SOME STUDIES ON SPERMATOGONIAL STEM CELLS AND REPRODUCTION IN MALE DOGS

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Table of abbreviations

PGCs	Primordial germ cells
As or SSCs	A single spermatogonia or spermatogonial stem cells
Ap	A paired spermatogonia
Aal	A aligned spermatogonia
In Spg	Intermediate spermatogonia
B Spg	Type B spermatogonia
GFP	Green fluorescence protein
HSCs	Hematopoietic stem cells
ESCs	Embryonic stem cells
MACS	Magnetic Activated cell sorting
IF	Immunofluorescence
GDNF	Glial cell line-derived neurotrophic factor
Gfrα-1	GDNF family receptor α-1
Ret	Rearranged during transfection, it is the transmembrane unit of GDNF receptor
PGP 9.5	Protein gene product 9.5
PCNA	Proliferating cell nuclear antigen
Ub	ubiquitin
Oct-4	Octamer-4
Plzf	Promyelocytic leukemia zinc-finger
bFGF	Basic fibroblast growth factor
EGF	Epidermal growth factor
LIF	Leukemia inhibitory factor
MEFs	Mouse embryonic fibroblasts
mDF	Modified Davidson’s fluid
PFA	Paraformaldehyde
BF	Bouin’s fluid
HIER	Heat-induced epitope retrieval
TBST	Tris buffered saline tween-20
BSA	Bovine serum albumin
FBS	Fetal bovine serum

INTRODUCTION

Although the dog is an important domestic animal, there are surprisingly few thorough reports in the literature regarding testis structure and function in this species (Soares *et al.* 2009). Spermatogenesis dynamics in dogs has received nearly no attention by reproductive biologists. Therefore, in this current study we are attempting to get a closer look at the origin of spermatogenesis in dogs by characterization and isolation of canine spermatogonial stem cells.

Mammalian spermatogenesis is the development of spermatogonial stem cells "SSCs" into spermatozoa over an extended period of time within the seminiferous tubule boundaries of the testis (Hess & de Franca 2009). These cells are capable of self-renewal to maintain the pool of stem cells throughout life, or they differentiate to generate a large number of germ cells. A balance between SSCs self-renewal and differentiation is therefore essential to maintain normal spermatogenesis and fertility throughout life.

Most of our understanding of SSCs behavior is derived from studies on non-primate mammals (mice and rats). The biology of SSCs in higher mammals has been investigated in the monkey (Hermann *et al.* 2007), pig (Luo *et al.* 2006; Goel *et al.* 2007), and bull (Izadyar *et al.* 2002; Aponte *et al.* 2008), but no studies have investigated canine SSCs. So far, research in this area has been restricted to short-term cultures of mixed germ cells derived from canine testis followed by transplantation into mouse testes to study cross-species transplantation efficiency (Dobrinski *et al.* 1999), and transplantations into dog testes for transgenesis studies (Kim *et al.* 2008). Therefore, research is needed toward understanding the mechanisms of self-renewal and differentiation of SSCs in dogs.

Since the 17th century, dogs have been used as models in physiological and metabolic studies. Because of obvious parameters such as lifespan and size, they are an important intermediate species between human and the mouse (Kirchhoff 2002). The dog is biologically more comparable to humans than mouse, with respect to stem cell kinetics, hematopoietic demand and responsiveness to cytokines (Horn *et al.* 2004). For example, a mouse produces as many erythrocytes in its lifetime as a human produces in one day or a dog in two-and-half days (Abkowitz *et al.* 1996). Of the 400 known hereditary canine diseases, more than half have an equivalent human disease, including cardiomyopathies, muscular dystrophy, prostate cancer (Starkey *et al.* 2005), blindness (Acland *et al.* 2005), and bleeding disorders (Tsai *et al.* 2007). More than 400 different dog breeds provide a wide range of genetic variation similar to human populations. Many pure bred dogs have unique traits including susceptibility to certain genetic diseases. Thus the dog is an outstanding model for comparative genetic investigations to elucidate human diseases.

