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—Original Article—

Determination phase at transition of gonocytes to spermatogonial stem cells improves establishment efficiency of spermatogonial stem cells in domestic cats

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Abstract. The development of germ cells has not been entirely documented in the cat especially the transition phase of the gonocyte to the spermatogonial stem cell (G/SSC). The aims of study were to examine testicular development and to identify the G/SSC transition in order to isolate and culture SSCs *in vitro*. Testes were divided into 3 groups according to donor age (I, < 4 months; II, 4–6 months; and III, > 6 months). In Exp. 1, we studied testicular development by histology, transmission electron microscopy and immunohistochemistry. In Exp. 2, we determined the expression of GFRα-1, DDX-4 and c-kit and performed flow cytometry. The SSCs isolated from groups II and III were characterized by RT-PCR and TEM (Exp. 3). Chronological changes in the G/SSC transition were demonstrated. The size, morphology and ultrastructure of SSCs were distinguishable from those of gonocytes. The results demonstrated that group II contained the highest numbers of SSCs per seminiferous cord/tubule (17.66 ± 2.20%) and GFRα-1$^+$ cells (14.89 ± 5.66%) compared with the other groups. The findings coincided with an increased efficiency of SSC derivation in group II compared with group III (74.33 ± 2.64% *vs*. 23.33 ± 2.23%). The colonies expressed mRNA for *GFRA1*, *ZBTB16*, *RET* and *POU5F1*. Our study found that the G/SSC transition occurs at 4–6 months of age. This period is useful for isolation and improves the establishment efficiency of cat SSCs *in vitro*.
Key words: Development, *In vitro* culture, Spermatogonial stem cell, Transition phase

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In male mammals, primordial germ cells derived from the extraembryonic ectoderm essentially develop into gonocytes, the origin of the male germline lineage, in the seminiferous cord before birth [1]. The gonocytes subsequently reproliferate and relocate to the privileged testicular niche formed by primitive Sertoli cells by day 12.5–14 of mouse embryo development [2, 3]. In rodents, the transformation of gonocyte to the spermatogonial stem cell (SSC) or transition phase of the gonocyte/spermatogonial stem cell (G/SSC) takes place in the seminiferous cord shortly after birth. This transition phase is, in part, mediated by Notch signaling and anti-MÜllerian hormone from active Sertoli cells of developing and mature testes [4, 5]. However, the heterogeneous population of gonocytes in terms of their maturation and apoptosis results in variably committed programs of G/SSC transition after the quiescent period of the testicular cell development [6, 7]. The mature gonocytes migrate down to the periphery of the seminiferous cord by KITL/KIT signaling, and then some POU5F1-expressing gonocytes are committed to be SSCs with self-renewal ability [8]. These SSCs form and settle at the basement membrane of the seminiferous cord [9]. The SSCs, thereafter, play a key role in regulation of the balance of stemness and differentiation of SSC populations in mammals.

SSC culture is a promising technique for genetic preservation and restoration of male germ cells in humans and animals. Additionally, it may be useful for *in vitro* production of male gametes. In felid species, SSC technology can serve as a potential tools for germ cell banking in domestic and wild felids. Moreover, the cat genome is highly conserved and similar to that of humans. It is therefore possible to use the cat as a model for inherited and infectious diseases such as acquired immune deficiency syndrome (AIDS) [10]. However, SSC research in the domestic cat and other felids has been very scant.

The G/SSC transition is short and occurs rapidly during days 3–6 and 5–8 after birth in the mouse and rat, respectively. By contrast to the findings in rodents, the human G/SSC phase generally occurs between 3–12 months after birth [5, 7, 11]. Thus, the duration of

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the G/SSC transition is largely variable among species, and this is predominantly because of the differences in the quiescent period of the gonocytes. The quiescent period of the gonocytes is important as the checkpoint of gonocytes capable of undergoing transformation into SSCs. Although the SSCs resume the mitosis to increase their stemness activity, the numbers of SSCs within the testis is extremely low (approximately 0.03% of total testicular cells) [12]. Until recently, informative data on testicular development, particularly the G/SSC transition, in relation to derivation of SSCs in domestic cats has been very limited.

Gonocytes have a unique and uniform in morphology. Generally, they are large and have prominent nucleoli and low complexity of cytoplasmic organelles [6], and they migrate to the basal lamina of the seminiferous cord by the cytoplasmic projection [9, 13]. Spermatogonia are defined by their small flattened shape with round to oval nucleus. There are 2 main populations of chromatin distribution, homogenous chromatins and condensation of granular chromatins [14]. It has been reported that puberty (complete spermatogenesis) in the cat takes place around 8–12 months of age with the spermatogenic cycle of 46.8 days (4.5 cycles) [15]. However, information on pre- and postpubertal testicular development in the cat has been scarce [16]. Given that only a specific stage of testicular development can enrich the SSC population, study of the G/SSC transition is essential for isolation and culture of SSCs.

