cell proliferation, cells were treated with mitomycin C (MMC, Sigma) diluted to a concentration of 10 µg/ml in culture medium for 3 hours at 37°C. MMC-treated medium was removed and cells were washed two times with 1X Dulbecco phosphate-buffered saline without calcium and magnesium (PBS(-), Corning Cellgro). Cells were digested with 0.25% (w/v) trypsin-1

mM ethylenediaminetetraacetic acid (trypsin-EDTA, Invitrogen) for 3 to 5 minutes. Trypsin was inactivated with an equal volume of STO or C166 culture medium. Cells were centrifuged for 6 minutes at 160 x*g* and resuspended in culture medium. An equal volume of 2X freezing medium (culture medium with 10% (v/v) dimethyl sulphoxide (DMSO, Sigma)) was slowly added to the cell suspension. Cell suspensions were aliquoted into 2.0 ml cryovials (Sarstedt) placed in a "Mr. Frosty" container (Nalgene), and stored in a -80°C freezer. After 24 hours, cell vials were transferred into liquid nitrogen for long-term storage.

Primary cat fetal fibroblasts (cFFs) were derived as described by Gómez et al. (2010) with minor modification. Three cat fetuses were obtained from one gravid female at approximately 35 days gestation following ovariohysterectomy for non-research related reasons. Fetuses were excised from the amniotic sacs and rinsed with DPBS(-). Fetal viscera were removed and remaining tissue was minced into small pieces and incubated with trypsin-EDTA for 20 minutes at 37°C in 5% CO_2 in air. Cells were centrifuged for 5 minutes at 160 x*g* and resuspended in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) GlutaMAX™ (Gibco), 100 u/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured in 75 cm^2 flasks (Corning) at 37°C in 5% CO_2 in air. Following 7 to 10 days of culture, cFF monolayers were disaggregated with trypsin-EDTA, and resuspended in culture medium. An equal volume of 2X freezing medium was slowly added to the cell suspension. Cell suspensions were aliquoted into 2.0 ml cryovials, placed in a "Mr. Frosty" container, and stored in a -80°C freezer. After 24 hours, cryovials were transferred to liquid nitrogen for long-term storage. Vials of cFFs were warmed in a 37°C water bath and swirled gently until thawed. Cells were expanded for 1 to 2 weeks (passaged every 3 days)

and blocked as described above, with the exception that MMC concentration was increased to 40 µg/ml and treatment time was increased to 5 hours. These changes were made based on a report that cFFs require a higher dosage of MMC to inhibit cell proliferation (Gómez et al., 2010). Blocked cFFs were dissociated and frozen as described above.

As needed, vials of feeder layers were thawed as described above. Cell concentration was determined using a hemocytometer and cell viability assessed by trypan blue dye (BioWhittaker) exclusion (Strober, 2001). STO and cFF cells were seeded at a concentration of 2 x 10^5 cells per well, and C166 cells at 1 x 10^5 cells per well, of a 12-well plate (Costar) coated with 0.1% gelatin. Feeder cells were routinely maintained at 37°C in 5% CO_2 in air and were cultured 2 to 4 days before co-culturing with cat testis cells. Immediately before introducing cat testis cells, cell culture medium was removed and wells were washed twice with DPBS containing calcium and magnesium (DBS+) to remove residual FBS.

Collection of Testes

Domestic cat testicles were obtained from local spay-neuter clinics. Immediately following surgery, tissue was aseptically wrapped in gauze pads moistened with DPBS(+) and stored at 4°C. Tissue was processed within 24 hours of surgery, and generally, within the first 6 hours. A portion of each testis was fixed in 4% formaldehyde and embedded in paraffin. Donor age was recorded if known or estimated by the attending veterinarian. Age group (peripubertal) was then confirmed by histological analysis of hematoxylin and eosin stained testis sections for progression through spermatogenesis.

Enzymatic Digestion of Testicular Tissue

Testis cells were isolated from peripubertal cats (age 4 to 5 months, n = 6) who had not yet initiated spermatogenesis. Using a modified two-step enzymatic digestion (Honaramooz et al., 2002), testicular tissue was dispersed to obtain a single-cell suspension. Testicles were rinsed in Hank's Balanced Salt Solution without calcium or magnesium (HBSS, Gibco) and mechanically dissected to remove the vaginal tunic, vas deferens, and epididymis. The testis was bisected and pointed forceps were used to remove the tunica albuginea. The tissue was minced into small pieces and grossly apparent connective tissue was removed. Tissue was incubated with 2 mg/ml collagenase (Sigma) for up to 30 minutes at 37°C in a shaking incubator at approximately 110 oscillations per minute. Tissue was washed twice with HBSS and incubated with 0.25% (w/v) trypsin (Invitrogen) supplemented with 7 mg/ml deoxyribonuclease I (DNase I, Sigma) at a 1:4 ratio for 5 to 10 minutes at 37°C in a shaking incubator. Ten percent (v/v) FBS was added to inactivate trypsin before passing through a 70 μm mesh filter (Fisher Scientific). Cell concentration was determined using a hemocytometer and cell viability assessed by trypan blue dye exclusion (Strober, 2001).

Enrichment of Cat SSCs with Differential Plating

To perform differential plating, dissociated testis cells were suspended in a basic cell culture medium of DMEM supplemented with 10% (v/v) FBS, 1% (v/v) GlutaMAX™, 100 u/ml penicillin, and 100 μg/ml streptomycin. The cells were plated at a concentration of 5×10^5 cells per ml (10 ml per dish) on 100 mm untreated cell culture dishes (Corning). The cells were incubated for 2 hours at 37°C in 5% CO_2 in air. Cells remaining in suspension at the end of the culture period were collected for cell culture initiation.

Culture of Putative Cat SSCs

Immediately following differential plating, cells were washed twice with serum-free culture medium (SFM) (modified from Kubota et al. (2008)) in which growth factors were omitted (Tables 4.1 and 4.2). Cell concentration was determined and cells were resuspended in complete (i.e., with growth factors) SFM at a concentration of 7×10^4 cells per ml. One ml of cell suspension was added per well of a 12-well plate containing a feeder cell monolayer (STO, C166, or cFF). Cat testis cultures were maintained in mixed gas (6% O_2, 5% CO_2, 89% N_2) at 37°C. Medium was changed every other day.

Selected cultures were passaged with a combination of enzymatic and mechanical techniques. Enzymatic digestion was performed by treating cells with Accutase™ (Innovative Cell Technologies) for 5 to 10 minutes at room temperature. When somatic cell overgrowth was noted, differential plating was performed as described above, except that complete SFM was used. Mechanical passaging was performed by removing clump-forming germ cells from the feeder layer using a fixed-volume bulb pipette (EZ Squeeze, Research Instruments). Regardless of the technique, cells were resuspended in complete SFM and pipetted several times before subculturing onto fresh C166 feeder cells. The timing of each subculture was determined based on the number and size of colonies, and condition of feeder cells.

Colony Morphology Assessment

Colonies were examined every other day with a Leica DMIL inverted microscope in the integrated modulation contrast (IMC) mode. Images were captured using a digital camera (model HDR-SR1, Sony Corporation) mounted on the trinocular eyepiece.

Component	Catalog Number (Manufacturer)	Final Concentration in Medium
Basal medium		
Dulbecco modified Eagle medium	12561 (Gibco)	
Bovine serum albumin	A3803 (Sigma)	2 mg/ml
Penicillin-streptomycin	15140-122 (Gibco)	50 units/ml; 50 µg/mL
Insulin-transferrin-sodium selenite	I1884 (Sigma)	5 µg/mL; 5 µg/mL; 5 ng/ml
Free fatty acid mixture[a]		7.6 mEq/L
GlutaMAX™	35050-061 (Gibco)	2 mM
Hydroxyethyl piperazineethanesulfonic acid (HEPES)	H0887 (Sigma)	10mM
Putrescine	P5780 (Sigma)	60 µM
2-Mercaptoethanol	02194705 (MP Biomedicals)	50 µM
Growth Factors		
Recombinant human GDNF[b]	212-GD-050 (R&D Systems)	20 ng/ml
Recombinant rat GFR alpha1-Fc chimera[c]	60-GR-100 (R&D Systems)	150 ng/ml
Recombinant human basic fibroblast growth factor	354060 (BDBiosciences)	1 ng/ml

[a] See Table 4.2

[b]Recombinant human glial cell line–derived neurotrophic family

[c]Recombinant rat glial cell line–derived neurotrophic family recetor alpha-1 Fc chimera

TABLE 4.1 Composition of cat serum-free culture media (modified from Kubota et al. (2008)). Basal medium was prepared in advance and stored at 4°C for up to three weeks. Immediately before use, growth factors were added to basal medium.

Free fatty acid	Catalog Number (Manufacturer)	Final Concentration in Medium
Linoleic Acid	L1012 (Sigma)	35.6 mM
Linolenic acid	L2376 (Sigma)	5.6 mM
Oleic Acid	01008 (Sigma)	13.4 mM
Palmitic Acid	P0500 (Sigma)	31.0 mM
Palmitoleic acid	P9417 (Sigma)	2.8 mM
Stearic Acid	S4751 (Sigma)	76.9 mM

TABLE 4.2 Composition of free fatty acid (FFA) mixture. FFAs were diluted in absolute ethanol to make stock solutions.

Cluster Forming Activity Assay

Assay was performed as described by Yeh et al. (2007) with minor modification. At 5 days in vitro (DIV), cultures were fixed with 4% methanol-free formaldehyde (Polysciences, Inc.) for 15 minutes at room temperature. Clusters within each well were manually counted and were defined as a colony of at least 6 cells, aggregated in a three-dimensional structure with tightly compacted cells such that individual cell borders are not readily discerned. For each feeder layer, data were obtained from four independent experiments, and three wells of a 12-well plate were counted for each experiment. An incomplete factorial design was applied such that six cats were used in total, and not every cat was exposed to all feeder cell types. To distinguish clusters derived from cat testicular cells and feeder layer cells, one to two wells per experiment contained only feeder cells treated with complete SFM. Data are expressed as the mean ± standard error of the number of cluster per 10^5 input cells. Data were analyzed using a mixed model ANOVA in PROC MIXED of SAS (Version 9.3, SAS Institute). Initially, the statistical model used included feeder layer as a fixed factor and cat as a random factor. However, the random effect of cat was removed from the model due to a high degree of non-significance ($p > 0.60$). Differences between treatments were compared using the Tukey-Kramer adjustment for pair-wise mean comparisons. Significance was declared using an alpha of 0.05.

Fluorescent Immunocytochemistry

Cells were fixed in 12 well plates with 4% methanol-free formaldehyde for 15 minutes at room temperature, gently washed 3 times with immunocytochemistry (ICC) wash buffer (1X DPBS without calcium or magnesium (DPBS(-)) and 0.05% (v/v) Tween-20),

and permeabilized with 0.2% (v/v) Triton-X 100 and 0.1% (v/v) Tween-20 diluted in DPBS(-) for 9 minutes at room temperature. Sections were blocked with MAXblock Blocking Medium (Active Motif) for 2 hours at room temperature. Primary antibodies were diluted in MAXbind Staining Medium (Active Motif) and incubated overnight at 4°C on a rotary shaker. See Table 4.3 for a complete list of antibodies and concentrations used. Wells were washed in ICC wash buffer three times and then incubated with species- and isotype-specific fluorescent–conjugated secondary antibodies (Tables 4.3) in MAXbind Staining Medium solution for 1 hour at room temperature on a rotary shaker. Wells were washed in ICC washing buffer three times and Hoechst 33342 (Sigma) was used to visualize cell nuclei. Cells were overlaid with 2 ml DPBS(-) following wash steps. Fluorescent imaging was performed using a Leica DMI 6000B inverted microscope, and images were acquired using the Leica Application Suite Advanced Fluorescence software (LAS AF, Leica) with a black and white Leica DFC 365 FX camera. Images were pseudocolored in LAS AF and exported to Photoshop CS6 for image processing. As a negative control, primary antibodies against the protein of interest were omitted. A representative control image of germ cell clumps cultured on C166 cells is shown in Supplementary Figure 7.

