DEPARTAMENTO DE CIÊNCIAS DA VIDA

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A Cat Problem: Characterization of Feline Spermatogonial Stem Cells

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A Cat Problem: Characterization of Feline Spermatogonial Stem Cells

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Paula Cristina Cardoso Mota (Centro de Neurociências e Biologia Celular) e com co-orientação do Professor Doutor João Ramalho-Santos (Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra)

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INDEX

ABBREVIATIONS

AP	Alkaline phosphatase
APS	Ammonium Persulfate
Bcl6b	B cell CLL/lymphoma 6, member b
bFGF	basic fibroblast growth factor
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
BTB	Blood-testis barrier
CLAP	Protease Inhibitor Cocktail
Csf1	Colony stimulating factor 1
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
DAB	3,3-diaminobenzidine tetrahydrochloride
DAPI	4',6-diamidino-2-phenylindole
DBA	*Dolichos biflorus agglutinin*
DDX4	DEAD box protein 4
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal growth factor
ESCs	Embryonic stem cells
FACS	Fluorescence activated cell sorting
FGF2	Fibroblast growth factor 2
FoxO1	Forkhead box protein O1
FSH	Follicle-stimulating hormone
GDNF	Glia cell-derived neurotrophic factor
GFRα-1	Gdnf family receptor alpha-1
GPR125	G-protein coupled receptor 125
HRP	Horseradish-peroxidase
ICC	Immunocytochemistry

ICSI	Intracytoplasmic sperm injection
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-1β	Interleukin-1 beta
IUCN	International Union for Conservation of Nature and Natural Resources
LH	Luteinizing hormone
MACS	Magnetic activated cell sorting
NEAA	Nonessential aminoacids
NICD	Notch intracytoplasmic domain
OCT4	Octamer-binding transcription factor 4
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered solution
PGCs	Primordial germ cells
PGP9.5	Protein gene product 9.5/ Ubiquitin carboxy-terminal hydrolase 1
PI3K	Phosphoinositide 3-kinase
Plzf	Promyelocytic leukaemia zinc-finger
PMCs	Peritubular myoid cells
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
RET	Rearranged during transfection
RIPA	Radio-Immunoprecipitation Assay
RT	Room temperature
SALL4	Sal-like protein 4
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
SSCs	Spermatogonial stem cells
SSEA-1	Stage-specific embryonic antigen 1
SSEA-4	Stage-specific embryonic antigen 4
Taf4b	TATA box binding protein (TBP)-associated factor 4b
TBS	Tris buffer solution
TEMED	N,N,N',N'-Tetramethyllenediamine
TGF-β	Transforming growth factor beta

THY-1	Thymus cell antigen 1
TNF-α	Tumor necrosis factor alpha
TRA-1-60	Tumor-rejection antigens -1-60
TRA-1-81	Tumor-rejection antigens -1-81
UCHL1	Ubiquitin carboxy-terminal hydrolase 1/ Protein gene product 9.5
WB	Western Blot

ABSTRACT

According to the IUCN Red List of Threatened Species 70% of all felid species are at risk, including the Iberian lynx (*Lynx pardinus*). Environmental contaminants, presence of a high percentage of morphologically abnormal spermatozoa (teratospermia), premature death and poaching are some of the factors that compromise fertility and reproduction. In order to overcome these issues, some artificial reproduction techniques are being developed and tested, namely sperm cryopreservation, testicular tissue xenografting and the more promising spermatogonial stem cell (SSC) transplantation.

Despite these developments in assisted reproduction techniques to recover fertility/genetic variability from rare felids, there are still a few gaps in terms of the basic physiology of male gametogenesis that need to be addressed. In fact, in felids the Stem/progenitor cells capable of supporting a sustained spermatogenesis have yet to be clearly identified, both morphologically and biochemically. This has made the purification of a testicular isolate containing only spermatogonial stem cells for posterior transplantation impossible.

Therefore, our aim for this work was to identify, with a certain degree of confidence, the population of domestic cat spermatogonial stem cells. To guarantee this goal we applied different methodologies to reveal markers for these cells and their possible regulation by factors that are external to the seminiferous tubule.

After testing, in domestic cat testis tissue, a long list of antibodies used in other species to detect putative spermatogonial stem cells, three main conclusions could be exctracted from the work. Firstly, protein PGP9.5 is expressed in the entire spermatogonial population as well as in the early meiotic germ cells (pre-leptotene), which denies its use as a SSC marker in adult animals. Secondly DBA lectin (*Dolichos biflorus agglutinin)* can be considered as a putative marker of cat SSCs. The DBA^+ cells appear next to the basal membrane, in low number, and either isolated or in arranged in clones of 2 cells to 8 cells, all characteristics described in SSCs of other species. Finally, contrarily to what has been observed in other species, the SSC niche in the cat seems to be greatly influenced by Leydig cells.

As a final message, domestic cat spermatogonial stem cell population seems to present some of the mouse SSCs characteristics but also seems to share characteristics with higher mammals SSCs.

Keywords: Felids, spermatogonial population characterization, spermatogonial stem cells markers, PGP9.5, DBA.

RESUMO

De acordo com a *Red List of Threatened Species* da IUCN, 70% das espécies de felinos estão em risco de extinção, incluindo o lince ibérico (*Lynx pardinus). Os* contaminantes ambientais, a elevada percentagem de espermatozóides morfologicamente anormais (teratospermia) e por outro lado a morte prematura e caça ilegal são alguns dos factores que influenciam a fertilidade e reprodução. Numa tentativa de combater a situação, novas estratégias estão a ser desenvolvidas, nomeadamente a criopreservação de espermatozóides, o xenotransplante de tecido testicular e a mais promissora, o transplante de células espermatogoniais estaminais (SSC).

Apesar de todo o empenho depositado no desenvolvimento de técnicas de reprodução assistida para a recuperação da fertilidade/variabilidade genética de felinos selvagens, há ainda alguns aspectos por clarificar relativamente à fisiologia básica da gametogénese masculina. De facto, nos felinos a célula estaminal/progenitora, capaz de sustentar uma espermatogénese contínua, não foi ainda claramente identificada, tanto morfologicamente quer bioquimicamente, facto que torna impossível o isolamento duma população pura de espermatogónias indiferenciadas para posterior transplante.

Como tal o nosso objectivo geral para este trabalho era identificar, com um certo grau de confiança, a população de células estaminais espermatogoniais de gato. Para isso desenvolvemos várias metodologias para revelar estas células e a sua possível regulação por factores externos ao túbulo seminífero. Após testar em tecido testicular de gato doméstico uma longa lista de potenciais marcadores de células estaminais espermatogoniais observados noutras espécies, chegamos a três conclusões principais. Primeiro, a proteína PGP9.5 (protein gene product 9.5) é expressa em toda a população de células espermatogoniais, assim como em células em inicio de meiose (pre-leptotenos), o que impede a sua utilização como uma marcador de células espermatogoniais estaminais em animais adultos. Como segunda conclusão, a lectina DBA (*Dolichos biflorus agglutinin)* pode ser considerada um potencial marcador de células espermatogoniais estaminais no gato. As células marcadas com a lectina DBA localizam-se junto à membrana basal dos túbulos seminíferos, correspondem a uma pequena população de células espermatogoniais e encontram-se isoladas ou em clonas de 2 a 8 células,

características também observadas noutras espécies.

Finalmente e contrariamente ao observado noutras espécies, o nicho de células espermatogoniais estaminais no gato parece ser influenciado pelas células de Leydig.

Como mensagem final deste trabalho podemos dizer que as células espermatogoniais estaminais de gato parecem apresentar algumas das características observadas nas suas congéneres de ratinho mas também compartilham características com as células espermatogoniais estaminais de outros mamíferos superiores.

Palavras-chave: Felinos, caracterização da população espermatogonial, marcadores de células estaminais espermatogoniais, PGP9.5 e DBA.

INTRODUCTION

I. Endangered Felids

A. Species current Situation

According to the Red List of Threatened Species of the IUCN (International Union for Conservation of Nature and Natural Resources), from the 36 felid species, 70% fall into classification such as near threatened, threatened, vulnerable, endangered and even critically endangered, as is the case with the Iberian lynx (*Lynx pardinus*). This situation is further aggravated by the fact that captive breeding programs present low success rates (Nowell & Jackson 1996).

Habitat loss, poaching, premature death, teratospermia [high percentage of spermatozoa with abnormal morphology) (Howard *et al.* 1984)] and environmental contaminants (reviewed in Mota *et al.* 2009) represent some of the factors that compromise fertility and reproduction in felid species.

B. Ongoing Strategies to recover Fertility

Given the scenario described above, several artificial reproduction techniques have been attempted in order to retrieve fertility/genetic variability in endangered felids, namely sperm cryopreservation (Ganan *et al.* 2009), xenografting [donor ex-vivo spermatogenesis in a recipient animal from a different species; (Snedaker *et al.* 2004; Mota *et al.* 2012)] and germ cell transplantation [where isolated germ cells from a donor animal are introduced in the testis of a recipient animal; (Silva *et al.* 2012)].

Despite the potential success of these techniques they have limitations. With sperm cryopreservation the main issue results from the fact that sperm cells are terminally differentiated haploid cells that no longer have the ability to proliferate, restricting this technique to a limited number of breeding attempts (Snedaker *et al.* 2004). Sperm is also impossible to retrieve in immature animals that have yet to reach puberty. Xenografting, although has led to the recovery of immature sperm from xenografts, has only been successfully carried out using testicular material from pre-pubertal animals. Moreover,

xenografts can only generate few sperm cells that cannot undergo the process of maturation in the epididymis, which makes ICSI (intracytoplasmic sperm injection) the only possible artificial reproduction technique (Snedaker *et al.* 2004; Mota *et al.* 2012). These issues, together with the fact that in xenografted testis tissue sperm production may take up to 36 weeks (Snedaker *et al.* 2004), further demonstrates the limitations of the technique. Contrarily, isolated germ cell transplantation overcomes some of the previously described limitations. Firstly, both immature and adult animals qualify for this technique given that spermatogonial stem cells (SSCs) can be collected from both. Also, because the increased numbers of sperm cells that are produced in this situation can undergo natural epididymal maturation, other assisted reproduction techniques, including artificial insemination as well as *in vitro* fertilization may be used to fertilize the oocyte. Lastly, sperm cells can easily and repeatedly be collected by electro-ejaculation, whereas xenografts must be digested so sperm can be retrieved (reviewed in Kim *et al.* 2006). On the other hand, phylogenetic distance between donor and recipient animal has proven to be a limitation to the xenotransplantation of SSC technique given the fact that undifferentiated germ cells from higher mammals transplanted to rodent testis permanently colonize the seminiferous tubules but do not originate differentiated germ cells (Kim *et al.* 2006).

Also the available methods to increase colonization of the donor germ cells, busulfan treatment and testis x-irradiation, may induce disease in the host animal (Silva *et al.* 2012). Despite the advantages of germ cell transplantation over the previous techniques, a few pitfalls still persist. Although xenotransplantation of a mix of ocelot germ cells to the recipient testis of a domestic cat has led to the retrieval of ocelot testicular sperm a few weeks after, no follow up studies were carried out to verify if any permanent colonization of the recipient testis by the donor spermatogonial stem cells had occurred as well as if the produced sperm possessed any fertility capacity (Silva *et al.* 2012).

II. Stem Cells in the Male Reproductive System

A. Origin of Spermatogonial Stem Cells

During fetal development, shortly before the epiblast of the blastocyst forms the three germ layers (ectoderm, mesoderm and endoderm), a reduced number of the pluripotent epiblast cells differentiate into primordial germ cells (PGCs) (reviewed in Phillips *et al.* 2010). In mouse embryos, by 7 days *post coitum* (dpc), about 100 alkaline-phosphatase positive PGCs can be detected in the extra embryonic mesoderm (reviewed in Tokas *et al.* 2011). Later in development PGCs migrate from the base of the allantois, along the hindgut, finally reaching the genital ridges (Aponte *et al.* 2005). It's during this phase that a first wave of proliferation occurs and three thousand PGCs finally colonize the genital ridges (Bendel-Stenzel *et al.* 1998). Once there, Sertoli cells surround PGCs forming the seminiferous cords (Aponte *et al.* 2005). In mouse and rat testis, around 13.5 dpc, PGCs suffer a second phase of mitotic divisions in the seminiferous cords that ends, after a few days, with the arrest of the cells in the G0/G1 phase of the cell cycle (De Felici 2009). At this phase, these cells are designated as gonocytes or pre-spermatogonia (reviewed in Kolasa *et al.* 2012).

These cells, described by Kim and colleagues as round-shaped germ cells with prominent nuclei (Kim *et al.* 2010), undergo a new cycle of mitosis and similarly to PGCs, in mice and rats, arrest in the G0/G1 phase of the cell cycle only resuming proliferation after they have migrated to the basal lamina of the seminiferous cords (Kim *et al.* 2010), as spermatogonial stem cells. The homing of the cells to the basal membrane, is connected to the CXCL12/CXCR4 signaling pathway, that also participates in the maintenance of the undifferentiated state of SSCs, with only a small fraction of the mouse undifferentiated spermatogonia expressing the CXCL12 receptor [CXCR4; (Yang *et al.* 2013)]. In mice and rats the differentiation of gonocytes into SSCs shortly after birth marks the start of spermatogenesis (Aponte *et al.* 2005) while in several mammal species this differentiation occurs during a prolonged pre-pubertal phase (Albert *et al.* 2010)

In adulthood, SSCs are located on the basal membrane of the seminiferous tubules surrounded by Sertoli cells and other germ cells. They are in close contact with peritubular myoid cells and all the components of the interstitial spaces - macrophages, blood vessels, lymphatic vessels, veins fibers and Leydig cells, the cells responsible for testosterone production (Alberts 2002).

B. Testicular Niche

1. General Concepts

Spermatogonial stem cells are located and maintained on the basal membrane of the seminiferous tubules by a specialized microenvironment designated “niche” (reviewed in Kolasa *et al.* 2012). Schofield first described this concept in 1978 for the hematopoietic system (Schofield 1978). This microenvironment is composed by somatic cells, extracellular matrix components and local soluble factors that regulate cell fate (Caires *et al.* 2010; reviewed in Phillips *et al.* 2010). Sertoli cells, the only somatic cells present within the seminiferous tubules, release paracrine factors, participating in the regulation of the self-renewal activity of SSCs as well as their differentiation (Meng *et al.* 2000; Kubota *et al.* 2004; Kostereva & Hofmann 2008). In mice and rat, the basal membrane with their integrins are responsible for anchoring SSCs to this region, while the vascular network and the interstitial cells (Leydig cells, peritubular myoid cells and macrophages) together provide extrinsic stimuli crucial for the localization of undifferentiated spermatogonia along specific portions of the basal membrane (Yoshida *et al.* 2007). Therefore, it is the integration of all these signals that leads to the self-renewal of SSCs and their maintenance in an undifferentiated state. These extrinsic signals modulate SSC intrinsic ones, such as kinases, transcription factors and second messengers, to guarantee homeostasis (Kostereva & Hofmann 2008).

This niche is essential to regulate some properties of SSCs (Kostereva & Hofmann 2008; de Rooij 2009; Hermo *et al.* 2010), as well as to maintain

testicular homeostasis by balancing spermatogonial differentiation and self-renewal.

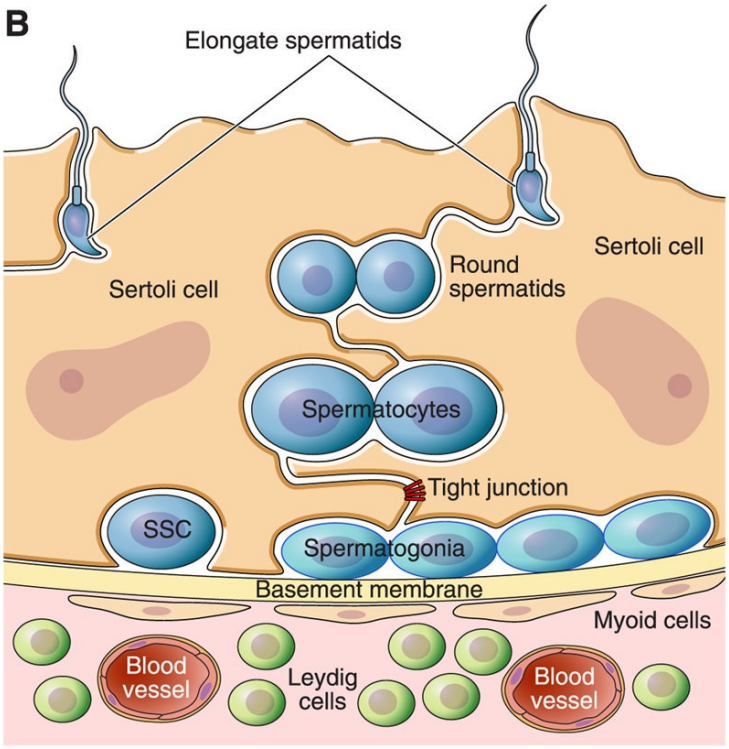

Figure 1. Copied from Oatley & Brinster 2011 – Schematic illustration of niche unit.

2. Influence by Sertoli Cells

Postnatally the seminiferous tubules continue to grow in length mainly due to the simultaneous proliferation and migration of Sertoli and spermatogonial stem cells. At puberty, Sertoli cell proliferation stops and a process of maturation begins so that Sertoli cells can support spermatogonial differentiation (Kyurkchiev *et al.* 2012). Mature Sertoli cells are polarized columnar epithelial cells located and distributed along the basal membrane of the seminiferous tubules, and whose cytoplasmic ramifications extend from the base of the epithelium to the tubule lumen (Griswold 1998). These cells are considered the most important somatic cells for germ cell support and differentiation, playing roles in the formation, maintenance and regulation of the blood-testis barrier (BTB), nourishing germ cells and mediating external signals fundamental for a correct spermatogenesis (reviewed in Phillips *et al.*

2010; Kyurkchiev *et al.* 2012). For the fulfillment of these functions a tight communication between Sertoli cells and between Sertoli and germ cells is required (Mruk & Cheng 2004). Indeed, the presence of thigh junctions between adjacent Sertoli cells participates in the formation of the BTB, separating the seminiferous tubule in basal (outer side, in contact with blood and lymph) and adluminal (inner side, isolated from blood and lymph) compartments [Figure 1; (Kyurkchiev *et al.* 2012)]. This creates an immune privileged environment in the adluminal compartment, which is vital for the haploid germ cells (reviewed in Phillips *et al.* 2010).

a) Cues for SSC Self-renewal

In order to maintain a normal and continuous spermatogenesis, both the processes of self-renewal and differentiation of SSCs must be precisely regulated by intrinsic gene expression in response to extrinsic signals, such as soluble factors or adhesion molecules from the surrounding microenvironment (Brinster 2002).

Sertoli cells start to secrete GDNF (glia cell-derived neurotrophic factor), a member of the transforming growth factor-β superfamily, immediately after birth (Meng *et al.* 2000). GDNF is the major factor responsible for the maintenance and self-renewal of SSCs *in vivo* as well as *in vitro* (reviewed in Kostereva & Hofmann 2008). Other factors secreted by Sertoli cells, such as basic fibroblast growth factor (bFGF or FGF2) and epidermal growth factor (EGF) are required and act synergistically to support *in vitro* proliferation of SSCs in mice (Kubota *et al.* 2004). In 2006, Naughton and co-workers demonstrated that the *in vivo* disruption of GDNF-mediated signaling in *Gdnf*-, *Gfr-α*-, and *Ret*- deficient mice resulted in a lack of SSC self-renewal with a progressive loss of spermatogenic capacity due to germ cell depletion, therefore enhancing the importance of this signaling pathway (Naughton *et al.* 2006).