In vitro culture of SSCs is only well understood in the mouse and rat in terms of mechanisms that control SSC signalling and self-renewal ability [17–21]. Of several factors contributing to successful isolation and propagation of SSCs, age of donor at SSC isolation, specific growth factors and types of culture system (such as feeder *vs*. feeder-free or serum *vs*. serum-free system) centrally control the fate of SSCs *in vitro* [22]. However, the effects of these factors on *in vitro* culture of SSCs in other domestic species remain unclear. The G/SSC phase occurs in a time- and species-specific manners. This phase of testicular development improves the efficiency of SSC isolation and culture by enrichment of the SSC population with minimal contamination of other differentiated germ cells during culture [19, 23–26]. Our previous study demonstrated that SSCs could be isolated from pubertal cats, and the resultant SSC-like colonies had SSC activity [27]. However, the efficiency of establishment of SSCs *in vitro* is still limited, and this is probably due to a lack of information concerning the G/SSC transition. This study therefore aimed to examine chronological changes in testicular cell development in order to define the specific period of the G/SSC transition that would be useful for increased efficiency of isolation and culture of SSCs in domestic cats.

Materials and Methods

Experiment 1: The transition of testicular germ cells during different phases of postnatal testicular development

Testes obtained from domestic cats (*Felis catus*) were collected and categorized into 3 groups depending on donor ages: less than 4 months (group I; neonatal (n = 2), 2.5 months (n = 1), 3 months (n = 3)), 4 to 6 months (group II; 4 months (n = 3), 5 months (n = 3)) and more than 6 months (group III; 7 months (n = 2), 8 months (n = 1), 9 months (n = 1), 12 months (n = 1), 24 months (n = 1)). Data concerning testicular weight and presence of epididymal sperm were also recorded. The cat testes were subjected to descriptive analysis for conventional histology (H&E staining), immunohistochemistry for apoptosis and proliferation using a TUNEL assay and Ki-67 and examination of their ultrastructure by transmission electron microscopy (TEM). The average percentage of gonocytes and spermatogonia per tubule was calculated from 30 counted cross sections of seminiferous cords and tubules.

Experiment 2: Phenotypic analysis of testicular cells with SSC markers and flow cytometric analysis of the SSC population

Exp. 2.1 To identify the SSC marker, GFRα-1 (GDNF family receptor α-1), DDX-4, (DEAD (Asp-Glu-Ala-Asp) box polypeptide 4) and c-kit were used for immunohistochemistry.

Exp. 2.2 Testes of group II (n = 5) and group III (n = 5) were collected from 10 cats and used in this experiment. The testes were dissociated by a modified two-step enzymatic digestion. The dissociated testicular cells were fixed and stained with SSC markers. The immunolabelled cells were examined by flow cytometry.

Experiment 3: In vitro culture and identification of derived SSC colonies

Independent testes from group II (n = 17) and group III (n = 18) were dissociated and cultured as previously described [27]. This experiment was designed to observe the SSC morphology and activity. The SSC colonies (5 colonies/group) were manually collected with a fine-ended glass pipette, and the mRNA expression of GDNF family receptor α-1 (*GFRA1*), Zinc finger and BTB domain containing 16 (*ZBTB16*), ret proto-oncogene (*RET*) and POU class 5 homeobox 1 (*POU5F1*) was determined using RT-PCR. The ultrastructure of SSC colonies was also analyzed by TEM.

Animals

Cat testes were obtained from domestic cats (*Felis catus*) following routinely castration at the Small Animal Teaching Hospital of the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. This study was ethically performed and approved by the Chulalongkorn University of Animal Care and Use Committee.

The testes were transported in a 0.9% (w/v) sodium chloride solution (Thai Otsuka Pharmaceutical, Bangkok, Thailand) supplemented with 1% (v/v) penicillin-streptomycin (Gibco, Life Technologies, Grand Island, NY, USA) at room temperature. After the surrounded tissues were separated and discarded from the testes, the testes were individually weighed and used for further analysis. For extraction of epididymal sperm, the caudal epididymides were separated from surrounding tissue and then cut into small pieces. The presence of epididymal sperm was observed under a light microscope at × 400 magnification.

Testicular histology

Testes were fixed in 4% (w/v) paraformaldehyde (VWR BDH Prolabo, Poole, UK) in phosphate-buffered saline (PBS) for 24 h, rinsed with 70% ethanol, embedded in paraffin, sectioned at a thickness of 4 μm and layered on a silane-coated glass slide.

