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## Isolation, Genetic Manipulation, and Transplantation of Canine Spermatogonial Stem Cells: Progress Toward Transgenesis Through the Male Germ Line

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### Abstract

The dog is recognized as a highly predictive model for pre-clinical research. Its size, life span, physiology and genetics more closely match human parameters than do those of the mouse model. Investigations of the genetic basis of disease and of new regenerative treatments have frequently taken advantage of canine models. However, full utility of this model hasn't been realized because of the lack of easy transgenesis. Blastocyst-mediated transgenic technology developed in mice has been very slow to translate to larger animals, and somatic cell nuclear transfer remains technically challenging, expensive, and low yield. Spermatogonial stem cell (SSC) transplantation, which does not involve manipulation of ova or blastocysts, has proven to be an effective alternative approach for generating transgenic offspring in rodents, and in some large animals. Our recent demonstration that canine testis cells can engraft in a host testis, and generate donor-derived sperm, suggests that SSC transplantation may offer a similar avenue to transgenesis in the canine model. Here, we explore the potential of SSC transplantation in dogs as a means of generating canine transgenic models for pre-clinical models of genetic diseases. Specifically, we 1) established markers for identification and tracking canine spermatogonial cells; 2) established methods for enrichment and genetic manipulation of these cells; 3) described their behavior in culture; and 4) demonstrated engraftment of genetically manipulated SSC, and production of transgenic sperm. These findings help set the stage for generation of transgenic canine models via SSC transplantation.

### Introduction

Spermatogonial stem cells (SSC) are the stem cells in testis that generate spermatozoa throughout the adult life of the male. As with all true stem cells, SSC can undergo both self-

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#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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renewal, and differentiation divisions, thereby maintaining a static population of stem cells, while generating a constant supply of spermatozoa. In mice, these cells can be isolated and expanded indefinitely without genetic drift or loss of stem cell potential (Shinohara *et al.* 2000a, Shinohara & Brinster 2000, Shinohara *et al.* 2000b, Kanatsu-Shinohara *et al.* 2003, Nagano *et al.* 2003, Kubota *et al.* 2004, Hamra *et al.* 2005, Kanatsu-Shinohara *et al.* 2005, Oatley & Brinster 2006, Hamra *et al.* 2008, Oatley & Brinster 2008, Oatley *et al.* 2010). They can be genetically manipulated efficiently by transduction and transfection (Kanatsu-Shinohara *et al.* 2004, Hamra *et al.* 2005, Kanatsu-Shinohara *et al.* 2006, Kanatsu-Shinohara & Shinohara 2007, Takehashi *et al.* 2010, Kanatsu-Shinohara *et al.* 2011). SSC provide an alternative approach for the generation of transgenic mice. When transplanted into the testes of sterile male mice, SSC efficiently repopulate the seminiferous tissue, and re-initiate spermatogenesis. The offspring of these males carry the genetic properties of the donor cells (Kanatsu-Shinohara *et al.* 2004, Kanatsu-Shinohara *et al.* 2006, Kanatsu-Shinohara & Shinohara 2007, Takehashi *et al.* 2010, Kanatsu-Shinohara *et al.* 2011). Mouse SSC can also be converted into pluripotent cells, without genetic manipulation (Guan *et al.* 2006, Conrad *et al.* 2008, Izadyar *et al.* 2008, Golestaneh *et al.* 2009b, Mizrak *et al.* 2010). These germ line-derived pluripotent cells (gPS) acquire an expression profile similar to embryonic stem cells (ESC; (Silva *et al.* 2009)), and are functionally indistinguishable from ESC: They form complex teratomas, differentiate into three germ layers in culture, and contribute to all the tissues of mice generated from chimeric blastocysts (Takehashi *et al.* 2007, Conrad *et al.* 2008, Izadyar *et al.* 2008). Thus, at least in the mouse, SSCs provide an unusually versatile source of material for stem cell and developmental research, including transgenic animal technology, and stem cell-based cell therapy.

It would be extremely valuable to translate this technology to large animals for modeling human diseases. The SSC could be used directly to generate transgenic animal models for preclinical research, and the gPS would serve to bypass both the ethical concerns regarding ESC and the potential of genetic anomalies created in the multi-gene insertion approaches to generating “induced pluripotent stem” (iPS) cells. Other approaches to transgenic models in large animals have been very difficult and inefficient. While multiple lines of canine (Hayes *et al.* 2008, Wilcox *et al.* 2009), and other large animal (Kumar De *et al.* 2011, Vassiliev *et al.* 2011, Kim *et al.* 2012) embryonic stem cells (ESCs) have been reported, all non-rodent lines tend to show genetic drift, and loss of pluripotency over time (Yang *et al.* 2010, Gerwe *et al.* 2011). In addition, demonstration of germ-line transmission and generation of transgenic large-animal models from ESC have been largely unsuccessful. Genetically chimeric pigs have been produced recently (West *et al.* 2010) by implanting iPS into early embryos, but this approach has not yet succeeded in other large animals. Several transgenic dogs (Hong *et al.* 2009, Hong *et al.* 2011), and other large-animal models (An *et al.* 2012, Giraldo *et al.* 2012, Jung *et al.* 2012) have been generated through somatic nuclear transfer but, so far, this approach has been extremely labor, cost and animal intensive.

Several authors have reported isolation and short-term culture of SPG from large animals (Kim *et al.* 2006, Rodriguez-Sosa *et al.* 2006, Goel *et al.* 2007, Hermann *et al.* 2007, Aponte *et al.* 2008) and humans (Wu *et al.* 2009a), as well as conversion of these cells into pluripotent gPS cells (Golestaneh *et al.* 2009b). SSC transplantation, and subsequent donor sperm production has now been reported in pigs, sheep, bulls, goats, monkeys and dogs (Izadyar *et al.* 2003, Kim *et al.* 2008, Herrid *et al.* 2011, Jahnukainen *et al.* 2011, Hermann *et al.* 2012, Zeng *et al.* 2012, Zeng *et al.* 2013). More importantly, SSC transplants in both sheep (Herrid *et al.* 2009) and goats (Honaramooz *et al.* 2003) have led to the birth of donor-derived offspring through normal mating. So this technology is clearly applicable to large-animals.

In this light, the canine has major potential as a pre-clinical transgenic model for two major reasons. First, research with the canine model has proven to be highly translatable to the clinical setting. This model better reflects the size, life span, physiology, and genetics of humans than does the mouse model (Tsai *et al.* 2007). The canine model is also much more cost-effective than primate models. It is a primary preclinical model for hematopoietic stem cell transplantation ((Lupu & Storb 2007)), gene therapy (Nowend *et al.* 2011, Okazuka *et al.* 2011) and cancer research (Gordon & Khanna 2010, Rowell *et al.* 2011). The dog displays a large repertoire of naturally occurring genetic diseases with human counterparts (Athanasίου *et al.* 1995, Lingaas *et al.* 2003, Acland *et al.* 2005) and where data are available, the same genes are involved in both species (Tsai *et al.* 2007). Second, the ability of canine SSC to engraft and reinitiate spermatogenesis in a recipient testis has been demonstrated (Kim *et al.* 2008). So far, canine SSC activity has only been shown in freshly prepared, un-fractionated testis cells. It remains to be demonstrated that canine SSC can retain engraftment potential after purification, genetic manipulation, culture, and cryopreservation.

Here, we describe, for the first time, the identification, isolation, culture, and genetic manipulation of canine SSC. We demonstrate engraftment and generation of mature transgenic sperm by canine SSC transplanted into the testes of recipient males. A true SSC can only be distinguished from committed and differentiating spermatogonia by its ability to engraft. Therefore, the term, “SSC”, will be reserved henceforth for true stem cells, while the more general term “spermatogonia” (SPG) will be used when that distinction is not verifiable.