Among different animal models for studying testicular tumors, canine seminomas are currently considered the most informative model for human spermatocytic seminomas (Looijenga et al. 2007). A recent study provided evidence of testicular dysgenesis syndrome (TDS) in dogs (Grieco *et al.* 2008). In this study, canine TDS lesions showed typical histological features of human TDS. In humans, TDS are reproductive disorders (cryptorchidism, hypospadias, decreased semen quality and seminoma) that have become more prevalent during the last 50 years. Also, TDS precedes the development of testicular cancer. For these reasons dogs represent a good model for studying SSCs biology, which will lead to understanding the etiology of testicular germ cell tumor formation, so that

more specific therapies can be developed. Moreover, understanding the biology of SSCs in dogs will enable us to develop male dog contraceptives by exploring nonsurgical alternatives as possible answer to dog overpopulation.

New technologies of manipulating male germline stem cells have been developed since the SSCs have been isolated and maintained in cell culture. Spermatogonial stem cell transplantation (SSCT) is one of the most powerful of all, first reported in mouse (Brinster & Zimmermann 1994). The technique allows in-vivo propagation of transplanted SSCs into infertile host testes. The procedure had a great impact in the field of male fertility restoration, germline gene therapy, conservation of endangered species, and production of transgenic animals. Canine embryonic stem cells have been recently characterized (Hayes *et al.* 2008; Wilcox *et al.* 2009). This achievement would allow the generation of transgenic dogs; however genetic manipulation of embryonic stem cells is complicated in comparison to manipulating germline stem cells. The successful production of donor-derived dog sperm after SSCs transplantation would enhance the production of transgenic dogs; therefore a full utilization of this species as a genetic model will be readily available.

This project aims at characterization and isolation of canine spermatogonial stem cells, and to establish a cell culture system for those cells. Isolation and identification of SSCs are a prerequisite for culture, genetic manipulation and/or transplantation studies. Previous reports in mouse showed that SSCs could proliferate in long-term cultures. Specific growth factors (fibroblast growth factor, epidermal growth factor, leukemia inhibitory factor, glial cell line-derived neurotrophic factor) support long-term survival of mouse SSCs in-vitro (Kanatsu-Shinohara *et al.* 2003). The same result was obtained in bovines by Aponte and colleagues (Aponte *et al.* 2008). Therefore, our hypothesis is that dog SSCs can proliferate in-vitro for a long time (3- 5 months) in the conditions indicated by those studies. This length of time will be sufficient to investigate the mechanisms of dog SSCs self-renewal and differentiation.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

OVERVIEW OF SPERMATOGENESIS

Spermatogenesis is a complex process that includes expansion and maturation of spermatogonial cells into spermatozoa over extended period of time (Kierszenbaum 1994; Griswold 1995). In the mammalian testis, the development of the germ line after birth involves four distinct events: stem cells self-renewal that maintains the continuous production of germ cells throughout life; spermatogonia proliferation and differentiation that amplifies the number of premeiotic cells; meiosis in spermatocytes; and spermiogenesis, which is the morphological differentiation of round spermatids into spermatozoa.

Huckins & Oakberg proposed the most acceptable scheme of spermatogonial self-renewal and differentiation (Huckins & Oakberg 1978). In this model (Figure 1), the A_s (A single) spermatogonia are considered to be the stem cells of spermatogenesis. Upon division of A_s spermatogonia, the daughter cells can either separate and become 2 independent stem cells or they can remain connected by an intercellular bridge and become A_{pr} (A paired) spermatogonia. The A_{pr} spermatogonia divide further to form chains of 4,8 or 16 A_{al} (A aligned) spermatogonia. A_{al} spermatogonia divide into differentiating type A spermatogonia (A1-A4) that further develop into intermediate (In) spermatogonial then into B spermatogonia. Finally type B spermatogonia differentiate into primary spermatocytes that will enter the process of meiosis to produce haploid spermatids and spermatozoa. Spermatozoa are the vehicles by which male genetic information is transmitted to successive generations.