GDNF signaling occurs through a multicomponent receptor complex composed by the co-receptor GFRα-1 (Gdnf family receptor alpha-1) and the

Ret tyrosine kinase transmembrane receptor (reviewed in Kostereva & Hofmann 2008). The binding of GDNF leads to the activation of multiple signaling pathways (Airaksinen & Saarma 2002) including activation of several kinases from the Src family (Braydich-Stolle *et al.* 2007) and of Ras (He *et al.* 2008).

Tadokoro and co-workers have long established that the expression of GDNF and subsequent proliferation of SSCs was dependent on Sertoli cells stimulation by FSH [Follicle-stimulating hormone; (Tadokoro *et al.* 2002)]. Simon and colleagues later confirmed this by demonstrating that FSH increases the level of GDNF in a Sertoli cell line. These authors also reported that FGF2, TNF-α (tumor necrosis factor alpha) and IL-1β (interleukin-1 beta) are required for *in vitro* GDNF production [Figure 2; (Simon *et al.* 2007)].

Other transcription factors necessary for SSC self-renewal and maintenance have been identified, namely Bcl6b (B cell CLL⁄ lymphoma 6, member b), Taf4b (TATA box binding protein (TBP)-associated factor 4b) and Plzf [promyelocytic leukaemia zinc-finger; (reviewed in Kostereva & Hofmann 2008)]. Although the exact function of these transcriptions factors remains unclear, it is believed that Plzf represses SSC differentiation in wild type mice, since in, mutant or knockout mice, its loss leads to a shift from SSC self-renewal towards differentiation. It is believed that repression of the c-Kit receptor transcription by Plzf, shown by Filipponi and colleagues, contributes towards this regulation (Filipponi *et al.* 2007).

b) Regulation of SSC Differentiation

Although the determinant factor of the self-renewal/differentiation switch isn't yet clearly elucidated, several studies have tackled the mechanisms behind the control of SSC differentiation. The c-Kit receptor involvement in this process was the first mechanism described. According to Dolci and colleagues, the binding of the Kit ligand, also denominated as SCF (Stem cell factor - secreted by Sertoli cells), to the c-Kit receptor present in differentiating spermatogonia leads to the up-regulation of early meiotic genes in these cells (Dolci *et al.* 2001).

Furthermore, in 2001, two other groups of researchers described the involvement of the Notch receptor and its ligands, Jagged-1 and Jagged-2, in germ cell differentiation (Dirami *et al.* 2001; Hayashi *et al.* 2001). Dirami and colleagues demonstrated, using immunocytochemistry, that the Notch receptor is activated in specific cell types and that its ligands are secreted by Sertoli cells (Dirami *et al.* 2001).

3. Influence by Interstitial Cells

The interstitial tissue between and adjacent to the seminiferous tubules contains blood and lymph vessels, macrophages, Leydig and myoid peritubular cells (Caires *et al.* 2010).

Several studies have described the cross-talk between Sertoli and these cells during spermatogenesis, meaning that both Leydig and peritubular myoid cells (PMCs) contribute to the maintenance of the SSC niche through their interaction with Sertoli cells (Sadava *et al.* 2011). The main and most studied function of Leydig cells is the secretion of testosterone, in response to pituitary Luteinizing hormone (LH). Notwithstanding, recent studies have shown the capacity of these cells to produce the colony stimulating factor 1 (Csf1), a specific regulator of SSC self-renewal (Silvan *et al.* 2013), contributing therefore to the testicular stem cell niche (Oatley *et al.* 2009). The same involvement has been described for peritubular myoid cells (PMCs). PMCs are elongated flat cells that surround the seminiferous tubules, presenting features of both fibroblasts and smooth muscle cells (reviewed in Diez-Torre *et al.* 2011). Until recently the contractile ability of these cells, driving the testicular fluid containing immotile spermatozoa towards the rete testis (Davidoff *et al.* 1990; Romano *et al.* 2005) was thought to be their only function. However, recent studies have extended their functions to include a role in male gonad development as well as in the maintenance of spermatogenesis (Schell *et al.* 2008) through the secretion of certain factors, such as fibroblast growth factors (El Ramy *et al.* 2005), TGF-β (Konrad *et al.* 2000) and extracellular matrix (ECM) components (Ailenberg *et al.* 1988).

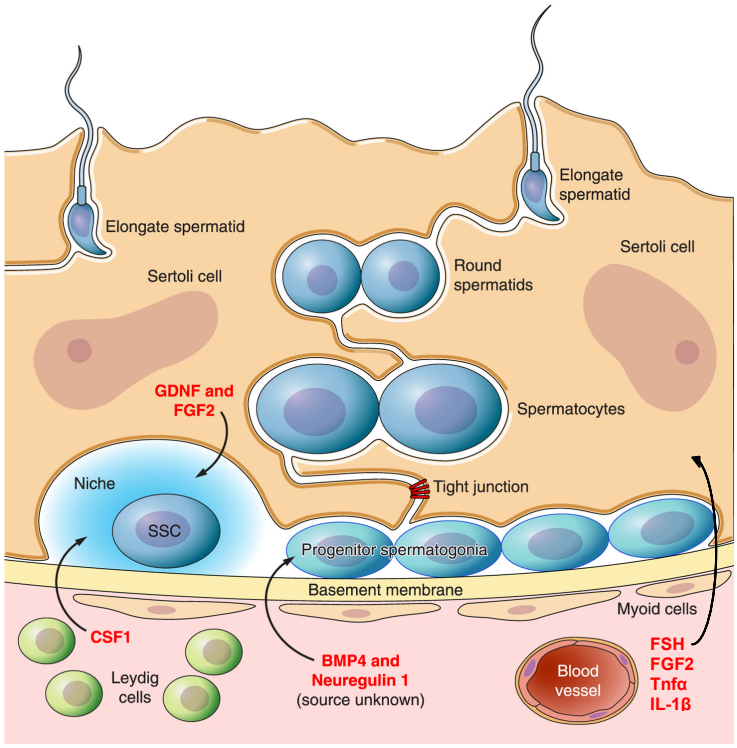

Figure 2. Adapted from Oatley & Brinster 2011 – Schematic illustration of GDNF and its regulators on SSCs.

C. Types of Spermatogonia

In 1978, Holtzer and colleagues stated that, similarly to other adult stem cell systems, spermatogonial stem cells themselves are not the ones ultimately responsible for the generation of differentiated sperm cells, but that this is the role of progenitor cells (Holtzer, 1978). Therefore, it is established that stem cells act as a regenerative reserve; although almost mitotically quiescent they maintain a solid population, while progenitors cells act as a functional reserve providing differentiated cells. However the relation between these three types of spermatogonia (stem, progenitor and differentiating) varies with species.

In the case of rodents, seven generations of type A spermatogonia have been described and subdivided into A_{single} (A_s), A_{paired} (A_{pr}), $A_{aligned}$ (A_{al}) and A1-A4 (Dettin *et al.* 2003; Ehmcke *et al.* 2006) with A_s spermatogonia considered the true spermatogonial stem cells with the ability to self-renew (Huckins 1971; Ehmcke *et al.* 2006). When these cells divide they give rise to A_{pr} spermatogonia. At this stage, these A_{pr} spermatogonia can either 1) complete cytokinesis and produce two new A_s spermatogonia or 2) maintain the

intracellular cytoplasmatic bridges (due to incomplete cytokinesis) and divide again giving rise to a chain of four A_{al} spermatogonia (reviewed in Phillips *et al.* 2010). Further mitotic divisions of A_{al} spermatogonia create chains of 8, 16 and 32 A_{al} spermatogonia (reviewed in Kolasa *et al.* 2012). The A_{pr} and A_{al} spermatogonia expand their clones in an asynchronous manner and independently of the seminiferous epithelium cycle (de Rooij 1998). In 2007, Nakagawa and co-workers have demonstrated the ability of progenitors A_{pr} and A_{al} spermatogonia to acts as potential stem cells with self-renewal ability in cases of injury or natural cell depletion (Nakagawa *et al.* 2007).

In non-human primate mammals, and particularly in the well-studied Rhesus monkey (de Rooij *et al.* 1986; Ehmcke *et al.* 2005a; Ehmcke *et al.* 2005b), the situation is different. The A spermatogonia have been divided into two subtypes: A_{dark} and A_{pale} spermatogonia (reviewed in Ehmcke *et al.* 2006) due to their nuclear staining with hematoxylin (Clermont 1966), and even though both these cells are referred as spermatogonial stem cells, their biological functions are distinct (reviewed in Tokas *et al.* 2011). A_{dark} spermatogonia have been defined as the "true" spermatogonial stem cells, acting as a regenerative reserve (reviewed in Ehmcke *et al.* 2006; Amann 2008). In normal circumstances, these cells are quiescent [(low mitotic activity; (Aponte *et al.* 2005)]. A_{pale} spermatogonia, despite having also self-renewing capacity, act as progenitor cells with the main purpose of continual production of differentiating spermatogonia and are therefore considered a functional reserve (reviewed in Ehmcke *et al.* 2006). These cells are highly proliferative, dividing continuously during each spermatogenic cycle (Clermont 1969). These undifferentiated spermatogonia (true SSCs and progenitor cells) need to be distinguished from differentiating spermatogonia.

Three types of differentiating spermatogonia are common to the majority of species: type A spermatogonia, intermediate spermatogonia and type B spermatogonia (reviewed in Phillips *et al.* 2010). In the mouse model and in contrast to the stem and progenitors spermatogonia, differentiating A1, A2, A3, A4, intermediate and B spermatogonia divide synchronously and are found in specific stages of the seminiferous cycle (reviewed in Phillips *et al.* 2010).

In the Rhesus monkey, upon mitosis, A_{pale} spermatogonia divide into to two new B1 spermatogonia. Further mitotic divisions lead to the production of four B2 spermatogonia, eight B3 spermatogonia and, ultimately, sixteen B4 spermatogonia. Lastly, these B4 spermatogonia differentiate in spermatocytes, which enter meiosis producing spermatids (reviewed in Ehmcke *et al.* 2006; Hermann *et al.* 2010). In humans, after the divisions of A_{pale} spermatogonia, only one generation of B spermatogonia is produced before giving rise to spermatocytes. This means that only two mitotic divisions occur, producing four spermatocytes that, ultimately, produce sixteen spermatids (reviewed in Ehmcke *et al.* 2006).

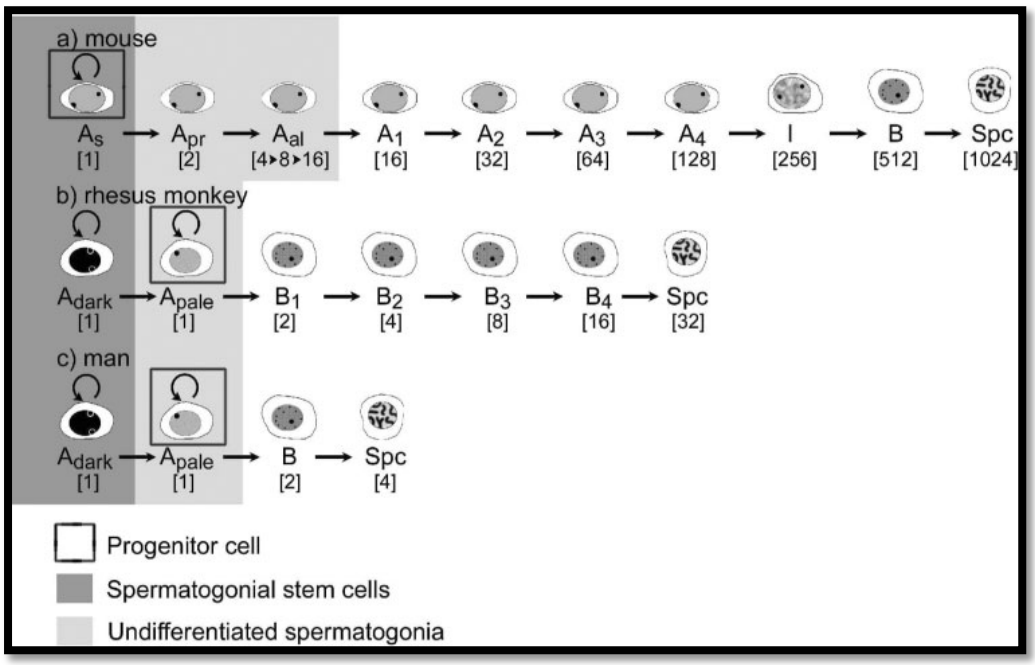

Figure 3. Schematic overview of spermatogoniogenesis in different mammalian species (copied from Ehmcke et al. 2006). The number between brackets corresponds to the theoretical number of cells formed per each spermatogenic cycle if germ cell loss (apoptosis) does not occur.

D. Molecular Markers of Spermatogonial Stem Cells

Germ cell therapies are being increasingly performed and may represent an important tool for assisted reproduction in endangered and genetically valuable species. However, before this technology can be applied directly in (endangered) animals there is a crescent need for the identification of germ

cell-specific markers, that will allow the selection of more purified SSC populations and also, a better analysis of the success of *in vitro/in vivo* culture systems (Vansandt *et al.* 2012). These intracellular and cell surface markers will also represent an important tool for the study of self-renewal, proliferation, differentiation and survival properties of SSCs. While some cell surface markers may be used in the isolation and purification of undifferentiated spermatogonia, intracellular markers, for example transcription factors and some enzymes, are important for the identification of such cell populations in experimental assays such as immunocytochemistry, immunohistochemistry and whole mounts.

Though some spermatogonial markers are species-specific, others are thought to be conserved among a variety of species (reviewed in Kolasa *et al.* 2012). In Table 1 we present some of the markers already described and the species where they were observed.

The first markers described were two laminin receptors - integrin α6 and integrin β1, identified after germ cells sorted using these markers presented a higher colonization rate in transplantation assays (Aponte *et al.* 2005). As cell surface receptors that mediate cell-cell and cell-matrix interactions, they were also described to be required for proliferation, differentiation, survival and migration of SSCs (Kanatsu-Shinohara *et al.* 2004; Hermo *et al.* 2010). Although these markers have been successfully used to enrich a population of SSCs (Aponte *et al.* 2005), they do not seem to be specific for SSCs since they were detected in both undifferentiated as well as in differentiating spermatogonia (Shinohara *et al.* 1999; Kanatsu-Shinohara *et al.* 2008b).

GDNF (glial cell-line derived neurotrophic factor) is necessary for SSC self-renewal, as mentioned above (Meng *et al.* 2000), making GFRα-1 (GDNF family receptor alpha 1) a candidate marker for SSCs in a variety of species.

Table 1. Undifferentiated spermatogonial markers described for several species.

Antigen	Spermatogonia	Species
α6-integrin	Germ cells on the BM	Mouse (Shinohara *et al.* 1999), human (Nickkholgh *et al.* 2014) and bull
β1-integrin	Germ cells on the BM	Mouse (Shinohara *et al.* 1999)
GFRα-1	A_s-A_{al4}, A_d, A_p, B1, B2	Mouse (He *et al.* 2007), rhesus macaque (Hermann *et al.* 2007), human (He *et al.* 2010), bull (Oatley *et al.* 2004) and domestic cat (Vansandt *et al.* 2012; Tiptanavattana *et al.* 2013)
c-KIT	A_{al}-RS	Rodents (Prabhu *et al.* 2006) and goat (Heidari *et al.* 2012)
THY-1	Few germ cells on the BM***	Mouse (Kubota *et al.* 2004), rhesus macaque (Hermann *et al.* 2009), bull (Reding *et al.* 2010), human (He *et al.* 2010) and domestic cat (Vansandt *et al.* 2012)
GPR125	A_s-A_{al}, rare germ cells on the BM	Mouse (Seandel *et al.* 2007), human (He *et al.* 2010) and domestic cat (Vansandt *et al.* 2012)
PLZF	A_s-A_{al}, A_d, A_p, B1, B2, germ cells on BM	Mouse (Buaas *et al.* 2004), pig (Goel *et al.* 2007) and domestic cat (Vansandt *et al.* 2012)
DDX4	A_s-RS	Mouse (Tanaka *et al.* 2000), rhesus macaque (Albert *et al.* 2010), human (Castrillon *et al.* 2000) and domestic cat (Mota *et al.* 2012)
OCT4	A_s-A_{al}, germ cells on BM	Mouse (Ohmura *et al.* 2004), rat (Ryu *et al.* 2005), hamster (Kanatsu-Shinohara *et al.* 2008a), pig (Luo *et al.* 2006) and domestic cat (Vansandt *et al.* 2012)
LIN28	A_s-A_{al}, rare germ cells on the BM	Mouse (Zheng *et al.* 2009), marmoset, rhesus macaque and human (Aeckerle *et al.* 2012)
PGP9.5	Germ cells on the BM	Mouse (Kon *et al.* 1999), rhesus macaque (Tokunaga *et al.* 1999), human (He *et al.* 2010), pig (Luo *et al.* 2006), sheep (Rodriguez-Sosa *et al.* 2006), goat (Heidari *et al.* 2012), bovine (Wrobel 2000) and domestic cat (Mota *et al.* 2012)
α-linked GalNac and β - linked GalNAc (DBA Lectin)	PGCs, gonocytes and few spermatogonia****	Bovine (Ertl & Wrobel 1992; Goel *et al.* 2010; Nasiri *et al.* 2012), pig, horse and llama (Klisch *et al.* 2011)
SSEA-1	A_s-A_{al}, rare germ cells on the BM	Pig (Goel *et al.* 2007), boar (Kim *et al.* 2013), domestic cat (Powell *et al.* 2011)
SSEA-4	A_s-A_{al}, rare germ cells on the BM	Human (Izadyar *et al.* 2011), non-human primates (Muller *et al.* 2008) and domestic cat (Powell *et al.* 2011)
TRA-1-60	Few cells on the BM	Marmoset (Muller *et al.* 2008) and domestic cat (Powell *et al.* 2011)
TRA-1-81	Few cells on the BM	Porcine (Kim *et al.* 2010), non-human primates (Muller *et al.* 2008) and domestic cat (Powell *et al.* 2011)
SALL4	A_s-A_{sl}, A_d, A_p, B	Mouse, marmoset and human (Eildermann *et al.* 2012)
FoxO1	Undifferentiated spermatogonia	Mouse (Goertz *et al.* 2011)

Adapted from (Valli *et al.* 2014). A_s – A single spermatogonia; A_{pr} – A paired spermatogonia; A_{al} – A aligned spermatogonia; A_d – A dark spermatogonia; A_p – A pale spermatogonia; B – type B spermatogonia; BM – basement membrane; RS – round spermatids.

In the mouse, this marker specifically labels A_s, A_{pr}, A_{al} spermatogonia (Meng *et al.* 2000), however, in a study performed by Hofmann and colleagues, it was observed that approximately half of the GFRα-1 positive cells were also c-Kit-positive (a marker of differentiating germ cells), which means that GFRα-1 labels undifferentiated as well as early differentiating spermatogonia, reducing its specificity for SSCs (Hofmann *et al.* 2005).

As mentioned, c-Kit a receptor for the Steel factor (Stem cell factor; SCF) shown to be required for spermatogonial proliferation, survival and adhesion to Sertoli cells (reviewed in Tokas *et al.* 2011), is only expressed in differentiating spermatogonia (Schrans-Stassen *et al.* 1999; Shinohara *et al.* 2000; Prabhu *et al.* 2006), and may be used in the enrichment/isolation of SSCs by negative selection [for example GFRα-1^+/c-Kit^-; (Aponte *et al.* 2005)].