Alkaline Phosphatase Activity Staining

Alkaline phosphatase (AP) activity was detected using the Alkaline Phosphatase Staining Kit II (Stemgent), according to manufacturer's instructions. Briefly, cells were fixed with 4% (v/v) paraformaldehyde for 4 minutes. Cells were developed with AP substrate for approximately 6 minutes in the dark. Once color was noted, the reaction was stopped by rinsing wells with DPBS(-). Cells were immediately imaged with a Leica DMIL.

Primary Antibody				Secondary Antibody*				
Target	Isotype	Source	Dilution	Host	Reactivity	Isotype	Conjugate	Dilution
OCT4	Mouse IgG_1	Millipore	120	Goat	Mouse	IgG_1	647	200
PLZF	Goat IgG	Santa Cruz	50	Donkey	Goat	IgG	488	400
UCHL1	Rabbit IgG	Novus	200	Goat	Rabbit	IgG	594	400
Vimentin	Mouse IgG_1	Abcam	1000	Goat	Mouse	IgG	647	400

*All secondary antibodies are Alexa Fluor dyes purchased from Molecular Probes

Table 4.3 List of antibodies used in triple label fluorescent ICC analysis.

inverted microscope in bright field mode. Images were captured using a digital camera (model HDR-SR1, Sony Corporation) mounted on the trinocular eyepiece

Results

Feeder Cell Type Influences Colony Morphology

Testis cells isolated from peripubertal cats and enriched for SSCs with differential plating were cultured on three different feeder layers. For each feeder cell type, testis cells derived from four cats (minimum three wells of a 12-well plate) were examined. When cultured on STOs, cat testis cells generally remained as single cells (Figure 4.1 A). Most of the colonies that did form exhibited a flattened appearance (Figure 4.1 B). The cells within a colony appeared loosely associated, as evidenced by clear cellular borders. Rarely, three dimensional colonies typical of SSC-containing germ cell clumps seen in other species (Kubota et al., 2004b; Kubota et al., 2011; Ryu et al., 2005) were observed (Figure 4.1 C). These colonies preferentially formed on top of the flattened, larger-celled colonies. As compared to the STOs, more colonies were present in the cFF feeder layer group (Figure 4.1 D-E). Some appeared flattened and loosely associated as noted with the STO feeders. However, many exhibited germ cell clump colony morphology with a rounded, three dimensional structure and tight intercellular contacts. Typically, between 10 and 30 cells were present in a colony. As shown in Figure 4.1 F, several chains of germ cells were also noted, indicative of differentiation (de Rooij, 2001; Huckins and Oakberg, 1978). Germ cell clumps were larger and more numerous when primary cat testis cells were cultured on C166 feeder cells (Figure 4.1 G-H). The large and tightly compacted nature of these

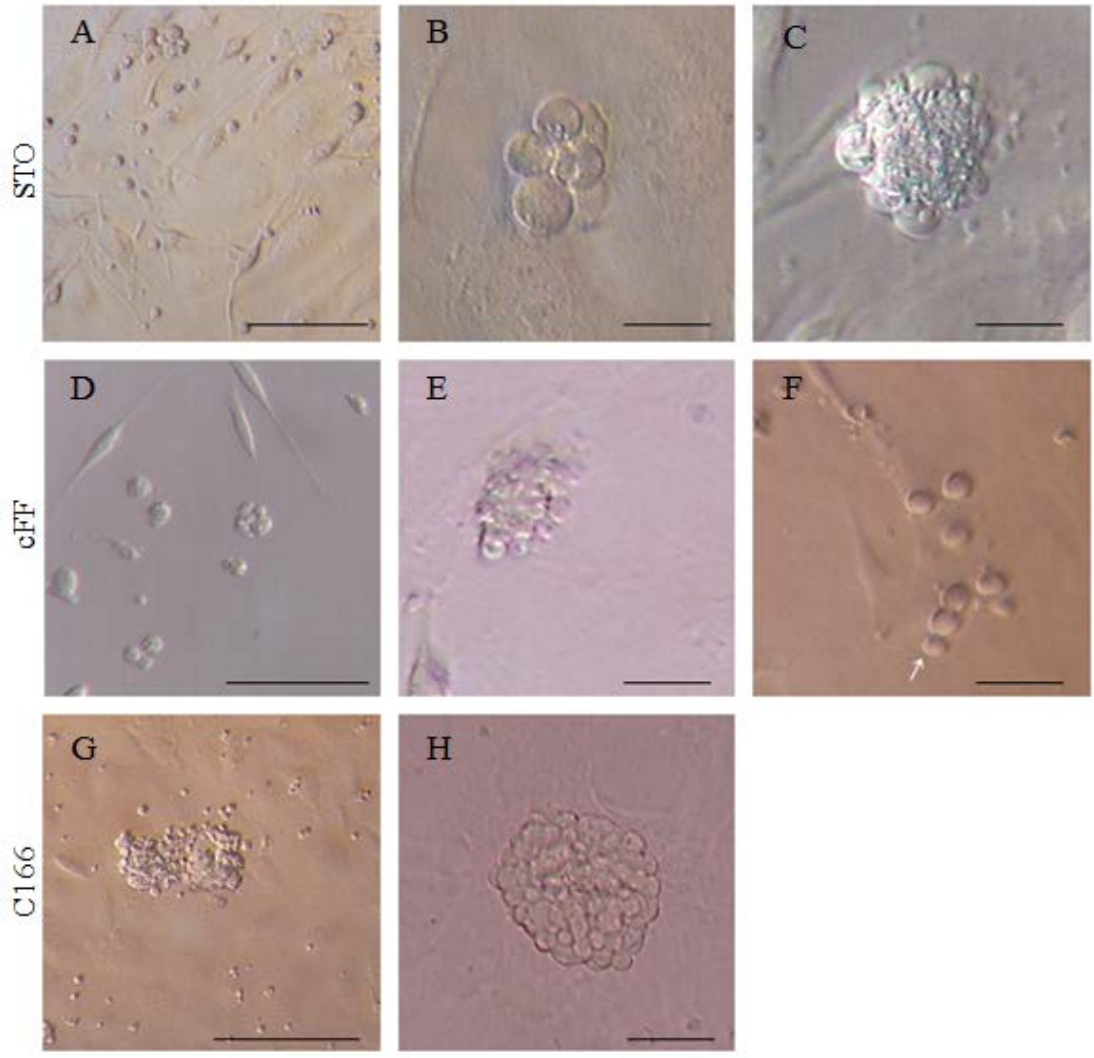

Figure 4.1 Culture of peripubertal cat testis cells. Testis cells were cultured on mouse STO cells (**A**-**C**), cFFs (**D**-**F**), and mouse C166 endothelial cells (**G**-**H**). (**A**, **D**, **G**) Appearance of cultured cells. (**B**, **E**, **H**) High magnification image of typical cell colony morphology. (**C**) Rarely, colonies of smaller, tightly connected cells would form on top of flattened, larger cells. (**F**) Chain of differentiating germ cells (arrow). (**A**, **D**, **G**) Scale bar; 100 µm. (**B**, **C**, **E**, **F**, **H**) Scale bar; 20 µm.

colonies prevented accurate cell number quantification; however, germ cell clumps were estimated to contain 20 to 80 cells.

To confirm that the clump-forming colonies were indeed germ cells, we used ICC to investigate the expression of three SSC markers: ubiquitin carboxy-terminal hydrolase 1 (UCHL1) (Kwon et al., 2004; Luo et al., 2006; Reding et al., 2010), promyelocytic leukemia zinc-finger (PLZF) (Filipponi et al., 2007; Reding et al., 2010), and octamer-binding transcription factor 4 (OCT4, also known as POU5F1) (Dann et al., 2008; Kubota et al., 2011; Ryu et al., 2005). Consistent with our morphological assessment of STO-cultured cells, most UCHL1-positive cells were present as single cells (Figure 4.2). A low level of PLZF and OCT4 expression was also noted in the UCHL1-positive cells. Flattened colonies containing large, loosely-connected cells did not express SSC markers. In the cFF feeder cell group, many of the cells within the germ cell clumps stained positive for all three SSC markers (Figure 4.3). Other cells within the clumps stained positive for a subset of the markers, and others still did not express any of the SSC markers examined. Similarly in the C166-cultured cells, the clump-forming colony cells co-expressed the three SSC markers (Figure 4.4). Particularly in the larger colonies, a gradient expression pattern was present, with the highest expression towards the perimeter of the colony. These results demonstrate that the clump-forming colonies consist of undifferentiated spermatogonia, a subset of which are likely cat SSCs.

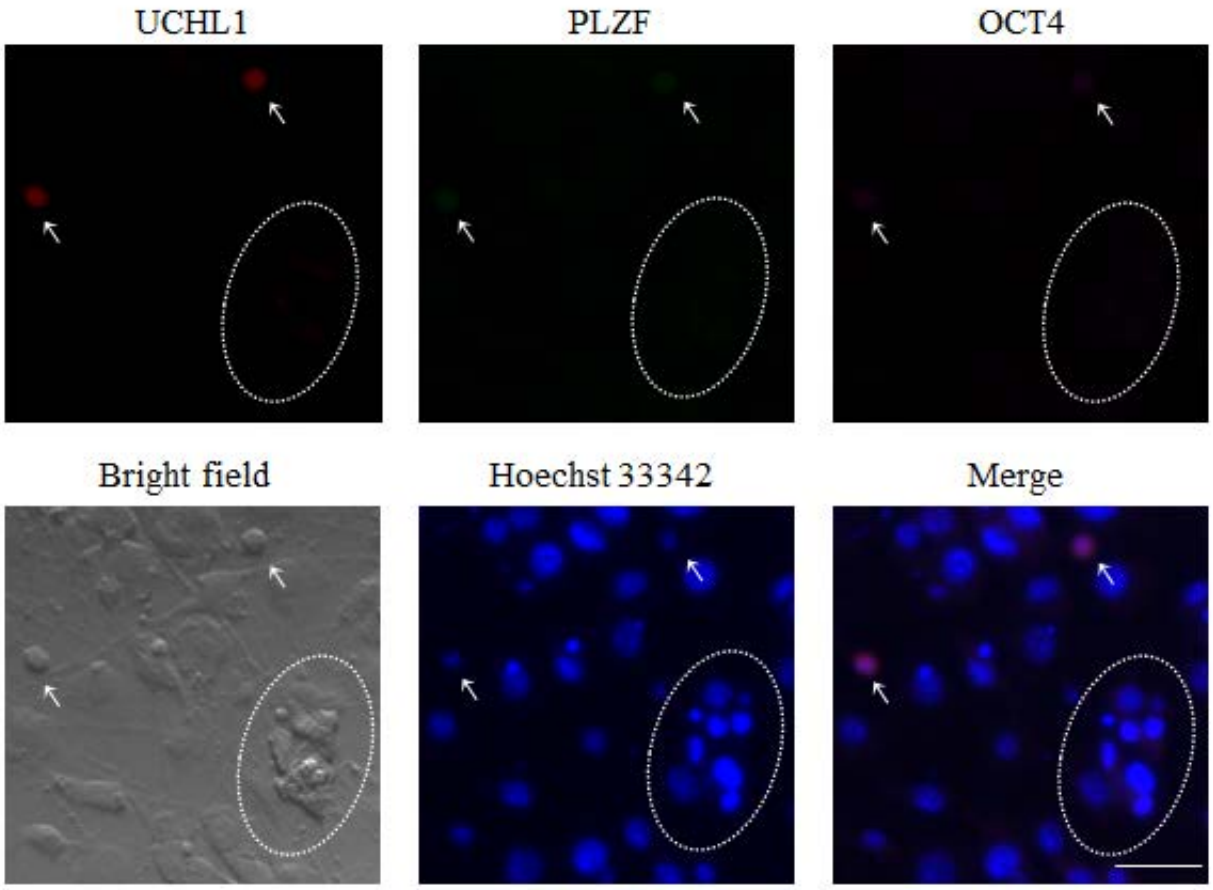

Figure 4.2 Co-localization of SSC markers in peripubertal cat testis cells cultured on STO cells. Representative fluorescent immunocytochemistry images of UCHL1, PLZF, and OCT4 protein expression in cultured cells. Cells were counterstained with Hoechst 33342 to mark cell nuclei. Arrows indicate cells positive for UCHL1. These cells also weakly expressed PLZF and OCT4. Note the lack of marker expression on flattened colony cells (dashed circle). Merge: merged image of all fluorescent panels. Scale bar; 50 µm.