A routine histological study was performed on each tissue section by hematoxylin and eosin (H&E) staining. For immunohistochemistry

(IHC), the expressions of SSC-related markers (Ki-67, DDX-4, GFRα-1 and c-kit) were detected by chain polymer-conjugated with peroxidase system. In short, slides were heated at 121 C for 15 min in citric acid buffer (VWR BDH Prolabo, Poole, UK; pH = 6.0) supplemented with either 0.02% (v/v) Tween-20 (for Ki-67 and DDX-4) or 0.03% (v/v) Triton X-100 (for GFRα-1 and c-kit). Hydrogen peroxide (1%, v/v) in methanol (VWR BDH Prolabo) was used to block endogenous peroxidase. The sections were incubated with 2% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) in PBS and subsequently with primary antibodies diluted in sterile PBS at 4 C for 24 h. Dako REAL™ EnVision™ Detection System (Dako, Glostrup, Denmark) was used at room temperature for 45 min. The sections were immersed in DAB and counterstained with Mayer's hematoxylin. The primary antibodies were mouse monoclonal Ki-67 (1:100, Dako), rabbit polyclonal DDX-4 (1:100, Abcam, Cambridge, MA, USA), rabbit polyclonal GFRα-1 (1:50, Abcam) and rabbit polyclonal c-kit (1:300, Dako). The primary antibodies were replaced with sterile PBS as for negative controls. For the TUNEL assay (ApopTag® Peroxidase In Situ, EMD Millipore, Temecula, CA, USA), the antigen was retrieved by 20 µg/ml (w/v) proteinase K digestion at room temperature for 15 min and quenched as previously described. The procedures were performed in accordance with the manufacturer's recommendation.

The morphologies of different stages of testicular germ cells and the expression of protein markers were observed using a BX5 light microscope (Olympus, Shinjuku, Japan). The percentage of testicular cells per tubule (gonocyte, spermatogonium and Sertoli cell) was calculated from a total of 30 centered cross-sectioned seminiferous cords/tubules. Photomicrographs were recorded using the cellSens software (Olympus) and Adobe Photoshop CS6 Version 13.0.1 (Adobe Systems, San Jose, CA, USA).

TEM

Testes, which were cut into small blocks, and SSC colonies were fixed in 3% (v/v) glutaraldehyde in 0.1 M Na-phosphate buffer (pH = 7.2) for 24 h, and then after washing in fresh buffer, they were fixed in 1% (w/v) osmium tetroxide for 1 h. The tissues were further dehydrated with gradient concentrations of ethanol and embedded in Epon (TAAB Laboratories Equipment Ltd, Aldermaston, UK). The Epon-embedded blocks were cut into semithin and ultrathin sections on an ultramicrotome (Reichert Ultracut S, Leica, Microsystems, Wetzlar, Germany). The semithin sections (1 µm thickness), which were stained with 1% (v/v) basic toluidine blue, were used for identifying an area of interest with an approximate size of 200 × 200 µm. Ultrathin sections (60 nm thickness) were prepared from the area of interest, contrasted with uranyl acetate (30 min) and lead citrate (10 min) and then observed using a transmission electron microscope (Philips CM 100 TEM, Philips Electron Optics BV, Eindhoven, The Netherlands)

Flow cytometry

The dissociated testicular cells were fixed in 4% (w/v) paraformaldehyde (VWR BDH Prolabo) in sterile PBS for 24 h at 4 C and then maintained in sterile PBS for further processing. Testicular cell suspensions were incubated with rabbit polyclonal GFRα-1 (1:50, Abcam) for 24 h at 4 C and washed with sterile PBS 2 times. The secondary antibody was Alexa Fluor® 488 goat anti-rabbit IgG (H+L) antibody (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA). The suspensions maintained in sterile PBS were analyzed using a BD FACSCalibur™ (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). For the negative control, the GFRα-1 primary antibody was replaced with sterile PBS to determine the baseline level of expression.

Culture of spermatogonial stem cells

The SSC culture medium modified from Kanatsu-Shinohara *et al*. [18] was used in this study. Dissociated testicular cells were counted and cultured at a final concentration of 2×10^5 cells/ml at 37 C in x a humidified incubator with 5% CO_2 in air. The SSC colonies were cultured in a feeder-free system and observed daily for morphology and proliferative characteristics using a phase-contrast microscope (CKX41, Olympus) for 4 weeks as previously described [27].

mRNA expression of SSCs cultured in vitro

A total of 5 SSC colonies were collected and extracted for total RNA using an Absolutely RNA Nanoprep Kit (Stratagene™, Agilent Technologies, Santa Clara, CA, USA). The extracted RNA was reversely transcribed using a SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) for cDNA synthesis (RT+). The negative control (RT–) was prepared by removal of Superscript™ III reverse transcriptase. The synthesized cDNA was used as the template for PCR.