Moreover, GPR125 (G-protein coupled receptor 125), an orphan receptor of the adhesion family present in A_s, A_{pr} and A_{al} spermatogonia in the mouse (Seandel *et al.* 2007), was used by He and co-workers to successfully isolate and purify A_{dark} and A_{pale} spermatogonia in humans using MACS (He *et al.* 2010).

Finally, in the category of cell surface markers, some molecules commonly used to identify undifferentiated pluripotent cells are the glycan cell surface molecules, stage-specific embryonic antigens -1 and -4 (SSEA-1 and SSEA-4) and the tumor-rejection antigens -1-60 and -1-81 (TRA-1-60 and TRA-1-81). These are believed to play an important role in the communication between a stem cell and its niche (Haltiwanger 2002). In 2008 Muller and colleagues (Muller *et al.* 2008) established the potential of SSEA-4 and TRA-1-81 for the isolation/purification of undifferentiated spermatogonia in human and non-human primates. Later, Kokkinaki and co-workers successfully isolated human undifferentiated spermatogonia using SSEA-4 and MACS (Kokkinaki *et al.* 2011).

Among the intracellular markers used to confirm the identity of the undifferentiated spermatogonia, we can find PLZF, DDX4, OCT4, Lin28, FoxO1, PGP9.5 and SALL4. PLZF (promyelocytic leukaemia zinc-finger) is an oncoprotein that belongs to the family of zinc-finger transcription factors

(Ching *et al.* 2010). As mentioned above, PLZF participates in the regulation of the SSC self-renewal ability and stem cell pool (Buaas *et al.* 2004; Aponte *et al.* 2005) by directly repressing the transcription of c-Kit (Filipponi *et al.* 2007). Its expression seems to be restricted to A_s, A_{pr} and A_{al} spermatogonia in mice (Buaas *et al.* 2004; Costoya *et al.* 2004).

DDX4 (VASA homologue), an ATP-dependent RNA helicase that plays a central role in the translational regulation of many important mRNAs, is highly related to the proliferative activity of spermatogonia, being found not only in undifferentiated, but also in differentiating spermatogonia in the mouse (Tanaka *et al.* 2000; Toyooka *et al.* 2000). Its absence drastically reduces the proliferation capacity of germ cells (Spradling *et al.* 2001). However, as this protein is also expressed in more differentiated germ cells, such as spermatocytes and round spermatids it cannot be used to identify the spermatogonia population in germ cells isolated from adult animals (Fujiwara *et al.* 1994b)

LIN28, a small RNA-binding pluripotent stem cell factor, is specifically expressed in mouse undifferentiated A spermatogonia (A_s, A_{pr} and A_{al} spermatogonia; (Zheng *et al.* 2009). Aeckerle and co-workers also described its presence in both marmoset and human adult testis (Aeckerle *et al.* 2012), contrarily to two independent studies (Cao *et al.* 2011; Gillis *et al.* 2011) where it was reported the absence of Lin28 in adult testis.

Another transcription factor described to label undifferentiated spermatogonia is FoxO1 (Forkhead box protein O1). In 2011 Goertz and colleagues have demonstrated its ability to label gonocytes as well as undifferentiated spermatogonia and highlighted the importance of this transcription factor in balancing SSC self-renewal and differentiation by showing *in vivo* that SSC FoxO1 depletion inactivated spermatogenesis in mice testis (Goertz *et al.* 2011).

UCHL1 (ubiquitin carboxy-terminal hydrolase 1), also known as PGP9.5 [protein gene product 9.5; (Heidari *et al.* 2012)] was initially described as a neuron-specific protein important in the nonlysosomal proteolytic pathway (Kon *et al.* 1999). However, it is expressed in the testis and has been used to identify undifferentiated spermatogonia in several species.

SALL4 (Sal-like protein 4; zinc-finger transcription factor) belongs to the transcriptional network that regulates pluripotency, being involved in the modulation of Oct4 expression (Zhang *et al.* 2006) and directly interacting with Nanog (Rao *et al.* 2010) e Plzf (Hobbs *et al.* 2012). This transcription factor is important for stem cell maintenance in various organs during embryonic development; however its expression becomes restricted to gonads postnatally, playing an important role in normal spermatogenesis (Gassei & Orwig 2013).

Finally, DBA, a molecule used to identify undifferentiated spermatogonia is in fact a lectin from *Dolichos biflorus agglutinin (horse gram*). Like other lectins, it has an affinity for sugar residues present in glycoprotein and glycolipids, but specifically binds to two types of glycans α-linked N-acetylgalactosamine (GalNac) and β-linked GalNac (Klisch *et al.* 2011). DBA has been used, not only to identify, but also to isolate undifferentiated spermatogonia, using methods such as MACS and FACS (Fluorescence activated cell sorting) from bovine testis (Herrid *et al.* 2009), since it detects both internal and membrane bound glycans.

III. Spermatogenesis and Spermatogonial Stem Cells in Felines

A. Spermatogenesis

In all mammalian species spermatogenesis is a cyclic and highly regulated continuous process that maintains the production of differentiated sperm throughout the life of mature males (Sadava *et al.* 2011). This is one of the most productive self-renewal systems in the male body, leading to the daily production of millions of mature sperm (Costa *et al.* 2006). In domestic cats, this process begins around 4 months, after a pre-pubertal period, and at 8 months these animals are consistently producing sperm (Tsutsui *et al.* 2004).

Spermatogenesis consists in three sequential stages: spermatogoniogenesis - the proliferative phase, in which spermatogonia undergo several mitotic divisions; meiosis - when the genetic material of spermatocytes is duplicated, recombined and segregated and lastly the differentiation phase - spermiogenesis – where round spermatids derived from secondary spermatocytes undergo several morphological modifications leading to the production of a terminally differentiated, haploid and elongated sperm cells (reviewed in Costa *et al.* 2006).

As mentioned in the first phase spermatogonial germ cells undergo successive mitoses (Holstein *et al.* 2003). These cell divisions are frequently incomplete due to incomplete cytoplasmic division (cytokinesis), although nuclear division (karyokinesis) is complete (Johnson & Everitt 2007). Due to the incomplete cytokinesis, cytoplasmic bridges connect the daughter cells resulting in a syncytium of spermatogonial cells currently denominated by clone cells (Holstein *et al.* 2003). In the cat, Blanco-Rodriguez described the presence of six generations of spermatogonia after labeling the cells in S phase with BrdU [Bromodeoxyuridine – nucleotide analog; (Blanco-Rodriguez 2002)]. In disagreement, França and Godinho, relating the number of preleptotene spermatocytes formed for each type A spermatogonia estimated the occurrence of four to five spermatogonial generations (França & Godinho 2003). An abnormal, yet common, feature of this phase in the cat is the elimination of an entire syncytium of spermatogonia that leads to the occurrence of “missing generations”, although the molecular mechanisms behind this phenomena are still unknown (França & Godinho 2003).

In meiosis, the primary spermatocytes enter the first meiotic pro-phase which is very prolonged and consist of several different stages (Holstein *et al.* 2003). The Leptotene stage, which involves chromatin condensation, is followed by chromosome thickening and homologous chromosome pairing (Zygotene stage), in preparation for *crossing-over and* chromatid exchange (Pachytene stage). This is the longest phase also characterized by increases in both nuclear and cytoplasmic volume. Finally, in the Diplotene stage, the chromosomes become tightly bound to their homologue at the chiasmata, the section where *crossing-over* and the exchange of DNA occurs (Snustad &

Simmons 2008). Meiosis I is completed after the separation of homologous chromosomes to opposite edges of the cells (through metaphase, anaphase, telophase and incomplete cytokinesis) and, as result, one primary spermatocyte gives rise to two secondary spermatocytes. Finally, the secondary spermatocytes originate the round spermatids that contain a single set of chromosomes via meiosis II (Johnson & Everitt 2007). In 2002, Blanco-Rodriguez, described the occurrence of an increased apoptosis during profase I of domestic cat germ cells, mainly in zygotene and pachytene spermatocytes (Blanco-Rodriguez 2002), contrarily to other species such as stallions were significant degeneration of B spermatogonia was observed (reviewed in Johnson *et al.* 2000). This was also supported by the low meiotic index (2.8 instead of 4) described by França and Godinho (2003) in the domestic cat.

During the last phase of spermatogenesis, different modifications take place in the spermatids. The chromatin condenses, the acrosome cap (enzyme reservoir) is formed and attached to the nucleus, flagellum structures are formed (Holstein *et al.* 2003) and spermatids lose a big part of their cytoplasm (residual bodies), which is phagocytized by Sertoli cells. Once this process is concluded, terminally differentiated sperm cells are formed and undergo another process denominated spermiation, in other words, these cells are released into the lumen of seminiferous tubules towards the epididymis (De Kretser *et al.* 1998). Abnormalities during this last phase of spermatogenesis may result in the production of teratospermic ejaculates, very common in felid species, and characterized by a high percentage of morphologically abnormal sperm. The molecular mechanisms behind this process are still unknown, but a few factors are believed to interfere in the regulation of the numbers of structurally normal sperm. In 2004, Neubauer and colleagues studied the occurrence of this phenomenon using a domestic cat model (Neubauer *et al.* 2004) and observed that teratospermic animals had a higher output of sperm, compared to normospermic cats. Overall, the gain in sperm output in teratospermic animals was possible at the expense of sperm quality, which was associated with a germ cell overload of Sertoli cells and also due to the defective elimination of abnormal cell (impaired phagocytosis and apoptosis).

For all mammallian species the spermatogenic cycle is organized/divided in stages. These stages, each one containing different cell associations, are classified based on general composition of the seminiferous tubules, occurrence of meiosis, changes in location and shape of the spermatid nuclei (Berndtson 1977), development of the acrosome and also the morphology of the developing spermatids (Leblond & Clermont 1952; Russell *et al.* 1990).

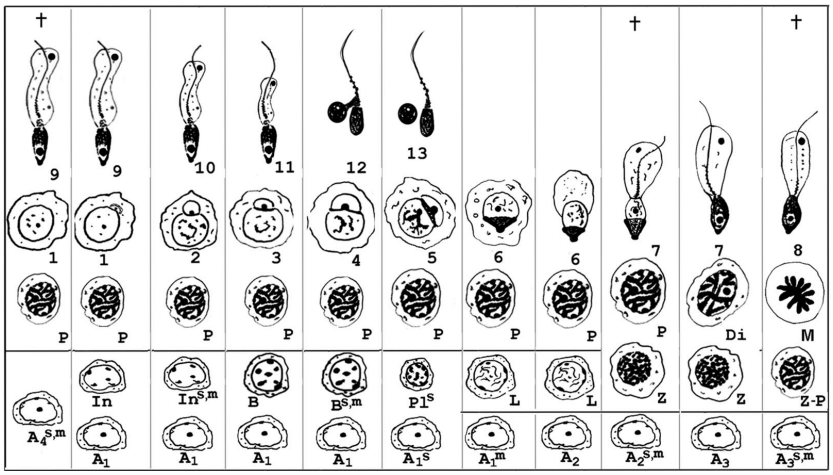

Figure 4. Schematic representation of the spermatogenic cycle by Blanco-Rodriguez (2002) and by França and Godinho (2003)

The disappearance of a specific cell association and its reappearance in the same area of the seminiferous tubule constitutes the seminiferous epithelium cycle, and the time required for this process defines its duration (Leblond & Clermont 1952). Böhme and Pier performed the first study, dividing the spermatogenic cycle in 8 stages; state I was defined by the appearance of round spermatids, stage IV corresponded to spermiation and stage VIII to meiosis (Bohme G & Berl 1986). More recently, França and Godinho also described that the seminiferous epithelium cycle of the domestic cat could be divided into 8 stages, with a few differences; stage I corresponded to the absence of elongated spermatids, stage IV corresponded to the meiotic division of spermatocytes and stage VIII to spermiation. These authors also referred that the frequency of different stages varied in a species-specific

manner and that in the cat the spermatogonial, meiotic and spermiogenic phases took approximately one third of the spermatogenic process, each. Furthermore, the length of the spermatogenic cycle was also determined with each seminiferous epithelium cycle taking 10.6 days, and in agreement with the notion of 4.5 epithelium seminiferous cycles per spermatogenic cycle, 40.6 days are required for the entire spermatogenic process to take place (França & Godinho 2003). Blanco-Rodriguez also described the occurrence of eight stages but ordered and further divided them in sub-stages in a manner more similar to the mouse seminiferous epithelium cycle (Blanco-Rodriguez 2002; Blanco-Rodriguez *et al.* 2003).

B. Spermatogonial Stem Cells

1. Types of Spermatogonia and possible Expansion Model

Contrarily to mouse, Rhesus monkey and humans, whose spermatogoniogenic models are well established, in cats the same does not occur. Despite attempts to describe the spermatogonial clonal expansion and cell types produced, the identification of the spermatogonial stem cell as well as the progenitor cell, and their regulatory mechanisms, is lacking. Therefore, it is yet to establish if the spermatogenic process in felids resembles more the mice model or the model described for higher order mammals. Several studies performed in domestic cats as well as in wild felids simply described/divided the spermatogonial cells into type A, intermediate and B spermatogonia, with no indication regarding which cells might act as spermatogonial stem/progenitor cells and which cells are differentiated cells. There was also no further evidence of the existence of different subtypes of type A or B spermatogonia (Blanco-Rodriguez 2002; França & Godinho 2003; Balarini *et al.* 2012), as is the case in other species (e.g. mouse and *Rhesus monkey*). Indeed, the identity both morphological and biochemical of the felid stem/progenitor cells capable of supporting sustained spermatogenesis is of vital importance for the success of some recovery strategies, namely for germ

cell transplantation techniques. In this technique only a small number of germ cells can be injected through the efferent ducts or directly in the rete testis. If a mixed germ cell suspension is injected in the testis, only a small portion of cells will be able to attach and invade the seminiferous epithelium to the basal membrane, were through proliferation and migration, will colonize the adjacent stem cell niches.

Thus, transplanting a purified population of SSCs/undifferentiated spermatogonia rather than a mixture of testicular cells will increase the odds of recipient testis colonization and sustained spermatogenesis.

Taking everything into consideration, there is an increasing need for a general improvement regarding identification of specific markers for felids as well as a defined characterization of the undifferentiated spermatogonia.

2. Germ Cell Transplantation Experimental Studies

As mentioned already, given the current status of endangered felids, several recovery strategies have been attempted to overcome the situation. However the latest developments have been focused on the germ cell transplantation technique.

In 2006, Kim and colleagues carried out the first attempt of germ cell transplantation using a mixture of domestic cat spermatogenic cells (i.e. type A, In and B spermatogonia; spermatocytes, spermatids and spermatozoa). These cells were successfully transplanted into the testis of a recipient mice, however, production of differentiated sperm cells did not occur. This phenomenon has been observed in other species and authors have attributed it to the phylogenetic distance between donor and recipient animal. In these experiments the donor germ cells were able to colonize and proliferate within the host seminiferous tubules, but were incapable of undergoing differentiation (Kim *et al.* 2006). Another limitation of this first study was the fact that they did not performed any enrichment protocol for more undifferentiated germ cells, and therefore, the percentage of germ cells with colonization capacity in the mixture of all the testicular cells was very low.

More recently, Silva and colleagues performed a study with the same aim,

however this time the germ cells were transplanted from an ocelot (*Leopardus pardalis*) to a domestic cat [*Felis catus*) (Silva *et al.* 2012)], two phylogenetic-related species, which allowed the production of ocelot sperm in the seminiferous tubules of the domestic cat. Despite the major improvements in comparison to the previous study, the authors failed to clearly control some aspects. For example, they used differential platting and density gradient centrifugation to enrich the cell suspensions in undifferentiated germ cells. This fraction presented 70% of type A spermatogonia (characterized histologically) and an unknown percentage of GFRα-1 positive cells (characterized by immunocytochemistry). However, this isn't an indisputable result, since GFRα-1 labeling has not been satisfactorily proven to be a marker of undifferentiated spermatogonia in the cat and, as mentioned above, has also been observed in differentiating spermatogonia in other species (Hofmann *et al.* 2005).

Even though the germ cells transplanted were able to differentiate in sperm cells, the spermatogenic development was only monitored until 13 weeks after the transplantation, corresponding only to two spermatogenic cycles. These may be supported by the presence of progenitor/differentiated spermatogonia, and therefore, no evidence of a sustained and permanent spermatogenesis supported by spermatogonial stem cells was given. Also, no tests were performed to check the fertilization capacity of the sperm produced.

Although promising, these studies failed to confirm the real nature (stem, progenitors or undifferentiated spermatogonia) of the transplanted cells, did not indicate the colonization rate and did not perform longtime follow-up as well as monitor efficacy of donor spermatogenesis in the recipient testis.

However we can deduce from all the studies conducted in the domestic cat and in other species that, transplanting a purified population of s/undifferentiated spermatogonia rather than a mixture of testicular cells, will increase the odds of recipient testis colonization and sustained spermatogenesis.

The studies also point to an increasing need for a general improvement regarding identification of specific markers for felids as well as a defined characterization of the undifferentiated spermatogonia.

3. Molecular Markers of Cat Spermatogonial Stem Cells

Out of the entire spermatogonial cell markers described previously, only a few have been described and used for various purposes in felids. In 2012, Vansandt and colleagues aimed to determine whether SSC markers initially identified in rodents would also be useful in the identification of felid SSCs. Although these authors were able to observe testis mRNA expression of some common markers, such as Thy1, GPR125, GFRα-1, Plzf, UCHL1 (or PGP9.5) and OCT4 using RT-PCR, there were no differences between young and adult animals or between whole testis and enriched germ cells single cell suspensions (Vansandt *et al.* 2012). These observations go against the expected results: 1) young animals present a higher SSCs to total germ cells ratio than adult animals and therefore should present higher levels of the SSC markers; and 2) an enriched germ cell suspension, with decreased contamination by somatic cells, would also present higher values for these markers than the whole testis single cell suspension. Also, no follow up studies were performed in order to confirm if these mRNA transcripts are producing proteins and if the protein expression was restricted to a subpopulation of cells adjacent to the basal membrane of the seminiferous tubules.

PGP9.5, a marker of undifferentiated spermatogonia in primates and bovines, was detected in all types of spermatogonia in paraffin-embebbed and cryoprocessed sections of adult domestic cat testis [(anti-PGP9.5; Z5116, Dako, Denmark) (Mota *et al.* 2012)] and therefore cannot be used to characterize SSCs in adult testis. Also in the same study DDX4, a marker of immature and undifferentiated germ cells in other species, was observed in germ cells ranging from spermatocytes (zygotene stage) to round spermatids in adult testis (Mota *et al.* 2012).

Using immunohistochemical assays Powell and colleagues described staining for SSEA-1, SSEA-4, TRA-1-60, TRA-1-81, and Oct-4 specifically at the basement membrane of the seminiferous tubules in both adult and prepubertal testes. The GPR125 marker was detected at the basement

membrane of the seminiferous tubules and across the seminiferous tubule section, as well as GFRα-1 (Powell *et al.* 2011), contrarily to what was described previously (Silva *et al.* 2012). Flow cytometry quantification of labeled cells showed a very small portion of SSEA-1 (7%) and SSEA-4 (3%) positive cells, making these pluripotency markers serious candidates for domestic cat SSC markers (Powell *et al.* 2011).

Although promising the variable results obtained, regarding domestic cat spermatogonial stem cell markers, in the different techniques used and studies performed attest the necessity of further studies in this area. The description of a reliable marker for domestic cat spermatogonial stem cells will be useful not only in transplantation assays but also in studies of factors affecting spermatogenesis and in the development of an *in vitro* system to produce sperm.