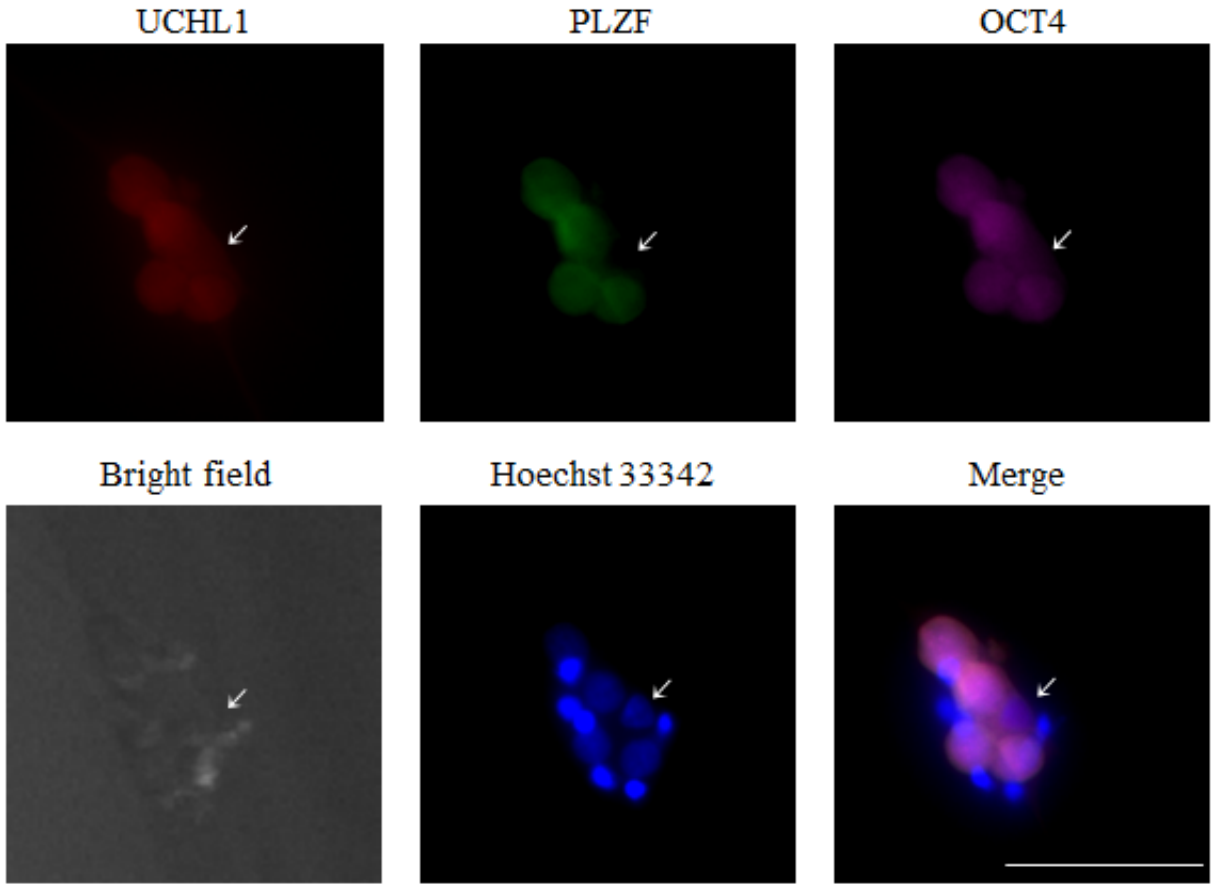

Figure 4.3 Co-localization of SSC markers in peripubertal cat testis cells cultured on cFF cells. Representative fluorescent immunocytochemistry images of UCHL1, PLZF, and OCT4 protein localizing to the same subset of cells within a germ cell clump. Cells were counterstained with Hoechst 33342 to mark cell nuclei. Arrow indicates cell positive for UCHL1 and OCT4, but not PLZF. Merge: merged image of all fluorescent panels. Scale bar; 50 µm.

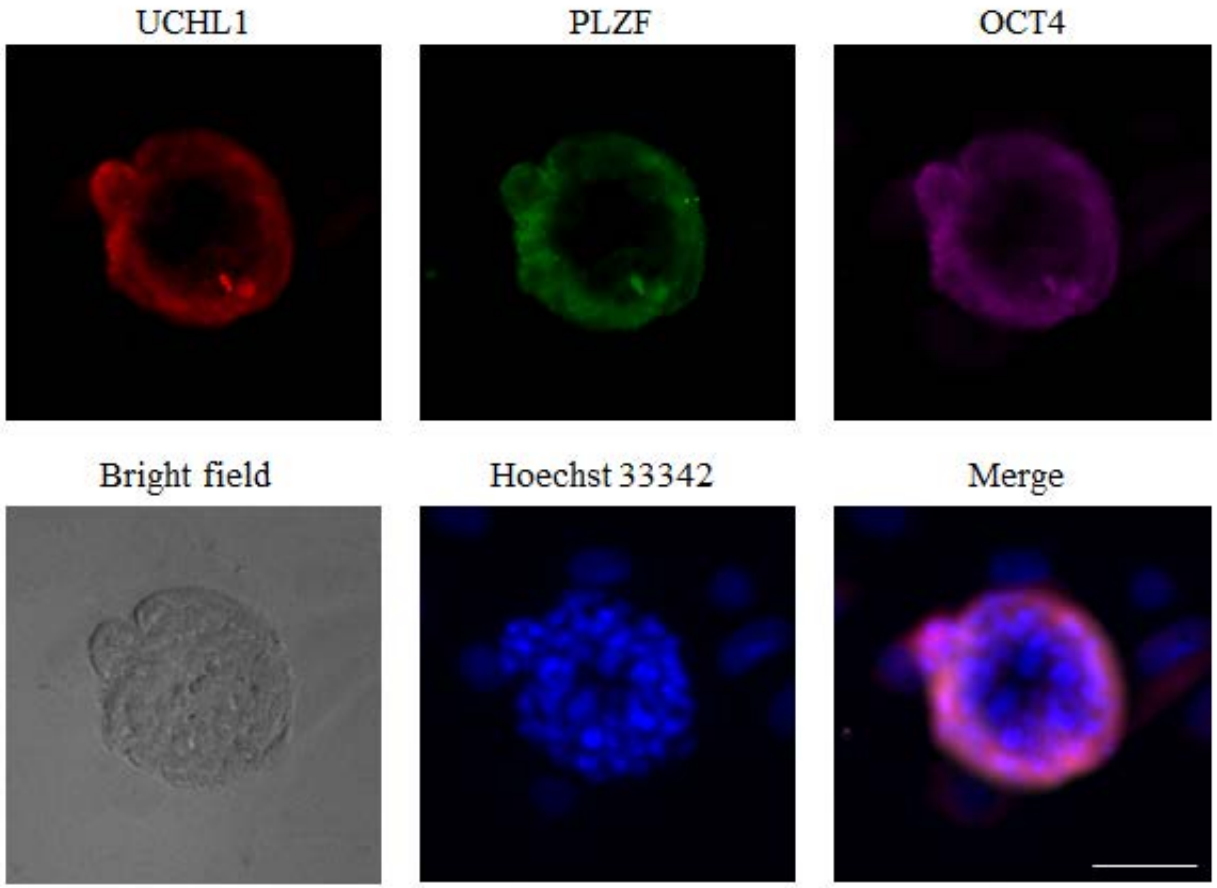

Figure 4.4 Co-localization of SSC markers in peripubertal cat testis cells cultured on C166 cells. Representative fluorescent immunocytochemistry images of UCHL1, PLZF, and OCT4 protein localizing to the same subset of cells within germ cell clumps. Cells were counterstained with Hoechst 33342 to mark cell nuclei. Merge: merged image of all fluorescent panels. Scale bar; 50 µm.

Germ Cell Clump Establishment Varies with Feeder Cell Type

It has been demonstrated in the mouse that the number of clusters—defined as colonies exhibiting germ cell clump morphology—established during a short-term (i.e., less than one week) culture period accurately reflects the SSC content of the culture (Yeh et al., 2007). Therefore, we performed a cluster forming activity assay to estimate the number of SSCs supported by each type of feeder cell. As shown in Figure 4.5 B, cat testis cells established a significantly higher number of clusters with C166 feeder cells (77.4 ± 13.8 colonies per 10^5 cells input), as compared to STOs (3.5 ± 1.1 colonies, $p = 0.0003$) or cFFs (22.7 ± 1.0 colonies, $p = 0.0024$). There was no significant difference between the STO and cFF cells' cluster numbers ($p = 0.2570$). No clusters were present in the control wells containing only feeder cells (Figure 4.5 A), demonstrating that the clusters were derived from the cat testis cells.

Subcultured Cat Germ Cells Maintained SSC Characteristics

Because the C166 cells showed the best potential for supporting cat SSC propagation, all subculture experiments were carried out using C166 feeder cells. Cultures established from two individuals were serial passaged to assess the ability of our culture system to support long-term germ cell clump propagation. The first passage (P1) was performed at 12 DIV. Germ cell clumps with characteristic grape-like morphology were apparent within 4 days (Figure 4.6 A-C). Additionally, several sharp-edged and tightly-compacted colonies were also present (Figure 4.6 D-F). These colonies were larger and grew much faster than the germ cell clumps. Both types of colonies persisted with each subculture. AP activity was variably present in germ cell clumps and compacted colonies