PCR was performed with RT+ and RT– using GoTaq® Green Master Mix (Promega, Fitchburg, WI, USA). Briefly, the PCR reaction consisted of initial denaturation (2 min at 95 C), 30 cycles of PCR cycling (30 sec at 95 C, 30 sec at the annealing temperature for each primer and 30 sec at 72 C; Table 1) and final extension (2 min at 72 C). The PCR products were electrophoresed in 1% (w/v) agarose gel (Bio-Rad, Hercules, CA, USA) and 5% (v/v) RedSafe™ nucleic acid stain (iNtRON Biotechnology, Gyeonggi-do, Republic of Korea) in TBE buffer. The amplified products were examined under UV light using a gel documentation system (Syngene, CB, UK). The primers used in this study are described in Table 1.

Statistical analysis

The data are presented as the mean±SEM. Data were analyzed with SPSS version 20.0.0 (IBM, Armonk, NY, USA). The statistical differences between groups were tested using the analysis of variance and the Bonferroni post hoc test (Exp. 1) or the independent samples Student's t-test (Exp. 2 and 3). A P-value ($P < 0.05$) was set to determine statistical significance.

Results

The transition of testicular germ cells during different phases of postnatal testicular development

The histologically cell types in cat testes observed in the different age groups (groups I, II and III; Fig. 1) were gradually identified within the seminiferous cords/tubules of the testes as development of the testicular germ cells advanced. This included the development of testicular germ cells from gonocytes and the transition of undifferentiated spermatogonia (SSCs) to spermatocytes, round spermatids,

Table 1. Sense and antisense of primers description of target genes used for SSC characterization

Gene	Sequence (5'–3' orientation)	Amplicon size (bp)	Annealing temperature (C)	Reference
GFRA1	Sense: CAACTGCCAGCCAGAGTCAA	250	60	[27]
	Antisense: AGCCATTGCCAAAGGCTTGA			
ZBTB16	Sense: GCAAGAAGTTCAGCCTCAAGC	119	63	[27]
	Antisense: GCTTGATCATGGCCGAGTAGTC			
*RET**	Sense: TGTGCATGACTACAGGCTGG	193	63	
	Antisense: CCTGCTCACAGTGAAGGTGT			
POU5F1	Sense: TGAGAGGCAACCTGGAGAAC	112	55	[51]
	Antisense: AACCACACTCGGACCACATC			
GAPDH	Sense: GGAGAAAGCTGCCAAATATG	191	55	[52]
	Antisense: CAGGAAATGAGCTTGACAAAGTGG			

* Newly designed primers from XM_003994195.2.

elongated spermatids and spermatozoa, respectively. During early testicular development (group I), gonocytes were predominantly found in the seminiferous cords. Pleomorphic gonocytes had an average size of 17.17 ± 2.82 μm (range of 10.9–25.77 μm). A prominent nucleolus and pseudopod were also observed. At the ultrastructural level, the gonocyte's nuclei displayed relatively homogenous euchromatin, and their cytoplasm only contained only few organelles (Fig. 2A). However, a small numbers of cells already had heterochromatin clumps within the nucleus. The mitotic activity was present along with proliferative gonocytes and Sertoli cells, as determined by the expression of the proliferation marker (Ki-67) (Fig. 1). This stage of testicular development had a higher percentage of gonocytes per seminiferous cord (15.49 ± 3.34) compared with those of testes of group II (7.52 ± 1.79) and group III (0.11 ± 0.10) (Fig. 2b, $P < 0.001$). In addition, apoptotic marker (TUNEL-positive cells) was expressed in phagocytic Sertoli cells only and was occasionally expressed in the gonocytes (Fig. 1).