GENERAL OBJECTIVES

As referred above, the worrying scenario regarding endangered feline species contributes to an increased need for feline reproduction development and in particular spermatogonial stem cell characterization for posterior sorting and transplantation. Our overall goal was, therefore, to identify possible markers for these cells and characterize their behavior as a population. To achieve this we went on to:

- Define possible spermatogonial/germ cell markers,
- Identify markers for restricted subpopulations of spermatogonia,
- Study the correlation of the spermatogonial populations with the spermatogenic cycle,
- Study the influence of the extra tubular environment in the spermatogonial subpopulations,
- Test the applicability of the spermatogonial markers in *in vitro* assays.

MATERIALS AND METHODS

I. Animals

Cat testes were obtained at local veterinarians clinics from cats castrated under general anesthesia. The tissue was kept in ice-cold transport medium [DMEM/F12 medium (31330-038, Gibco Life Technologies) supplemented with 1% nonessential aminoacids (NEAA, M7145-100ml, Sigma-Aldrich), 2% Fungizone (15290-026, Gibco Life Technologies) and 2% antibiotics - penicillin/streptomycin (15140-122, Gibco Life Technologies)] until further manipulation.

1. Immunohistochemistry

Cat testes were fixed in Bouin's (HT10132, Sigma-Aldrich) solution for 24 hours and then transferred and stored in 70% ethanol. Testis tissue was latter embedded in paraffin wax and cut into 5 µm thick cross-sections and stored until further use. Testis tissue sections were deparaffinized (Xylene, 2x 10 minutes) and rehydrated in a graded series of ethanol (99% ethanol 2x 10 minutes; 96%, 80% and 70% ethanol for 10 minutes each; tap water for 15 minutes, distilled water for 1-2 minutes and washed in Tris buffered solution (TBS – 90g NaCl, 61g Trizma base, pH 7.6), 2x 5 minutes). For antigen retrieval, sections were heated in a water bath in either citrate buffer (1.47g Tris sodium citrate, 0.25 ml Tween 20, pH=6) or Tris-EDTA buffer (1.21g Trizma base and 0.37g EDTA, pH=9) for 10 minutes at 100°C, followed by a cooling period until a temperature of 35º-40ºC (approx. 20 minutes) was reached. A treatment with hydrogen peroxide (3% for 15 minutes) was performed to abolish endogenous peroxidase activity, followed by washing steps with distilled water and TBS. Blocking of nonspecific antibody binding was accomplished with incubation of the tissue sections with 25% normal goat serum (G9023, Sigma-Aldrich) diluted in TBS with 0.5% BSA (A7906, Sigma-Aldrich) for 30 minutes at room temperature. The slides were incubated with primary antibody overnight at 4°C in a humidified chamber (according to

Table 2). For unspecific labeling controls, sections were incubated in blocking solution containing 25% goat serum and 0.5%BSA+TBS without primary antibody (negative control) or with rabbit IgG (I5006, Sigma-Aldrich; control antibody).

Table 2. Antibodies used in immunohistochemistry assays.

Primary Antibody			
Antibody	Concentration	Manufacture	Secondary Antibody
PGP9.5	1:100-1:250	[Z5116] Dako, Glostrup, Denmark	Goat anti-rabbit-biotinilated Goat anti-rabbit-horseradish peroxidase
LIN28	1:20	[NBP2-19355] Novus Biologicals, Littleton, CO [ab46020] Abcam, Cambridge, UK	Goat anti-rabbit-biotinilated
DDX4	1:50-1:100	[ab13840] Abcam, Cambridge, UK	Goat anti-rabbit-biotinilated
GFRα-1	1:20-1:100	[sc-10716] Santa Cruz Biotechnology, Santa Cruz, CA	Goat anti-rabbit-biotinilated
α6-integrin	1:20-1:100	[sc-10730] Santa Cruz Biotechnology, Santa Cruz, CA	Goat anti-rabbit-biotinilated
GPR125	1:20-1:500	[ab51705] Abcam, Cambridge, UK	Goat anti-rabbit-biotinilated
SSEA-4	1:100	[#MAB4304] Millipore, Billerica, MA	Goat anti-mouse-biotinilated
c-KIT	1:20-1:500	[sc-168] Santa Cruz Biotechnology, Santa Cruz, CA	Goat anti-rabbit-biotinilated
FoxO1	1:100	[#2880], Cell Signalling Technology, Danvers, MA	Goat anti-rabbit-biotinilated
SALL4	1:50	[ab57577] Abcam, Cambridge, UK	Goat anti-mouse-biotinilated
CXCR4	1:100	[ab2074] Abcam, Cambridge, UK	Goat anti-rabbit-biotinilated
CXCL12	1:100	[sc-28876] Santa Cruz Biotechnology, Santa Cruz, CA	Goat anti-rabbit-biotinilated
Lectins	Concentration	Manufacture	Detection System
DBA	15µg/ml	[L6533] Sigma-Aldrich, St Louis, MO	streptavidin-horseradish-peroxidase (ST-HRP)

The following washing steps were carried out in TBS (3 x 5 minutes). The secondary antibodies (1:200, RT) used according to Table 2 were incubated for 1 hour. After a washing step (3x 5 minutes TBS) the sections were incubated with streptavidin-horseradish peroxidase (ST-HRP)/ streptavidin-alkaline phosphatase (ST-AP) for 30 minutes (1:500 and 1:150, respectively,

at RT), except when anti-rabbit-HRP was used as secondary antibody. All these were washed and staining was finally visualized after incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB, in urea buffer; D4168, Sigma-Aldrich) and Fast Red TR/Napthol AS-MX Phosphatase (4-Chloro-2-methylbenzenediazonium/3-Hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate, in Tris buffer; F4648, Sigma-Aldrich), respectively, until color developed as observed under microscope. Positive staining appeared as a brown (HRP - DAB) or as a red precipitate (AP - TR/Napthol) in the cells.
After washing the slides in deionized water, samples were counterstained with hematoxylin for 5-10 minutes and washed in tap water for 15 minutes. After a quick step in distilled water slides were mounted with a aqueous based mounting medium (VectaMount, H-550 Vector Laboratories, Inc. Burlingame, CA, USA).

B. Morphometric Analysis

Morphometric analysis was performed for positive spermatogonial markers, this included PGP9.5 and DBA lectin. Quantification of the number of labeled cells was performed using *Image J* free software. A grid with a fixed size (five square inches) was placed over several images taken with Leica Microscope DM4000 at 200x magnification from testis sections from 5 to 8 animals, stained with DBA and PGP9.5, respectively. The positive cells were manually identified, counted and related to the area determined according to the grid.
As previously mentioned, the extra-tubular components in direct contact with the pool of spermatogonial stem cells might influence several aspects of their dynamic, from stem maintenance to their differentiation. To assess any possible correlations, morphometric analysis was performed using round seminiferous tubules, and counting the number of DBA^+ cells present near each defined region - near Leydig cells, near another seminiferous tubule and near interstitial space with or without blood vessels.
Briefly, several images of rounded shaped, staged seminiferous tubules were acquired with Leica Microscope DM4000 at 100x magnification. For every staged seminiferous tubule, the angle of the different compartments

surrounding the seminiferous tubule was measure as shown in Figure 5 using *Image J* free software. This was performed for at least 10 seminiferous tubules of each stage described for domestic cat spermatogenesis (approximately 80 tubule sections per animal, for 4 animals).

After that, the number of DBA labeled cells in each region was counted and divided by the angle of the region to normalize the data.

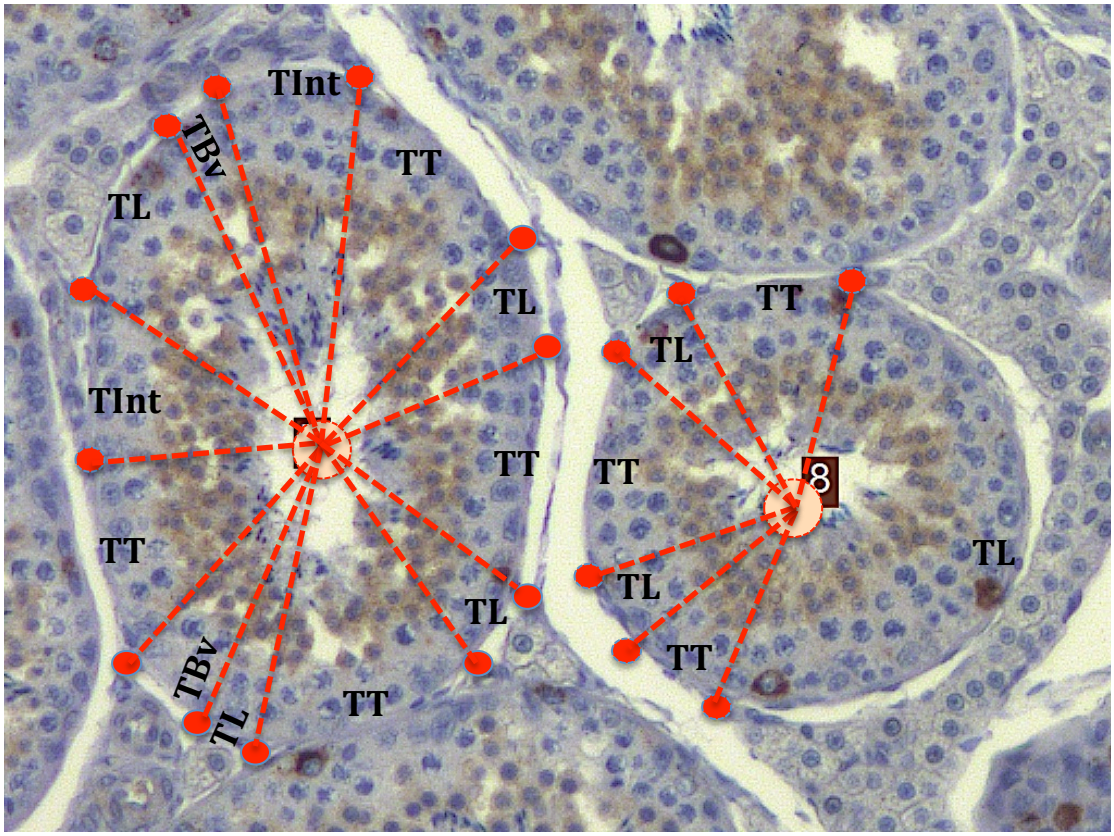

Figure 5. Schematic representation of the morphometric analysis performed in domestic cat seminiferous tubules cross-sections. TBv: tubule-blood vessel region, TT: tubule-tubule region; TL: tubule-Leydig cell region and TInt: tubule-interstial space containing blood vessels region.

II. Whole Mount Staining

Domestic cat testis tissue was partially digested using a digestion medium containing 10 mg of collagenase diluted in DMEM/F12 (31330-038, Gibco - Life Tecnhologies, NY, USA) for 10 minutes at 37ºC with manual agitation every 2-3 minutes. Afterwards, the tubules were gently separated using thin

tweezers and immersed in Bouin's fixative solution for 24 hours and then transferred and stored in 70% ethanol.

Briefly, whole mount staining was performed by rehydrating pieces of testis tissue in a graded series of ethanol: 70% ethanol 3 times for 10 minutes, 50% and 35% ethanol for 15 minutes, each, and finally in distilled water for 15 minutes.

Further isolation of seminiferous tubules and antigen retrieval were performed with a solution of hydrogen chloride (for 30 minutes at room temperature) followed by 0.1% trypsin (for 1 hour at 37ºC). After washed (3 x 10 min) with TBS, the seminiferous tubules suspensions were incubated with the primary antibody, rabbit anti-PGP 9.5 ([Z5116] 1:100, Dako, Denmark) or with the lectin DBA ([L6533] 15 µg/ml, Sigma- Aldrich) overnight at 4°C with agitation, followed by washing steps in TBS (3 x 10 minutes). For secondary antibodies, the tissues incubated with anti-PGP9.5 were incubated with goat anti-rabbit-biotinilated, followed by incubation with streptavidin-horseradish-peroxidase (ST-HRP) and those incubated with the lectin DBA were directly incubated with ST-HRP for 1 hour at room temperature. Following incubation and washing steps (3 x 10 minutes in TBS), all tissue pieces were stained using DAB system for 5 to 15 minutes and mounted in an aqueous mounting medium.

A. Morphometric Analysis

Once again morphometric analysis was performed for spermatogonial cells labeled with DBA lectin. Quantification of the number of labeled cells was performed using *Image J* free software. A grid with a fixed size (five square inches) was placed over several images taken with Leica Microscope DM4000 at 200x magnification from whole mounts of 3 animals. The positive cell-clones were manually identified, counted and related to the area determined according to the grid.

III. Germ cell isolation

As mentioned, all testes from male domestic cats were obtained from local veterinary clinics following routine castration procedures. After transportation testes were weighted and decapsulated. One quarter of one testis was fixed in Bouin's solution, one quarter used for protein extraction (dipped in liquid nitrogen and stored at -80ºC) and the remaining tissue was placed in basic medium - DMEM/F12 medium (31330-038, Gibco Life Technologies) supplemented with 1% nonessential aminoacids (NEAA, M7145-100ml, Sigma-Aldrich), 1% Fungizone (15290-026, Gibco Life Technologies) and 1% antibiotics penicillin /streptomycin (15140-122, Gibco Life Technologies) - and finely minced with scissors until approximately 1 mm cubic size pieces were obtained. This tissue was transferred to a digestion medium containing DMEM/F12, collagenase (1mg/ml; C2674, Sigma-Aldrich) and DNAse (15U/ml; D4547, Sigma-Aldrich), followed by incubation in a 37ºC water bath for 25 minutes with manual agitation every 2-3 minutes. The cell suspension was divided in two portions. One was used for protein extraction and cell analysis (procedure described below) and the tubules in the other portion of cell suspension were separated from interstitial cells by elutriation at unit gravity for 5 minutes. The supernatant was removed and tubules were resuspended in a second digestion medium containing DMEM/F12, collagenase (1mg/ml), hyaluronidase (1mg/ml; H3506, Sigma-Aldrich) and DNAse (15U/ml). This mix was shacked every 2-3 minutes and aspirated every 5 minutes with a 1 ml-pipet-tip (20x) until the solution turned milky and bigger cells lumps were absent (incubation in a 37ºC water bath for 15 minutes). Cells were centrifuged at 200 g for 10 minutes at 4ºC, the supernatant discarded and cells resuspended in basic medium. Cells were divided, half were used to extract protein and the remaining was allowed to adhere to Poly-L-lysine coated coverslips for 30 minutes in 24-well plate. Afterwards cells were fixed with 4% formaldehyde for 30 minutes and finally stored in PBS with 0.01% sodium azide.

IV. Immunocytochemistry

The cell fractions obtained from the testicular digestion were used to perform some immunocytochemistry with some of the markers used in the immunohistochemistry experiments to assess the presence of spermatogonia in the different digestion steps. Briefly, the cell fractions were permeabilized with 1% Triton-X in PBS for 30 minutes at RT followed by a blocking step with PBS plus 100 mM glycine and 0.1% BSA. Incubation with primary antibody diluted in blocking reagent, rabbit anti-PGP9.5 (1:100 [Z5116] Dako, Denmark) and with the lectin DBA (15µg/ml, [L6533] Sigma-Aldrich, St Louis, MO) was performed for 1 hour at 37ºC. The cells were then washed in PBS plus 0.1% Triton-X (3x 5 minutes with agitation), followed by secondary antibody incubation with anti-rabbit-Alexa488 and streptavidin-Alexa564 solution for 1 hour at RT in the dark. After, the "secondary antibody solution" was aspirated and replaced with a DAPI solution (1 µg/ml) for another 10 minutes in the dark. Following a washing step (3x 5 minutes PBS plus 0.1% Triton-X) the cells were mounted using mounting medium (Vectashield, Vector Laboratories, Inc. Burlingame, CA, USA).

V. Western Blot

Proteins extracts were prepared from whole testis tissue and after the first and second digestion steps (enzymatic digestion explained below). Briefly, small pieces of tissue, placed in a mortar, were grounded with a pestle while a small quantity of liquid nitrogen was added, until a powder was achieved. After that, approximately 20 mg of tissue were weighed and placed in 400 µl of lysis buffer - RIPA (R0278-50ML, Sigma-Aldrich), PMSF (78830-5G, Sigma-Aldrich) and CLAP (AEBSF–[4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride]; Aprotinin; Bestatin hydrochloride; E-64–[N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide]; Leupeptin hemisulfate salt and Pepstatin A; P8340-1ML, Sigma-Aldrich), vortexed and placed on ice and on a shaker for 2 hour. At the end the samples were centrifuged (20 minutes

at 12000rpm and 4ºC) and the pellet discarded.

Protein extraction from isolated cells followed the same protocol. Briefly, isolated cells were added to lysis buffer (100 µl/1.5 million cells), vortexed and placed on ice and on a shaker for 30 minutes. After, samples were centrifuged (20 minutes at 12000rpm and 4ºC) and the pellet discarded. Protein determination was performed using the Pierce BSA method (Pierce® BCA Protein Assay Kit #9981L) according to the manufacture instructions. Protein extracts were separated with a SDS-PAGE Electrophoresis System, transferred onto PVDF membrane and probed with antibodies against several markers. Briefly, 30 µg protein samples were resuspended in sample buffer - 950 µl 2x Laemmli buffer (Bio Rad #161-0737) and 50 µl β-mercaptoheptanol (M7522, Sigma-Aldrich - loaded and electrophoresed on 8 and 12% Tris gels (Table 3) according to the target proteins' molecular weight.

Table 3. Composition of the gels used in Western Blot assays.

Resolving Gel	**8%**	**12%**
Distilled water	4.05 ml	3.3 ml
1.5M Tris pH 8.8 (BioRad #161-0798)	1.875 ml	1.875 ml
10% SDS	75 µl	75 µl
40% Acrylamide (BioRad #161-0148)	1.5 ml	2.25 ml
30% APS (BioRad #161-0700)	112.5 µl	112.5 µl
TEMED (BioRad #161-0800)	11.25 µl	11.25 µl

Stacking Gel	**2 Gel**	**1 Gel**
Distilled water	6.4 ml	3.2 ml
0.5M Tris pH 6.8 (BioRad #161-0799)	2.5 ml	1.25 ml
10% SDS	100 µl	50 µl
40% Acrylamide (BioRad #161-0148)	1 ml	0.5 ml
30% APS (BioRad #161-0700)	50 µl	25 µl
TEMED (BioRad #161-0800)	10 µl	5 µl

Protein transfer was performed after PVDF membranes were activated (1-2 minutes in methanol, 5 minutes in distilled water and 15 minutes in transfer

buffer - 25mM Tris, 190mM Glycine and 200ml methanol) at a constant voltage according to gel type (12% gel – 100V:90minutes and 8% gel – 70V:3hours) at 4ºC. After transfer PVDF membranes were washed in TBS (24.2g Trizma base and 1.37g NaCl) and blocked with 5% nonfat milk (Bio Rad #170-6404) for 1 hour at RT on a shaker. Incubation with primary antibodies against the antigens described in Table 4 was performed overnight at 4ºC. After that, the membranes were washed in TBS-T [TBS plus 0.1% Tween 20 (3 x 15 minutes)] and incubated with a 1:3000 dilution of a secondary solution (in accordance with the species of the primary antibody) conjugated with horseradish peroxidase or streptavidin-horseradish-peroxidase (HRP) solution (DBA lectin) for 1 hour. Finally, the membranes were again washed in TBS-T (3 x 15 minutes) and developed with the ECL system (Bio Rad #170-5070) according to the manufacturer's instructions.

Table 4. Antibodies used in Western Blot assays.