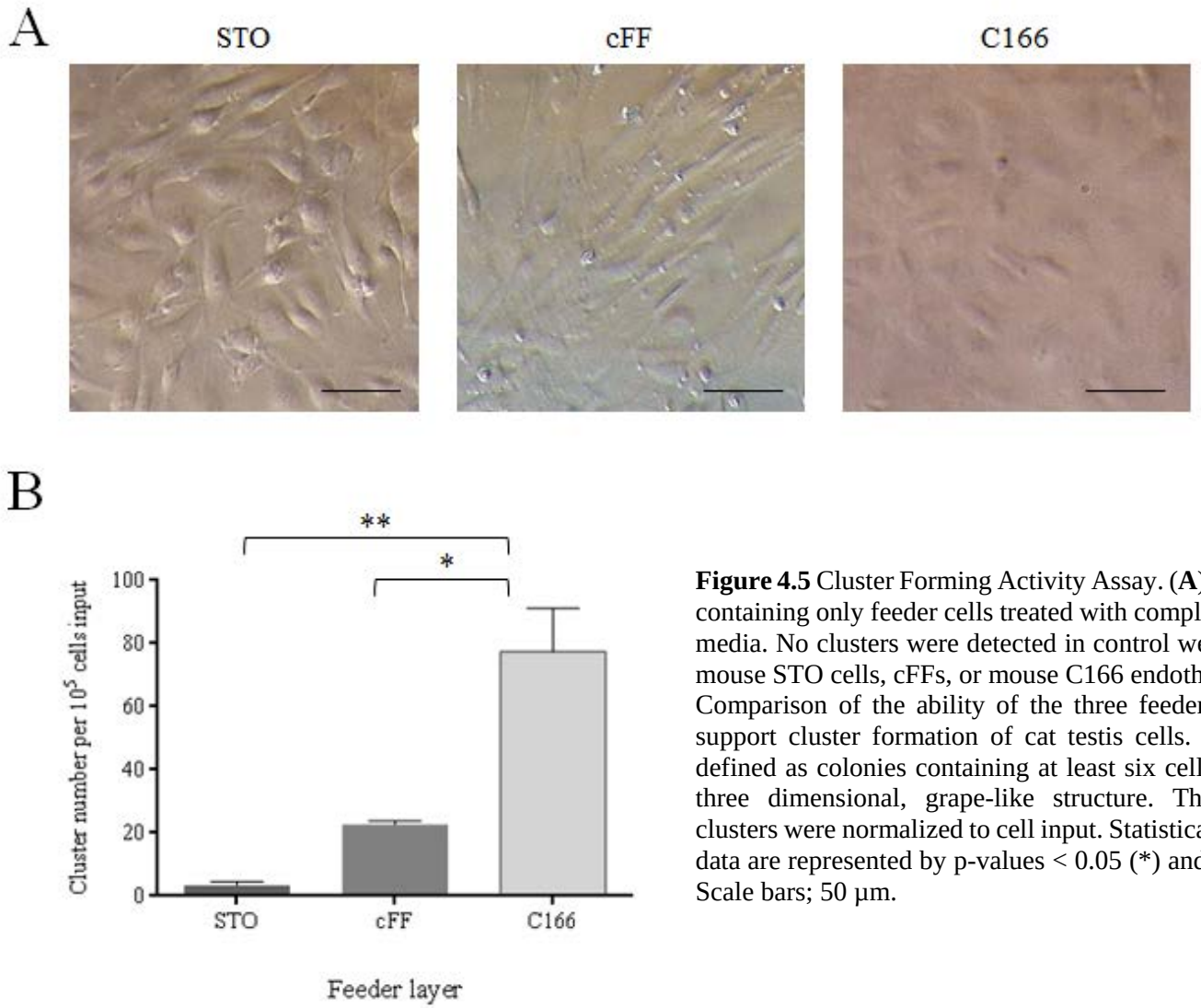

Figure 4.5 Cluster Forming Activity Assay. (**A**) Control wells containing only feeder cells treated with complete serum-free media. No clusters were detected in control wells containing mouse STO cells, cFFs, or mouse C166 endothelial cells. (**B**) Comparison of the ability of the three feeder cell types to support cluster formation of cat testis cells. Clusters were defined as colonies containing at least six cells displaying a three dimensional, grape-like structure. The number of clusters were normalized to cell input. Statistically significant data are represented by p-values < 0.05 (*) and < 0.001 (**). Scale bars; 50 µm.

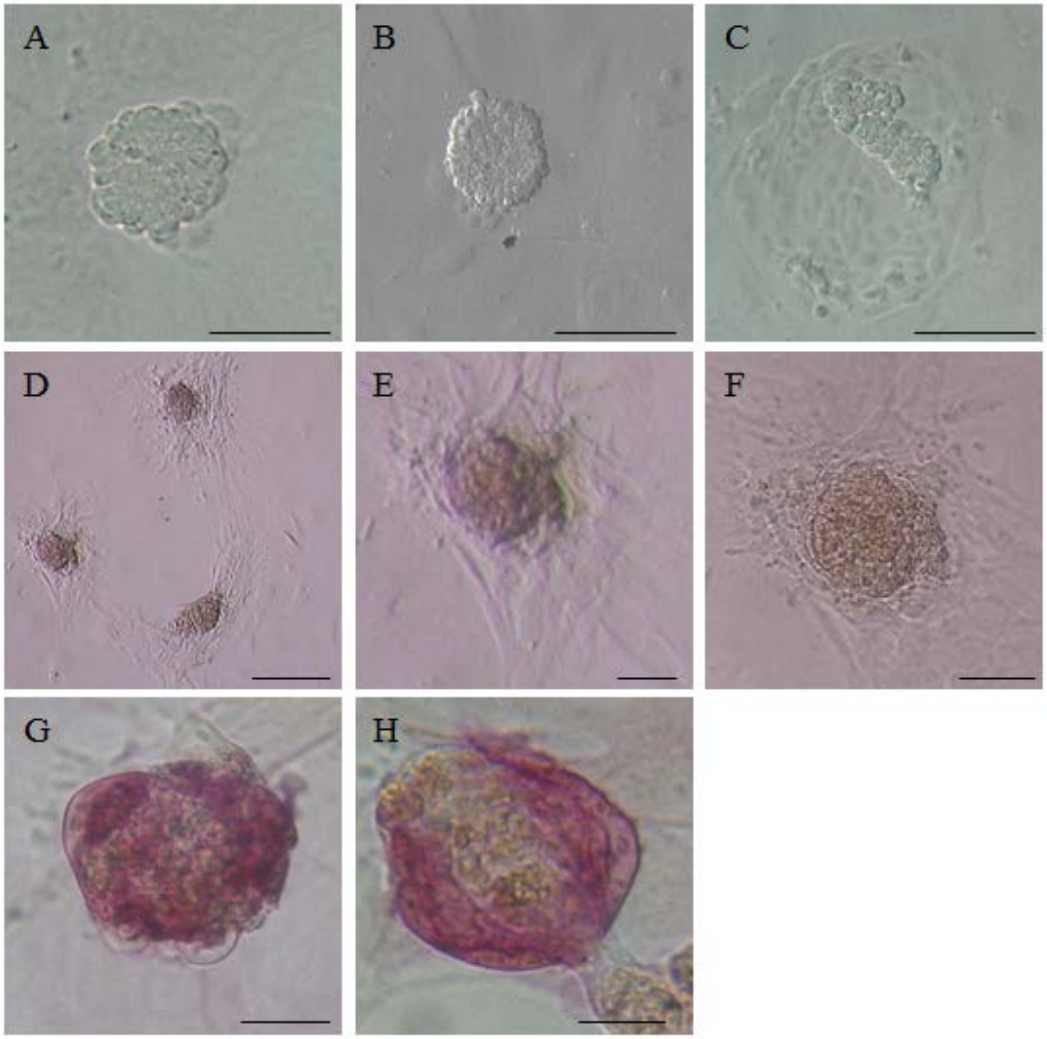

Figure 4.6 Characterization of passaged cat testis cells. (**A-C**) Germ cell clumps were present approximately 4 days after the first passage (**A**) and consistently reestablished following subculture onto fresh mouse C166 feeder cells (**B-C**). (**D-F**) Larger, well- circumscribed colonies with tightly compacted cells were also present at each passage. Alkaline phosphatase activity in germ cell clumps (**G**) and compacted colonies (H). (**A-C**, **E-F**) Scale bar; 50 µm. (**D**) Scale bar; 200 µm. (**G-H**) Scale bar; 20 µm.

(Figure 4.6 G-H). Typically, a mix of positive and negative cells was seen. However, colonies of each type that were negative for AP activity were also observed (data not shown).

To further characterize the cell colonies, ICC was repeated at 28 DIV. As shown in Figure 4.7 A, germ cell clumps maintained expression of all 3 SSC makers. In a separate set of wells, cells were stained for two of the SSC markers (UCHL1 and OCT4) and vimentin, an intermediate filament specifically expressed by Sertoli cells (Steger et al., 1996). As expected, germ cell colonies positive for UCHL1 and OCT4 did not express vimentin (Figure 4.7 B, top panel). Additionally, several of the compacted colonies stained positive for vimentin, but did not express UCHL1 or PLZF (Figure 4.7 B, bottom panel).

Discussion

Reported here is the first study evaluating the effect of feeder layer type on cat SSC culture. Although STO cells are capable of supporting long-term culture of mouse and rat SSCs (Kubota et al., 2004b; Ryu et al., 2005), STO cells may not be as supportive in non-rodent species (Kubota et al., 2011) and there is a rich body of literature demonstrating the profound effect feeder cell choice can have on the success of a SSC culture system (Aponte et al., 2006; Harkey et al., 2013; Kuijk et al., 2009; Lee et al., 2013; Mirzapour et al., 2012; Nasiri et al., 2012; Oatley et al., 2004; Yu et al., 2014). Indeed our study demonstrated that colony morphology was highly affected by the type of feeder cell. When cultured on the STO feeder layer, cat germ cells remained as single cells (Figure 4.1 A-C). Grapelike clusters of cells were able to establish on both cFF and C166 feeder cells (Figure 4.1 D-H). Based on their distinct morphological appearance, which is well conserved in other

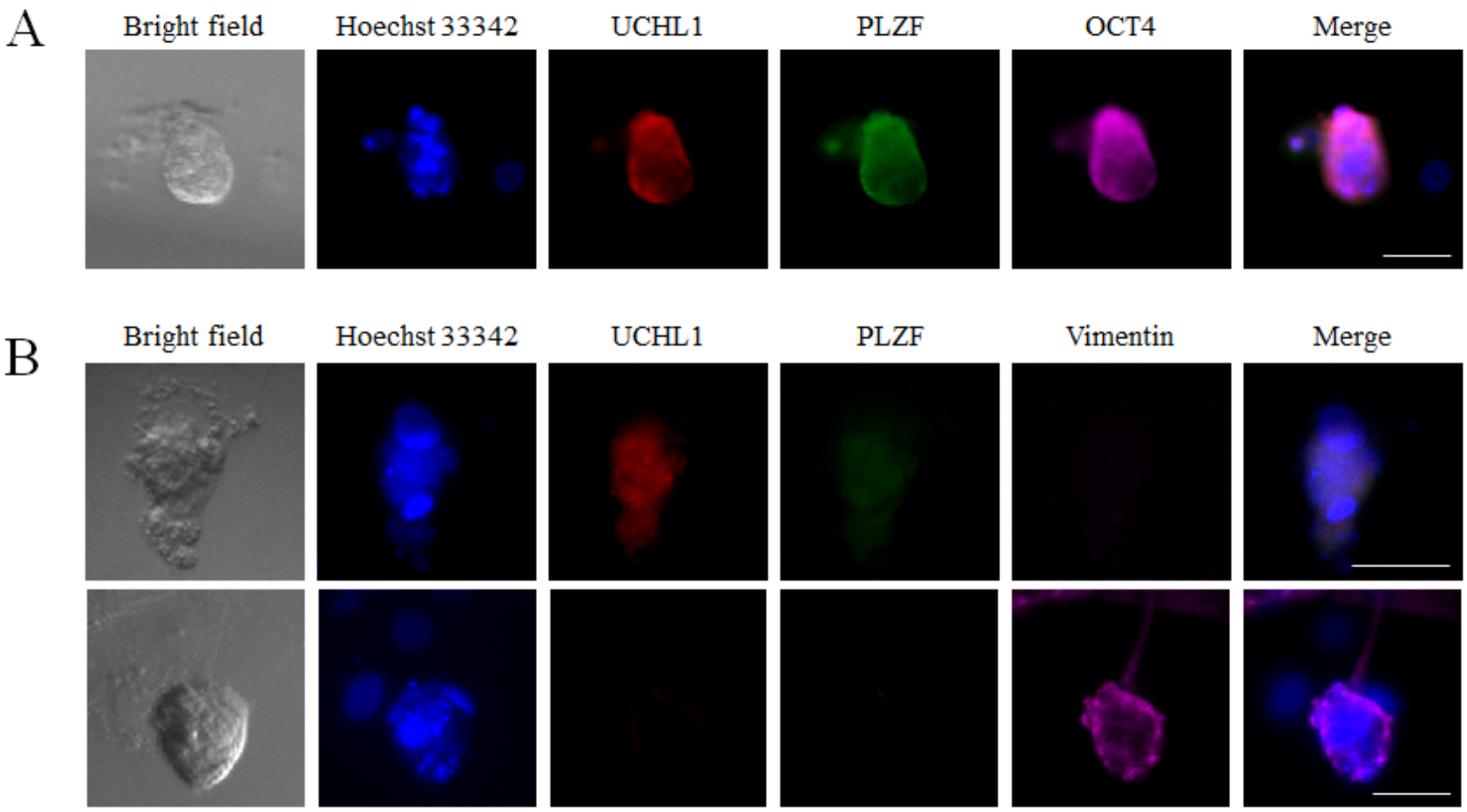

Figure 4.7 Co-localization study of P2 (28 DIV) cat testis cells cultured on C166 cells. Representative fluorescent immunocytochemistry images of (A) UCHL1, PLZF, and OCT4 or (B) UCHL1, PLZF, and vimentin expression in cultured cells. Cells were counterstained with Hoechst 33342 to mark cell nuclei. Merge: merged image of all fluorescent panels. Scale bar; 50 µm.

mammals (Kubota et al., 2004b; Kubota et al., 2011; Ryu et al., 2005; Sadri-Ardekani et al., 2009), we determined these were germ cell clumps which contained SSCs.

