Journal of Reproduction and Development, Vol. 59, No 2, 2013

—Original Article—

Characterization and *In Vitro* Culture of Putative Spermatogonial Stem Cells Derived from Feline Testicular Tissue

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Abstract. Spermatogonial stem cells (SSCs) function to regulate the balance of self-renewal and differentiation of male gametes. SSCs have been successfully isolated and cultured *in vitro* in several species, but not in feline. Therefore, in this study, we aimed to culture and characterize feline SSCs. In experiment 1, testes (n=5) from different pubertal domestic cats were cryosectioned and fluorescently immunolabeled to examine the expression of SSC (GFRα-1), differentiated spermatogonium (c-kit) and germ cell (DDX-4) markers. In experiments 2 and 3, testicular cells were digested and subsequently cultured *in vitro*. The resultant presumptive SSC colonies were then collected for SSC identification (experiment 2), or further cultured *in vitro* on feeder cells (experiment 3). Morphology, gene expression and immunofluorescence were used to identify the SSCs. Experiment 1 demonstrated that varying types of spermatogenic cells existed and expressed different germ cell/SSC markers. A rare population of putative SSCs located at the basement membrane of the seminiferous tubules was specifically identified by co-expression of GFRα-1 and DDX-4. Following enzymatic digestion, grape-like colonies formed by 13-15 days of culture. These colonies expressed *GFRA1* and *ZBTB16,* but did not express *KIT*. Although we successfully isolated and cultured feline SSCs *in vitro*, the SSCs could only be maintained for 57 days. In conclusion, this study demonstrates, for the first time, that putative SSCs from testes of pubertal domestic cats can be isolated and cultured *in vitro*. These cells exhibited SSC morphology and expressed SSC-specific genes. However, long-term culture of these putative SSCs was compromised.
Key words: Domestic cat, Gene expression, *In vitro* culture, Isolation, Spermatogonial stem cells

(**J. Reprod. Dev. 59:** 189–195, 2013)

Spermatogonial stem cells (SSCs) play a central role in perpetuating the genetic information via spermatogenesis throughout adulthood, as long as functional SSCs are present within the seminiferous tubules of the testis. These cells share some molecular features and have capability to differentiate into three germ layer lineages [1–4]. Therefore, they hold great promise, not only for treating male-related infertility, by *in vitro* spermatogenesis [5], but also for cellular differentiation, which could be useful for patient-specific cell therapy [1, 6]. It is also believed that SSCs may be useful for gamete banking for males with a valuable genetic background, which could be used for future propagation, differentiation and cell transplantation.

Within the testis, the SSCs are located at the basement membrane of the seminiferous tubules, and are entrapped by the stem cell niche, comprising the contacting domain of Sertoli cells, vascular structure, interstitial cells and noncellular portions [7]. This SSC niche communicates with internal and external testicular factors, which are important in maintaining SSC properties. Factors necessary for the propagation of SSCs *in vitro* are largely unknown and may differ between species. Identification of these factors is important for development of successful culture conditions for SSCs. Furthermore, the numbers of SSCs within the testis are extremely low (e.g., approximately 0.02–0.03% of mouse testicular cells) [8]. These shortcomings could be addressed by identification of SSC markers and also by examining the factors that regulate the fate of SSCs during *in vitro* culture. Although putative SSC markers, such as GDNF family receptor α-1 (GFRα-1), $\alpha_6\beta_1$-integrins, epithelial cell adhesion molecule (EpCAM), promyelocytic leukemia zinc finger (PLZF; ZBTB16), thymus cell antigen-1 (Thy-1, CD90), LIN28, E-cadherin type 1 (CDH1), POU domain class 5 homeobox 1 (POU5F1) and Nanos 2 and 3 are promising candidates for purification and characterization of SSCs in a number of species [9–19], the definite characterization of "true" SSCs is confirmed if these cells can colonize and produce sperm following transplantation into the recipient's seminiferous tubules [5]. To date, GFRα-1 receptor is mostly used as a consensus marker for SSC identification in several species, including rodents [20]. GFRα-1/Ret is a co-receptor of GDNF, an SSC factor that plays a central role in regulating *in vivo* and *in vitro* SSC activity [21]. GDNF is often added to SSC culture medium, although successful culture of SSCs with this factor varies considerably between species [4, 10, 22–26]. Several factors have been shown to improve the success of SSC culture, such as the culture medium, age of donor and the culture system used [26]. In the domestic cat, information regarding the factors regulating SSCs *in vivo*, and the techniques for identification, isolation and *in vitro* culture of SSC is currently lacking. The objectives of this study were therefore to characterize SSC germ cell markers and to examine the efficacy of *in vitro* culture in domestic cats.