Antibody	Concentration	Manufacture
PGP9.5	1:200	[Z5116] Dako, Denmark
LIN28	1:1000	[NBP2-19355] Novus Biologicals, Littleton, CO
DDX4	1:1000	[ab13840] Abcam, Cambridge, UK
GFRα-1	1:200	[sc-10716] Santa Cruz Biotechnology, Santa Cruz, CA
c-KIT	1:200	[sc-168] Santa Cruz Biotechnology, Santa Cruz, CA

VI. Statistical Analysis

Statistical analysis was performed using SPSS (Statistical Package for the Social Sciences Program), version 21.00, software for mac (SPSS Inc., Chicago, IL, USA). All variables were checked for normal distribution.

Population comparisons were performed using K independent samples non-parametric test (Kruskal-Wallis H test) to analyze the tubule region effect.

Further discrimination between group differences was assessed by Mann Whitney U-test. $p<0.05$ was considered significant (results are presented as Mean ± S.E.M. of the number of experiences indicated).

Correlations (Pearson) were calculated to assess a relation between testis weight and DBA^+ cells in cross-sections of testis tissue.

Graphical representation was perfumed using SPSS and Excel, version 14.4.1 for mac.

RESULTS AND DISCUSSION

I. Germ Cell Markers

As previously mentioned, numerous markers have already been tested in several species and a few in the cat. In this work we probed domestic cat testis tissue with several different markers aiming to label and identify undifferentiated spermatogonia/spermatogonial stem cells. However, as we will further describe, our assays provided a variety of results.

A. Unsuccessful Markers in Domestic Cat

1. SSEA-4, c-Kit and GRP125

Although SSEA-4 has been described in the cat (Powell *et al.* 2011) our antibody, tested by immunohistochemistry and using human testicular tissue as an accepted positive control, didn't recognize any antigen on testicular cells from either cat or human (Figure 6 A-B). We were unable to test the antibody described in the previous study (Powell *et al.* 2011) since only abstracts of the work presented in congresses were available. As conclusion, we cannot discard this marker only the antibody we used since no positive staining was observed in the positive control used.

The c-Kit antibody, tested as a possible negative selection marker, used in this study has successfully labeled differentiating germ cells in other species. However, in the cat only a general unspecific labeling was observed either in low or high concentrations of antibody (Figure 6 C-D). Western blot analysis revealed labeling of several protein bands (data not shown) and discards the use of this antibody in domestic cat.

Even though the presence of GPR125 mRNA has been described in isolated cat testicular cells (Vansandt *et al.* 2012) and that the antibody we used has successfully labeled undifferentiated human spermatogonia (He *et al.* 2010), similarly to c-Kit, the surface receptor GPR125 presented a high unspecific

labeling in all testicular cells in the several concentrations tested (Figure 6 E-F) rendering the use of this antibody in feline SSC identification studies impossible.

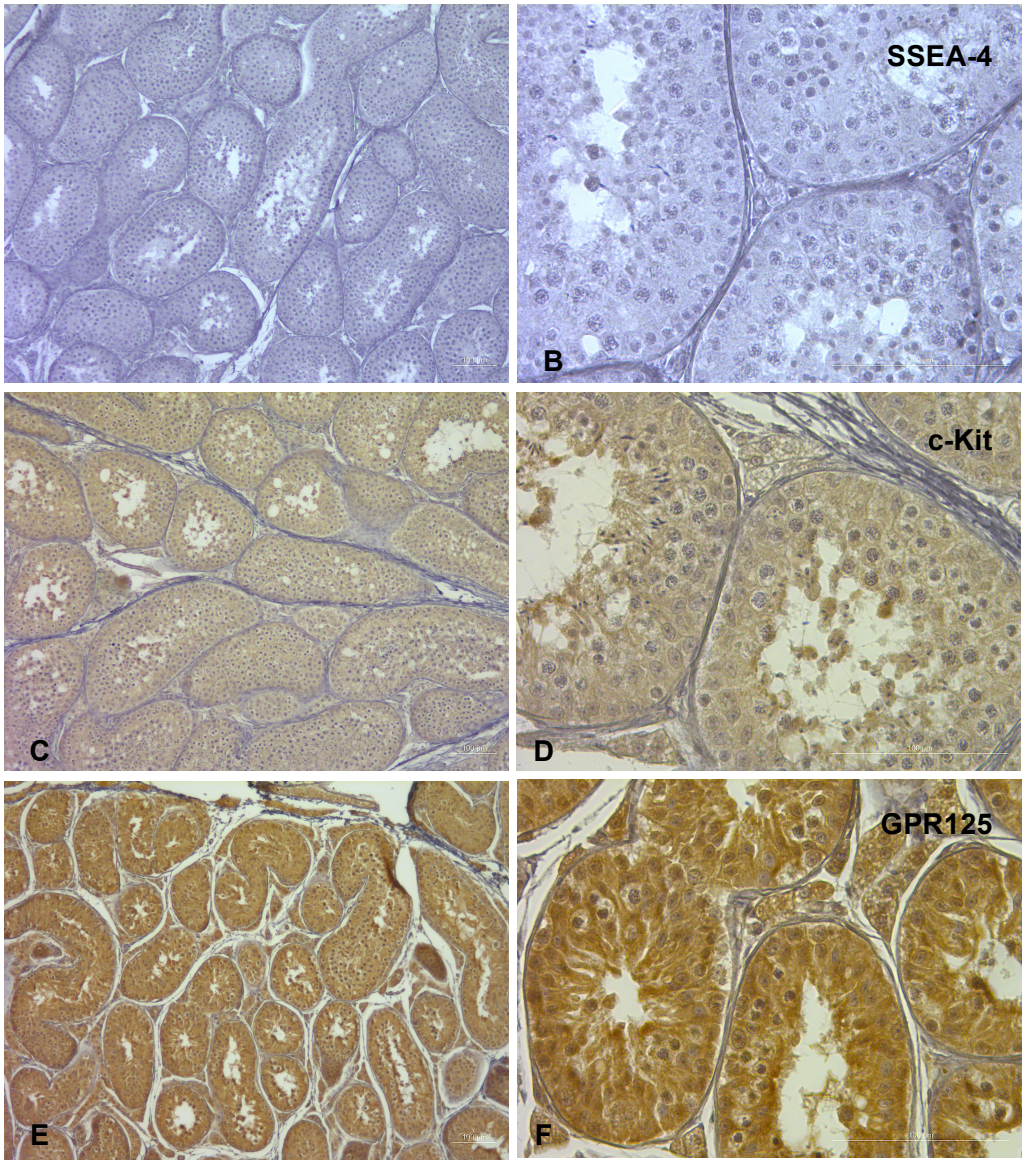

Figure 6. Unsuccessful labeling of germ cell in adult domestic cat testis. (A - B) show the absence of staining when an anti-SSEA-4 antibody was used. In contrary, an overall strong unspecific labeling of c-Kit (C-D), and of GPR125 (E-F). A, C and E – 100x magnification and B, D and F – 400x magnification.

2. α6-integrin

Alpha-6 integrin was first described in 1999 by Shinohara and colleagues as a potential marker of undifferentiated spermatogonia since a germ population enriched for this factor presented a higher colonization rate when transplanted into host mouse testis (Shinohara *et al.* 1999). However, immunohistochemistry in some of our domestic cat testis samples has revealed a Sertoli cell-labeling pattern (Figure 7 A-B). This same pattern has also been observed in other species, including rat (Salanova *et al.* 1998) and denies this protein as marker of SSCs in the cat. As shown in Figure 7 (C-D), in some of the samples the antibody didn't produce any labeling. This may be due to slight differences in fixative medium/time used in the testis samples.

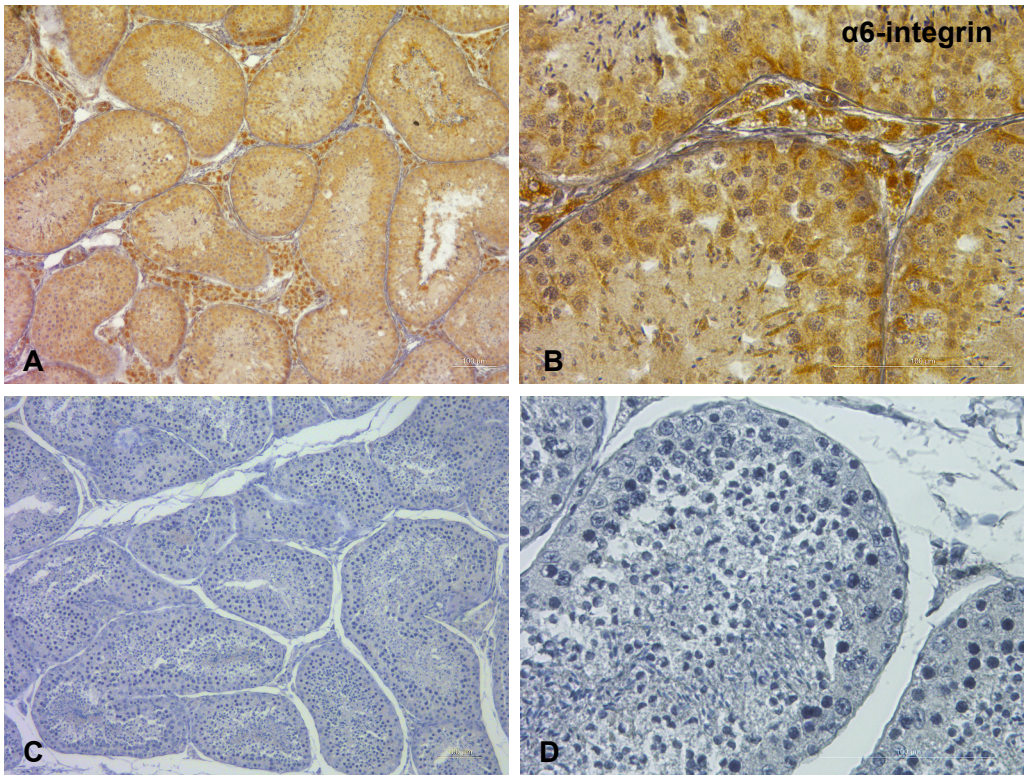

Figure 7. α6-integrin staining in adult domestic cat testis. (A - B) reveal a Sertoli cell-labeling pattern (dark brown precipitate) on a high background unspecific labeling, 100x and 400x magnification, respectively and (C-D) show no labeling of testicular cells (100x and 400x magnification, respectively).

3. Lin28

The pluripotency factor, Lin28 labels A_s and 2-4 cell-clones of A_{al} spermatogonia in the mouse testis (Shinohara *et al.* 1999). Using two different antibodies, one for the full-length protein (ab46020) and another for a region between aminoacids 1 and 180 of Lin28 (NBP2-19355), we observed either unspecific labeling of all seminiferous tubules (Figure 8 A-B) cells or a remarkably specific Sertoli cell-cytoplasmic pattern with the second antibody used for Lin28 (Figure 8 C-D), respectively. Western blot analysis revealed labeling of a band within the expected molecular weight and a strong labeling of an additional band between 50 and 75 kDa (Figure 9) raising questions regarding the specificity of the polyclonal rabbit antibody. The Sertoli cell pattern obtained may thus be due to labeling of the protein corresponding to the unspecific labeling. If we had observed any labeling of germ cells, confirmation could only be achieved using a blocking peptide for this specific antibody.

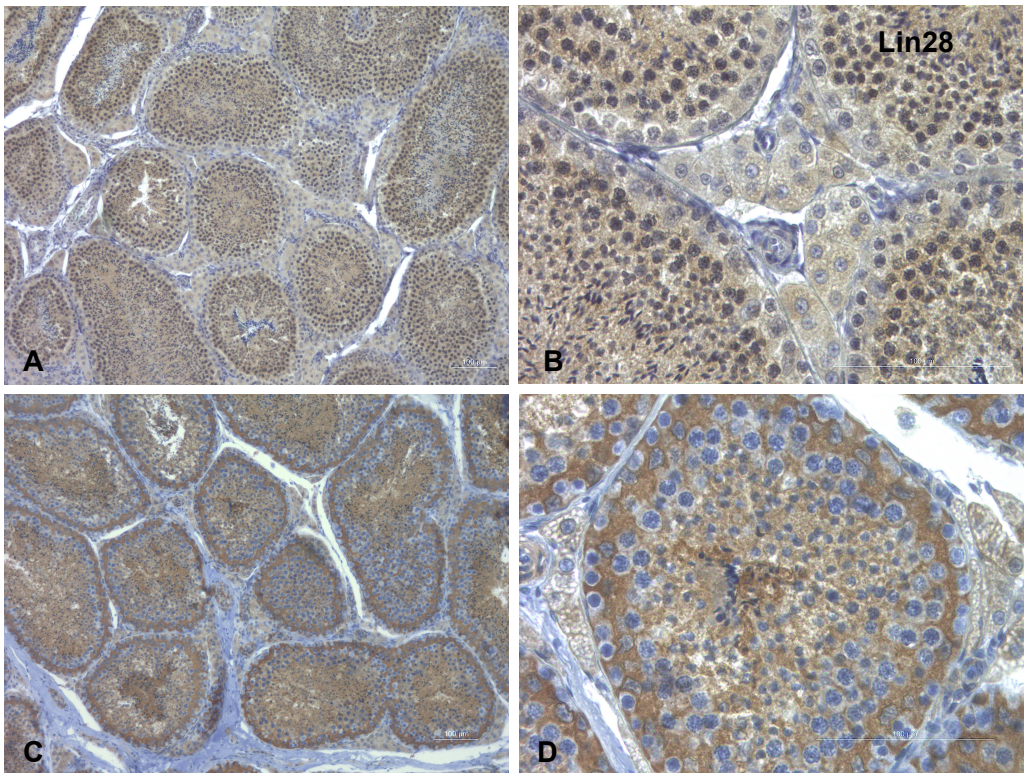

Figure 8. Two types of unspecific Lin28 labeling in adult domestic cat testis. (A - B) reveal an overall seminiferous tubule unspecific labeling using the anti-Lin28 ab46020 antibody (100 x and 400x magnification, respectively) and (C-D) show a Sertoli cell-cytoplasmic labeling pattern (C-D, 100x and 400x magnification, respectively), using NBP2-19355 anti-Lin28 antibody.

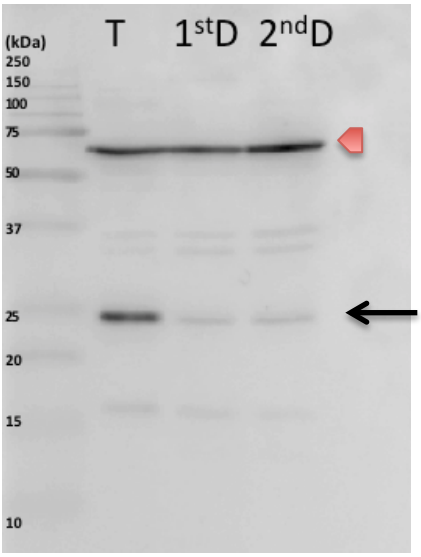

Figure 9. Western blot analysis of testicular tissue and isolated cells with anti-Lin28 NBP2-19355. The black arrow points to a labeled band, in the expected molecular weight (23-29 kDa), in the tissue and testicular cell fractions of isolated cells. Red arrowhead highlights the presence of an unspecific band between 50 and 75 kDa.
T- tissue lysate, 1^{st}D – testicular cell lysate from first digestion and 2^{nd}D – testicular cell lysate from second digestion.

4. GFRα-1

Although the GDNF signaling pathway is conserved among species, and both mRNA and GFRα-1 protein have been detected in domestic cat testis (Silva *et al.* 2012; Vansandt *et al.* 2012), our experiments didn't reveal any specific labeling (Figure 10). Since we used the same antibody as Silva and colleagues these were unexpected results. The explanation for our poor results could reside in the different tissue processing. In Silva's work (Silva *et al.* 2012), GFRα-1 labeling was obtained after staining frozen sections of isolated germ cells and Paraplast embedded sections of domestic cat testis tissue. However, since we tested the same antibody in unrelated techniques, such as immunocytochemistry and Western blot, where once again no labeling was detected (data not shown), the cause for the lack of labeling may be due to a defective lot of antibody.

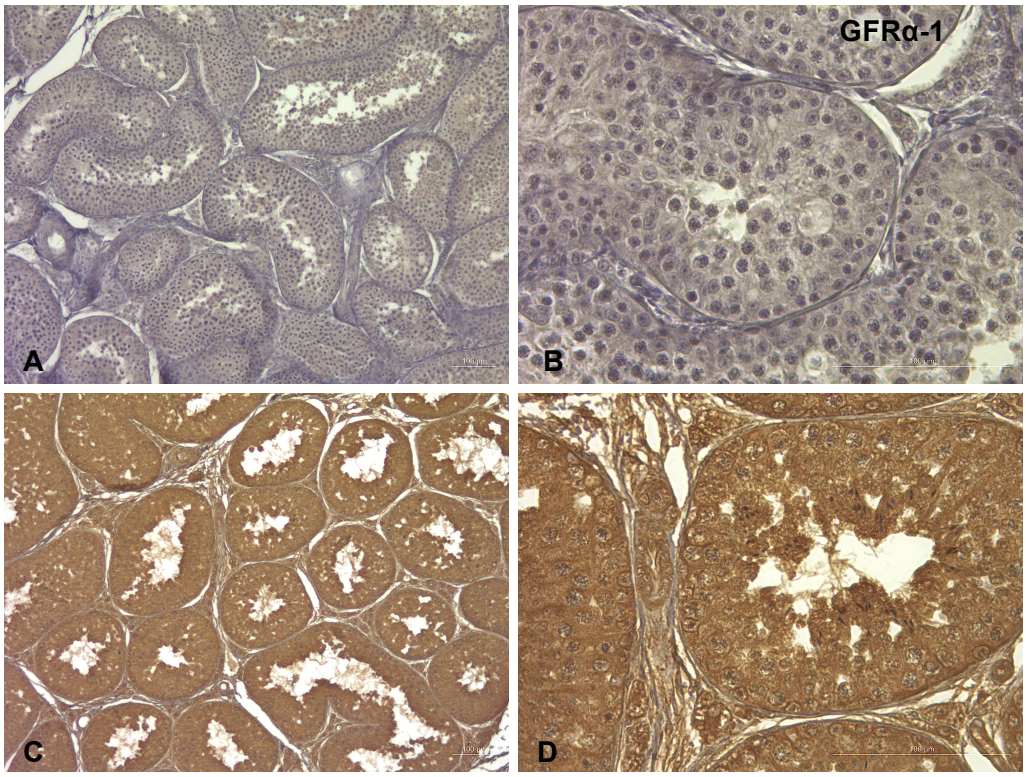

Figure 10. Two outcomes of GFRα-1 labeling in adult domestic cat testis. (A - B) reveal the absence of GFRα-1 staining, 100 x and 400x magnification, respectively and (C-D) show a strong unspecific labeling of the whole testis tissue when a higher concentration of the antibody was used (1:20), 100x and 400x magnification, respectively).