Spermatogenesis is an interactive process, in which a close interaction of the germ cells with somatic Sertoli cells is critical for successful production of spermatozoa. This interaction forms a complex three-dimensional architecture referred to as the seminiferous epithelium, which is supported by a basement membrane generated from contribution of both Sertoli and outlying peritubular myoid cells (Oatley & Brinster 2008). The seminiferous epithelium is divided into a basal compartment, exposed to many lymph and blood substances, and an adluminal compartment, to which blood substances have limited access. These two compartments are established by the tight junctions formed between adjacent Sertoli cells, which are the major component of the blood-testis barrier (Clermont 1972; Russell *et al.* 1983).

Little is known about the mechanisms that drive the different events of spermatogenesis, in particular the cues that mediate the decision of spermatogonial stem cells to self-renew or differentiate. Spermatogonial stem cells “SSCs” are maintained in specialized microenvironments called “niche” (Watt & Hogan 2000; Scadden 2006; Kostereva & Hofmann 2008). Sertoli cells are the main component of the niche by physically supporting the spermatogonial stem cells and providing them with growth factors. In addition, adhesion molecules that connect the SSCs to the basement membrane, and cellular components of the interstitium between the tubules, mainly Leydig cells, are important regulators of the niche function (Hofmann 2008). Yoshida *et al* recently shown that undifferentiated type A spermatogonia were preferentially localized to tubular regions

adjacent to blood vessels and interstitium (Yoshida *et al.* 2007). In this study the authors used transgenic mice, in which undifferentiated type A spermatogonia expressing a green fluorescence (GFP), the movement of these cells was monitored by real-time imaging over 96 hours. Clusters of undifferentiated type A spermatogonia were found adjacent to vasculature/interstitium after images of serial sections of seminiferous tubules were reconstructed in three-dimension.

The same study also showed that at the transition into differentiating spermatogonia (A_1-A_4) the cells migrated out of these areas and dispersed over the entire basal compartment of the seminiferous epithelium. Yet, the relationship of the actual stem spermatogonia to the vasculature still unanswered question. A vasculature niche has been described for other stem and/or progenitor cells in other systems in which they are closely associated with the vascular cells (Palmer *et al.* 2000; Sugiyama *et al.* 2006; Nikolova *et al.* 2007). However, unlike stem cells in the brain, and bone marrow, spermatogonia are not in direct contact with the vasculature. The role of the vasculature appears to be indirect, since the A undifferentiated spermatogonia are associated only with the vessels that are surrounded with interstitial cells (Yoshida *et al.* 2007).

Sertoli cells have receptors for follicle stimulating hormone (FSH), and testosterone (Sofikitis *et al.* 2008), which are the main hormonal regulators of spermatogenesis. The anterior pituitary gland produces and releases both FSH and LH in response to gonadotropin releasing hormone (GnRH) stimulation. Some studies have shown that inhibition of GnRH release during postnatal development in mice impairs SSCs proliferation, whereas in adults SSC proliferation is increased when GnRH is suppressed (Kanatsu-Shinohara *et al.* 2004b).

Because germ cells in meiotic stages are isolated from the extratubular environment by the blood-testis barrier, only paracrine factors produced by the Sertoli cells can induce or inhibit their differentiation and further development (Skinner & Griswold 2005). It has been discovered that Sertoli cells secrete paracrine factors that were originally identified in the brain to ensure differentiation, maintenance, and repair of neurons. This fact has provided a considerable advance in our understanding of the regulation of spermatogenesis (Cupp *et al.* 2000; Meng *et al.* 2000; Park *et al.* 2001).