Predominant changes in germ cell types were clearly demonstrated in the testis of group II, especially at 4 months of age. At this time point, the major populations of testicular cells in the seminiferous cord were gonocytes, spermatogonia and Sertoli cells. The gonocytes dramatically transformed into an undifferentiated type of spermatogonia (referred to SSCs) as determined by an increase in the nucleus to cytoplasm ratio (reduced cell size) with darkening of the cytoplasm and chromatin condensation. This cell transformation occurred simultaneous to migration of spermatogonia from the innermost structure to the periphery of the seminiferous cords. Occasionally, some cells were at the G to S phase and moving toward the periphery of the tubule (Fig. 1). The putative SSCs presented as round to oval cells that were localized to the basement membrane of the seminiferous cord. The mean size of these putative SSCs was 10.31 ± 0.89 μm (ranged of 8.11–13.55 μm). At this stage of postnatal testicular development, the mean percentage of SSCs per seminiferous cord/tubule (17.66 ± 2.20) was significantly higher than those for testes of groups I (2.18 ± 0.36) and III (6.45 ± 1.56) (Fig. 2B, $P < 0.001$). At the ultrastructural level, the nucleus of the SSCs was predominantly euchromatic, but small heterochromatic blocks were also observed. The SSCs displayed an increasing amount of mitochondria compacted within the gonocytes (i.e., group I testes). This finding coincided with the intensely stained cytoplasm of the SSCs observed in our H&E examination (Fig. 2A). For group II, histology demonstrated no expression of Ki-67 in some gonocytes and spermatogonia (Fig. 1). Apoptosis was predominantly observed in gonocytes, which were occasionally engulfed by Sertoli cells (Fig. 1). By 6 months of age (group III), initiation of meiosis of spermatogenesis took place, as indicated by the presence of prophase I spermatocytes within the seminiferous cords. With advanced age of the cats (group III), the development of seminiferous tubules occurred, and testicular germ cells at advanced stages were present at this time. While gonocytes were rarely present in all testicular samples, spermatogonia and spermatocytes were clearly the major testicular cell population in the seminiferous tubules. However, complete spermatogenesis was first observed in 9-month-old testes. The Ki-67 was found to be strictly expressed in spermatogonia. Apoptosis was episodically found in a small population of spermatogonia and premeiotic spermatocytes (Fig. 1).

Phenotypic analysis of testicular cells with SSC and testicular markers

Immunohistochemistry was used to study the expression of GFRα-1, DDX-4 and c-kit as SSC, germ cell and differentiated spermatogonium markers, respectively. In the seminiferous cords/tubules, the DDX-4 protein was localized in the cytoplasm of gonocytes, spermatogonia, primary and secondary spermatocytes and some of round spermatids (Fig. 3). The DDX-4 showed descriptively lower expression in both spermatogonia and primary spermatocytes when compared with the other cell types. The expression of c-kit was restricted to gonocytes and spermatogonia. The patterns for c-kit expression were different between gonocytes and spermatogonia in that the perinuclear membrane (golgi pattern) was predominantly found in gonocytes, while the c-kit protein was translocalized to express in the cytoplasm and plasma membrane of the spermatogonium (Fig. 3). GFRα-1 was essentially expressed in the plasma membrane and cytoplasm of the SSCs or spermatogonia (Fig. 3) but not in gonocytes.

Flow cytometric analysis of the SSC population

To determine the numbers of SSCs (GFRα-1-positive cells) within the testes, testes from groups II and III only were selected as group I testes did not contain SSCs. Of the total cells isolated from testicular

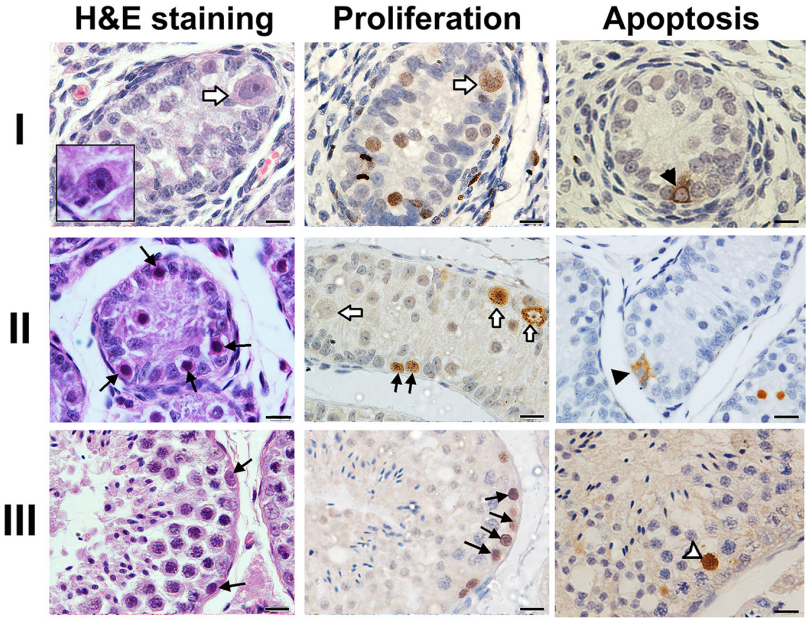

Fig. 1. Conventional histology (left panel), proliferative activity (Ki-67 expression, middle panel) and apoptotic activity (TUNEL assay, right panel) of cat testicular sections in groups I, II and III. The testicular section of group I (< 4 months) demonstrated a large proportion of gonocytes in the seminiferous cords. The gonocytes had a specialized cytoskeleton or pseudopod to move toward the basement membrane (closed up). These cells had high proliferative activity, while apoptotic gonocytes were occasionally phagocytized by Sertoli cells. Group II (4–6 months) presented mixed populations of gonocytes and spermatogonia in the seminiferous cords. Ki-67 activity was absent in some gonocytes and spermatogonia (black arrow). However, the rest of the gonocytes and spermatogonia expressed proliferative and apoptosis activity. Complete spermatogenesis was only observed in Group III testes. Spermatogonia and primary spermatocytes obviously expressed proliferative and apoptosis activities, respectively. Gonocyte, white arrow; spermatogonium, black arrow; phagocytizing Sertoli cell, black arrow head; primary spermatocyte, white arrowhead. Scale bars = 10 µm.