5. CXCR4 and CXCL12

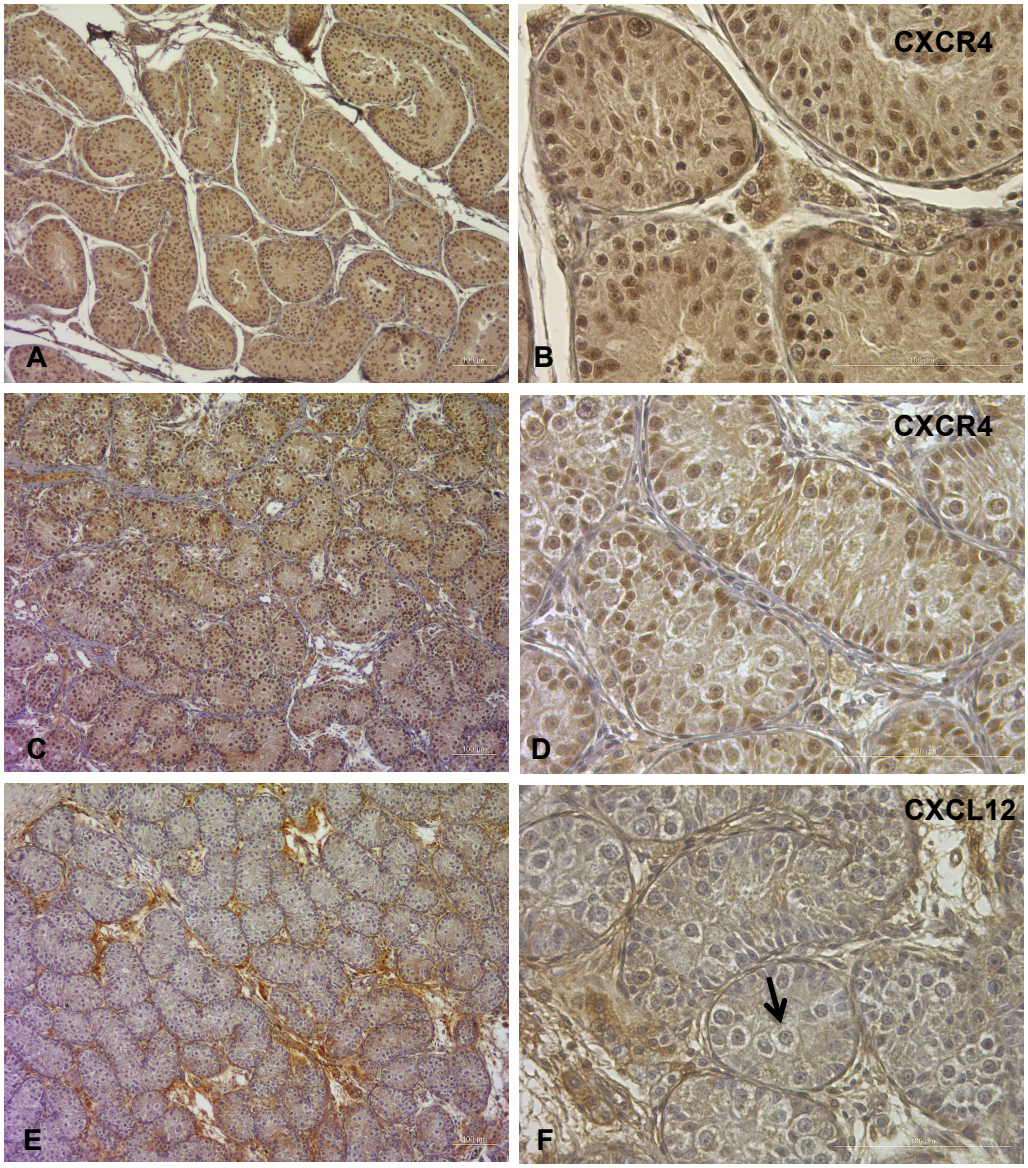

Figure 12. Unspecific labeling of the complex CXCR4/CXCL12. (A-D) reveal unspecific labeling of CXCR4 in pubertal (A-B) and immature (C-D) domestic cat testis sections (A and C - 100 x and B and D - 400x magnification, respectively). E-F show an unspecific labeling of the anti-CXCL12 when used in immature domestic cat testis, 100x and 400x, respectively; arrow points to a gonocyte in the center of the seminiferous tubule.

In 2013 Yang and co-works described the involvement of the CXCR4/CXCL12 complex in mouse undifferentiated germ cells migration and maintenance of these cells within the stem cell niche (Yang *et al.* 2013). Based on this knowledge we used an anti-CXCR4 and anti-CXCL12 rabbit polyclonal antibodies to probe immature and pubertal testis of domestic cats, where gonocyte and spermatogonial migration, respectively, can still be observed.

Once more we only observed unspecific labeling for both markers (Figure 11) but can only conclude that this antibody is unsuitable for IHC detection of domestic cat SSCs.

B. Successful Germ Cell Markers

1. DDX4

DDX4 (VASA homologue) was observed in cat testis sections (Figure 14) and protein extracts from isolated cells fractions (Figure 13).

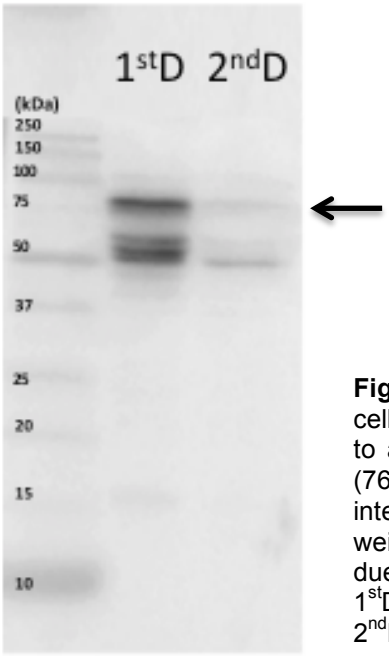

Figure 13. Western blot analysis of isolated testicular cells probed with anti-DDX4. The black arrow points to a labeled band in the expected molecular weight (76 kDa) for both fraction, although much more intense in the first digestion, as expected. The lower weight bands also labeled with the antibody may be due to peptides resulting from DDX4 degradation. 1^{st}D – testicular cell lysate from first digestion and 2^{nd}D – testicular cell lysate from second digestion.

Despite being described as a spermatogonia marker in other species, in the cat only more differentiated germ cells were labeled (from early spermatocytes to round spermatids, Figure 14). These observations are in accordance with previous reports from our group, also using the domestic cat as model (Mota *et al.* 2012) as well as from a study performed in murine (Fujiwara *et al.* 1994a). We should also point that the labeling intensity varied with antibody lot number indicative of polyclonal variability between lots. Although not suitable for spermatogonial identification, DDX4 continues to be a useful marker to recognize more differentiated germ cells in testicular cell suspensions.

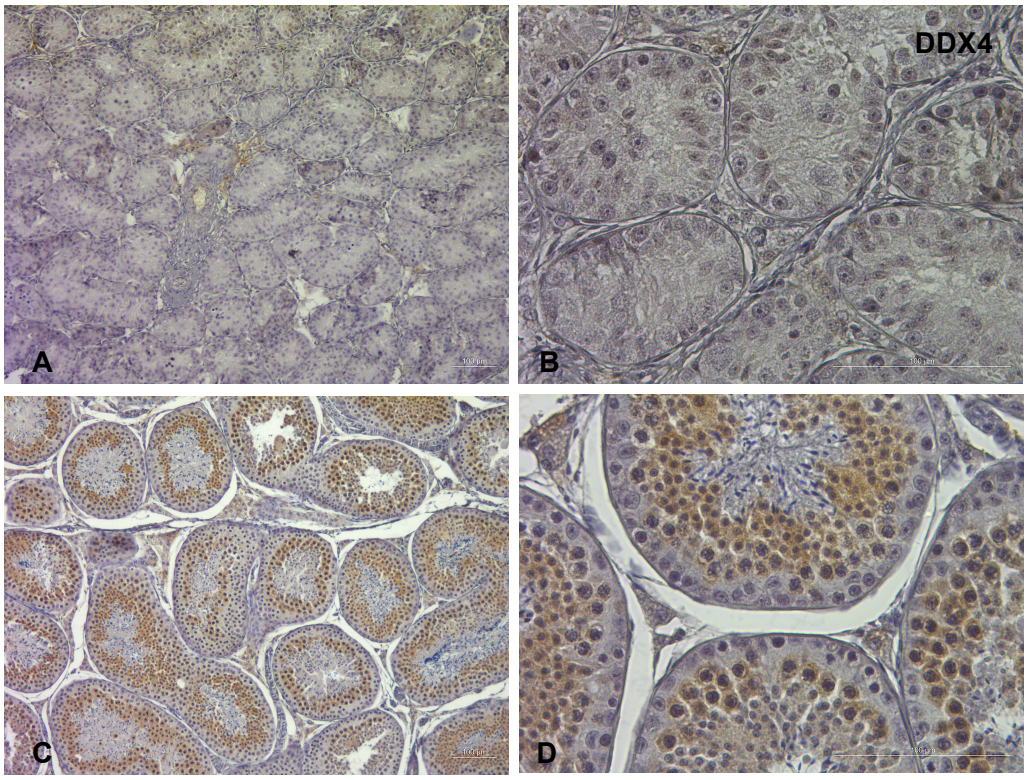

Figure 14. DDX4 labeling in domestic cat testis tissue cross-sections. (A and C) 100x and (B and D) 400x magnification. In immature domestic cat testis sections no labeling of germ cells was observed (A-B). In adult animals (C-D) it is possible to observe labeling of meiotic germ cells but no brown precipitate can be visualized in the basal compartment of the seminiferous tubules where the spermatogonial population resides.

C. Successful Spermatogonial Markers

1. PGP9.5

As described in the introduction PGP9.5/UCHL-1has been recognized as a marker of undifferentiated spermatogonia in species such as bovine (Wrobel 2000), monkey (Tokunaga *et al.* 1999) and humans (Valli *et al.* 2014). However, mice Sertoli cells also express the protein (Kwon 2004). In domestic cat seminiferous tubules it has been observed in the nucleus and/or cytoplasm of all spermatogonia subtypes (Mota *et al.* 2012). This results where confirmed in this study, as we can observe in Figure 15 (E-F), and extended by the observation that gonocyte/pre-spermatogonia are labeled with anti-PGP9.5 antibody at cytoplasm level (big cells with a big nucleus at the center of the seminiferous tubules, Figure 15 A-B) and also, although with

less affinity, the pre-leptotene spermatocytes before they cross to the adluminal compartment (Figure 15 C-F). Furthermore, we observed no variation in labeling intensity in spermatogonia present in all age categories (immature, pubertal and adult).

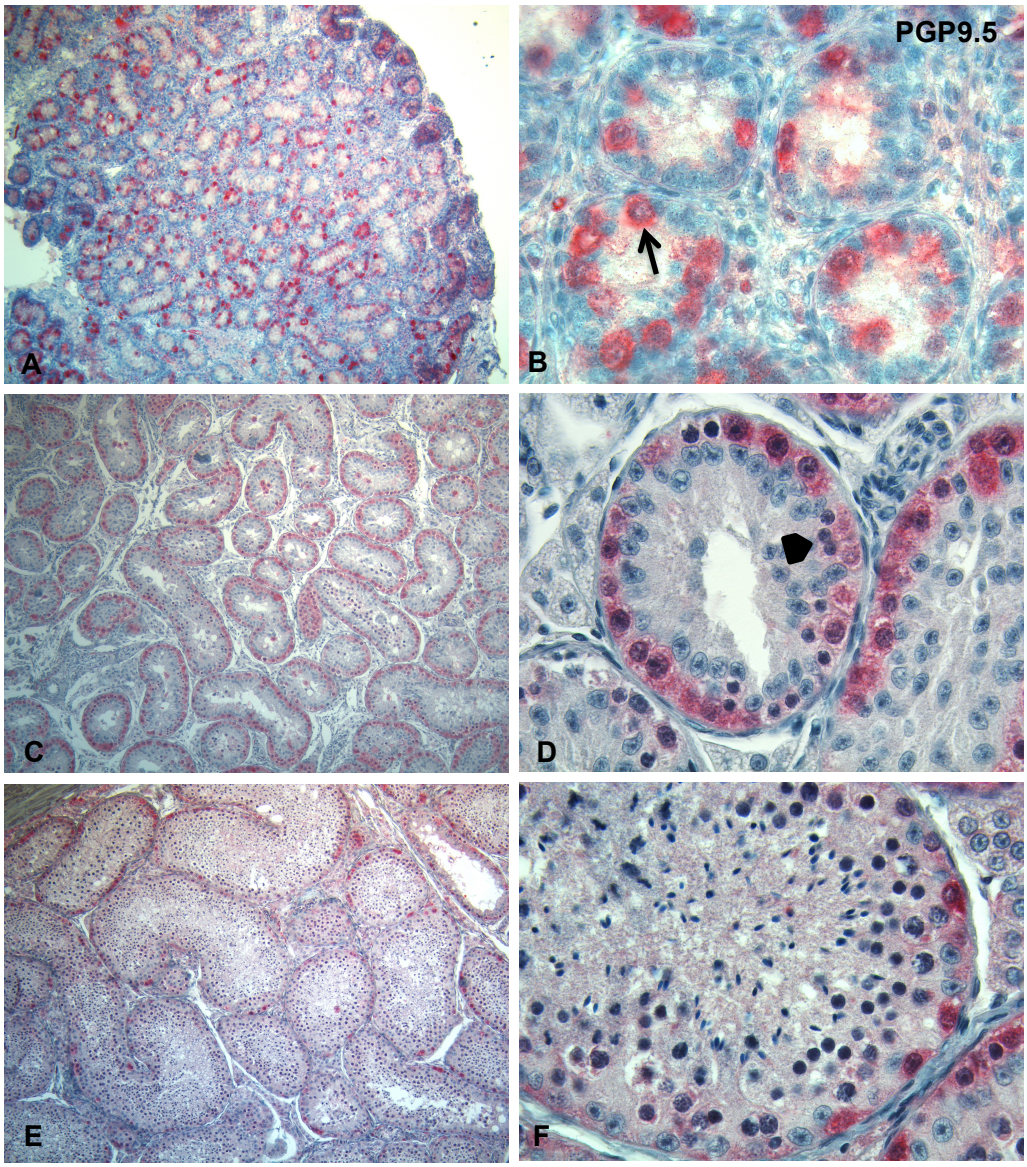

Figure 15. PGP9.5 labeling pattern. Red staining (alkaline phosphatase/ TR/Napthol system) of more primitive cells (Gonocytes) could be observed in an immature animal (arrow in B). (C-F) Red staining shows labeling of all spermatogonia as well as, pre-leptotene spermatocytes (arrows in D) in the tubule of a pubertal animal (C-D), as well as in adult animals (E-F). A-C-E 100x and B-D-F 600x magnification.

The specificity of this antibody to recognize the domestic cat protein was further demonstrated by the presence of a strong-labeled band at the expected molecular weight (Western blot analysis, Figure 16).

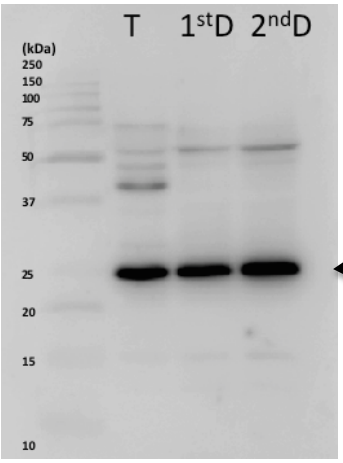

Figure 16. Western blot analysis of testicular protein using PGP9.5 (24.9 kDa). The black arrow points to a highly labeled band, in the expected molecular weight, in all cell fractions.
T- tissue lysate, 1stD – testicular cell lysate from first digestion and 2ndD – testicular cell lysate from second digestion.

Using the whole mount staining technique, we observed large clones of spermatogonia labeled with PGP9.5 antibody confirming the presence of the protein in all spermatogonial subtypes and pre-leptotene, nonetheless preventing the visualization of the clonal expansion (number of cell divisions per undifferentiated spermatogonia, Figure 17).

Although this marker cannot discriminate between undifferentiated and differentiated spermatogonia cells, it is still a valuable tool to study domestic cat spermatogenesis, *in vivo* and *in vitro,* due to its consistent results.

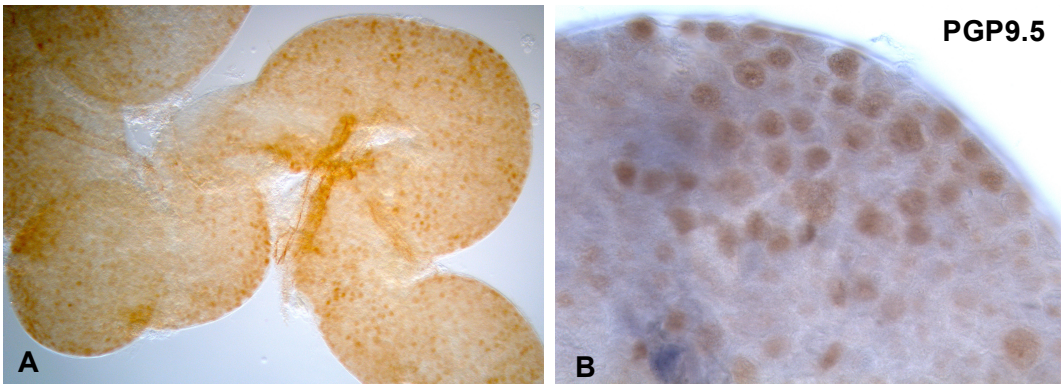

Figure 17. Whole mount staining of adult cat testis with anti-PGP9.5 and revealed with the DAB system (brown precipitate). In figure A (100x magnification) we can observe a convoluted seminiferous tubule with a high number of labeled cells (drown dots) In a higher magnification (600x) big clones of spermatogonia (brown labeled cells) can be observed in the surface of the seminiferous tubule.

D. Successful Spermatogonial Stem Cell Markers

1. Lectin DBA (*Dolichos biflorus agglutinin)*

In our search for undifferentiated spermatogonia surface marker we came across a study from Herrid et al. (2007) where they described the use of the lectin *Dolichos biflorus agglutinin* (*Horse gram*: DBA) to label and isolate undifferentiated bovine spermatogonia. This lectin has also been used in pig and chicken to detect primordial germ cells (Takagi *et al.* 1997) and gonocytes in pig (Goel *et al.* 2007).

Our first immunohistochemistry experiments showed few labeled cells in close contact to the basal membrane of adult domestic cat seminiferous tubules. The low number of labeled cells was already a good indicative of an undifferentiated marker but the presence of isolated strongly labeled cells emphasized this hypothesis (Figure 18 E-F). Since the previously mentioned studies reported staining of more primitive germ cells, we further tested the lectin DBA in immature and pubertal animals. Surprisingly, our experiments provided opposite results. We observed a very light, almost inexistent staining in gonocytes/pre-spermatogonia in immature animals (Figure 18 A-B). The intensity of staining increased in prepubertal animals (Figure 18 C-D), reaching its maximum in fully active seminiferous tubules (Figure 18 E-F).