Received: August 24, 2012
Accepted: December 19, 2012
Published online in J-STAGE: January 25, 2013

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Materials and Methods

All chemicals used in this study were purchased from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise indicated.

Experimental designs

Experiment 1– Immunolabeling of germ cell, SSC and differentiating spermatogonium markers: A total of 5 pubertal cat testes were cryosectioned and then fluorescently labeled with 1) an SSC marker (GFRα-1, GDNF family receptor α-1), 2) a germ cell marker (DDX-4, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4), and 3) a differentiated spermatogonium marker (c-kit, CD-117). Secondary antibody staining without primary antibody was used as a negative control. The immunofluorescently labeled samples were then examined using fluorescent microscopy. The characteristics and localization of each marker were explained by descriptive analysis.

Experiment 2– Identification of feline SSCs cultured *in vitro*: This study was performed to observe the characteristics and proliferative activity of feline SSCs. Dissociated testicular cells were cultured in a SSC culture medium. The free-floating presumptive SSC colonies (as shown in Fig. 3a, replicate I = 37 colonies; II = 25 colonies) were manually collected with a fine-ended glass pipette and tested for the expression of spermatogonial marker genes (*GFRA1*, GDNF family receptor α-1; *ZBTB16*, Zinc finger and BTB domain containing 16; and *KIT*, c-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) using RT-PCR (reverse transcriptase-polymerase chain reaction). Gene expression of both dissociated testicular cells and presumptive SSC colonies were analyzed. To verify the designed primers, the PCR products of testicular cells (positive control tissue) were sequenced and blasted in GenBank to determine the nucleotide homology. Occasionally, some SSC colonies were also immunolabeled with GFRα-1 to detect protein expression.

Experiment 3–Culture of feline SSCs: The feline SSCs were isolated and cultured as described in experiment 2; however, the SSC colonies were further cultured on feeder layers (CF-1 MEFs/Sertoli cells). The success of *in vitro* culture was assessed daily for colony morphology and growth characteristics using a phase-contrast microscope (CKX41, Olympus, Shinjuku, Japan).

Sample collection and immunolabeling of germ cell, SSC and differentiating spermatogonium markers

The testes (weighing between 0.3–0.5 g) were obtained from pubertal domestic cats (of unknown age) following routine castration at the Veterinary Public Health Division of the Bangkok Metropolitan Administration, Bangkok, Thailand. They were transported in 0.9% (w/v) normal saline solution at room temperature (approximately 30 C) to the laboratory. The epididymides were dissected and cut into 2–3 pieces. The presence of motile sperm observed after smearing the epididymides onto a glass slide indicated the complete spermatogenesis of pubertal cat's testes. After extraneous tissues were dissected from the testes, they were then fixed in 4% (w/v) paraformaldehyde for 24 h. The testes were maintained in 20% (w/v) sucrose in phosphate buffered saline solution (PBS) until being processed. Testicular tissues to be used for cryosectioning were first frozen in OCT compound (Jung, Wetzlar, Germany). Cryosections were sectioned at 7 µm using a Cryostat-microtome (Leica Microsystems, Wetzlar, Germany). To perform immunolabeling, the sections were first incubated in PBS supplemented with 2% (w/v) bovine serum albumin (BSA) and 5% (v/v) normal goat serum in order to block nonspecific antigens. The sections were incubated with mouse monoclonal GFRα-1 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit polyclonal c-kit (1:300, Dako, Carpinteria, CA, USA) antibodies at 4 C overnight or in rabbit polyclonal DDX-4 (1:100, Abcam, Cambridge, MA, USA) antibody at 37 C for 1 hour. After washing twice with PBS, the sections were labeled with the secondary antibodies at 37 C for 1 hour using goat anti-mouse IgG TRIT-C at a dilution of 1:250 (for GFRα-1) and goat anti-rabbit IgG FIT-C at a dilution of 1:100 (for c-kit and DDX-4). 4',6-Diamidino-2-phenylindole (DAPI) was used to label DNA. The fluorescently labeled samples were then examined under an epifluorescent microscope (BX5, Olympus, Tokyo, Japan). Photomicrographs of individual fluorescent channels were recorded using the DP2-BSW program (Olympus) and merged using Adobe Photoshop CS5 Version 12.0 (Adobe Systems, San Jose, CA, USA).