## Materials and Methods

### Reagents

Hanks' balanced salt solution (HBSS: cat #14175), penicillin/streptomycin (15140-122), B27-vitamin A supplement (12587-010), trypsin-EDTA (25200-056), Superscript II reverse transcriptase (18064-014), all Alexa-tagged secondary antibodies (Supplemental Table I), Dispase (17105-041), dispase (17105-041), mouse LIF (LIF2005), and heat-inactivated Fetal Bovine Serum (FBS) (10082147), were all from Invitrogen (Eugene, OR). Biosprint 96 blood DNA isolation kits (940057), and RNAeasy RNA isolation mini kits (74104), were from Qiagen (Valencia, CA). Type 4 collagenase (LS004212) and hyaluronidase (LS005475) were from Worthington (Lakewood, NJ). Primary antibodies were purchased as indicated in Supplementary Table I. DME/F12 medium (SH30023.01) and buffered formalin (SF98-4) were from Fisher Scientific (Waltham, MA). Primer synthesis was done by Operon (Huntsville, AL). Trypsin (T4799), DNase I (D4513-1VL), laminin (L2020), gelatin (G1890-100G), L-Glutamine (G6392-10L), BSA (A9576-50ml), donkey serum (D9663-10ML), and human FGF2 (F0291-25UG) were from Sigma (St. Louis, MO). Advantage 2 polymerase mix (639202) was from Clontech (Mountain View, CA). Human EGF (354052) was from BD Biosciences. Human GDNF (212-GD-050) was from Roche Biochemicals (Indianapolis IN). Rat soluble GFRA1 (560-GR-100), human BDNF (284-BD-025), human NT3 (267-N3-025), human NT4 (268-N4-025), human Pleiotropin (252-PL-250), and human WNT3A (5036-WIN-010) were all from R&D Systems (Minneapolis, MN). Target retrieval solution (S1699) was from DAKO (Carpinteria, CA). Falcon Tissue culture flasks and plates were from BD Biosciences (San Jose, CA).

### Canine testis tissues

Testes were obtained from the Seattle Spay and Neuter Clinic, immediately following routine neutering for unrelated purposes. Tissue was collected from a variety of breeds in the

age range of 3 to 12 months. Testes were transported in sterile Hanks Buffered Saline on ice, and used within 2 hours of castration.

### Isolation and culture of SPG

Testes from 3–5 month old (pre-pubertal) dogs were washed twice in fresh, sterile HBSS. The testis parenchyma, containing the testicular cords, was dissected away from the tunica vaginalis and tunica albuginea, epididymis, and other non-seminiferous tissue, washed twice in HBSS, minced to < 1 mm pieces, and digested for 30 minutes at 37°C in 30 ml DME/F12 medium containing 1 mg/ml type 4 collagenase and 33 µg/ml DNase I. Tissue was dispersed into single cells and small lengths of cords by vigorous pipetting, then filtered through sterile gauze. The filtrate was washed twice in HBSS by centrifugation at 200 × g for 5 minutes. The washed cells and cord segments were plated in 225 cm<sup>2</sup> culture flasks at an estimated 5×10<sup>7</sup> cells per flask in DMEM/F12 medium containing 10% FBS, 50 units/ml penicillin, and 50 µg/ml streptomycin. At 3 days culture, dead cells were removed by replacing the medium with HBSS. Loose cells were then dislodged from the tissue culture layer by 15–20 vigorous horizontal raps of the flask against a styrofoam block. The loose cells were pooled, and selected for non-adhesion to gelatin by 2 rounds of plating for 2 hours on a gelatin-coated 10 cm tissue culture dish and re-collection of all non-adherent cells. The remaining loose cells were either used directly for analysis, or cryo-preserved under liquid nitrogen in 70% DME/F12, 20% FBS, 10% DMSO. Cells were cultured in either of two basic media: A) Complete DMEM/F12: DME/F12 with 10% FBS and Pen/Strep or B) SG: a serum-free medium optimized for rat SSC culture (Wu *et al.* 2009b). Additional modifications to the culture conditions, including substratum, feeder cells, serum level, and growth factors, are specified in Supplementary Table II. Mouse Embryonic Feeder Cells (MEFs) were expanded from 14 day mouse embryos, mitotically inactivated by γ irradiation (3000 Rad), and plated at 25,000 cells/cm<sup>2</sup> 1 day prior to addition of SPG. Testis fibroblast feeder cells were adherent cells from 3 month old canine testis, expanded for at least 3 passages and at least 10 days of constant culture, then irradiated at 3000 Rad and plated at 25,000 cells/cm<sup>2</sup> 1 day prior to addition of SPG. Cultures were maintained at 37°C in a humidified tissue culture incubator with 5% CO<sub>2</sub>, and media were changed every 48 hours.

### Lentiviral constructs

Self-inactivating lentiviral reporter construct, PL-SIN-EF1a-EGFP (Hotta *et al.* 2009), driven by the widely expressed mouse EF1α promoter, was purchased through Addgene. A second self-inactivating lentiviral construct carrying a widely expressed PGK- mCherry reporter (pRCH-PCh-W), was obtained from the Viral Vector Production Core at Fred Hutchinson Cancer Research Center. Packaging vector, psPAX2 (plasmid 12260) and vsv-G envelope vector, pMD2.G (plasmid 12259) were from the Trono lab and purchased from Addgene ([http://www.addgene.org/Didier\\_Trono](http://www.addgene.org/Didier_Trono)).

### Lentiviral Transduction of SPG

PL-SIN-EF1a-EGFP and pRCH-PCh-W were packaged in HEK293 cells as VSVG-pseudotyped lentiviral particles according to Addgene protocol E.2 (<http://www.addgene.org>), filtered through a .45 µM filter (Millipore, Billerica, MA), concentrated by centrifugation at 13,000g for 15 hours, and stored in aliquots at –80°C. Infectious particle concentrations were determined by titered infection of HEK293 cells (15 hours transduction in the presence of 4 µg/ml polybrene on 70% confluent cells), followed by determination of percentage of reporter-expressing cells at 48 hours post-infection. Final titers were 1–2×10<sup>7</sup> infectious units/ml. Transduction of freshly isolated SPG (“loose cells”) was performed by incubating 0.5–2×10<sup>6</sup> cells with virus (moi = 5–15) in 1 ml transduction medium (DMEM/F12/10%FBS/4 µg/ml polybrene) for 15 hours in a tissue culture incubator. Cells were then

washed twice with HBSS, and plated in SG medium. Expression of reporter genes was monitored by flow cytometry and by epifluorescence microscopy.

### Histology and Immunohistochemistry

Seminiferous tissue was cut into 1 cm cubes, fixed for 72 hours in buffered formalin, embedded in paraffin and sectioned at 1.5  $\mu$ m thickness. Sections were deparaffinized in 3 washes of xylene, rehydrated through an ethanol series (100%, 100%, 85%, 70%, water), and washed twice in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6) all for 10 minute intervals. Antigen retrieval was performed by a 20 minute incubation at 95°C in a pre-warmed target retrieval buffer (DAKO Carpinteria, CA), followed by 3 washes in HBSS. Treated sections were incubated for 30 minutes in blocking buffer (TBST + 5% BSA and 5% Donkey Serum), then for 15 hours at 4°C in blocking buffer plus primary antibody (see Supplementary Table I for antibodies and concentrations). Sections were washed 5  $\times$  3 minutes in TBST, incubated 2 hours at RT in a 1000 X dilution of an appropriate Alexa Fluor-tagged donkey secondary antibody (Supplemental Table I) in TBST, washed similarly, and observed by epifluorescent microscopy. Cultured cells were fixed on the plate for 20 minutes in 10% buffered formalin, and probed as described for post-antigen retrieval sections. Dolichos biflorus agglutinin (DBA) mediated localization of  $\alpha$ -D-N-acetyl-galactosamine was performed as described (Izadyar *et al.* 2002).

### Microscopy

Phase and Epifluorescent microscopy were done on an Eclipse Ti-U inverted microscope equipped with several phase lenses, a TI-FL Epi-fl epi-illumination system, and digital DS-Qi1Mc monochrome camera (Nikon). Images were recorded and pseudo-colored using NIS-Elements D3.2 software from Nikon and color-merged with Photoshop software (Adobe).

### Flow cytometry analysis

For dual reporter expression studies, cells were washed twice with HBSS, dissociated with Trypsin-EDTA for 5 minutes at room temperature, washed twice with HBSS and observed by flow cytometry on a FACS Calibur flow cytometer (BD Biosciences). For comparison of GFP reporter expression to VASA immunolocalization, cells were washed twice with HBSS, dissociated with Trypsin-EDTA for 5 minutes at room temperature, fixed for 5 minutes in 10% buffered formalin, washed 3 times in HBSS, blocked 10 minutes in blocking buffer, incubated at RT for 1 hour in blocking buffer containing anti VASA antibody (Supplemental Table I) at 100 X dilution, re-washed and incubated with donkey anti rabbit-Alexa 594 secondary antibody (Supplemental Table I), re-washed, and observed by flow cytometry as above. All washes were performed by re-suspension in HBSS and centrifugation at 200  $\times$  g for 5 minutes.