As another line of evidence for this hypothesis, ICC was performed using three SSC markers previously validated in our lab (Chapter 2). UCHL1 marks a subset of spermatogonia in the mammalian testis, and it has been suggested that down-regulation of UCHL1 helps trigger SSC differentiation (Luo et al., 2009). PLZF is a spermatogonia-specific transcription factor in the testis that is directly involved in self-renewal and maintenance of the SSC pool (Buaas et al., 2004; Costoya et al., 2004). OCT4 also plays an important role in SSC self-renewal, through a separate pathway from PLZF (Dann et al., 2008). ICC analysis confirmed that the single cells present in the STO treatment group were spermatogonia, as evidenced by their UCHL1 expression (Figure 4.2). The low level of PLZF and OCT4 expression likely reflected that these cells had begun differentiating. Many of the cells within germ cell clumps of the cFF and C166 treatment groups expressed all three markers (Figures 4.3 and 4.4) , which is characteristic of SSCs in other species (Kubota et al., 2004b; Kubota et al., 2011; Ryu et al., 2005).

It is important to note that an unequivocal SSC-specific marker has not been identified in any species. All of the markers used in our study are believed to be variably expressed by more differentiated spermatogonia as well (Chapter 2). However, the co-expression of all three markers provides an additional line of evidence that subsets of these cells are bona fide SSCs. Correspondingly, not every cell within a clump is a SSC. Clump colonies contain a heterogeneous population of cells, and as the SSCs divide, they are also producing cells committed to differentiation (de Rooij, 2006; Kanatsu-Shinohara et al., 2005a; Yeh et al., 2007). The cell within the cFF germ cell clump that did not express PLZF

may be one such example of a differentiating SSC daughter cell (Figure 4.3, arrow). Similarly, the larger germ cell clumps in the C166 treatment group showed a differential staining pattern (Figure 4.4). It is reasonable to assume that the inner cells of a large colony would not have as much access to the SFM which contains growth factors essential for SSC renewal. Alternatively, the halo effect seen in the larger colonies could also be an artifact imposed by the inability of antibodies to penetrate the full depth of the colony. Regardless, ICC analysis confirmed our original morphological assessment that the clumps represented germ cell colonies containing SSCs.

The CFA assay introduced by Yeh et al. (2007) exploits the distinct grape-like morphology of germ cell clusters to semi-quantitatively measure the amount of SSC activity in a culture. Importantly, the ability of this assay to faithfully reflect the number of SSCs in culture was confirmed using SSC transplant experiments (Yeh et al., 2007). Taking advantage of this assay, we quantified the number of germ cell clumps in the three feeder systems, adhering to a strict set of morphological criteria as described in the methods section. As shown in Figure 4.5 B, C166 cells were far superior to STO or cFF feeder cells in supporting cluster formation in the cat. Furthermore, control wells containing only the feeder cells were also treated with complete SFM. No clusters were observed in these wells, confirming that the clusters were derived from cat testis cells (Figure 4.5A). These results are highly suggestive that C166 cells support the establishment of SSC colonies in the cat.

An important measure of the regenerative capacity of the cultured SSCs is their ability to be propagated *in vitro*. To that end, cultures from two individuals established on C166 feeders were serial passaged. Germ cell clumps consistently re-established following each subculture (Figure 4.6 A-C). The clumps displayed variable AP activity (Figure 4.6

G), which is a widely used stem cell marker present in undifferentiated embryonic stem cells embryonic germ cells, and induced pluripotent stem cells (Matsui et al., 1992; Resnick et al., 1992; Takahashi and Yamanaka, 2006), as well as SSCs (Kubota et al., 2004b; Kubota et al., 2011; Ryu et al., 2005). ICC analysis performed after the second passage (28 DIV) confirmed that the germ cell clumps were still strongly expressing SSC markers (Figure 4.7 A). The lack of vimentin expression further verified that the clumps were not derived from somatic cells (Figure 4.7 B, top panel).

Interestingly during subculture, a second type of colony emerged. These colonies were larger, faster growing, tightly-compacted, and well-circumscribed (Figure 4.6 D-F). Because several of the colonies were AP-positive (Figure 4.6 H), we hypothesized these colonies could be multipotent germline stem cells (mGSCs), which are reported to spontaneously arise in neonatal mouse SSC cultures (Kanatsu-Shinohara et al., 2004a). However, ICC revealed that none of the well-circumscribed colonies expressed OCT4 (data not shown). Given OCT4's critical role in the regulation of pluripotent stem cells (Boyer et al., 2005; Niwa et al., 2000), it is highly unlikely these colonies were mGSCs. A far more likely explanation is that these colonies were derived from testis somatic cells. Both Sertoli (Carreau et al., 1996) and peritubular myoid (Chapin et al., 1987) cells have been reported to display AP activity *in vitro*. Indeed, ICC analysis revealed that several colonies expressed vimentin (a Sertoli cell marker), but not UCHL1 or PLZF (Figure 4.7 B, bottom panel).

At the time of writing, cultures from two cats have been maintained on C166 cells for 78 DIV (5-6 passages). Throughout the entire culture period, germ cell clumps have continued to proliferate. This is a significant milestone as the only report of SSC culture in

the cat experienced a significant decline in proliferative activity at 30 DIV, and complete colony degeneration by 57 DIV (Tiptanavattana et al., 2013). It remains unknown if the authors' choice of feeder cells (mouse embryonic fibroblasts and homologous Sertoli cells) contributed to the culture cessation.

It is interesting to note that our results are consistent with the finding by Kubota et al. (2011) that C166 cells, and not STO cells, support the *in vitro* propagation of rabbit SSCs. Notably, rabbits are the only non-rodent species to be successfully cultured long-term (Kubota et al., 2011). Whether the ability of C166 cells to support SSC *in vitro* propagation is only shared between the cat and rabbit, or if it represents a broader requirement of non-rodent species is an interesting point to consider. Certainly, this could be a critical finding towards the establishment of more long-term SSC cultures in non-rodent systems. Although our results cannot directly be extrapolated to other species, we suggest that feeder cell type should be evaluated before attempting to establish a long-term SSC culture system, and the use of C166 cells warrants consideration.

Taken together, our results demonstrate that C166 feeder cells facilitate the establishment and proliferation of germ cell clumps in the cat. Following several passages, colonies maintained a distinctive grape-like morphology characteristic of SSCs in other species. Furthermore, these colonies displayed AP activity and retained expression of SSC markers. Further studies, including a SSC transplant assay are required to confirm the presence of SSCs in our culture system. Nonetheless, our study represents an important first step towards the achievement and optimization of a long-term SSC culture system in the cat.

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Chapter 5: Summary and Future Directions

The overall objective of the dissertation was to develop spermatogonial stem cell (SSC) technology in the domestic cat, as a model for rare and endangered felids. A requisite first step was the identification of molecular markers that could reliably detect the SSC population. While a variety of markers have been described in other species, they have not been validated in the cat. Therefore, the first study (Chapter 2) focused on the characterization of six well-conserved SSC markers: thymus cell antigen 1 (THY1), G-protein coupled receptor 125 (GPR125), glial cell line–derived neurotrophic factor family receptor alpha 1 (GFRalpha1), promyelocytic leukemia zinc finger (PLZF), ubiquitin carboxy-terminal hydrolase 1 (UCHL1), and octamer-binding protein 4 (OCT4). Using endpoint reverse transcription-polymerase chain reaction (RT-PCR), we were able to confirm transcript expression for all six markers in the cat testis. Fluorescent immunohistochemistry (IHC) demonstrated that UCHL1, PLZF, and OCT4 proteins were co-expressed by a subpopulation of undifferentiated spermatogonia that most likely includes the SSCs. Importantly, the expression pattern of our markers was conserved in the cheetah (*Acinonyx jubatus*) and Amur leopard (*Panthera pardus orientalis*), confirming the utility of the domestic cat as a model for wild felids. Notably, the Amur leopard is part of the first felid lineage to diverge from the domestic cat and cheetah (Johnson et al., 2006). Thus, our findings strongly suggest that SSC markers may be conserved across all felids.

Given the age range of the wild felid donors (8 to 18 years), it is encouraging to note that SSCs were detected in their testes. Animals in captivity typically live longer than in the wild. One unfortunate consequence of this is the onset of reproductive senescence, which precludes the ability to collect and bank semen from aged individuals (Painer et al.,

2014; Swanson, 2006). Indeed, one of three donors was not producing spermatozoa at the time of euthanasia. It has been demonstrated in the mouse that infertility in older males results from a failure of the SSC niche, rather than the SSC itself (Ryu et al., 2006). The significance of this finding is that, although there are less SSCs in an aged individual, the SSCs that remain are still functional. In all species examined to date, SSCs can be cryopreserved using a simple freezing procedure (Avarbock et al., 1996; Brook et al., 2001; Clouthier et al., 1996; Dobrinski et al., 1999a, 2000; Izadyar et al., 2002a; Nagano et al., 2001; Ogawa et al., 1999). It would be reasonable to assume a similar level of success could be achieved in the cat. Given the enormous potential of SSCs to rescue genotypes of males that would otherwise be lost, future studies optimizing the cryopreservation of felid SSCs is warranted.

If genetic resource banks expand to include SSCs, an important question to address is how these cells will be used to propagate animals. As the precursors to spermatozoa, SSCs have not yet acquired fertilization competence. One potential avenue to mature the SSCs is *in vitro* spermatogenesis. Sperm-like cells have been obtained in rodents (Kubota et al., 2004b; Lee et al., 2006b), cattle (Lee et al., 2001) and humans (Sousa et al., 2002; Tesarik et al., 1998). However, the low efficiency and dependence on complex reproductive technologies (i.e., intracytoplasmic sperm injection, embryo transfer) limit its utility in wildlife conservation.

An intriguing alternative is germ cell transplantation. When SSCs are transplanted into the testis of a host animal of the same species, the donor SSCs are able to colonize the seminiferous tubules and reinitiate spermatogenesis (Brinster and Zimmermann, 1994). This ability allows for epididymal maturation and the production of fertilization-competent

spermatozoa. When considering this technique in the context of wildlife conservation, same-species transplants are not desirable as the host animal's own genetics would be lost. SSC xenotransplantations are possible. For example, transplants from rat to mouse (Clouthier et al., 1996), and mouse to rat (Zhang et al., 2003b) have led to the production of normal spermatozoa in the host. When domestic cat SSCs are transplanted into a mouse testis, the cells are able to colonize the seminiferous tubule, but arrest before completing spermatogenesis (Kim et al., 2006). Hence, the taxonomic distance between donor and host is an important consideration. The domestic cat is an obvious candidate for xenotranplants from wild felids. Indeed, it has been demonstrated that domestic cat testes can support spermatogenesis of transplanted ocelot SSCs (Silva et al., 2012). It remains unknown whether the domestic cat would be a suitable host for the other felid species. It is encouraging to note that the genome and physiological mechanisms are relatively well conserved across felids (Brown, 2006; Johnson et al., 2006; Pelican et al., 2006).