Presumptive SSC colonies obtained from culture were fluorescently labeled with GFRα-1 (specific SSC marker). The colonies were first treated with 2% (w/v) BSA (bovine serum albumin) and 5% (v/v) normal goat serum in PBS. They were then incubated with mouse monoclonal GFRα-1 and the secondary antibody (goat anti-mouse IgG TRITC) as described above.

Isolation of testicular cells

Testes were obtained from pubertal cats after castration as previously described. Upon arrival, they were weighed and decapsulated from the tunica albuginea in Hanks' balanced salt solution (HBSS) containing 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamicin. Only testes weighing between 0.3–0.5 grams were used in this study. Feline testicular cells were digested as previously described by Ogawa *et al*. [27] and Anway *et al*. [28] with some modifications. A two-step enzymatic digestion was used in this study. In brief, the tunica albuginea was first removed from the testicular parenchyma, and the seminiferous tubules were then separated from the interstitial compartments by treating the testicular parenchyma for 15 min with 0.5 mg/ml type IV collagenase and 0.016 mg/ml DNase I (Roche, Indianapolis, IN, USA) in HBSS and placed in a shaking water bath (37 C, 135 strokes/min). The second enzymatic digestion was performed by incubating the separated seminiferous tubules at 37 C for 30 min with 0.04 mg/ml type IV collagenase, 0.03% (v/v) trypsin-EDTA (Gibco, Grand Island, NY, USA) and 0.001 mg/ml DNase I in HBSS. The digested contents were filtered through a nylon mesh (100 µm and 40 µm, respectively, BD Falcon™, Bedford, MA, USA). Finally, the cell suspension was washed with HBSS and centrifuged at 201 × *g* for 5 min at 4 C.

Assessment of testicular cell viability

Viability of the testicular cells was evaluated by labeling the cells with fluorescent probes and then visualized using an epifluorescence microscope (BX51, Olympus, Japan). Plasma membrane integrity was assessed using the non-membrane permeant DNA stain ethidium homodimer-1 (EthD-1; Molecular Probes, Eugene, OR, USA), while intracellular esterase enzyme activity was examined using calcein AM (Molecular Probes). A total of 200 cells were evaluated per testicular digestion. The examined cells were classified into 2 categories as

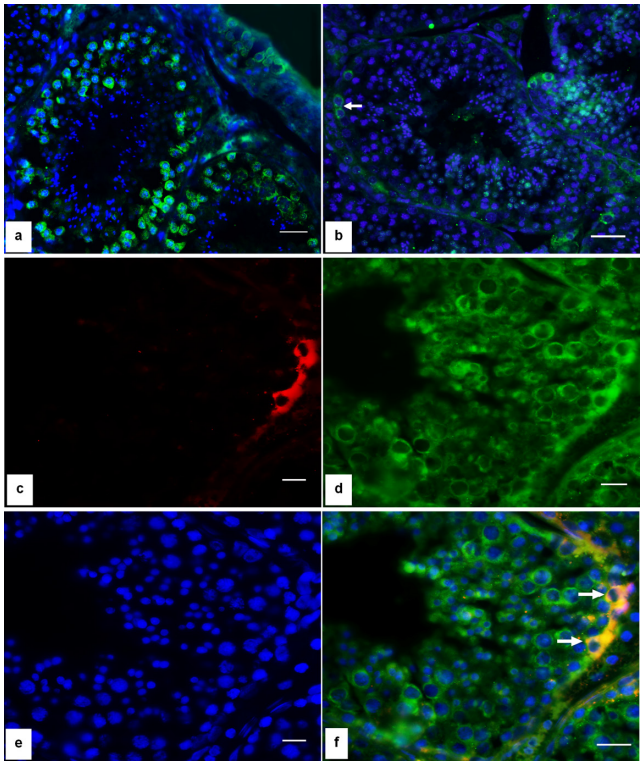

Fig. 1.

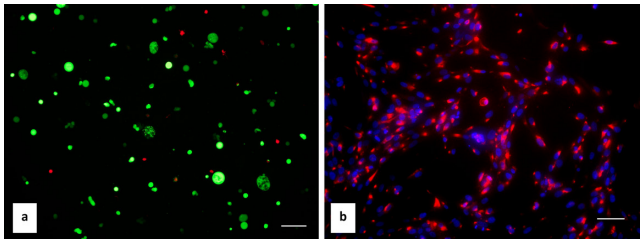

Fig. 2. a: The viability of testicular cells after enzymatic digestion. Calcein AM-positive (green) cells indicate the esterase enzyme activity (viable cells), while EthD-1 (red) binds specifically to the nucleus of membrane-disrupted testicular cells (dead cells). b: High proportions of cells positive for vimentin (red) were obtained after hypoosmotic shock treatment and cultured *in vitro*. This vimentin binds specifically to intermediate filaments of Sertoli cells. Scale bars=50 µm.