### RTPCR analysis of gene expression

Three separate experiments were performed using dissociated testicular cells from three different pre-pubertal dogs: a 16-week old Black Labrador, and two 10-weeks old German Shepherd littermates. For each testis sample, RNA was extracted from intact seminiferous tissue at day 0, from the total cultured cells at 3 days, from the “loose” and adherent cell populations isolated at day 3, and from duplicate loose and adherent populations after 30 days in culture. RNA from testis tissue and cultured cells was extracted using an RNeasy mini kit (Qiagen) according to manufacturer’s instructions, and quantitated by UV spectral analysis using a Nanodrop™ 1000 spectrophotometer (ThermoFisher Scientific Inc). Two  $\mu$ g RNA was reverse transcribed using oligo dT18 primers and Superscript II Reverse Transcriptase (Invitrogen) according to manufacturer’s instructions. Prior to expression analysis, cDNAs were normalized to beta Actin (ACTB) by adjusting the cDNA



concentrations until they gave similar signals by ACTB RTPCR. The adjusted cDNAs were used for all subsequent expression analysis. Primers for amplification of each specific mRNA target (Supplemental Table III) were designed to amplify a region spanning one or more introns of significant size, thus eliminating false positive signals from contaminating genomic DNA. Each primer pair was verified by cloning and sequencing the product amplified from testis cDNA. Primers were designed with a  $T_m$  of 72–74°C to accommodate a uniform two-step cycle of 20 sec at 94°C and 2 minutes at 68°C. Amplifications were performed as 20  $\mu$ l reactions containing 2  $\mu$ l normalized cDNA, primers at 200 nM, dNTPs at 200  $\mu$ M and Advantage 2 polymerase mix and buffer. Products were separated by agarose, gel electrophoresis and detected by ethidium bromide staining.

### Transplantation of SSC

Several preparations of donor SPG (Supplementary Table IV) were prepared and transduced with lentivirus, as described above, washed in serum-free and growth factor-free SG medium, and cryopreserved in SG / 20% BSA / 10% DMSO. On the day of transplantation, each cell preparation was thawed, washed 3 times in DME/F12, re-suspended in 1 ml DME/F12, and held at 4°C until use (1–3 hours). Transplantation of SPG and all animal care and handling was done at Cornell University, and all related protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Cornell University. Three 5- months-old hounds (Marshall BioResources, North Rose, NY, USA) had their testes subjected to focal external beam radiation to deplete their endogenous germ cells as described (Kim *et al.* 2008), except that the irradiation regimen was reduced from 3 consecutive days of 3 Gy/day, 8 weeks prior to transplant, to a single 3 Gy dose on the day of transplant. The smaller dose was designed to lower the apparently excessive toxicity to the testis tissues observed in our previous study, which had resulted in complete loss of libido, and almost no recovery of testis size, histological evidence of spermatogenesis, or sperm count. One ml of medium containing  $2\text{--}5 \times 10^5$  SPG was injected in retrograde fashion into the rete testis under the guidance of ultrasound scanning (Aloka 633, Colormetrics Medical Systems Inc., Wallingford, CT, USA) as previously described (Kim *et al.* 2008).

### VNTR analysis of donor/recipient sperm production

DNA was purified by silica binding from ejaculated sperm, using a Qiagen Biosprint magnetic bead DNA isolation kit, according to the manufacturer's instructions. Donor and recipient DNA standards were prepared respectively from testis tissue used to generate each SSC preparation, and blood from pre-transplant recipients. The contribution of recipient and donor sperm cells was quantified by fluorescent VNTR (Variable Number Tandem Repeat) analysis of microsatellite loci, as described (Scharf *et al.* 1995, Hilgendorf *et al.* 2005) and modified (Graves *et al.* 2007). Micro-satellite FH2001, was used to distinguish the DNA of recipients 795941 and 795917 from their respective donor cells. Microsatellites FH2611 and FH2199 were used to distinguish Recipient 795933 from its donor cells. The primers used for these VNTRs are listed in Supplemental Table V. In all cases standard curves showed a threshold of unequivocal detection of donor DNA at 1–2% of total.

### PCR detection of transgenic cells in sperm

Sperm DNA was analyzed for the presence of PL-SIN-EF1a-EGFP sequence by PCR. Three distinct regions within the construct were amplified in separate reactions as a means of corroborating low signal reactions (see Supplementary Table V for primer sequences and Fig. 7b for target positions). Amplification was in 20  $\mu$ l reactions containing 100 ng DNA, 200 nM primers, 200  $\mu$ M dNTPs, and 1X Advantage 2 polymerase mix and buffer. Reactions were run for 35–40 cycles under the 2-step conditions used for RTPCR. As standards for semi-quantitative estimation of vector copy #, reactions were run

simultaneously on a titration series of 0, 20, 60, 200, and 2000 copies of linearized PL-SIN-EF1a-EGFP plasmid (based on a plasmid size of 7601 base pairs, and a calculated mass of 7.78 atograms per molecule) in 100 ng canine DNA (estimated at 40,000 haploid genomes, based on a haploid canine genome size of  $2.5 \times 10^9$  base pairs or 2.56 pg (<http://www.genome.ucsc.edu/>)).

## Results

### Establishment of molecular markers for canine SPG

In order to identify and track canine SPG in culture, we screened a battery of commercially available antibodies to established murine and human SPG markers for specificity in canine pre-pubertal (4 months) testis. At this stage, the testicular cords present a simple architecture compared to mature testis ((Nayernia *et al.* 2004); see Fig. 1A). Only two cell types, the gonocytes/SPG and Sertoli cells, are packed in the dense cord-like structures, bound by a basement membrane. Both cell types are attached to the basement membrane at this age, but the Sertoli cell nuclei align along the periphery while the nuclei of the SPG are more centrally located (Nayernia *et al.* 2004). All SPG are SSC at this stage, since spermatogenesis has not yet started. Antibodies against reported SPG and SSC-specific target antigens; VASA (Noce *et al.* 2001), UCHL1 (Goel *et al.* 2007), DAZL (Anderson *et al.* 2007), PLZF (Jijiwa *et al.* 2008), AP180 (von Kopylow *et al.* 2010), POU5F1 (Anderson *et al.* 2007) and RET (Anderson *et al.* 2007, Tyagi *et al.* 2009) all marked the interior SSC of canine pre-pubertal testis (Fig. 1). In contrast, ACTA1 was enriched outside of the cords in peri-tubular myoid and Leydig cells (Fig. 1B–I). GATA4, known as a Sertoli cell marker in mice (Ketola *et al.* 2002) and pigs (McCoard *et al.* 2001), was confined to the peripheral Sertoli nuclei of the cords (Fig. 1J–L). The VASA antibody was the most reliable probe for SSC, yielding a consistent, strong signal in all experiments. Dual-label staining demonstrated co-localization of VASA, UCHL1, DAZL, PLZF, and AP180 in the same set of interior cells (Supplemental Fig. 1). Unfortunately we were unable to demonstrate SSC-specific labeling by antibodies against established murine SSC surface antigens (data not shown), such as GFRA1, B1 and A6 integrins, and GPR125, each of which could be useful for immunofluorescent sorting of mouse SSC (Shinohara *et al.* 1999, Bjarnadottir *et al.* 2004, Grisanti *et al.* 2009). Localization of cRet, another SPG surface antigen in mice ((Tyagi *et al.* 2009)), was sporadic in pre-pubertal canine testis, and appeared to be nuclear rather than cell-surface (Fig. 1G). The reported localization of  $\alpha$ -D-N-acetyl-galactosamine on the surface of porcine (Goel *et al.* 2007) and bovine (Izadyar *et al.* 2002) SSC also could not be confirmed in the dog using a similar dolichos biflorus agglutinin (DBA) binding assay.

The same probes were used to localize SPG in mature testis (Fig. 2). As the testis matures, a central lumen forms in the cords, creating seminiferous tubules. The SSCs are confined at the basement membrane by Sertoli cells. Successive advancing stages of male germ cell development (spermatogonia, spermatocytes, spermatids, and spermatozoa) advance spatially toward the lumen, whereas Sertoli cell processes interdigitate among the male germ cells, supporting the developmental process ((Nayernia *et al.* 2004); see Fig. 2A). Most of the SPG/SSC markers seen in pre-pubertal testis were more difficult to detect in mature testis. All immunofluorescence signals were lower in adults. However, VASA, UCHL1 and DAZL were all clearly expressed in cells located either at the basement membranes of tubules, or in layers near these peripheral cells (Fig. 2B–F), consistent with SPGs and early stages of spermatogenesis. As detected by immunohistochemistry, VASA expression appeared to be higher in differentiating SPGs and spermatocytes than in the most peripheral cells, including the SSC (see Supplemental Figure 4). This pattern is similar to that seen in Human and Rhesus testis (Castrillon *et al.* 2000, Hermann *et al.* 2007). Thus VASA may be

a poor marker for adult canine SSC, and a clear marker has not yet emerged. As indicated in Figure 2E–F, DAZL and UCHL1, may detect earlier stages than VASA, but the identity of the positive cells is not yet clear.