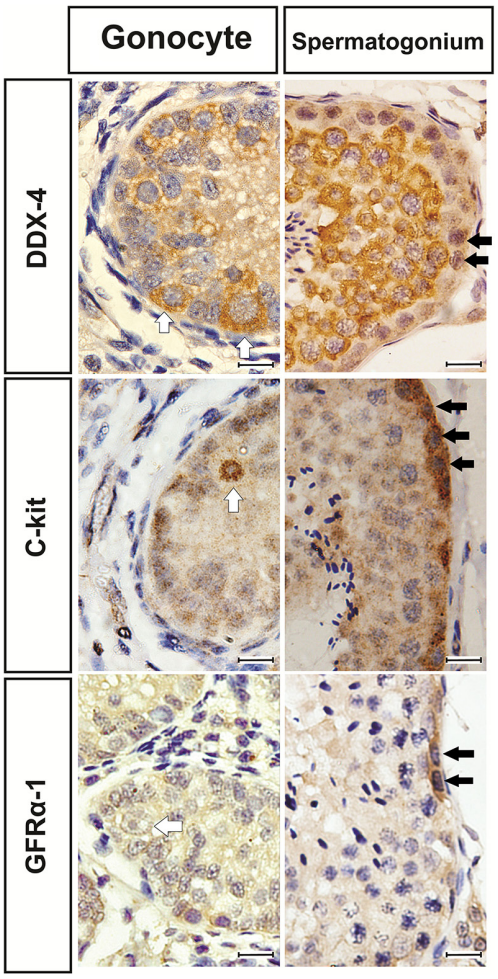

Fig. 3. The protein expression of DDX-4, c-kit and GFRα-1 in the cat testes (gonocytes, left panel; spermatogonia, right panel). The DDX-4 was expressed in the cytoplasm of all types of testicular germ cells. Spermatogonia and primary spermatocytes expressed DDX-4 at lower levels than secondary spermatocytes and round spermatids. Expression of c-kit was localized to the perinuclear area (golgi pattern) of the gonocyte, plasma membrane and cytoplasm of most spermatogonia. Single and paired spermatogonia expressed GFRα-1 in the plasma membrane and cytoplasm. Gonocyte, white arrow; spermatogonium, black arrow. Scale bars = 10 µm.

digestion, the percentage of GFRα-1$^+$ cells obtained from group II testes (14.89 ± 5.66) was significantly higher than that of group III testes (1.46 ± 1.64) ($P < 0.001$).

In vitro culture and identification of derived SSC colonies

Following testicular cell digestion and SSC culture, SSC colonies containing 2–6 cells were observed within 2 weeks. The SSCs gradually increased in cell number to approximately 12 cells around 4 weeks of culture. The SSC colonies were tightly packed, with small intercellular space. Regarding SSC culture, the SSC colonies obtained from group II testes had a higher success rate than those obtained from group III testes, as determined by the number of colonies observed at 4 weeks of culture (74.33 ± 2.64% *vs*. 23.33±2.23%, $P < 0.001$).

The SSCs from both groups expressed *GFRA1, ZBTB16* and *RET* and also demonstrated a typical ultrastructure morphology (TEM) (Fig. 4). TEM revealed that the SSCs had a round to oval nucleus with flattened cytoplasm. The nucleus contained euchromatin with a small number of heterochromatin clumps underneath the nuclear membrane, which was similar to the ultrastructure found in testicular tissues (Fig. 4).