Fig. 1. Expression of germ cell-specific (DDX-4), differentiated spermatogonium (c-kit) and, spermatogonial stem cell markers (GFRα-1) and co-localization between DDX-4 and GFRα-1 in cryosectioned feline testes. a: The expression of DDX-4 (green) was found in all stages of germ cells except some spermatids and Sertoli cells. DDX-4 expression was found within fine granules in the cytoplasm. b: c-kit was labeled at the plasma membrane and cytoplasm of differentiating spermatogonial cells (green). c-kit was expressed on 2-pairing cells (arrow). Multicolor photomicrographs illustrate the expressions of putative spermatogonial stem cells that highly expressed GFRα-1 (red, c), DDX-4 (green, d) and DAPI (blue, e). Co-expression of GFRα-1 and DDX-4 is shown by arrows (f). a and b: scale bars=30 µm. c–f: scale bars=10 µm.

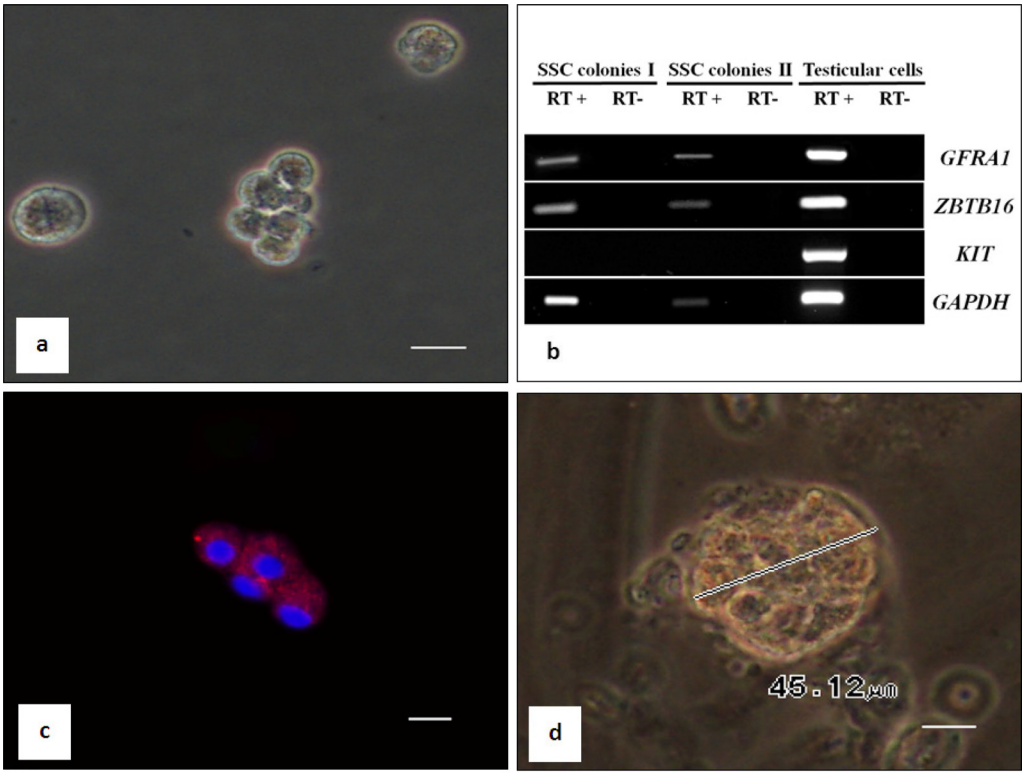

Fig. 3. SSC colonies were isolated and cultured *in vitro*. a: Floating "grape-like" SSC colonies were found between days 13–15 of *in vitro* culture. b: RT-PCR analysis of presumptive SSC colonies and testicular cells was performed. PCR product bands indicate that presumptive SSC colonies expressed *GFRA1* and *ZBTB16* but no *KIT* PCR product was observed. c: Immunolabeling of an SSC floating colony revealed the expression of GFRα-1 at the cell membrane and within the cytoplasm. d: An SSC colony (day 47 of *in vitro* culture) was cultured on mitomycin-treated Sertoli cells. Scale bars=10 µm.

either viable (intact plasma membrane: positive for calcein AM and negative for EthD-1) or dead cells (damaged plasma membrane, EthD-1 positive). Only testicular cell suspensions demonstrating more than 80% viable cells were used in this study.