### Isolation of canine SSC

Pre-pubertal testes (3–6 months) were used as the source of SSC, taking advantage of the lower cellular complexity of the tissue at this stage. Spermatogenesis has not yet begun, and SSC are the only germ-lineage cells in the testes. In contrast to the mouse, a standard two-step digestion of canine pre-pubertal testis tissue with collagenase, followed by collagenase plus trypsin, and/or hyaluronidase (Hamra *et al.* 2008) did not generate a usable single-cell suspension. Considerable cell lysis was observed within the cords before the basement membrane dissociated. Thus we chose a gentler collagenase-only digestion, and plated incompletely digested pieces of seminiferous cords (Fig. 3A). By 3 days, most cells had migrated out of the cord structures onto the plate surface. Most of the VASA+ cells were rounded, loosely adherent cells that resided on top of a super-confluent layer of fibroblast-like cells (Fig. 3B,C and 4A). These VASA+ cells could be dislodged by vigorous agitation of the flask, and harvested with the medium. The “loose” cells were then further enriched by depleting collagen-adhesive cells (Hamra *et al.* 2008), with 2 rounds of short-term culture on gelatin-coated plates. The final preparation typically contained about  $0.5\text{--}1 \times 10^6$  of 70% pure SPG, as judged by VASA immunohistochemistry (Fig. 4B) and by flow cytometry (Fig. 4C). Prior to enrichment, less than 3% of cells were VASA-positive, indicating an enrichment of about 23-fold. Cryo-preservation of the enriched cells, followed by thawing and washing, resulted in about 70% viability, based on trypan blue exclusion. RTPCR analysis of several SPG markers confirmed the enrichment of SPGs (Fig. 4D). Canine homologue mRNAs of the well-established mouse SPG markers, DAZL, AP180, PLZF, cRet, Piwi1 (Lee *et al.* 2006), VASA, POUF5, NCAM (Li *et al.* 1998, Kubota *et al.* 2004) Telomerase (TERT:(Riou *et al.* 2005)) and NANOS2 and 3 (Suzuki *et al.* 2009) all were enriched in the “loose cell” prep and depleted from the residual adherent cells, as compared to total testis cells. These data strongly support the hypothesis that the “loose cells” were canine SSC. Putative SPG marker RNAs; GFRA1, GPR125, ITGA6 and ITGB1 did not enrich with the canine SPGs, consistent with the lack of SPG-specific localization of the respective antigens by immunohistochemistry in canine testis (above). In addition UCHL1 mRNA was not enriched in the SPG pool, consistent with a post-transcriptional regulation in germ cells, or perhaps up-regulation of the gene in cultured adherent cells as reported previously (Luo *et al.* 2006).

### Gene transfer into canine SPG

To test the efficiency of gene transfer by lentivirus, freshly enriched SPG were transduced with lentiviral vectors containing either an EF1-GFP or a CMV-mCherry reporter, and analyzed by flow cytometry for reporter expression. When cells were transduced with both vectors, at a multiplicity of infection (MOI) of 5 each, and analyzed at 48 hours (Fig. 5A–B) about 32% of the cells expressed at least one reporter and 22% expressed both. Since fibroblasts tend to rapidly out-grow the SPG in these cultures, a second experiment focused on reporter expression within the VASA+ population. Cells were transduced at an MOI of 20 with the EF1-GFP reporter vector, then fixed, permeabilized and labeled with the VASA antibody at 96 hours for flow cytometry (Fig. 5C). The SPG population (retaining VASA expression) showed a transduction efficiency of 8.8% under the conditions used here, based on GFP expression. Thus it is possible to insert and express transgenes in these cells by standard lentiviral transduction at a significant level. Our data might underestimate the true transduction efficiency of SPG if a portion of the cells left the VASA+ pool (differentiated or died) during the incubation period.



## Behavior of canine SPG in culture

Conditions for in vitro survival and expansion of rodent SSC have been extensively studied, suggesting appropriate conditions to try for canine SPG culture. The essential factor for self-renewal-type expansion of rodent SSC in culture is Glial cell Derived Neurotrophic Factor (GDNF (Oatley *et al.* 2006)). However, EGF and  $\beta$ FGF and LIF enhance the proliferative effect of GDNF (Kanatsu-Shinohara *et al.* 2003). The inositol phosphate-anchored GDNF co-receptor, GFR $\alpha$ 1, which can be added effectively as a soluble agent, is essential in some strains of mice, suggesting it is a limiting factor in those strains (Kubota *et al.* 2004). Additional signaling factors, including BDNF (Pyle *et al.* 2006), FGF9 (DiNapoli *et al.* 2006), NT3 (Pyle *et al.* 2006), NT4 (Pyle *et al.* 2006), Pleiotropin (Soh *et al.* 2007), and Wnt3a (Golestaneh *et al.* 2009a) have been implicated as having facilitating roles in survival and/or self-renewal; either in SSC directly, or in the closely related ESC. Serum levels and feeder cells have both had mixed effects on SSC survival in the literature (Hamra *et al.* 2005). Matrix components, particularly laminin and collagen, are predicted to affect morphology, mobility, and regulation of SSC, based on the localization of these materials at the SSC niche, high levels of B1 and A6 integrins on SSCs and an affinity for laminin (Shinohara *et al.* 1999).

In an attempt to establish an optimal culture environment for canine SSC, the enriched cells were cultured under a variety of conditions (Supplemental Table I). Base conditions were either a serum free medium (SG), optimized for murine SSC culture (Wu *et al.* 2009b), or DME/F12 with 10% FBS. Additional variables included feeder cells (canine, murine, none), substratum (laminin, gelatin, none), altered serum content (10%, 1%, none), and a variety of growth factors implicated in the mouse literature as promoters of SSC self-renewal. As an initial criterion for successful SPG culture, we recorded presence or absence of VASA-positive cells after one month in culture. As seen in Supplemental Table I, all culture conditions that included the established required components for rodent SSC self-renewal (SG medium with GDNF, FGF2, EGF, soluble GFRA1, and LIF (Wu *et al.* 2009b)) maintained significant numbers of VASA-positive cells for at least a month (e.g. Fig. 6E). The individual components of this cocktail were not tested separately. Addition of feeder cells, matrix, or any of the other signaling molecules did not noticeably alter the number, staining intensity, morphology, or association of VASA-positive cells, as determined by microscopic observation over a 1-month period. Therefore all subsequent studies used culture condition #12 (SG medium with GDNF, FGF2, EGF, soluble GFRA1, LIF, and a laminin substratum).

In early cultures, most of the cells adhered to substratum, but maintained a relatively rounded morphology compared to the elongated fibroblast-like cells in the culture (Fig. 6A and B). The rounded cells were often arrayed as connected chains, reminiscent of the syncytial chains of type A-aligned spermatogonia in vivo (Hermann *et al.* 2009). By 8 days, fibroblasts had proliferated to form a confluent layer, and the less adherent SPG-derived cells had formed irregular flattened clumps on top of the fibroblasts (Fig. 6C). These clumps retain VASA expression for many weeks, as shown in Fig. 6D and E, and remain distinct from the rapidly expanding somatic cell population (Supplemental Figure 2). Similar clumps of cells with similar morphology expressed several SPG markers in culture, including DAZL, UCHL1 and AP180 as well as VASA. All localized to similar clumps of cells with similar rounded morphology and round nuclei (Fig 6F–I). The level of expression of a given marker varied significantly among members of the same clump (Fig 6D), and the relative expression of different SPG markers varied among closely associated cells in the clumps (Fig 6J–K). This might reflect varying stages of spermatogonial differentiation, as is the case in mature testis (see Fig. 2E). In fact, over 2 weeks of culture, cells expressing DAZL, UCHL1 or AP180 became relatively rare, while VASA-positive cells remained numerous

(data not shown). The germ-line-specific mRNA expression profiles seen at the time of isolation, was lost by 30 days (Supplemental Figure 3), consistent with the rapid expansion of somatic cells, and the probable loss of SPG over this period.