Felid germ cell transplants could also provide unique insights into gametogenesis. For example, there is a high incidence of teratospermia in felids (Wildt, 1994). While there is no doubt a genetic component, as felids with reduced genetic diversity produce more abnormal sperm (Wildt et al. 1987; Wildt et al., 1983), the exact etiology and mechanism are not known. A fundamental question is whether the defect arises from the germ cells, the somatic environment, or both. Transplantation experiments involving SSCs from a normospermic donor and a teratospermic host, as well as the reverse experiment, could help elucidate the cellular mechanisms responsible for teratospermia. If it turns out that transfer of teratospermic SSCs into a normospermic testis rescues the phenotype in part or

in full, then SSC transplants would be an invaluable management tool in species with high rates of teratospermia.

Because SSCs are an extremely rare cell population, Study 2 (Chapter 3) focused on techniques to enrich cat SSCs. Despite testing a battery of antibodies against four cell surface SSC markers, we were only able to identify one antibody (directed against E-cadherin) that displayed specific labeling in cat testis. Paradoxically, magnetic-activated cell sorting (MACS) of the E-cadherin-selected cells appeared to cause a reduction in the number of stem cells. Because the E-cadherin transcript (*CDH1*) was also reduced in the presumed enriched fraction, we concluded that the antibody was not labeling E-cadherin-positive cells during the MACS procedure. The reason for non-specific cell labeling is unknown, but is likely due to loss of antigenicity caused by enzymatic digestion of tissue and loss of the surface marker. Alternatively, the antibody may not have been suited for the application (i.e., Western blotting versus MACS), which is a frequent challenge in antibody-based applications.

In the absence of a suitable cell surface marker, testis cells were enriched with differential plating. Using reverse transcription quantitative-polymerase chain reaction (RT-qPCR), we demonstrated that a two hour, differential plating technique could enrich prepubertal cat testis for SSCs. In the adults, several SSC genes trended upwards, but did not achieve statistical significance, implying a low level of enrichment occurred. While our current level of enrichment is acceptable for prepubertal cats, as evidenced by our ability to establish SSC cultures (Chapter 4), a higher level of enrichment is desirable for adults, especially in the context of rescuing genotypes of aged males. Modifications to the

differential plating technique (e.g., increased plating time, reduced plating density, or use of a substrate such as gelatin) warrant further exploration.

The objective of our final study (Chapter 4) was to establish a culture system that can support the long-term propagation of cat SSCs. To that end, we first evaluated feeder layers from three different cell sources: STO cells were chosen because they are the main cell type used in the mouse culture system (Kubota et al., 2004b); the mouse endothelial line C166 cells were selected based on their ability to support the only non-rodent long-term SSC culture system (Kubota et al., 2011); and primary cat fetal fibroblasts served as the homologous feeder layer in our study. The cluster forming activity (CFA) assay (Yeh et al., 2007) demonstrated a clear effect of feeder cell type, with the C166 cells supporting the highest number of germ cell clumps. We further assessed the identity of the colonies with immunocytochemistry, which confirmed that the germ cell colonies were co-expressing three SSC markers (UCHL1, PLZF, and OCT4). Based on these results, C166 cells were used for subsequent culture experiments. Germ cell clumps established from two individuals have now been subcultured for 5-6 passages and cultures are still ongoing. At the time of writing, cells have been continuously cultured for 78 days in vitro. During that time, we confirmed that the colonies were still strongly expressing SSC markers. Furthermore, the colonies display alkaline phosphatase activity, which serves as a general stem cell marker (Matsui et al., 1992; Resnick et al., 1992; Takahashi and Yamanaka, 2006), and has been reported to be present in SSCs of other species (Kubota et al., 2004b; Kubota et al., 2011; Ryu et al., 2005).

Although the SSC transplant assay is considered the gold standard for SSC identification, there are two key advantages of the CFA assay. First, the transplant assay

requires several weeks to prepare host animals and a minimum two month post-transplantation waiting period to evaluate host testes for SSC colonization (Nagano et al., 1999; Zhang et al., 2003a). This makes it difficult to systematically assess changes to the culture system in a stepwise manner. Similarly, the long latent period precludes the transplant assay as an effective method to monitor culture progression. Results are obtained for the CFA assay within one week of culture initiation or passaging, thus greatly reducing the time required for data acquisition (Yeh et al., 2007). Second, the transplant assay is more laborious and technically challenging as it requires host animal preparation, donor cell labeling, microinjection into the rete testis or efferent ducts, and analysis of host testis (Oatley et al., 2002). This often limits the number of experimental conditions that can be investigated. The simpler nature of the CFA assay thus allows for more treatments to be explored. Of course, the CFA assay does not determine the regenerative capabilities of the cultured SSCs and unequivocal identification will still require an SSC transplant assay.

Although not formerly investigated, several aspects of our culture system likely played crucial roles in our success. First, the age of testis donors (peripubertal) was carefully selected to maximize the number of SSCs available for cell culture. Shinohara et al. (2001) reported that the stem cell population of an animal increases with age. However, enrichment of SSCs is less efficient in adults, due to the diluting effects of more advanced germ cells (Borjigin et al., 2010; Herrid et al., 2007; Izadyar et al., 2002b; Luo et al., 2006). Additionally once an animal reaches adulthood, the stem cell niche begins to age, causing a reduction in the SSC population (Ryu et al., 2006). Therefore, peripubertal animals—who have experienced significant testis growth but not yet started spermatogenesis—represent the ideal donor age (Luo et al., 2006). Second, our cultures system was serum-

free. Serum contains undefined materials and variation among batches can lead to inconsistent cell culture performance. Furthermore, serum is toxic for many cell types (Barnes and Sato, 1980; Enat et al., 1984) and similarly, has been demonstrated to have a detrimental effect on cultured SSCs (Kubota et al., 2004b). Third, enzymatic passaging was performed with Accutase™, which is gentler on cells than trypsin (Bajpai et al., 2008), and does not require the addition of fetal bovine serum to inactivate it. Fourth, cells were cultured in a low oxygen environment. This has been demonstrated to be beneficial to embryonic stem cells (Ezashi et al., 2005), and the reduced oxygen environment more closely reflects conditions within the SSC niche. Finally, minimum essential medium alpha, the preferred serum-free media used in rodent SSC culture, has been observed to be detrimental to cat follicular cells during culture by researchers at Smithsonian Conservation Biology Institute. It was found that cat follicles grew well in Dulbecco modified Eagle medium (DMEM). Therefore, we used DMEM in our cat SSC culture system.

Another important area to investigate is whether glial cell line-derived neurotrophic factor (GDNF), soluble GFRalpha1, and basic fibroblast growth factor (bFGF) are essential to the culture system, as has been demonstrated in other species (Kubota et al., 2004b; Kubota et al., 2011; Ryu et al., 2005). Certainly, proliferation of our colonies in the presence of the three growth factors suggests their importance in cat SSCs. However, additional experiments would be required to confirm this hypothesis.

In conclusion, the SSC technology explored in this dissertation has the potential to impact rare and endangered felids in a number of ways. Due to the enormous regenerative capacity of SSCs, cells can be expanded *in vitro* and thus, are virtually unlimited. This creates opportunities to rescue the genotypes of animals that die unexpectedly, or are too

young or too old to produce spermatozoa. SSCs are also very amenable to cryopreservation and could become routinely stored in genome resource banks. This will be particularly useful for the many felid species whose spermatozoa display severe cryosensitivities. Most importantly for future research purposes, SSC can be induced to differentiate *in vitro* which will allow developmental mechanisms to be explored. There is no doubt that future applications of SSC technology will provide unique insights into the mechanisms of spermatogenesis and, consequently, additional benefits to reproductive management programs.