Preparation of feeder cells

This study was designed to analyze 2 types of feeder cells that have been previously reported to support SSCs *in vitro,* i.e., CF-1 MEFs (mouse embryonic fibroblasts, CRL-1040, ATCC) and feline Sertoli feeder cells.

For isolation of feline Sertoli cells, the testicular cells were digested using the same procedure described above. Sertoli cells were isolated using a hypoosmotic shock technique as previously described by Anway *et al*. [28] with minor modifications. The Sertoli cells were then examined for cell purity by means of cell morphology using a phase contrast microscope (Olympus) and immunolabeling with vimentin (1:200). Sertoli cell morphology was typically recognized within a mixed population of testicular cells by their large cell size, extended cytoplasm and varied size of cytoplasmic vacuoles [29]. The purity of Sertoli feeder cells is shown in Fig. 2b. The CF-1 MEFs and Sertoli cells were cultured in knockout DMEM/F-12 (Gibco) supplemented with 10% (v/v) fetal bovine serum (2 mM GlutaMAX™ (Gibco) and 0.25% (w/v) Penicillin-Amphotericin B (Gibco). The CF-1 MEFs (passage 3–5) and Sertoli cell (passage 1) feeder cells were treated with mitomycin-C for 2.5 and 3.0 h, respectively.

Culture of spermatogonial stem cells

The SSC culture medium (modified from Kanatsu-Shinohara *et al*. [10]) used in this study was StemPro-34 SFM (Invitrogen, Carlsbad, CA, USA) supplemented with StemPro Supplement (Invitrogen), 25 µg/ml insulin, 100 µg/ml transferrin, 60 nM sodium selenite, 60 µM putrescine dihydrochloride, 6 mg/ml D-(+)-glucose, 30 mg/ml MEM sodium pyruvate, 1 µM DL-lactic acid, 5 mg/ml BSA, 2 mM GlutaMAX™ (Gibco), 0.5 µM 2-mercaptoethanol (Gibco), 1x MEM amino acids solution, 1x MEM nonessential amino acids solution, 10^{-4} M L-ascorbic acid, 10 µg/ml D-biotin, 0.25% (w/v) penicillin-amphotericin B (Gibco) and 1% (v/v) fetal bovine serum (FBS, Gibco). This culture medium was further supplemented with 20 ng/ml mouse epidermal growth factor (EGF), 10 ng/ml human basic fibroblast growth factor (FGF2, BioVision, Milpitas, CA, USA), 10^3 U/ml recombinant human leukemia inhibitory factor (rhLIF, Millipore, Temecula, CA, USA) and 50 ng/ml recombinant rat glial cell line-derived neurotrophic factor (rrGDNF, R&D Systems, Minneapolis, MN, USA).

Cell culture was performed at 37 C in a humidified environment of 5% CO_2 in air. Partial purification of the SSC population was performed using negative selection by plating onto 0.1% (w/v) gelatin coated-dishes 2–3 times. This procedure allows fibroblasts and other testicular somatic cells to attach to a Petri dish. Putative SSCs were then counted and cultured at a final concentration of 2×10^5 cells/ml on mitomycin-treated CF-1 MEFs. The SSC colonies were passaged manually every 2–3 weeks depending on the proliferation rate of the SSCs. For long-term culture of SSCs, the SSC colonies were co-cultured on Sertoli cells. The SSC colonies were observed daily for morphology and proliferative characteristics using a phase-contrast microscope (CKX41, Olympus).

RNA extraction, RT-PCR analysis and gene expression

Total cellular RNA was extracted from dissociated testicular cells and SSCs using an Absolutely RNA Nanoprep Kit (Stratagene™, Agilent Technologies, CA, USA). The extracted RNA was kept at –80 C until use. For reverse transcription (RT), first-strand cDNA was synthesized from total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen). The cDNA was stored at –20 C. These procedures were performed according to the manufacturer's instructions.

The Primer Express® Software v.3.0 (Applied Biosystems, Carlsbad, CA, USA) was used to design specific primers (*GFRA1*, *ZBTB16* and *KIT*). Conserved regions of the bovine *GFRA1* mRNA sequences (accession number: NM_001105411) and the dog *ZBTB16* mRNA sequences (accession number: XM_845250.3) were used to design primers. A domestic cat *KIT* mRNA sequence (accession number: NM_001009837.3) was used to design *KIT*. In all cases, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, 5'-GGAGAAAGCTGCCAAATATG-3' and 5'-CAGGAAATGAGCTTGACAAAGTGG-3') designed from previous study [30] was used as the internal control.