Although the marker-positive canine cells persisted for extended periods in culture, we have not yet been able to establish proliferating (non-differentiating) lines using available human and mouse growth factors. In one experiment a set of replicate plates from a single SPG preparation was cultured without passage for 30 days. Plates were fixed and stained for VASA at intervals and the VASA+ cells manually counted. There was no detectable loss or expansion of VASA+ cells over this experiment. So far, any attempt to passage SPG - clumps or entire populations leads to rapid loss of expression of the SPG markers.

### Transplantation of genetically manipulated SPG and production of transgenic sperm

The only rigorous way to verify that the isolated “loose” cells were SSC, and that these cells retained true SSC characteristics after genetic manipulation, is to demonstrate engraftment and donor spermatogenesis after transplantation into a host testis. To this end, 6 separate SPG preparations were transplanted into 6 recipient testes of 3 hounds. Four of the transplants, used cells that had been previously transduced with a retroviral reporter (see Supplemental Table IV). Recipient hounds were prepared by focal irradiation of the testes, and  $2-5 \times 10^5$  SPG were injected into the seminiferous tubules of each testis via retrograde injection into the rete testis. Due to the low recovery of spermatogenesis, and complete lack of libido observed in previous transplants (Kim *et al.* 2008), the irradiation dose in this study was reduced from 3 days of 3 Gy/day to a single dose of 3 Gy.

Post-procedure, the recipient dogs showed normal sexual maturation, including normal sized testes, normal libido and interest in female urine. They also trained easily for manual ejaculation, allowing for periodic collections of sperm. Upon castration at 14 months post-transplant, the testis histology showed normal spermatogenic activity throughout the seminiferous tissue (Supplemental Figure 4). Epididymal sperm content, determined at the time of castration, was in the normal range, varying from  $1.1-4.6 \times 10^8$  cells in the two dogs that received transgenic SPG (Supplemental Table IV).

Sperm samples were analyzed for donor DNA contribution at 4 months by VNTR. In replicate reactions, each animal showed donor signals at or near the threshold of detection (1% donor) by VNTR assay (e.g. Fig. 7A). Standard curves for donor chimerism indicated no reliable quantitative information below 1%, due to random noise. Similar equivocal VNTR evidence for donor sperm was obtained through 15 months post-transplant (data not shown). Thus, donor contribution in these dogs remained at or below 1%, and could not be accurately quantified by this method.

As a more sensitive alternative assay, sperm DNA was analyzed for the presence of the reporter vector, PL-SIN-EF1a-EGFP, by PCR in the two animals that received transgenic SPGs. As shown in Fig. 7B, 3 separate PCR reactions, amplifying 3 regions of the vector, were performed in parallel for internal verification. Both dogs yielded vector-positive sperm (Fig. 7C) by this assay, while sperm from the non-transduced dog was negative. Based on the included vector standards, the copy number was between 20 and 60 copies per 40,000 haploid cells. If we assume a vector copy number of 40 per 40,000 cells, an SPG transduction efficiency of 9% (shown above) and a vector copy/cell value of 1 (predicted from the low transduction efficiency), then we can estimate that about 0.1% of the total sperm were transgenic, and that about 1.0% were of donor origin. This calculated figure was consistent with our VNTR results, showing positive signals at the 1% detection threshold.

## Discussion

Our long-term goal is to develop a robust strategy for generating transgenic canine models. Traditional approaches to transgenics have focused on introducing transgenes either directly into oocytes and zygotes or indirectly into early embryos via replacement of inner cell mass cells with ES or cloned cells. These approaches all require the ability to harvest significant numbers of oocytes or embryos from the female reproductive tract, manipulate them in vitro without damage, and implant them efficiently into a surrogate mother. The difficulties in performing these tasks, the low yields and high costs of traditional cloning, and the absence of clear cut, genetically stable ES cells in most species, combined to make development of transgenesis in large animal models a very expensive and time consuming endeavor. The development of SSC transplantation in rodents, as an alternative to embryo-mediated gene transfer, opened a new opportunity to explore this relatively less invasive, more efficient and lower cost approach in canines and other larger animal models. Successful SSC transplantations in several large animals (Izadyar *et al.* 2003, Kim *et al.* 2008, Herrid *et al.* 2011, Jahnukainen *et al.* 2011, Hermann *et al.* 2012, Zeng *et al.* 2012), led us believe this approach could be applied to the dog model.

Previously, we demonstrated that canine SSC can be transplanted successfully, that they can re-establish regions of normal spermatogenesis, and that they can generate mature donor-derived sperm in a host testis (Kim *et al.* 2008). In that study, unfractionated seminiferous tubule cells were transplanted to a donor testis without enrichment, culture, or genetic manipulation. In order to exploit SSC transplantation as a viable approach to canine transgenesis, the efficiencies of SSC collection, gene transfer, engraftment, and donor sperm production will have to be optimized. Ideally, this may require clonal expansion of lines of transgenic SSC, to achieve sufficient cell numbers and genetic homogeneity to guarantee a high level of transgenic sperm production, and thus a practical ratio of transgenic to wild-type offspring. Here, we extended the previous findings by developing and assessing methods to isolate, culture, and genetically manipulate canine SPG. We then tested whether or not engraftment potential is maintained after these manipulations.

Based on the rich data available on SPG markers in rodents and other animals, we were able to identify similar antigens and mRNAs that uniquely identified SPG and their progeny in canine testis. Several of these markers, including VASA, PLZF, DAZL, UCHLI and AP180, were detectable, using commercially available antibodies, in prepubertal gonocytes, adult spermatogonia, and cultured SPG. These antibodies facilitated tracking SPG through isolation and culture. Interestingly, five cell surface molecules, (GFRA1, GPR125, ITGA6, ITGB1 and  $\alpha$ -D-N-acetyl-galactosamine), which have been useful for flow cytometry-based enrichment of SPG from other species, were either undetectable or non-specific for canine SPG. In the absence of a useful surface marker, we exploited a differential matrix affinity method for enrichment of canine SPG from pre-pubertal testis to 70% purity. The identity of this population was confirmed by the co-enrichment of mRNAs for 11 well-established SPG-specific genes. Lentiviral transduction of the enriched SPG yielded about 9% reporter-positive cells. Thus it is now possible to prepare and cryopreserve a population of  $0.5-1 \times 10^6$  70% pure canine SPG with 9% lentiviral marking from an average sized pre-pubertal dog.

When cultured under the optimal conditions established for mice, the enriched canine SPG persisted as VASA+ cells for several weeks. Under these conditions, some cells remain positive for several markers of SPG, including VASA, DAZL, UCHL1, AP180, and PLZF, suggesting that they may retain their SSC phenotype in culture. However, most of the VASA-positive cells lost expression of other primitive markers, suggesting loss of the SSC phenotype. Furthermore, there was no evidence of expansion of germ-line cells. The

standard methods used by mouse SSC investigators of serially passaging clumps of rounded cells led to loss of SPG markers, as did trypsin-mediated or manual passage of the cells. Extensive testing of culture conditions did not reveal any advantage conferred by addition of feeder cells, matrix components, or other growth factors reported to enhance self-renewal divisions of mouse SSC. Thus, while canine SPG can be maintained in culture for weeks, conditions for expansion remain to be established. For the most part, these results parallel those reported from other large animal spermatogonial cell cultures. Short term expansions of germ-line cells with SPG markers, reported for human (He *et al.* 2010), pig (Kuijk *et al.* 2009), and bovine (Aponte *et al.* 2008) suggest that large-animal SSC lines may soon be possible. However, in no case, has a stable line of SSC been reported from a large animal.

We have demonstrated, for the first time in dogs that transplantation of transgenic SPG can lead to engraftment in a host testis and transmit the transgene to mature spermatozoa. This extends the findings in our previous study (Kim *et al.* 2008), establishing engraftment of unfractionated, un-manipulated canine testis cells. In addition to the changes in donor cell manipulation this study significantly changed the preconditioning regimen for host testes. In the first study a fractionated dose of 9 Gy, 8 weeks prior to transplantation was used to eliminate endogenous stem cells. While this regimen was consistent with those used successfully in other large animals (Kim *et al.* 2006, Herrid *et al.* 2009, Jahnukainen *et al.* 2011, Zeng *et al.* 2012), it may have delivered too high a dose for the dog, since testis size, sperm count and libido did not recover and sperm had to be recovered from the epididymis. Therefore, we reduced the irradiation dose to 3 Gy in the present study. The lower radiation dose used resulted in complete recovery of libido, testis size, spermatogenesis, and sperm count. However, most of the recovery was undoubtedly from endogenous SSC that escaped the preconditioning, possibly inhibiting donor engraftment. The typical interval of several weeks between irradiation and transplant is based on the logic that tissue damage and inflammation caused by irradiation will interfere with homing of stem cells to their niche (Herrid *et al.* 2011). However, there is not a compelling body of empirical data to confirm this. Arguably, disruption of the tissue might actually facilitate entry of the SSC to their niche by disturbing the sertoli cell tight junctions that block entry to that niche (Takashima *et al.* 2011). Hematopoietic stem cell homing to bone marrow is actually enhanced, not blocked, by local irradiation 24 hr prior to infusion of the cells (Bastianutto *et al.* 2007). Thus we used a single dose of 3Gy, immediately prior to transplant. The optimal preconditioning regimen probably lies between those of our two studies.