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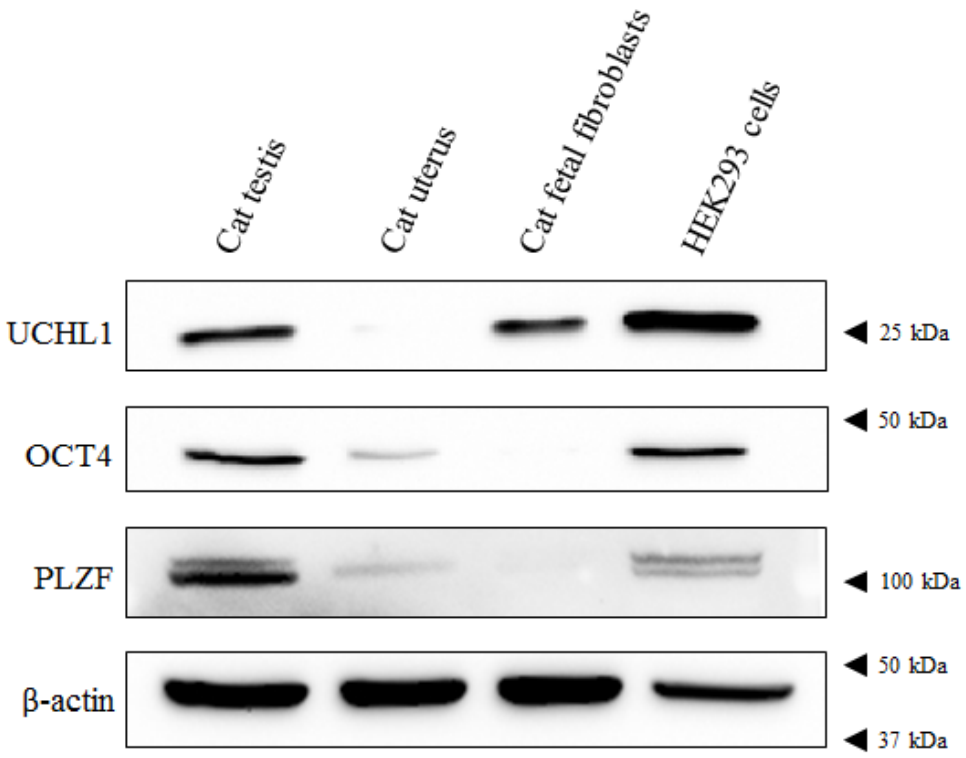
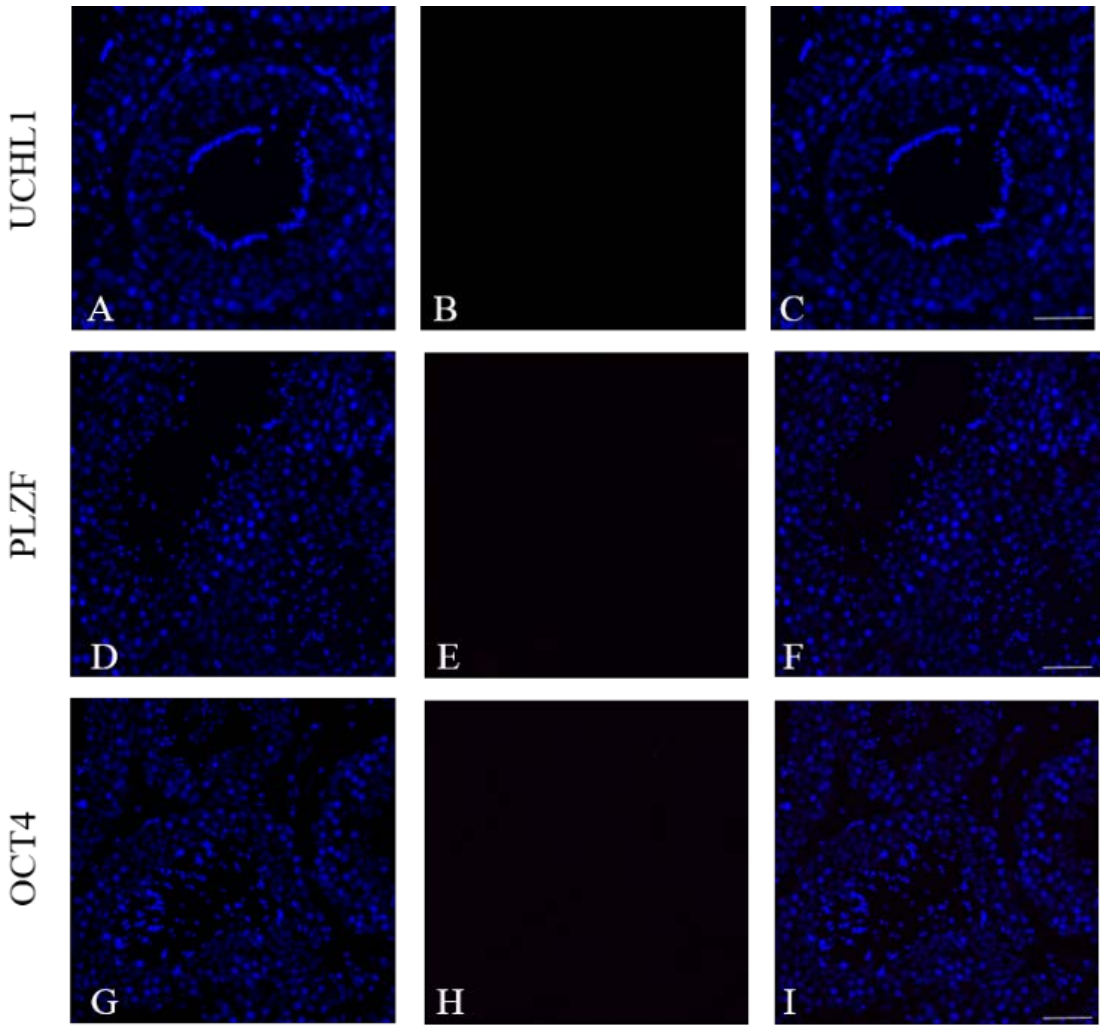
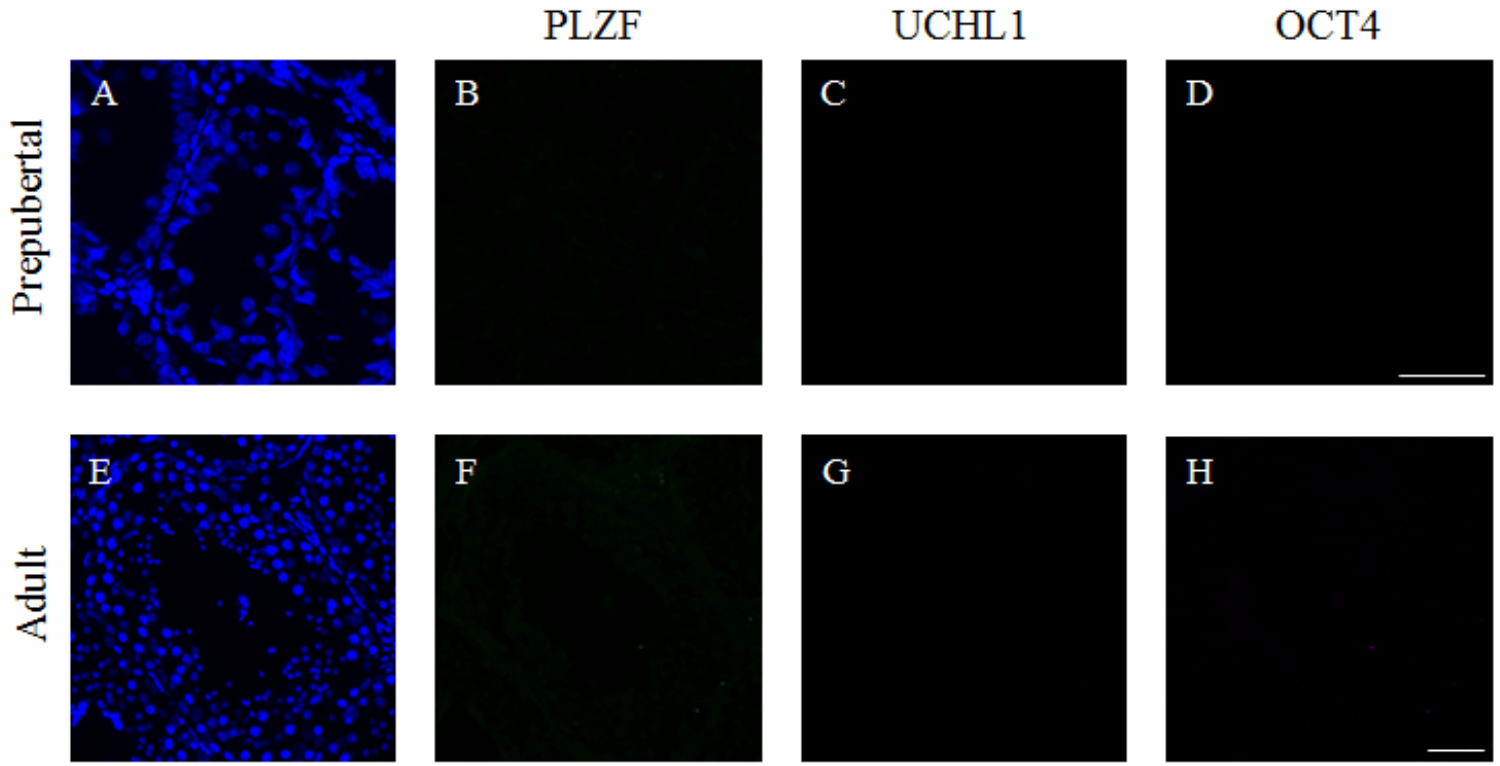
Appendix



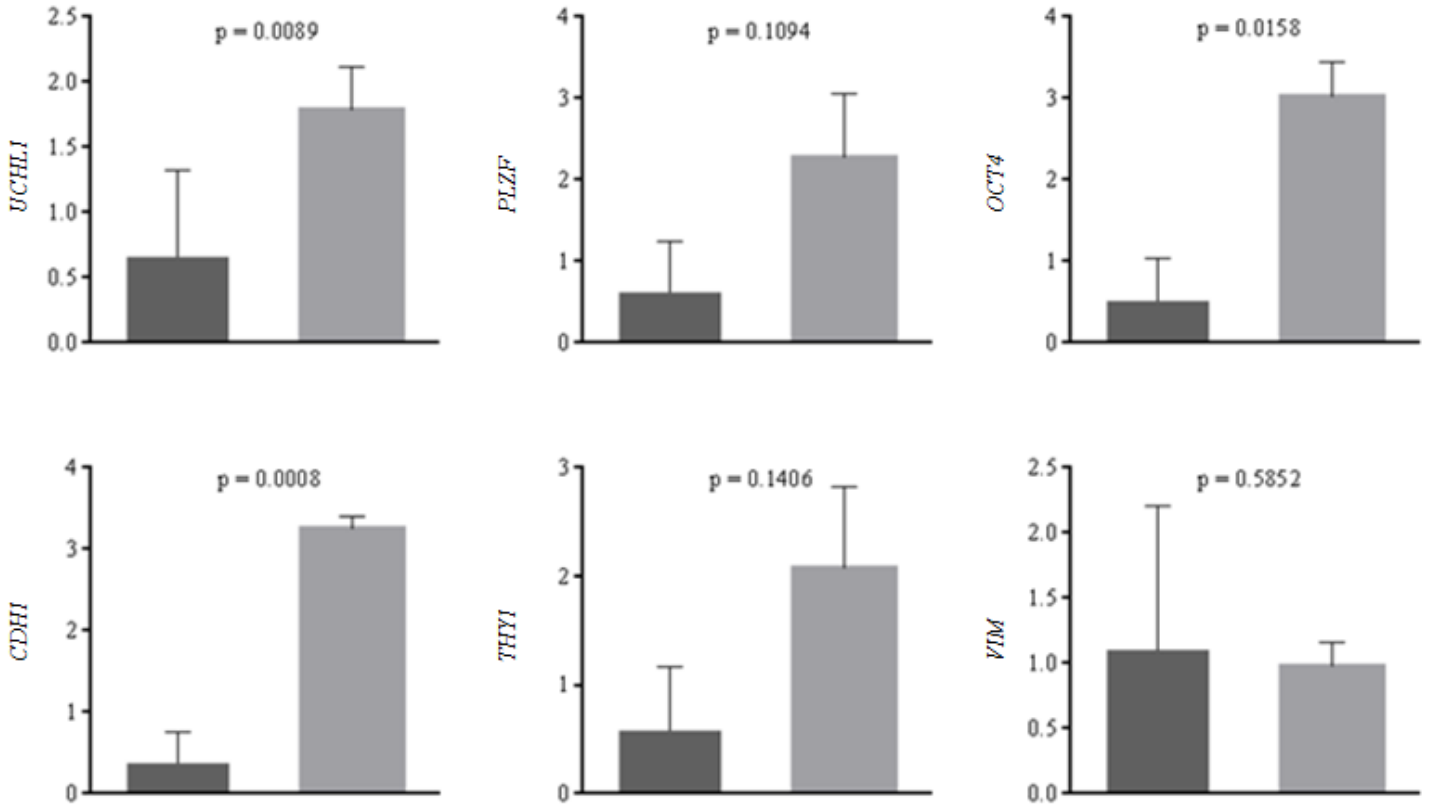
Supplementary Figure 1 Negative control Western blots. To confirm specificity of antibodies, Western blots were performed using predicted negative tissue and cell lines. Peripubertal cat testis (age 6 months) was included as a positive control. Forty micrograms of total protein were loaded for each sample. Blots were visualized with Supersignal West Pico chemiluminescent substrate. UCHL1 was expressed in both cell lines. However, no expression was noted in the uterus, consistent with previous reports that *in corpora* UCHL1 expression is limited to neuronal and gonadal tissues. OCT4 was strongly expressed in HEK293 cells, and to a lesser degree, in uterine tissue. No band was detected for cat fetal fibroblasts. Similarly, PLZF expression was high in HEK293 cells, lower in uterus, and absent in cat fetal fibroblasts.