PCR was performed on template cDNA containing either reverse transcriptase (RT+) or without reverse transcriptase (RT–) as a control. Briefly, the PCR reaction was performed as follows: 2 min at 95 C for initial denaturation, followed by 30 cycles of 30 sec at 95 C, 30 sec at annealing temperature for each primer (*GFRA1* [product length: 250 bp; accession number JX984462.1], 60 C, 5'-CAACTGCCAGCCAGAGTCAA-3' and 5'-AGCCATTGCCAAAGGCTTGA-3'; *ZBTB16* [product length: 119 bp; accession number HF678120], 63 C, 5'-GCAAGAAGTTCAGCCTCAAGC-3' and 5'-GCTTGATCATGGCCGAGTAGTC-3'; *KIT* [product length: 533 bp; accession number JX984463.1], 60 C, 5'-TCCTGCTCCGCGTCCAGACA-3' and 5'-CTTGCCCTTCCGGTCCGCAG-3') and 30 sec at 72 C. Incubation for 2 min at 72 C was used for the final extension. The PCR products were electrophoresed in 2% (w/v) agarose gel (Bio-Rad, Hercules, CA, USA) in TBE buffer containing 0.4 mg/ml ethidium bromide (Promega, Madison, WI, USA). The amplified products were examined under UV light using a Gel Documentation system (Syngene, Cambridge, CB, UK).

The PCR products (from testicular cells) were extracted using a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, CA, USA) and sequenced. The extracted products were confirmed for purity by electrophoresis using 1–2% agarose gel. The sequences derived from both strands were aligned using BioEdit Version 7.0.8.0 (T.A. Hall Software, Raleigh, NC, USA). These sequences were blasted in GenBank in order to determine the nucleotide identity compared with other species.

Statistical analysis

The expression of germ cell, SSC and differentiating spermatogonium markers (experiment 1 and 2) and SSC morphology (experiment 3) were descriptively analyzed. Viability of testicular cells after dissociation is expressed as a mean ± SD.

Results

Experiment 1: immunolabeling of germ cell, SSC and differentiating spermatogonium markers

Feline testicular cryosections were immunofluorescently labeled in order to verify the specificity of SSC, germ cell and differentiating spermatogonium markers in domestic cats. In general, testicular cryosections contained the various stages of spermatogenesis, and each type of spermatogenic cells differently expressed germ cell/SSC/differentiating spermatogonia makers. DDX-4 (germ cell marker) was expressed in spermatogonia, primary/secondary spermatocytes and some round spermatids (Fig. 1a). GFRα-1 (SSC marker) staining was predominantly found at the plasma membrane of a single cell located at the basement membrane of the seminiferous tubule (Fig. 1b). However, faint GFRα-1 expression was also occasionally observed in the cytoplasm. Expression of c-kit (differentiating spermatogonia marker) was found at the basement membrane of the seminiferous tubule, similar to GFRα-1. However, the numbers of c-kit-positive cells were found to be greater than those of GFRα-1. Furthermore, c-kit-positive cells were present, either as single cells or as pairs (Fig. 1c). A rare population of SSCs situated at the basement membrane of the seminiferous tubules was specifically identified by co-localization of GFRα-1 and DDX-4 (Fig. 1d).

Experiment 2: identification of feline SSCs cultured in vitro

After enzymatic isolation, the viability of dissociated testicular cells was 92.8 ± 1.9% (mean from 4 replicates) (Fig. 2a). They were subsequently cultured under modified SSC conditions. Approximately 7 days after cell seeding, 2-cell presumptive SSCs with incomplete cytokinesis were first observed. These paired cells continued to proliferate and form a tightly packed 'grape-like' structure containing 3-6 cells per SSC colony by day 13–15 of *in vitro* culture (Fig. 3a). In order to identify the SSCs, these SSC colonies were collected and examined for mRNA expression using RT-PCR. The results revealed that these colonies expressed *GFRA1* (SSC marker) and *ZBTB16* (early spermatogonium marker) but did not express *KIT* (differentiated spermatogonium marker) (Fig. 3b). This result was in an agreement with the finding that these colonies also strongly expressed GFRα-1 (Fig. 3c). In addition, the partial feline 3 mRNA nucleotide sequences (*GFRA1*, *ZBTB16*, *KIT*) obtained from RT-PCR were subsequently blasted to verify the nucleotide identity with the sequences previously reported in other species. The nucleotide identity of *GFRA1* mRNA sequences between feline and other species ranged from 86–94%. Moreover, the mRNA sequences of *ZBTB16* and *KIT* showed high nucleotide identity (92–99 and 99%, respectively) (Table 1).