Figure 1. The first steps of mammalian spermatogenesis

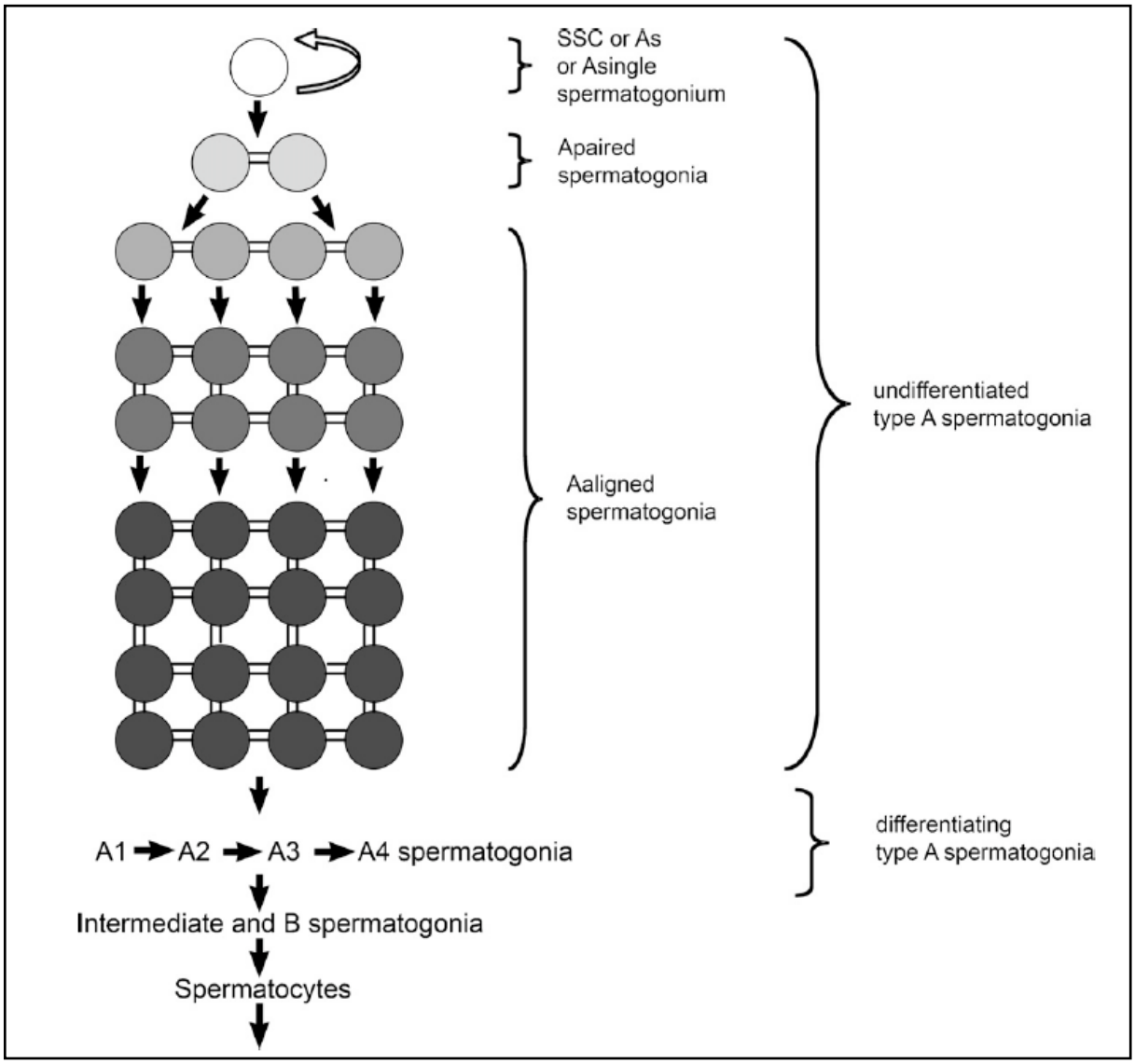


Diagram representing the first steps of spermatogenesis (according to Huckins & Oakberg scheme), in particular the different subtypes of A spermatogonia. The spermatogonial stem cell (SSC or A_s or Asingle spermatogonium is able to self-renew (curved arrow) or to differentiate into Apaired spermatogonia linked by an intercellular bridge (straight arrow). The Apaired spermatogonia subsequently proliferate into 4, 8, and 16 Aaligned spermatogonia, ultimately producing differentiating type A spermatogonia. The differentiating type A spermatogonia proliferate and differentiate to become spermatocytes that undergo meiosis, producing spermatids that will go through spermiogenesis. All spermatogenic cells differentiate as cohorts of units interconnected by intercellular bridges. Cited from (Hofmann 2008).