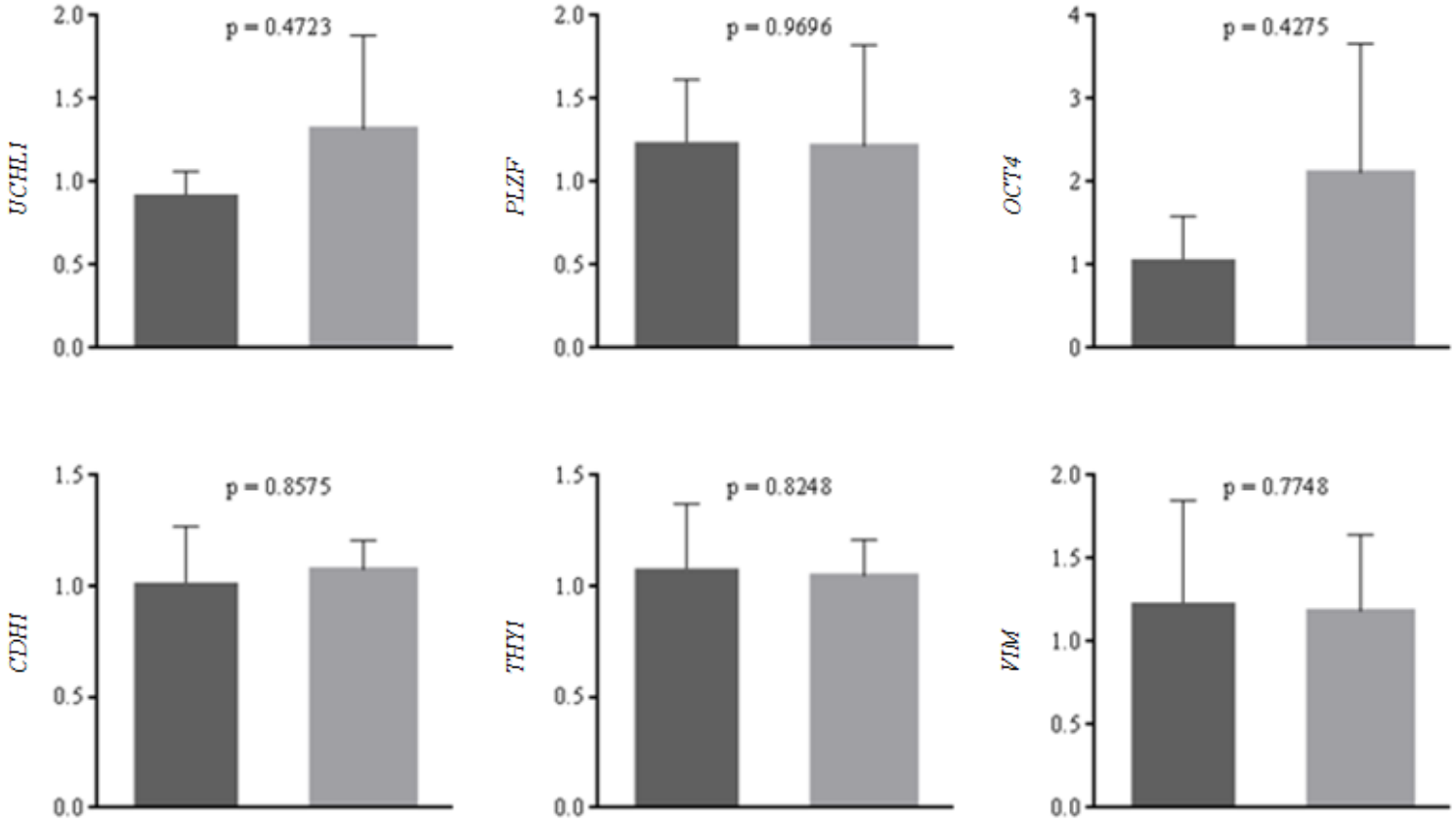
Supplementary Figure 2 Fluorescent immunohistochemistry single label negative controls. Control slides in which the primary antibody against UCHL1 (**B**), PLZF (**E**), and OCT4 (**H**) were omitted showed minimal immunoreactivity. (**A**, **D**, **G**) Hoechst 33342 to mark cell nuclei. (**C**, **F**, **I**) Merge of previous two images. Scale bars; 50 μm.



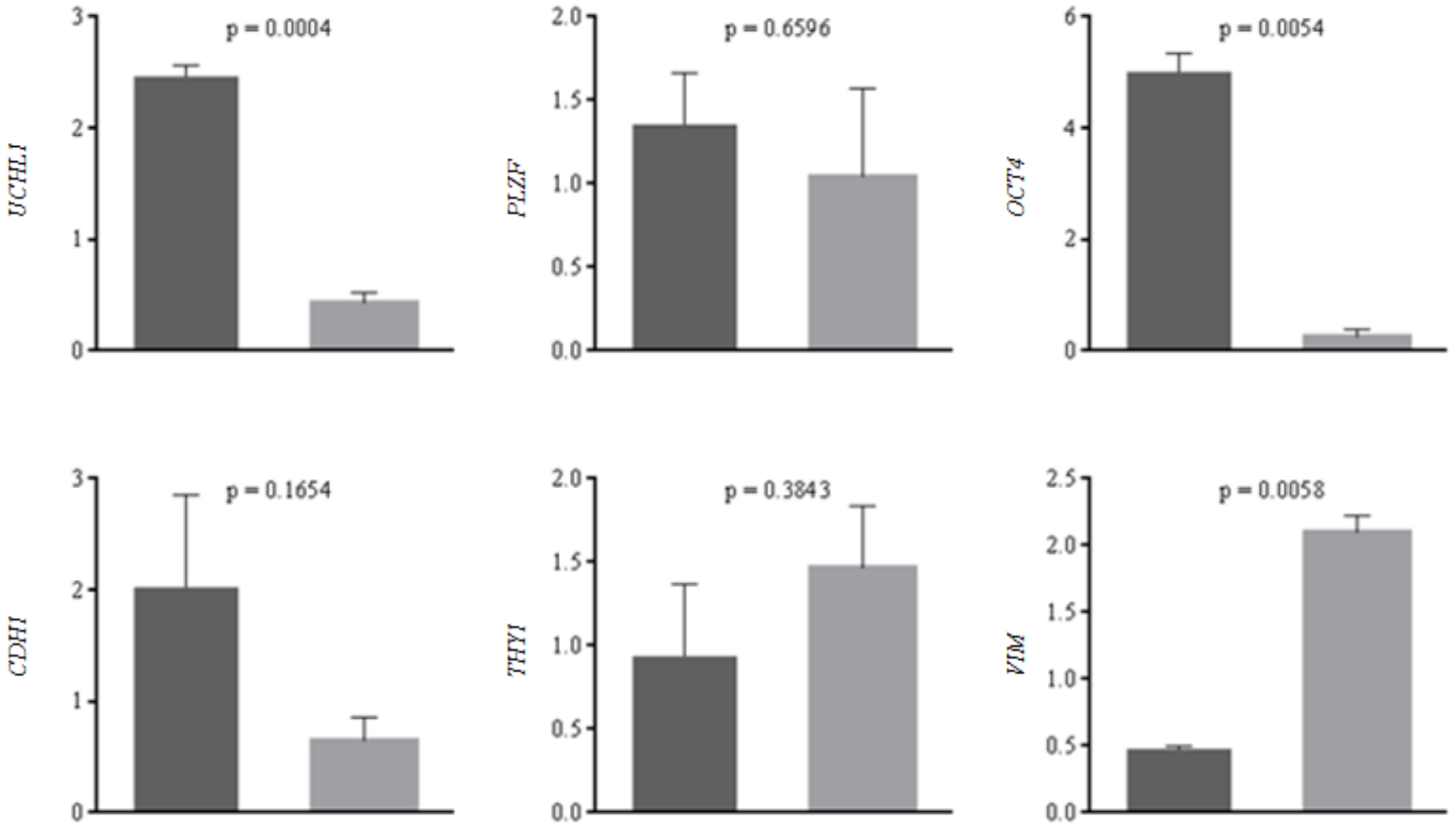
Supplementary Figure 3 Fluorescent immunohistochemistry co-localization negative controls. Control slides in which the primary antibody against PLZF (**B**, **F**), UCHL1 (**C**, **G**), and OCT4 (**D**, **H**) were omitted showed minimal immunoreactivity. (**A**, **E**) Hoechst 33342 to mark cell nuclei. (**A**-**D**) Prepubertal cat testis. (**G**-**H**) Adult cat testis. Scale bars; 50 µm



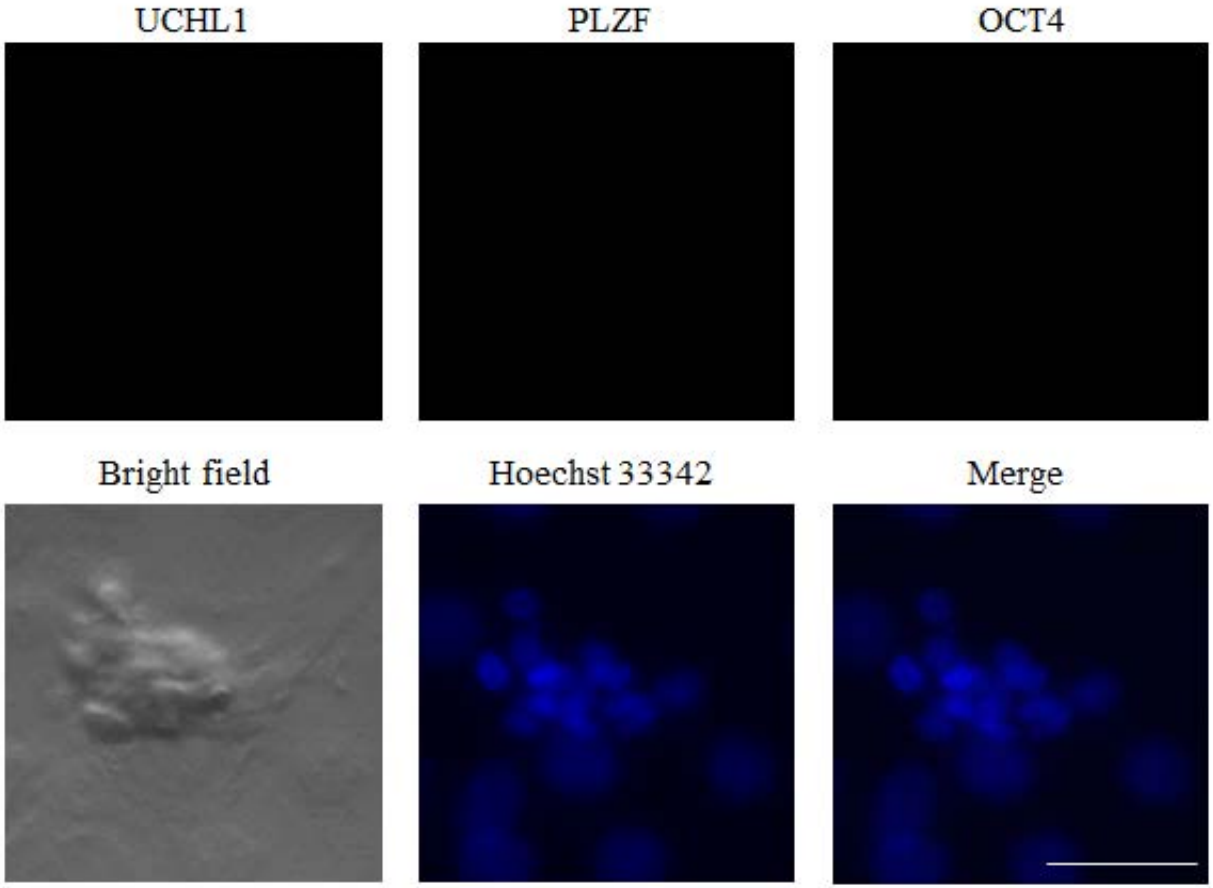
Supplementary Figure 4 RT-qPCR analysis of SSC (*UCHL1*, *PLZF*, *OCT4*, *CDH1*, and *THY1*) and Sertoli cell (*VIM*) gene transcripts using mRNA isolated from prepubertal cat testis before (dark bars) and after (light bars) differential plating. Four animals were used and analyses were performed in triplicate. Errors bars represent ± SE. The p-values are depicted on each graph.



Supplementary Figure 5 RT-qPCR analysis of SSC (*UCHL1*, *PLZF*, *OCT4*, *CDH1*, and *THY1*) and Sertoli cell (*VIM*) gene transcripts using mRNA isolated from adult cat testis before (dark bars) and after (light bars) differential plating. Three animals were used and analyses were performed in triplicate. Errors bars represent ± SE. The p-values are depicted on each graph.



Supplementary Figure 6 RT-qPCR analysis of SSC (*UCHL1*, *PLZF*, *OCT4*, *CDH1*, and *THY1*) and Sertoli cell (*VIM*) gene transcripts using mRNA isolated from adult cat testis before (dark bars) and after (light bars) differential plating and MACS. Testes from three animals were pooled for each replicate (n = 3) and analyses were performed in triplicate. Errors bars represent ± SE. The p-values are depicted on each graph.



Supplementary Figure 7 Fluorescent immunocytochemistry negative control. Control wells in which the primary antibody against UCHL1, PLZF, and OCT4 were omitted showed minimal immunoreactivity. Cells were counterstained with Hoechst 33342 to mark cell nuclei. Merge: merged image of all fluorescent panels. Scale bar; 50 µm.

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