Experiment 3: culture of feline SSCs

For long-term culture, both CF-1 MEFs and Sertoli cells were used as feeder cells. A total of 30 SSC colonies (each colony contained 3–6 cells) were first cocultured with CF-1 MEFs. Of these colonies, we found only 6 colonies loosely attached onto the CF-1 MEFs, and only 2 colonies (mean diameter: 46.5 μm) were observed following passage of the SSC colonies to new CF-1 feeders. Unfortunately, the proliferative activity appeared to decline by day 30 of *in vitro* culture (maximal diameter approximately 30 μm). We subsequently layered the colonies onto feline Sertoli cells as homologous derived-feeder cells (since Sertoli cell feeders have been reported to support the proliferation ability of SSCs) [31, 32]. These two colonies attached onto the Sertoli cell feeders. However, only one colony continued to proliferate. Its diameter increased to 45.12 μm by day 47 of *in vitro* culture (Fig. 3d), and the colony underwent degeneration at approximately 57 days of *in vitro* culture.

Discussion

In the current study, we reveal that spermatogenic cells within the seminiferous tubules of the feline testis differently expressed germ cell and SSC markers. Three markers (GFRα-1, c-kit and DDX-4) were used to define SSC, differentiated spermatogonium, and germ cell markers, respectively. Furthermore, we demonstrated for the first time that feline SSCs could be successfully isolated and cultured *in vitro*, although long-term culture of these SSCs was compromised.

In experiment 1, we examined the expression pattern of several markers used for identifying SSCs and differentiating spermatogonial cells, since the specific markers for SSCs remain elusive in domestic cats. From our results, it is clear that the GFRα-1 is a consensual SSC marker in domestic cats, as the expression pattern was similar to those of other species such as the rodent (expressed in A_{single} and possibly in A_{paired} spermatogonia) and monkey (expressed in A_{dark} and A_{pale} spermatogonia) [20, 33]. This marker has successfully been used to enrich (more than 90%) undifferentiated spermatogonial stem cells in the mouse [34, 35]. In addition, while only a rare population of GFRα-1-positive cells was found at the basement membrane of the seminiferous tubule (approximately 2–4 cells per cryosection), all of these cells also co-expressed DDX-4 (a specific germ cell maker) (Fig. 1d). However, since all stages of spermatogenic cells (except elongated spermatids and spermatozoa) were positive for DDX-4, the results confirm that this marker can only be used as a general germ cell maker [36, 37]. Indeed, co-localization of GFRα-1 and other SSC or undifferentiated spermatogonium markers such as $\alpha_6\beta_1$-integrins, POU5F1 and LIN28 would be required to conclusively identify the "true" SSCs in the domestic cat [9, 17–19, 38–41].

GFRα-1 and its co-receptor, RET tyrosine kinase located on the cell plasma membrane, are the specific binding sites of glial cell line-derived neurotrophic factor (GDNF) [42]. This ligand-receptor binding signals via the Ras/ERK1/2 pathway to stimulate DNA synthesis and cell proliferation, which in turn maintain function and survival of the SSCs both *in vivo* and *in vitro* [21, 22, 43, 44]. By contrast to GFRα-1, DDX-4 and c-kit were expressed in a more advanced stage of spermatogonia, similar to the pattern previously reported in rodents and juvenile rhesus macaques [20, 33, 45]. Moreover, expression of c-kit in feline testes was occasionally observed at the basal compartment of seminiferous tubules, as reported in adult rhesus macaques [46]. Although the expression of c-kit in undifferentiated spermatogonia (A_{paired} spermatogonia) is still controversial, we found in domestic cats, that c-kit was expressed in paired cells at the basement membrane of the seminiferous tubules, similar to the finding that c-kit was expressed in A_{paired} spermatogonia in other species (Fig. 1c) [34, 47].

Following enzymatic digestion of the feline testes, only small numbers of SSC-like colonies (3–6 cells) formed within approximately

Table 1. Sequence alignments of *GFRA1*, *ZBTB16* and *KIT* amplicon products with mRNA sequences previously reported in GenBank

Species	GenBank accession number	Nucleotide identity (%)
GFRA1 **(**product length: 250 bp**)**	JX984462.1	
Mouse (*Mus musculus*)	NM_010279.2	86
Rat (*Rattus norvegicus*)	NM_012959.1	88
Human (*Homo sapiens*)	NM_145793.3	93
Bovine (*Bos taurus*)	NM_001105411.1	92
Dog (*Canis Familiaris*)	XM_846994.2	94
ZBTB16 **(**product length: 119 bp**)**	HF678120*	
Mouse (*Mus musculus*)	NM_001033324.2	92
Rat (*Rattus norvegicus*)	NM_001013181.1	96
Human (*Homo sapiens*)	NM_006006.4	97
Bovine (*Bos taurus*)	NM_001037476.1	99
Dog (*Canis Familiaris*)	XM_845250.3	95
KIT **(**product length: 533 bp**)**	JX984463.1	
Cat (*Felis catus*)	NM_001009837.3	99

*ENA accession number.