ORIGIN OF SPERMATOGONIAL STEM CELLS

The embryonic origin of spermatogonial stem cells starts at day 7.0-7.5 of gestation in mammals, when primordial germ cells "PGCs" arise in the embryonic ectoderm. PGCs migrate from the embryonic ectoderm to the urogenital ridges and take part in formation of the embryonic gonad (Clermont & Perey 1957; McLaren 2003). The expression of the gene *sry* by Sertoli cells in the male initiates the development of seminiferous cords that encapsulate PGCs. Once PGCs reach the primitive gonads, they stop proliferation and they are called gonocytes or "pre-spermatogonia" (Byskov 1986).

Differentiation of gonocytes into spermatogonial stem cells occurs between days 0-6 postpartum in mice (Huckins & Clermont 1968; Bellve *et al.* 1977; de Rooij & Russell 2000). In other species, the transition period of gonocytes into SSCs is largely undefined and may occur over a period of several months in livestock animals or years in humans and other primates (Oatley & Brinster 2008). Many studies in mice showed that two different populations of gonocytes are present in the neonatal mouse testis. One subpopulation proceeds directly into differentiating spermatogonia "A2" without undergoing self-renewal, and completes the first wave of spermatogenesis. The second subpopulation transform into SSCs that provide the basis for all subsequent rounds of spermatogenesis (de Rooij 1998; de Rooij & Russell 2000; Yoshida *et al.* 2006). There is no data currently available about this process in other species.

IDENTITY OF SPERMATOGONIAL STEM CELLS

SSCs are the adult stem cells of the testis; these cells are defined by their function: 1) to reproduce themselves (self-renewal), 2) to generate progenitor spermatogonia that are committed to differentiation and 3) to perform the dual function throughout life (Ebata *et al.* 2008). Identification of SSCs is based on their unique activity as a stem cell. Their morphological characterization, cell-surface/intracellular markers and functional assay are the basic parameters used to identify SSC populations. The most direct assay to identify SSCs and examine their biological activity is functional transplantation that will be discussed below.

1. SSCs transplantation:

In the original assay, determination of SSCs identity is based on the ability of donor cells to re-establish spermatogenesis following injection into germ cell-depleted testis of a recipient mouse (Brinster & Avarbock 1994; Brinster & Zimmermann 1994; Nagano *et al.* 1999; Oatley & Brinster 2006). This system is well established for other adult stem cell populations, including hematopoietic stem cells "HSCs" (Harrison 1980), and neural stem cells (Blanpain *et al.* 2004).

The transplantation system (see Figure 2) of SSCs includes injection of a donor testicular cell suspension into seminiferous tubules of a recipient testis in which endogenous germ cells have been depleted by treatment with chemotoxic drugs (e.g., busulfan) or are naturally devoid of germ cells (e.g., W/Wv mutant males). Upon injection, only SSCs regenerate and maintain donor-derived spermatogenesis by colonizing the seminiferous