By our calculation, the enriched and genetically modified SSC used here engrafted almost as well as did the un-enriched, untreated canine cells of our previous study. Based on histology, only a 5–15% recovery of spermatogenesis was observed in the first study, after 9 Gy of total irradiation. This was supported by about a 10% of normal sperm count. Thus, the observed 20% donor sperm represented about 2% of normal sperm production. In contrast, after a 3 Gy dose in the present study, both histology and sperm count were normal, indicating a 100% recovery of spermatogenesis. Thus, the 1% donor sperm generated in these animals represented 1% of normal total germ cell activity and sperm production. Since a large fraction of endogenous SSC survived the present 3 Gy preconditioning, it is also likely that these cells inhibited homing and engraftment of donor cells, either by physical exclusion, or by homeostatic signaling mechanisms that regulate self-renewal (Caires *et al.* 2010, Caires *et al.* 2012). From this comparison, we conclude that the level of donor engraftment in these two studies was similar, in spite of differences in preconditioning of the recipient tissue or isolation and manipulation of the donor cells. Similar studies with other large animals have yielded variable levels of donor sperm production (Honaramooz *et al.* 2003, Izadyar *et al.* 2003, Herrid *et al.* 2009, Herrid *et al.* 2011, Jahnukainen *et al.* 2011, Hermann *et al.* 2012, Zeng *et al.* 2012, Zeng *et al.* 2013), ranging from less than 1% to as high as 30% in sheep (Herrid *et al.* 2009).

The relatively low donor sperm levels observed here must be improved before canine SSC transplantation can be exploited as a practical approach for transgenesis. Optimization of the preconditioning regimen may yield a large improvement. Another area worthy of improvement may be immune-compatibility. In this study, no attempt was made to match host and donor. The blood-testis barrier, which protects developing germ cells in the tubule lumen from immune surveillance, may not fully protect SSC. These cells reside outside the barrier formed by Sertoli cell tight junctions. However, there is also some degree of immune privilege outside the barrier, since immunologically unmatched SSC transplants in bull, (Izadyar *et al.* 2003), goat (Honaramooz *et al.* 2003), pig (Mikkola *et al.* 2006), as well as dog (Kim *et al.* 2008), have all led to engraftment. Of note, unrelated SSC transplants in bulls yield significantly lower levels of engraftment than autologous transplants (Izadyar *et al.* 2003), suggesting that work on this point might help improve efficiency. The importance of donor/host interactions for canine SSC transplants remains to be determined.

Finally, establishment of culture conditions for true clonal expansion of canine SSC, such as is routine in mice, will also be extremely useful. A uniform clone of transgenic SSC would yield a >10 fold increase in the percent of transgenic cells over a primary transduction efficiency of 9%. It would also facilitate unlimited expansion: a 100 fold increase in the number of total SSC injected, would not exceed the cell numbers injected in our first study. It would also facilitate selection confirmation and expansion of low frequency events, such as homologous recombination, prior to transplantation. All of these factors will greatly affect the level of transgenic sperm production, and hence, the efficiency of generating transgenic offspring. A major obstacle to expansion of canine SSC may be species-specific qualities of self-renewal regulation. First, the available non-canine growth factors used in this study may not be fully compatible with the canine receptors. Second, canine SSC may utilize one or more alternative signaling pathways not observed in mice. With these caveats in mind, we are presently exploring both the production of canine growth factors, particularly GDNF, and genetic manipulation of canine SSC to bypass the requirement for GDNF (Lee *et al.* 2007, Lee *et al.* 2009).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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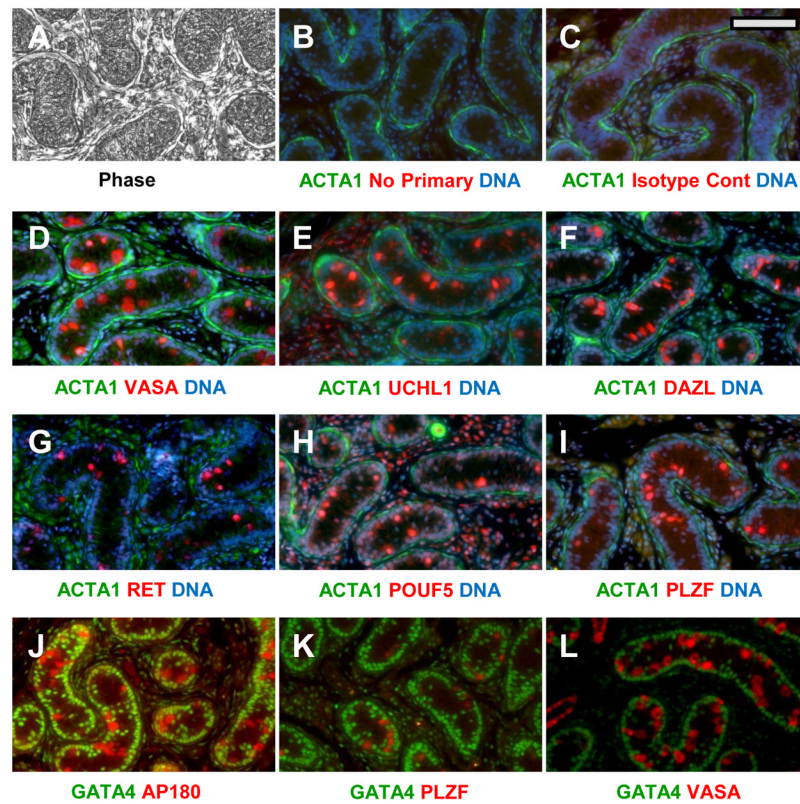
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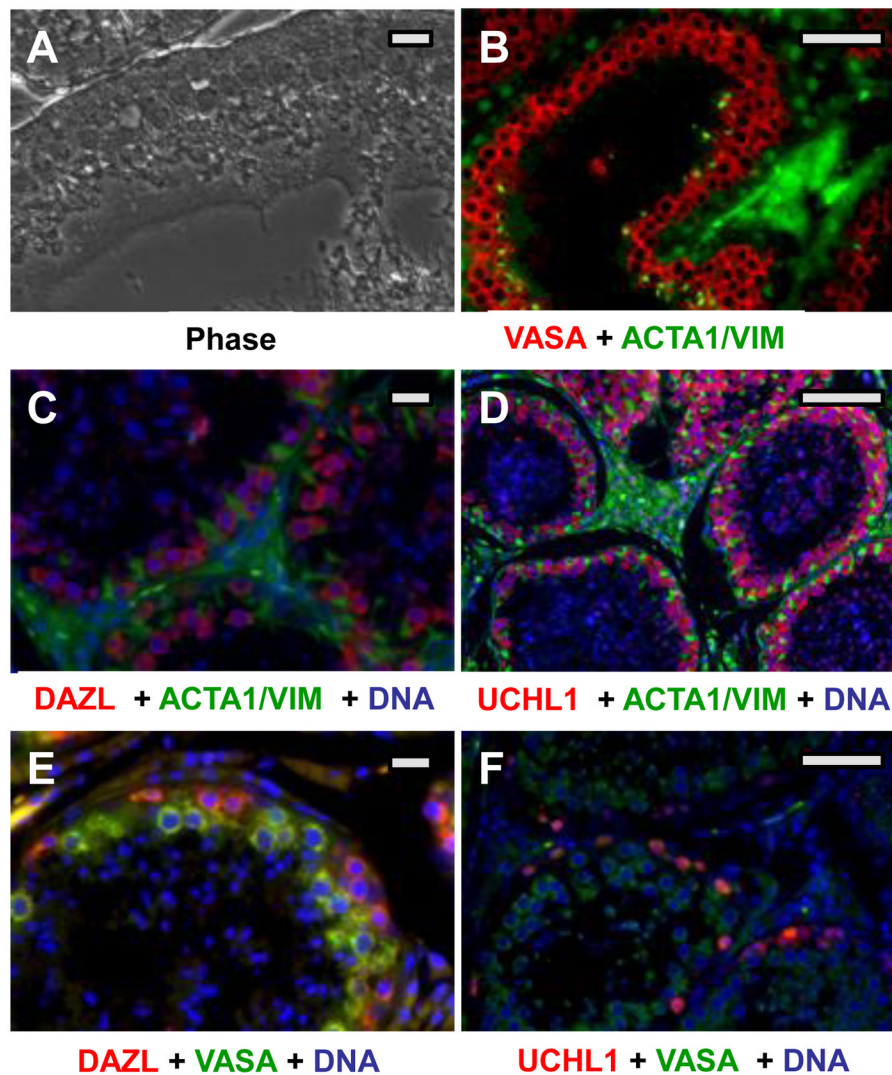