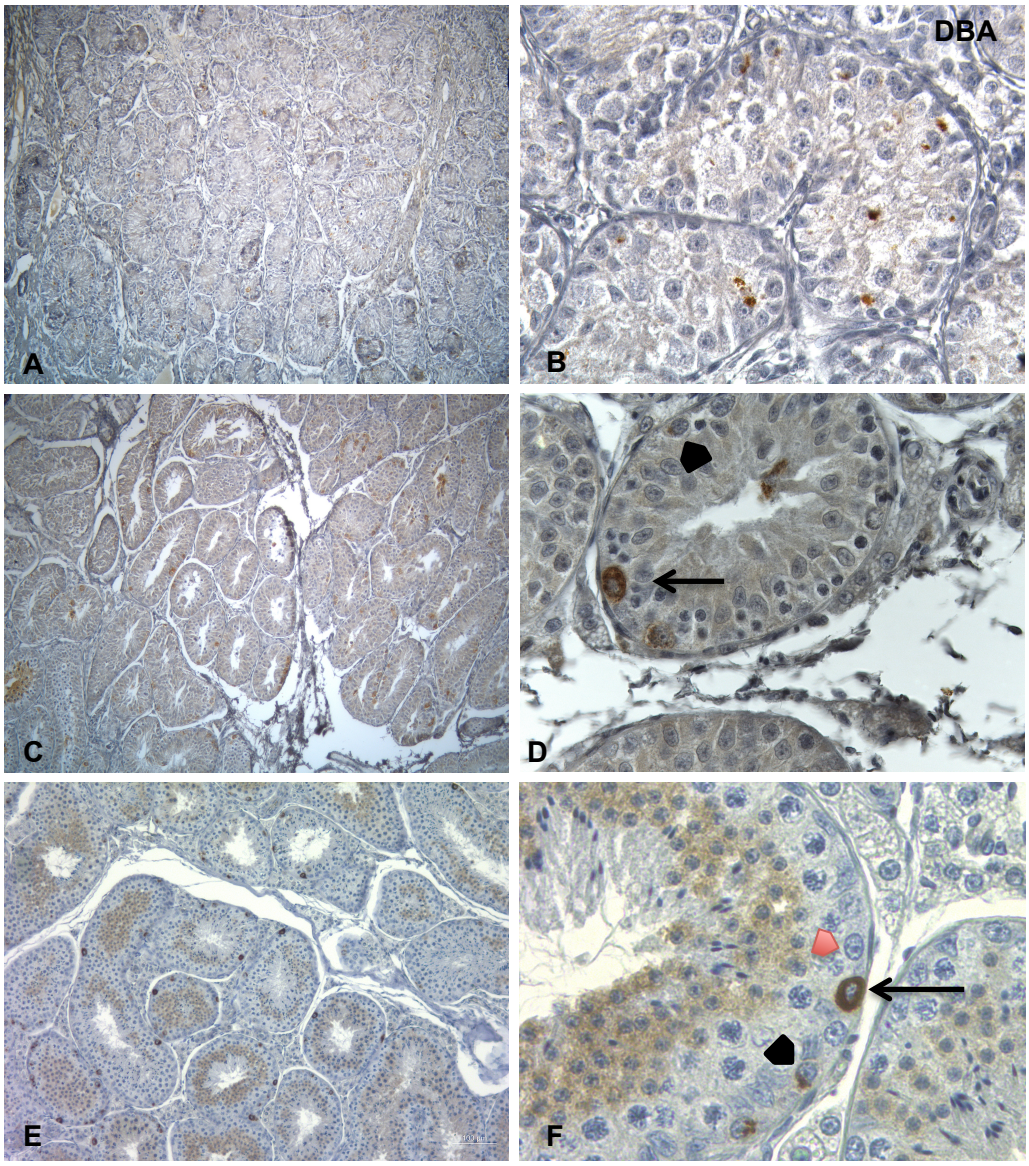

Figure 18. DBA labeling in testis of three different aged animals. (A-B) show very few germ cells lightly stained in an immature animal; the intensity of labeling increased in pubertal animals (C-D), reaching its maximum in relation to intensity and number of cells labeled in adult animals (E-F). In pubertal as well as in adult animals two intensities of labeling could be observed, strong staining (arrow) and light staining (black arrowhead). (A, C and E) – 100x and (B, D and F) – 600x magnification. Red arrowhead points towards unspecific labeling of round spermatids that wasn't constant in all animal testis sections tested (data not shown). Red arrow points to a spermatogonia not labeled by the DBA lectin.

A double immunostaining experiment using anti-PGP9.5 antibody and DBA lectin showed that all spermatogonial cells labeled with DBA are also positive for PGP9.5, but the population labeled with PGP9.5 is bigger than the one labeled with DBA. These results are in accordance with immunohistochemistry using only DBA, where some morphologically identified spermatogonia were not labeled with DBA (Figure 18 red arrow).

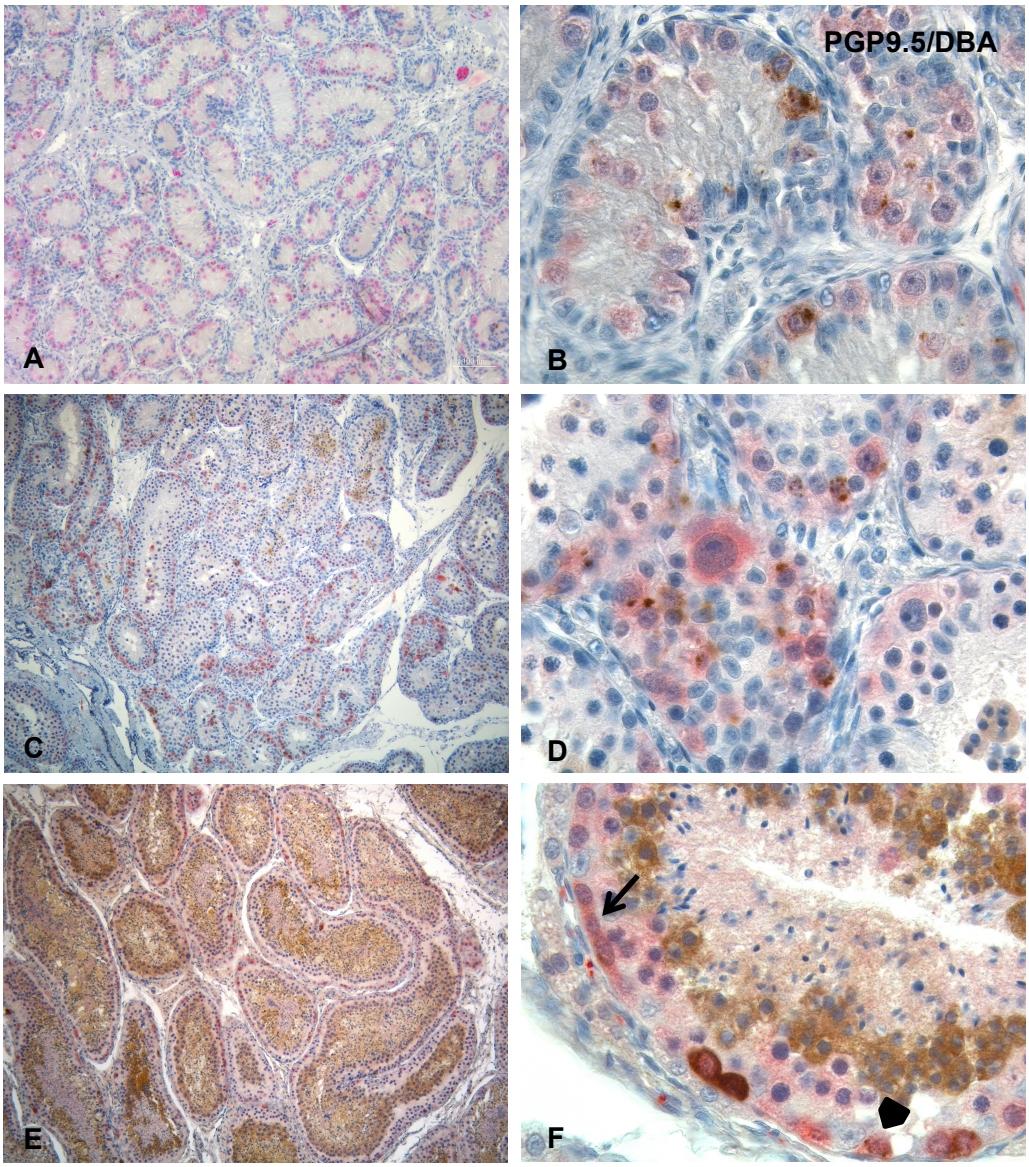

Figure 19. Double-staining with DBA and PGP9.5 in testis of three different aged animals. Very few germ cells are lightly stained with DBA (brown precipitate) while PGP9.5 labels the cytoplasm of all germ cells (red precipitate) in an immature (A-B) and pubertal animal (C-D). In adult animals (E-F) it is possible to characterize 3 populations – $PGP9.5^{+}/DBA^{-}$ red stained cells (black arrow); $PGP9.5^{+}$ low DBA^{+} (orange staining- arrowhead) and $PGP9.5^{+}$ strong DBA^{+} (brown cells in the basement membrane). A-C-E 100x magnification and B-D-F 600x magnification

DBA as well as PGP9.5 positive cells quantification in round seminiferous tubules indicated that the total number of DBA positive spermatogonia corresponds to 65±5% (Mean±SEM) of total spermatogonia population as detected by PGP9.5. Since within DBA positive population, the dark/strongly labeled cells represent approximately 8% of total labeled cells, this will correspond to 5.2% of the total spermatogonial population (PGP9.5 positive cells), a similar percentage to the one described for spermatogonial stem cells

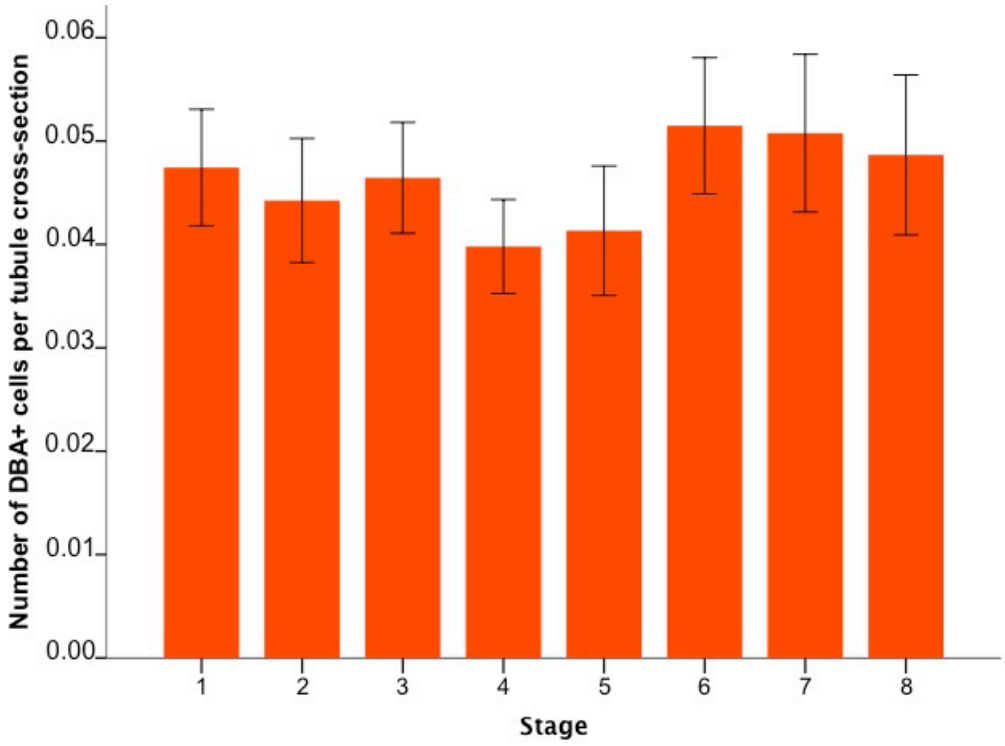

Figure 20. Variation of DBA$^+$ cells with seminiferous epithelium/tubule stage. No differences where observed in the number of DBA$^+$ cells between the seminiferous epithelium stages. Bar represents Mean±SEM of ≈ 10 seminiferous tubules cross-sections per animal for 4 animals.

in rat testis, namely 4% (Kubota *et al.* 2004). Additional arguments for the undifferentiated state of the cells labeled with DBA are the lack of relation between these and the seminiferous epithelium stage (Figure 20) as described for mice (reviewed in Ehmcke *et al.* 2006) and their preferential localization towards a specific region of the tubule, as described next.

Following Campos-Junior's protocol we determined the number of total DBA labeled cells and strongly DBA-labeled cells in the different regions of the seminiferous tubule cross-sections (Figure 5). We decided to further divide the interstitial region in two: one containing blood vessels and the other without close contact to these, given previous knowledge of clustering spermatogonial stem cell near the vasculature in mouse model (Yoshida *et al.* 2007). However, unexpectedly, in our results the region presenting higher

amounts of DBA labeled cells was in direct contact with Leydig cells (Figure 21). Regarding this latest aspect, other studies have showed a preference for other neighboring regions, namely interstitial region in the collared peccary [*Tayassu tajacu*; (Campos-Junior *et al.* 2012)] and donkey (Chiarini-Garcia *et al.* 2009). In the cat the association with Leydig cells may be due to the fact that the majority of the interstitial space is occupied with these cells and therefore there's no clear separation of both regions.

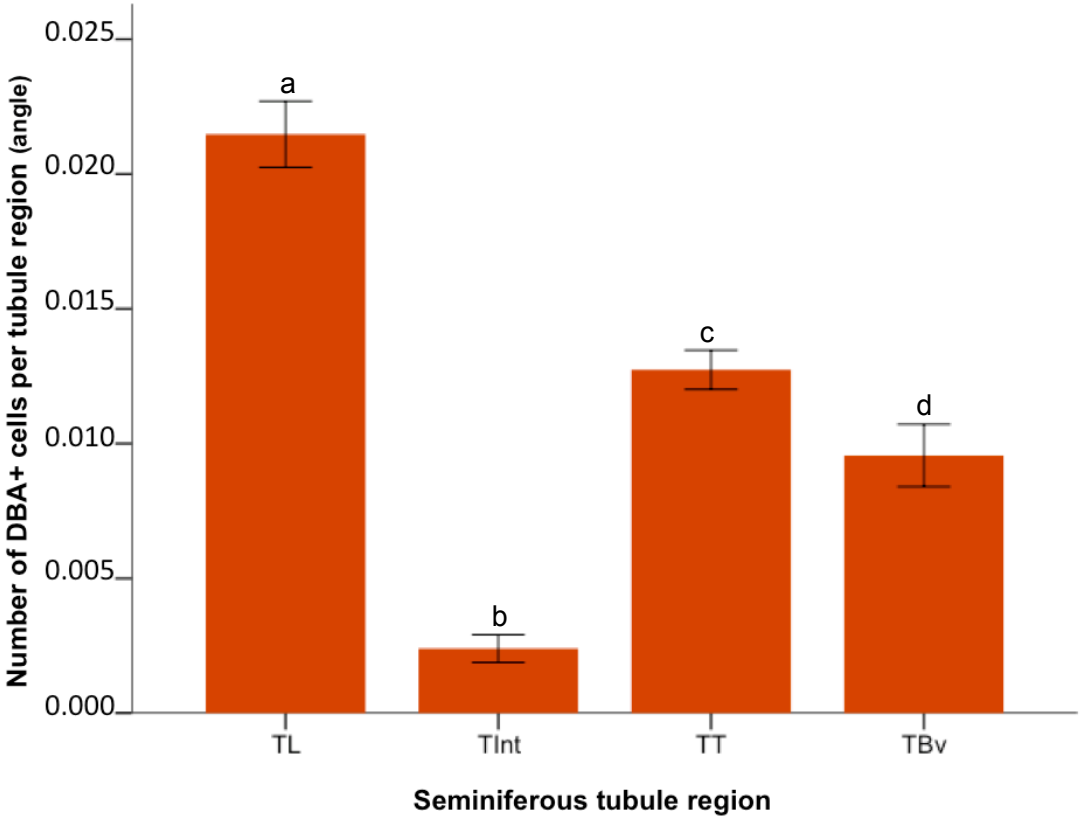

Figure 21. Effect of tubular region on the distribution of DBA^+ cells. Bar represents Mean±SEM of ≈ 80 seminiferous tubules cross-sections per animal determined for 4 animals. a, b ,c and d represent the statistically significant differences observed in the number of DBA positive cells between all seminiferous tubule regions ($p<0.001$). TL - Tubule-Leydig cells region; Tint – Tubule-interstitial region without blood vessels; TT – Tubule-tubule region; TBv – Tubule-interstitial region with blood vessels (see Figure 5 for further information).

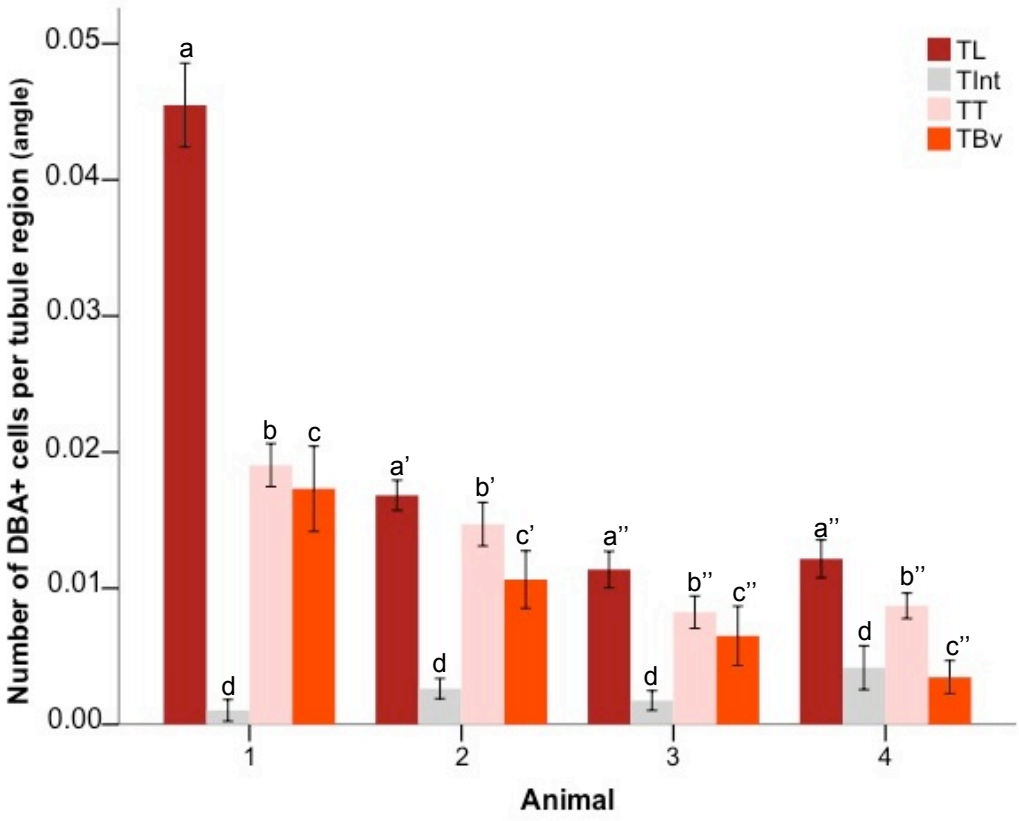

Figure 22. Variation of the number of DBA^{+} cells, and respective tubular localization, with the animal. Bar represents Mean±SEM of ≈ 80 seminiferous tubules cross-sections. a, a', a''/b, b', b''/c, c', c'' represent statistically significant differences for the tubule regions noted ($p<0.05$) between animals. TL - Tubule-Leydig cells region; Tint – Tubule-interstitial region without blood vessels; TT – Tubule-tubule region; TBv – Tubule-interstitial region with blood vessels (see Figure 5 for further information).

Studies using whole mounts have provided further information regarding DBA staining. Firstly, the general observation of the whole mounts reveals smaller cell-clones labeled with DBA when compared to those stained with PGP9.5 antibody (Figures 23 and 17, respectively) and confirms the variation in DBA labeled cells according to animal maturity observed in IHC (Figure 23 and 18.)