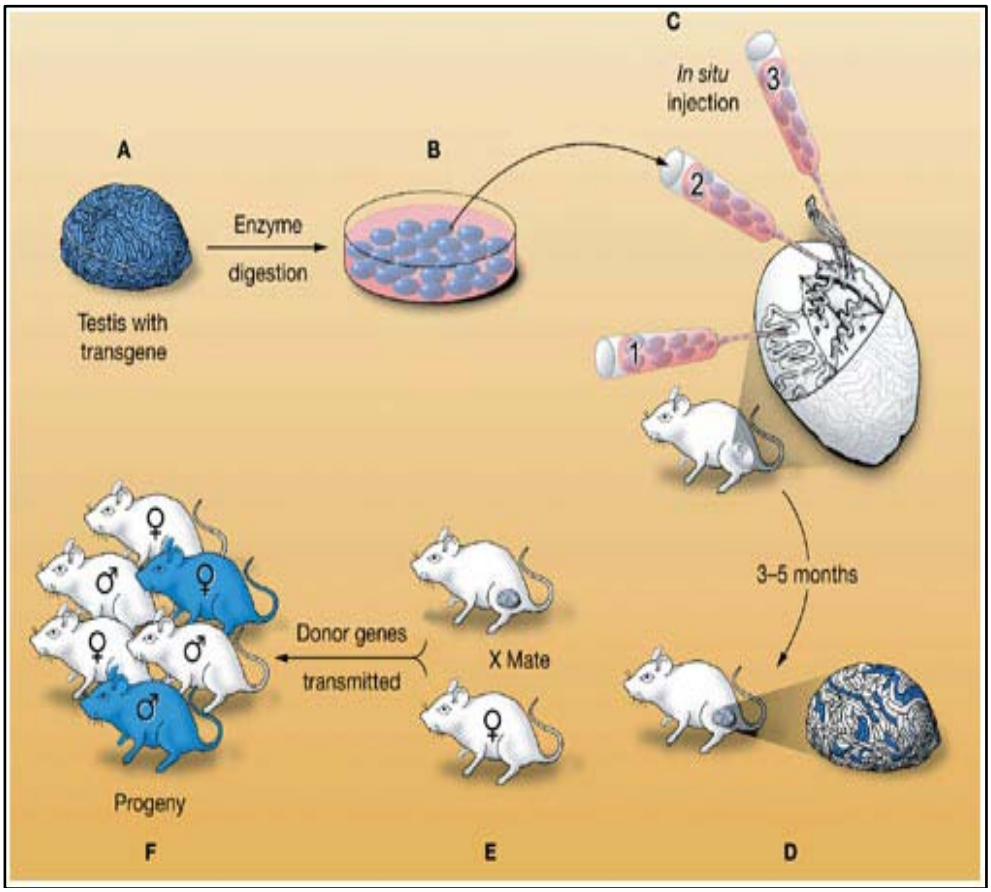
tubules. Therefore, spermatogonial transplantation allows us to identify SSCs using their function as a marker (Brinster & Zimmermann 1994). The technique of spermatogonial transplantation has been applied for rats (Jiang & Short 1995), monkeys (Schlatt et al. 1999), goats (Honaramooz et al. 2003; Honaramooz et al. 2008), pigs (Honaramooz et al. 2002), and dogs (Kim et al. 2008).

Allogeneic transplantation (transplantation between individuals of the same species) is indicated for practical and experimental purposes. A major extension of spermatogonial transplantation was achieved by the successful cross-species transplantation (xenogeneic transplantation) of SSCs.

A study by Clouthier *et al* reported successful establishment of rat spermatogenesis in mouse testis (Clouthier et al. 1996). Similarly, hamster sperm was produced by recipient mice (Ogawa et al. 1999). However, transplantation of germ cells from rabbit and dog (Dobrinski et al. 1999), bull (Oatley et al. 2002), primate (Nagano et al. 2001), and cat (Kim et al. 2006) into mouse testes showed colonization of stem cells but not a complete spermatogenesis. In spite of this result, SSCs are able to readily migrate from the tubule lumen to the basement membrane, which is the first step for a successful transplantation.

The technique of spermatogonial transplantation is also valuable because it allows quantification of SSCs. Upon transplantation; donor-derived spermatogenesis is reconstituted in the form of distinctive segments along the seminiferous tubules (Nagano *et al.* 1999). Recent studies, have demonstrated that each of these segments or “colonies” originates from one single stem cell (Zhang *et al.* 2003; Kanatsu-Shinohara *et al.* 2006b; Yeh *et al.* 2007). Consequently, by counting the number of colonies, the number of injected SSCs can be reliably quantified. The quantitative nature of the transplantation assay has been critical to determine SSCs identification markers (Ebata *et al.* 2008), which will be discussed in the following section.

Figure 2: SSCs transplantation in mice



(A) A single-cell suspension is prepared from the testes of a fertile male that expresses a reporter transgene. (B) The testis cells can be cultured in appropriate conditions. (C) Cells are microinjected into the seminiferous tubules of an infertile recipient male. There are three methods for microinjection: the micropipette can be inserted (1) directly into the seminiferous tubules, (2) into the rete testis, or (3) into an efferent duct. (D) Spermatogonial stem cells colonize the basement membrane of the tubules and generate donor-cell-derived spermatogenesis. Each stretch of cells in the seminiferous tubules of the recipient testis represents a spermatogenic colony derived from a single donor stem cell. (E) Mating the recipient male to a wild-type female resulted in donor-cell-derived spermatozoa fertilizing wild-type oocytes. (F) Progeny with the donor haplotype are produced. Cited from (Kubota & Brinster 2006).