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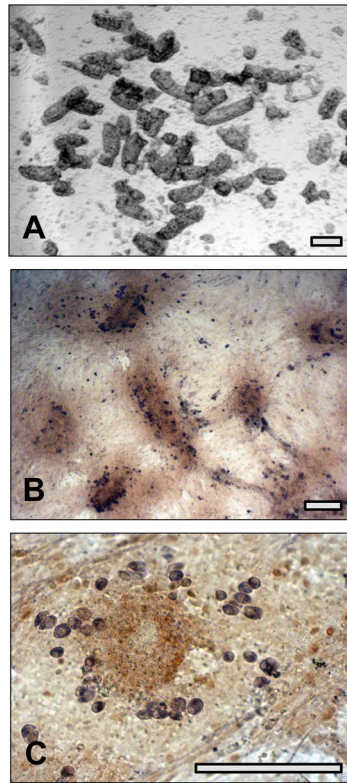
**Figure 1. Localization of SPG antigens in pre-pubertal canine testis**

(A) Phase image of tissue after deparaffinization. (B–L) Dual label probes in which putative SPG antigens (red) and somatic cell antigens (green) were marked. Putative SPG antigens (red) were probed with either rabbit (C–I) or goat (J–L) primary antibodies as indicated, followed by the appropriate Alexa-594 conjugated donkey secondary antibody. Somatic cells (green) were labeled either with mixed mouse antibodies against  $\alpha$ Actin and vimentin, (B–I) or GATA4 (J–L) followed by an anti-mouse Alexa 488 conjugate. The specific antibodies and dilutions used are shown in Supplemental Table I. Controls for background labeling were done identically except that they included either no primary antibody (B) or a rabbit isotype control (C). Specific antibodies and dilutions are listed in Supplemental Table I. Nuclei were counterstained blue with Hoechst 33342 in some sections (B–I). Bar = 100  $\mu$ m.



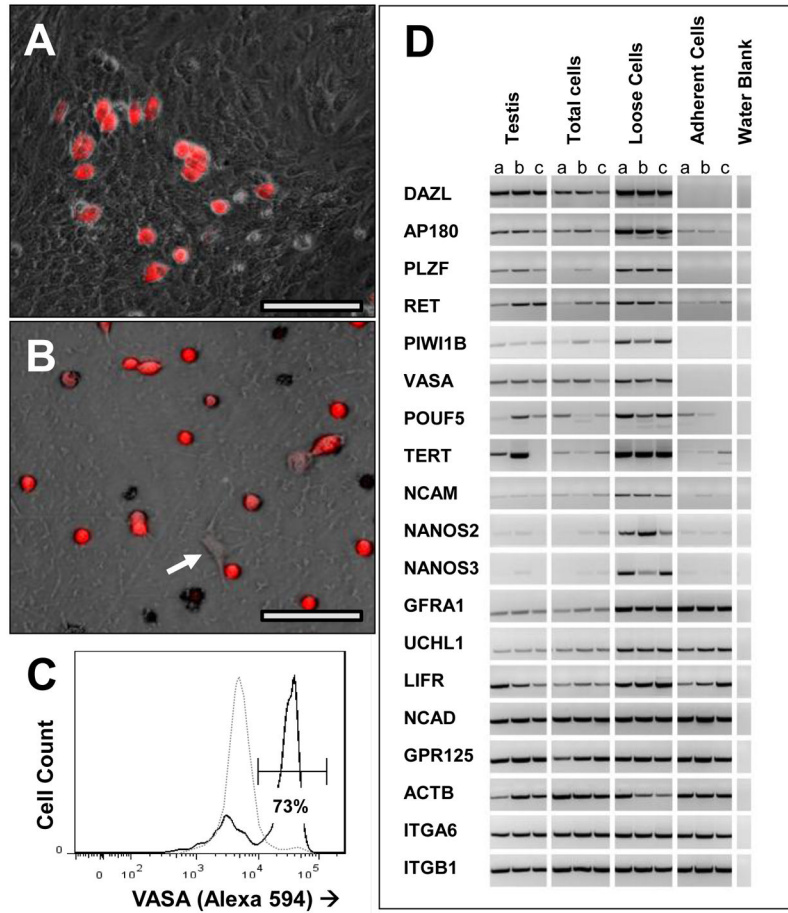
**Figure 2. Localization of SPG antigens in adult canine testis**

(A) Phase image of tissue after deparaffinization. (B–D) Dual label probes in which putative SPG antigens (red) and somatic cell antigens (green) were marked. Putative SPG antigens (red) were probed with either rabbit (B) or goat (C,D) primary antibodies as indicated, followed by the appropriate Alexa-594 conjugated donkey secondary antibody. Somatic cells (green) were labeled with mixed mouse antibodies against alpha actin and vimentin, followed by an anti-mouse Alexa 488 conjugate. (E,F) Dual label probes for two SPG antigens. Note that the UCHL1 staining intensity in F is much lower than in D, showing only the most strongly positive cells. Bars = 150  $\mu$ m.



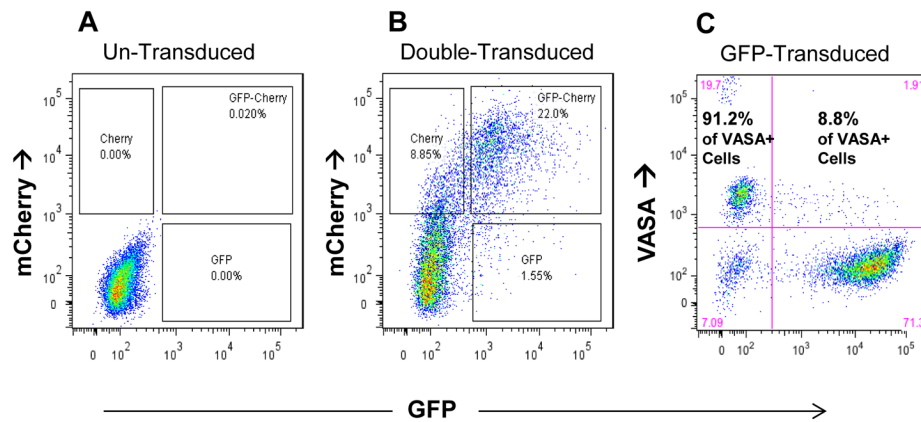
**Figure 3. Emergence of SPG as a “loose cell” population in cultures of total testis cells**  
(A) Bright field image of partially digested seminiferous tubules used to initiate culture. (B) Immunohistochemistry for VASA (purple) and GATA4 after 3 days culture of total testis cells. Note remnants of tubule pieces (dark brown) are firmly attached to substratum. (C) Higher power image of culture shown in (B). The VASA-positive cells are rounded and loosely scattered on top of the dense fibroblast-like lawn. Bars = 100  $\mu$ m.