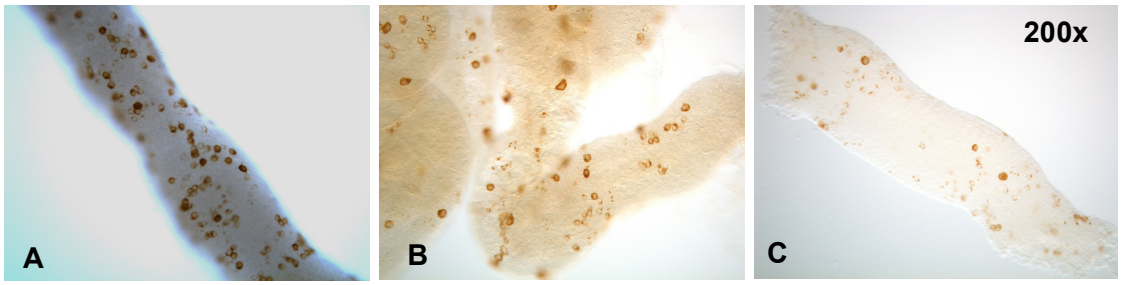

Figure 23. Whole mount staining of domestic cat seminiferous tubules. Figure shows the difference in the number of DBA labeled cells according to the animal maturity. (A) Adult cat testis – 3.97g, (B and C) Pubertal animals – 1.66 and 1.67, respectively.

Secondly, we could observe three types of DBA staining pattern: Cytoplasmatic and possibly cell membrane staining (strong staining), cell membrane with or without “Golgi” staining and finally only “Golgi” (Figure 24).

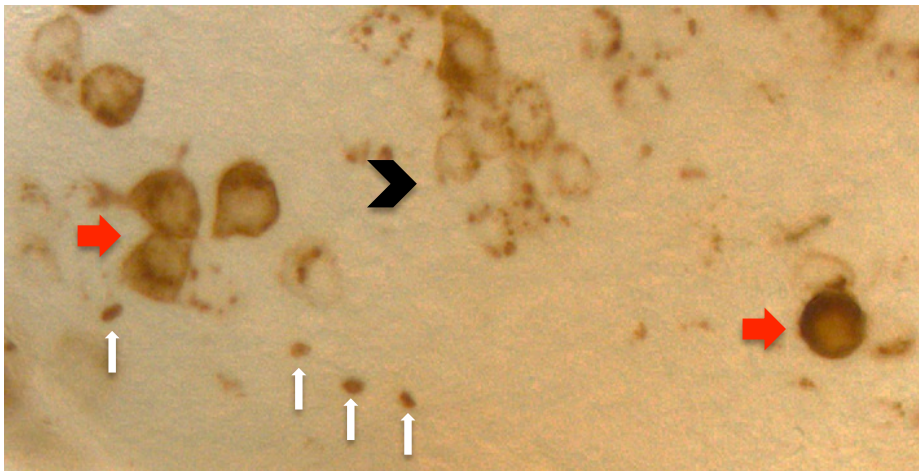

Figure 24. Close-up image of Whole mount DBA staining of domestic cat seminiferous tubules. Different staining patterns can be observed: strong cytoplasmic staining (red arrow); membrane staining (black arrowhead) and “Golgi-pattern” staining (white arrows).

Thirdly, whole mounts tridimensional viewing of the seminiferous tubules, allowed observation of strongly labeled isolated cells, 2 cell-clones also strongly labeled, 2 cell-clones with only one strongly labeled cell and bigger clones (four and above) with one or more strongly labeled cells (Figure 25) but always corresponding to small proportion of all labeled cells. The appearance of 2-cell clones with one strong and one lightly stained cell points towards the possibility of the occurrence of asymmetric divisions in domestic cat spermatogonia. Also, since bigger clones of cells present a lighter cell staining we assume that the staining decreases with cell divisions.

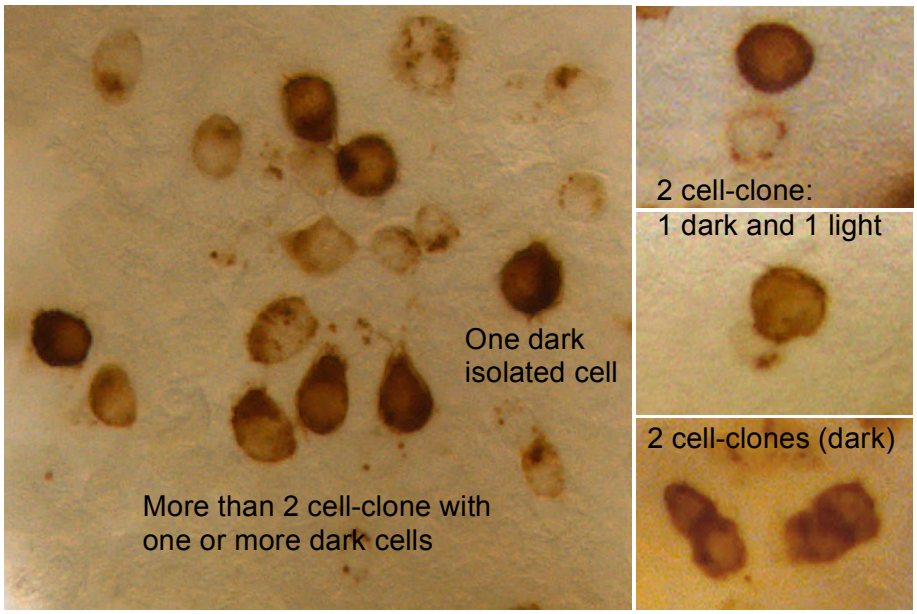

Figure 25. Close-up image of whole mount staining of domestic cat seminiferous tubules with representation of all the cell-clone types observed.

Furthermore, the finding that the majority (48%) of the strongly DBA-stained cells correspond to isolated cells contributes to the hypothesis that these cells represent the stem cell pool (Figure 26).

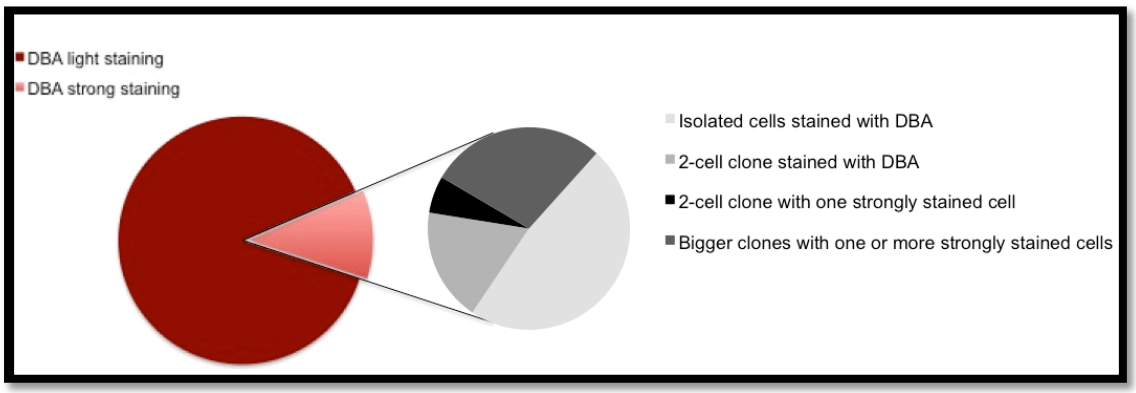

Figure 26. Schematic representation of the percentage of DBA stained cells/cell-clones observed in the 3 whole mounts experiments. In red/pink are represented the total number of DBA labeled cells and in greys the percentages of the cell-clones observed.

In humans, using the same whole mount technique and PGP9.5 as a marker of undifferentiated spermatogonia, expansion was observed until the 8-

cells/clone stage, while with the use of a marker of differentiated spermatogonia, c-Kit, 16 interconnected cells were detected (Valli *et al.* 2014). As domestic cat's spermatogonial clonal expansion has not been established yet, we cannot confirm that isolated cells represent the true stem cells, as in the mouse model, or that the 8 cells-clones with one or two strongly labeled cells correspond to clones containing spermatogonia with different developmental potential, as it has been proposed for Rhesus monkey (Figure 27).

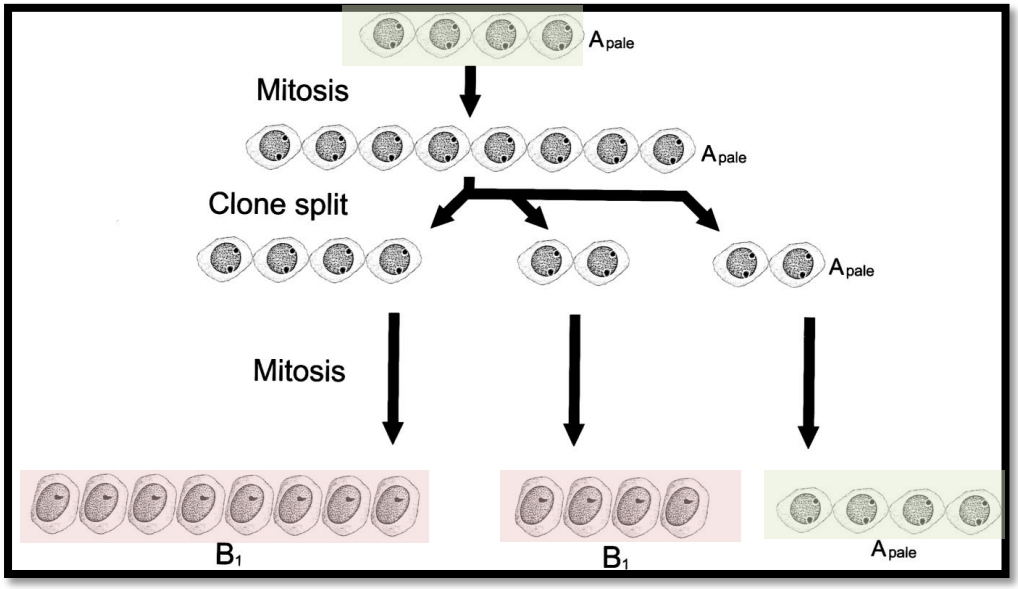

Figure 27. Proposed clonal expansion model for Rhesus monkey by Ehmcke and colleagues (2005). In this model the A_{pale} spermatogonia, organized in pairs or in quadruplets, proliferate resulting in a doubling of A_{pale} population. The subsequent quadruplets or eight-cell clones of A_{pale} spermatogonia are unstable, and therefore separate into pairs or quadruplets of A_{pale} spermatogonia. This generation of separated clones enters a second mitosis, giving rise to either B1 spermatogonia or A_{pale} spermatogonia. The four-cell clone of A_{pale} spermatogonia remain mitotically quiescent renewing the original A_{pale} population for this cycle of spermatogenesis while the B1 clones enter the subsequent differentiation steps (Ehmcke *et al.* 2005a). Light red squares indicate differentiated spermatogonia and light green squares correspond to undifferentiated spermatogonia.

In order to test the potential of DBA for cell isolation, given its affinity for glycan epitopes of surface proteins in other stem cells, we performed immunocytochemistry in isolated cells.

We could confirm DBA labeling on non-permeabilized (data not shown) and also in permeabilized cells. However, due to the possible increase in permeability caused by fixatives, we cannot discard DBA lectin recognition of

cytoplasmic glycans, therefore, freshly isolated/non-permeabilized cells should be used to confirm cell surface binding of DBA.

Like in IHC, DBA labeled cells positive for PGP9.5 (Figure 28), although the number of PGP9.5-labeled cells was higher then the number of DBA-positive cells. Contrarily to IHC observations, no spermatids labeling ($DBA^{+}/PGP9.5^{-}$) was observed.

Confirmation of SSC surface labeling together with absence of round spermatid labeling prompts the use of DBA lectin on sorting and purification experiments of spermatogonia subpopulations either through MACS, FACS or differential platting.

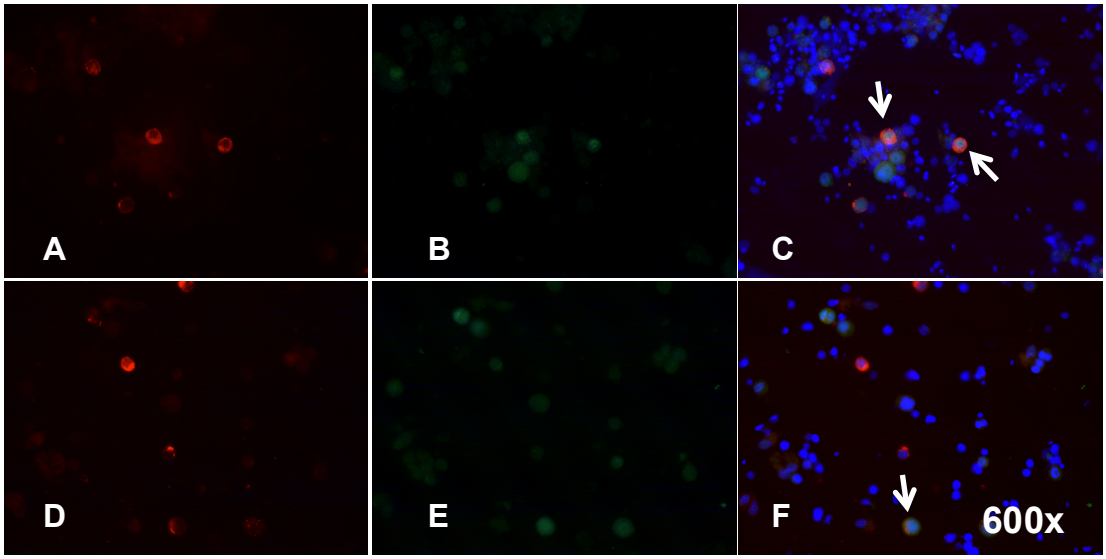

Figure 28. Immunocytochemistry experiment performed in isolated testicular cell fractions from domestic cat testis. (A-C) correspond to fractions from the first digestion and (D-F) correspond to fractions from the second testicular digestion. (A and D) represent DBA labeling and (B and E) are the cells positive for PGP9.5 staining. (C and F) are the final merged images (DBA and PGP9.5 staining), plus nucleus counterstaining with DAPI. White arrowheads point to spermatogonia labeled with both DBA and PGP9.5, 600x magnification.

CONCLUSIONS AND FUTURE PERSPECTIVES

Our goal with this work was to facilitate the recovery of endangered species' genetic potential, via selection and transplantation of a purified population of undifferentiated spermatogonia, through the study of a possible surface marker for SSCs. Although this may seem a stringent and easily achievable objective, the description of the work performed reveals several challenges.

The limited recognition of feline antigens by commercially available antibodies represented a major issue since every experiment performed was based on antigen-antibody binding. Also the scarce information regarding feline spermatogonial cells hampered the interpretation of the results obtained in some of the assays.

Nevertheless, we can propose with a certain level of confidence DBA lectin as a new marker for domestic cat, and possibly other felines, undifferentiated spermatogonial cells and that the stem cell niche might be under Leydig cell influence. This confidence is only disturbed by the observation that immature and pubertal animal present a lower labeling of spermatogonial cells, although this may indicate that the population of adult SSCs is only establish late in puberty.

As a resume Figure 29 represents the expression pattern of the successfully tested markers in our experiments using domestic cat testis tissue.

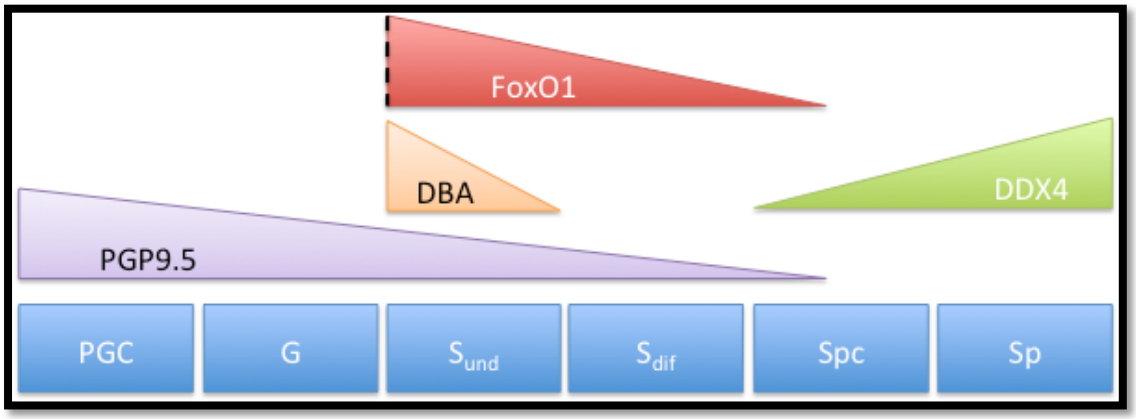

Figure 29. Schematic representation of the tested markers' expression in domestic cat testis. PGC – primordial germ cells, G- gonocytes, S_{und} - undifferentiated spermatogonia, S_{dif} – differentiating spermatogonia, Spc – spermatocytes and Sp- spermatids.

In future work we have to include the confirmation of DBA lectin as a spermatogonial stem cell marker through xenotransplantation assays. The observation of an increased rate of colonization after transplantation of an enriched population of DBA^+ cells into mouse host testis will be the definite prove of DBA as a domestic cat spermatogonial stem cell marker. Also, BrdU studies of spermatogonial clonal expansion could confirm that the isolated cells correspond to the long-term BrdU-labeled quiescent cells.

Its usefulness as a surface marker in purification techniques also needs to be accessed through the use of freshly isolated cells.

As ongoing work we can show two other antibodies that recognized domestic cat proteins localized in spermatogonia and other germ cells. FoxO1 apparently presents a staining pattern similar to PGP9.5 and SALL4 a more elaborated pattern since not all spermatogonia in each stage are labeled with the antibody and other germ cells, later in spermatogenesis, are also labeled with this antibody.

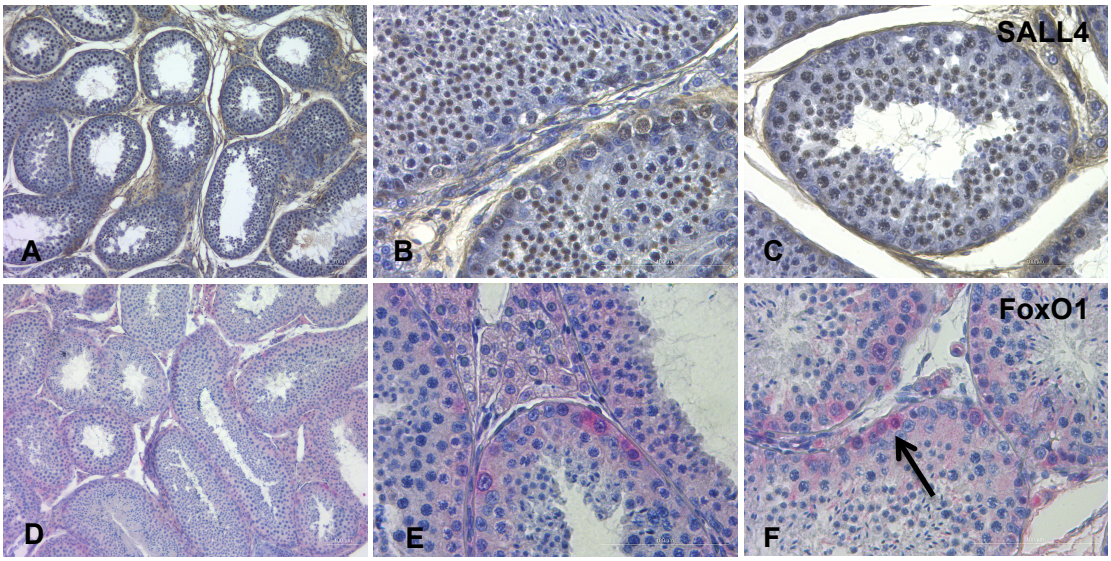

Figure 30. SALL4 and FoxO1 labeling of domestic cat testis tissue sections. (A and D) 100x (B-C and E-F) 600x. SALL4 staining (brown precipitate) was observed in the nucleus of germ cells. Although cytoplasmatic FoxO1 staining (red precipitate) has also been observed in men it points to tertiary variations of proteins between species. Black arrow points to FoxO1 labeled spermatogonia.

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