2 weeks in the modified culture system used in this study. This finding is in an agreement with experiment 1, where the numbers of GFRα-1-positive cells were low compared with other germ cells (DDX-4-positive cells). We confirmed for the first time that these colonies were SSCs by immunolabeling with a GFRα-1 fluorescent probe and also by mRNA expression of SSC-specific genes (*GFRA1* and *ZBTB16* mRNA). Furthermore, these cells did not express the differentiated spermatogonium marker (*KIT*). We also additionally demonstrated that the nucleotide sequences of these genes were similar to other species (Table 1), suggesting that these genes are relatively conserved between species. Nevertheless, although these genes have been shown to be potential markers for identification of rodent SSC subpopulations [13, 48, 49], there is a further requirement to determine other genes that may also be expressed in SSCs within the domestic cat. In fact, information regarding gene expression and cell signaling in feline SSCs has yet to be fully established. Furthermore, transplantation of the positive GFRα-1 colonies obtained in this study into the seminiferous tubules is still required in order to examine the biological assay of the SSCs in terms of colonization and *in vivo* spermatogenesis in the recipient testis. However, this SSC transplant technique has yet to be established, since cat mixed germ cells xenotransplanted into mouse testes colonized within the seminiferous tubules but failed to reinitiate sperm production [50]. Furthermore, long-term establishment of a germ cell-depleted model in tom cats remain unsuccessful [50, 51].

In the current study, we isolated and identified SSCs from domestic cats as a molecular assay. The SSC colonies were maintained *in vitro* for only approximately 57 days. This indicated that GDNF supplementation is not an exclusive factor for maintaining the self-renewal and function of SSCs *in vitro*, despite the five-fold increase (50 ng/ml) in GDNF concentration in our study compared with an original mouse SSC protocol [10]. GDNF is well recognized to increase SSC proliferation *in vitro* in a dose-dependent manner [43]. This proliferative activity of GDNF has been reported in a number of species including the mouse [22, 35], rat [24], hamster [26] and bull [25, 28, 52]. Moreover, Kanatsu-Shinohara *et al*. [26] revealed in the hamster, that addition of FGF2 was necessary to promote GDNF activation, while EGF supplementation adversely affected hamster SSCs. It is therefore essential to determine the interaction of these growth factors on derivation of feline SSCs, since the culture system may be species-specific. In addition to growth factor supplementation, feeder cell layers are also one of the critical factors determining the success of SSC derivation. For example, mouse embryonic fibroblasts (MEFs) and testicular somatic cells have been successfully used to support the culture of SSCs in rodents [1, 10], bovine [25, 28] and humans [32]. In the current study, we found that MEFs were not suitable for SSC culture, as only 2 colonies (of 30 colonies) were maintained, while the proliferative activity appeared to decrease over time. We therefore decided to transfer the two colonies to Sertoli cell feeders because this feeder type has been demonstrated to support SSCs *in vitro* in several species [31, 32]. We found that feline Sertoli cells could reactivate their proliferative activity and further support SSC growth for 57 days. Sertoli cells and SSCs interact *in vivo* by forming an SSC niche and by secreting GDNF and other growth factors to activate SSC proliferation [40, 53]. However, it is worth noting that the use of feeder cells to support SSC activity remains largely controversial because feeder-free culture systems have been demonstrated to be preferable for hamster SSCs rather than culture systems containing feeder cells [26, 48].

In the current study, we demonstrated for the first time that spermatogonial stem cells can be isolated from testes of pubertal domestic cats. These SSCs expressed SSC-specific genes and could be successfully cultured *in vitro*. However, long-term culture of these SSCs was compromised. Further studies investigating other factors that regulate the proliferation and senescence of SSCs in the domestic cat are required.

Acknowledgments

N Tiptanavattana is receiving a Ph.D. scholarship from "The Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej". This study was financially supported by "Integrated Innovation Academic Center: IIAC" Chulalongkorn University Centenary Academic Development Project (CU56-HR07), and Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (HR1166IB-55). We would like to thank the Veterinary Public Health Division of the Bangkok Metropolitan Administration, Thailand, for sample collection. The authors would also like to sincerely thank Ms Jutamas Ngopon for assisting with RT-PCR.

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