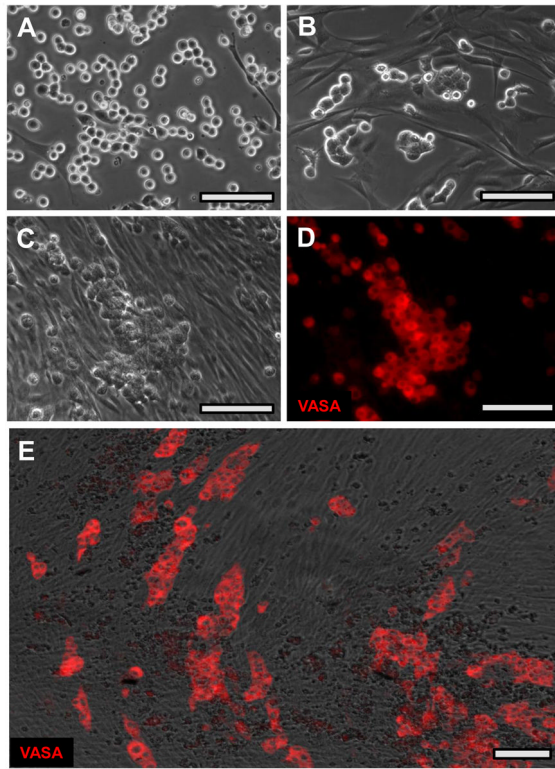
**Figure 4. Enrichment of SPG by differential adhesion**  
**(A)** Composite phase/ fluorescent image of 3 day testis cell culture before harvest of SPG. VASA-positive cells are shown in red. **(B)** Final preparation of SPG after harvest of “loose” cells from 3-day culture, and 2 rounds of differential adhesion to gelatin. The isolated cells were allowed to adhere to a laminin surface, and were then probed for VASA by immunofluorescence. The arrow indicates a VASA-negative fibroblast. **(C)** FLOW data showing VASA immunofluorescence before and after enrichment of SPG by differential adhesion. Dotted line: unfractionated testis cells, collected at day 3 of culture, prior to removal of “loose” cells. Cells (which had fully emerged from tubule structures by this time) were re-suspended by trypsin. Solid line: enriched SPG (loose cells), after 2 rounds of depletion of adherent cells from duplicate 3-day culture. Both cell populations were prepared for FACS analysis of VASA expression as described in Methods. **(D)** Co-enrichment of SPG -specific transcripts with the “loose” cell population. Candidate SPG -enriched transcripts were amplified by RTPCR from ACTB-normalized cDNAs extracted at three days of culture from total testis cells, enriched SPG (loose cells) and remaining adherent cells. ACTB RTPCR was included as an internal control. Each panel of 3 lanes represents three RTPCRs of 3 separate cell preparations derived from the same set of three donor testis cell suspensions. Lane **a** is from a 16 week old Black Lab. Lanes **b** and **c** are from 10 week old German Sheppard littermates. Primers are listed in Supplemental Table III. Bars = 50 um.

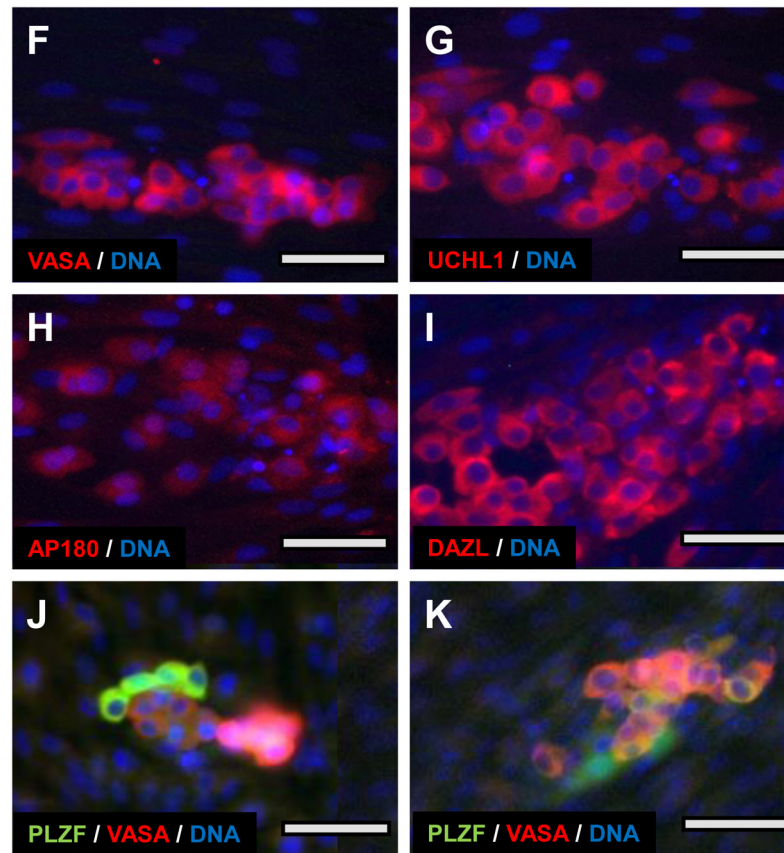




**Figure 5. Gene transfer and expression in SPG by lentiviral transduction**

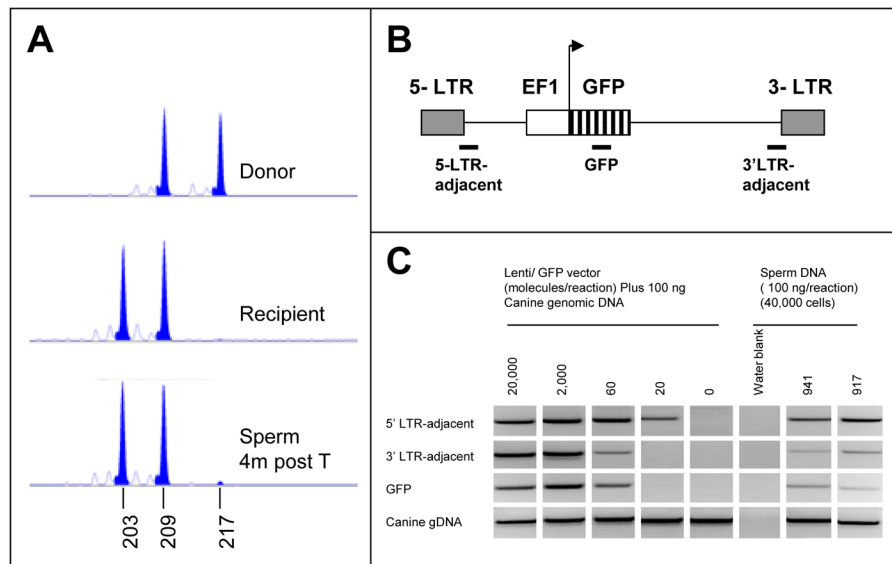
(A,B) Co- transduction with EF1-GFP and CMV-mCherry at an moi of 5 each. (A) FLOW diagram showing untreated control cells. (B) Same FLOW parameters showing expression of both vectors 4 days after a 15 hr transduction. (C) Expression of EFI-GFP in VASA-positive cells. Freshly isolated SPGs were transduced at moi of 20 for 15 hr and cultured for 4 days. Cells were fixed, permeabilized, probed for VASA using an Alexa-594 secondary antibody conjugate, and analyzed by FLOW cytometry. Two dimensional dot plot of VASA and GFP gated on total live cells. Large print indicates percent distribution of GFP-expressing cells within VASA+ population.





**Figure 6. Behavior of canine SPG in culture**

Enriched SPG from a 4 month old black lab were cultured under condition #12 (Supplemental Table II. (A–C) Phase images of SPG on day 1, 3, and 8 of culture, respectively. (D) Fluorescent image of same field as (C) probed for VASA. (E) Low power composite phase/fluorescence image of culture at 30 day showing clusters of tightly associated VASA-positive cells arranged on top of a confluent layer of fibroblast-like cells. (F–I) Localization of VASA (F), UCHL1 (G), AP180 (H) and DAZL (I) at two weeks. (J–K) Co-localization of PLZF (green) and VASA (red) within SPG clusters. Nuclei in F–K are counterstained (Blue) with Hoechst 33342. Bars = 50  $\mu$ m (A–E) or 25  $\mu$ m F–K).



### Figure 7. Engraftment of transgenic SSC

(A) At 4 months post-transplant, a sperm sample from recipient 795933, was tested for donor sperm contribution by VNTR. Microsatellite FM2611 was amplified from donor (A) and recipient (B) DNA as well as from post-transplant sperm. The donor-specific allele (217 base pair product) was also evident in the sperm DNA sample at a level of about 1%, as calculated by comparison of the areas under the donor and recipient-specific (203 base pair) peaks. (B) Diagram of EF1-GFP vector, showing three regions amplified for corroborated detection of vector. (C) PCR of EF1-GFP vector from ejaculated sperm of two transplanted dogs. Sperm was taken 14 months after each dog (941 and 917) had been transplanted with SPG that had been transduced with the EF1-GFP virus. 100 ng sperm DNA, representing approximately 40,000 haploid cells, were analyzed in three separate PCR reactions representing three regions of the vector. Control reactions contained 100 ng normal dog DNA plus a titrated number of copies of the vector. A fourth PCR of the canine Myc gene served as an internal control for genomic DNA. Primers are listed in Supplemental Table III.