Discussion

This study revealed that development of testicular germ cells occurred asynchronously depending on cell types and was donor-age dependent. Here, we reported for the first time that the G/SSC transition phase was primarily initiated by 4 months of age in domestic cats. In addition, this phase of testicular development was appropriated for SSC derivation, as this period contained the highest numbers of GFRα-1$^+$ SSCs and significantly improved the efficiency of SSC

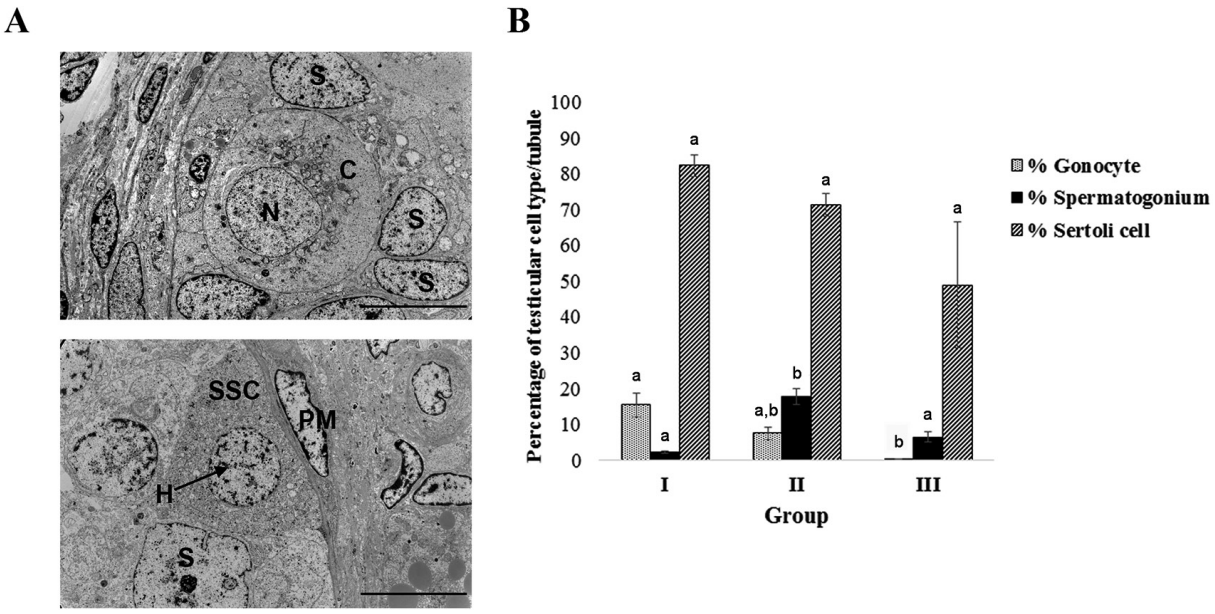

Fig. 2. Morphology and numbers of testicular germ cell types within seminiferous cords/tubules. A) The ultrastructure of the gonocyte (upper panel) and spermatogonium (lower panel). The gonocyte has abundant euchromatin chromatin in nucleus (N) and sparse organelles in the cytoplasm (C). The spermatogonium (SSC) has small clumps of heterochromatin (H) and numerous of mitochondria. B) Mean percentage of gonocytes and spermatogonia per seminiferous cord/tubule. Different letters indicate values that are significantly different ($P < 0.05$). S, Sertoli cell; PM, peritubular myoid cell. Different characters (a and b) within the types of cells indicate values that are significantly different ($P < 0.05$). Scale bars = 10 μm.

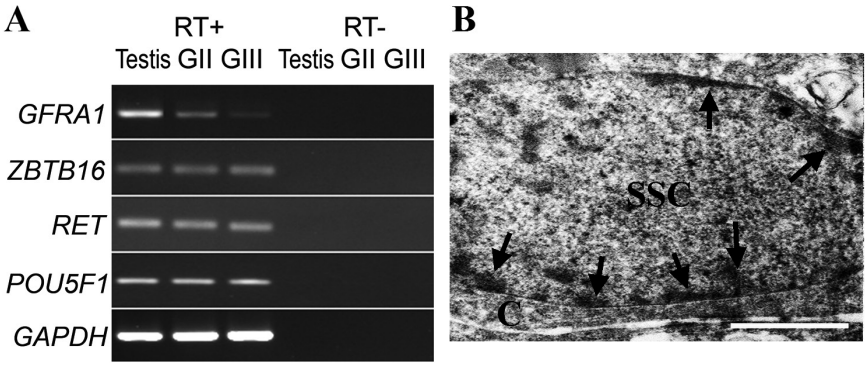

Fig. 4. The mRNA expression and ultrastructure of SSC colonies. A) The RT-PCR products from group II (GII) and III (GIII) testes. RT+ and RT– were performed to detect mRNA expression of SSC genes (*GFRA1, ZBTB16, RET* and *POU5F1*). B) The nuclear ultrastructure of *in vitro* cultured SSCs demonstrated a typical nuclear ultrastructure containing euchromatin and small clumps of heterochromatin (arrow). C, cytoplasm Scale bars = 1 μm.

colony formation.

In this study, we described chronological development of testicular germ cells in cat testes, as these cellular changes have been proposed to critically affect to successful derivation of SSCs *in vitro* [22]. It was clearly demonstrated that the development and transformation of feline gonocytes into testicular germ cells was a slow process when compared with a rodent model [2]. At birth, cat seminiferous cords contained an abundance of intracytoplasmically DDX-4-expressing gonocytes. However, a subpopulation of testicular cells with a cell-surface DDX-4 pattern, as previously reported in pigs was not demonstrated in this study [28]. According to our results, the gonocytes in group I testes (less than 4 months) had a high proliferation rate prior entering to the G/SSC phase. The relative percentage of gonocytes per tubule gradually decreased with the advancement of spermatogenesis as a consequence of an increase in apoptosis and decrease in proliferation activity of gonocytes in group II testes. This topographic transformation of gonocytes or G/SSC transition is similar to that in other studies previously reported [6, 7, 29]. Gonocytes become quiescent (mitotic arrest with apoptosis) before mitotic resumption and the G/SSC transition [30–32]. Although

the phase at the G/SSC transition precisely occurs in the mouse and rat [24], the quiescent period of gonocytes and the G/SSC phase vary and are controversial among species [33, 34]. The present study demonstrated that the process for the G/SSC transition in the cat was an asynchronous phenomenon throughout the seminiferous cord/tubule, which was dissimilar to rodents [23]. The mechanisms that control the quiescent period of the G/SCC transition are still unknown, but this period is required for marked molecular changes (such as downstream of pluripotent markers) [35, 36]. A previous study reported that active gonocytes migrated down to basement membrane of the seminiferous cord by KITL/KIT signaling prior the SSC transformation [8]. Furthermore, the c-kit receptor, which is well known as the plasma membrane receptor was localized to the perinuclear area (golgi pattern) of the gonocytes and then translocalized to the plasma membrane of spermatogonia. This golgi pattern of gonocytes was usually found in germ cells, but it has also be seen in gastrointestinal stromal tumor (GIST) cells in humans [37–39]. The morphology and ultrastructure of gonocytes within the seminiferous cords were remarkably distinguished from those of SSCs, in terms of morphology and the numbers of mitochondria. These enriched mitochondria in the SSCs appeared to be associated with darkened cytoplasm in H&E sections and helped to maintain a high level of mitochondrial activity in rat and mouse SSCs [26, 40], and they appeared to stimulate proliferation of the early phase of SSCs [41]. These feline SSCs expressed mRNA (*GFRA1*, *ZBTB16*, *RET* and *POU5F1*) and proteins (DDX-4 and GFRα-1) essentially important for SSC activities, which is similar to other reports [18, 27, 42].

The pubertal age of the cats in this study was determined to be around 9 months of age, as indicated by complete spermatogenesis and the presence of spermatozoa in the caudal epididymis. This finding is different from previous studies indicating that the pubertal period of cat ranges from 8 to 12 months [16]. The difference in pubertal period may be caused by differences in photo period, climate, animal care or nutritional status [43–47]. Regarding the meiosis phase, the apoptosis that occurred in spermatogonia and primary spermatocytes was intensively observed in the first wave of spermatogenesis. The apoptosis at this point is essential for regulation of the balance between the number of germ cells and Sertoli cells and also for reduction of impaired germ cells during meiosis [48, 49]. In this study, group II testes contained the highest percentage of morphological spermatogonia (referred to as SSCs) per cord/tubule as a result of the resumption of mitosis [23]. This stage of testicular development was therefore appropriate for enrichment of GFRα-1$^+$ cells. The percentage of spermatogonia per cord/tubule observed from histology was closely similar to the percentage of GFRα-1$^+$ testicular cells from flow cytometry (17.66 ± 2.20 *vs*. 14.89 ± 5.66). The high proportion of SSCs in the group II testes coincided with the improved success rate of SSC colony formation (74.33 ± 2.64%) when compared with a more advanced stage of testicular development (group III testes; 23.33 ± 2.23%, $P < 0.001$). This result, therefore, confirms the importance of the G/SSC phase transition and also possibly the negative effects of other testicular cells (differentiated germ cells and supporting testicular cells) on successful SSC culture [22]. However, maintaining SSC activities *in vitro* remains problematic for long-term culture of SSCs in domestic cats [27, 50]. Further study is required to underpin the mechanisms that essentially contribute to the multifaceted fate of SSC properties during SSC culture. It is also important to study whether additional SSC purification would really improve SSC establishment in the advanced stage of testicular development.

This study found that testicular development and the G/SSC transition phase in the cat testis occur at a specific time point. This transition phase is a synchronous phenomenon throughout the seminiferous tubules. Determination of this G/SSC transition successfully promotes the efficiency of SSC isolation and culture of SSCs *in vitro*.

Declaration of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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