Location and characterization of female germline stem cells (FGSCs) in juvenile porcine ovary

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Abstract

Objectives: Existence of germline stem cells (GSCs) in juvenile mammalian female ovaries has been drastically debated recently since reports that adult mouse ovaries still have mitotically active germ cells have been proposed. In addition, definitive location of such female germline stem cells (FGSCs) had not been demonstrated.

Materials and methods: We segregated porcine FGSCs mechanically from ovary cortex, and tested our hypotheses by utilizing immunofluorescent staining, qRT-PCR and western blotting.

Results: We attached emphasis to unambiguous location of FGSCs, which settle simultaneously in the theca. Dissected cells from porcine thecal layers maintained similar characteristics to mouse FGSCs and ESCs over 4-months *in vitro* culture.

Conclusion: These results may provide a new resource for the study of oogenesis and therapy for ovarian sterility.

Introduction

Germline stem cells (GSCs) play a uniquely important role in biology, providing a mechanism for sexual reproduction and passing on the genetic legacy from one generation to the next. They share some similar characteristics with embryonic stem cells (ESCs) (1), including in morphology, pluripotency and the capacity for germline transmission (2,3). Male germline stem cells (mGSCs) – spermatogonial stem cells (SSCs) – are to be found in seminiferous tubules, and are central to

spermatogenesis throughout life of the male mammal. They can be transfected to produce transgenic offspring by SSC transplantation (2,3).

In contrast, there were widely differing held notions that few female germline stem cells (FGSCs) exist in ovaries or that they cease division after birth in most mammalian species (4,5). Specially in recent reports, several lines of *in vivo* and *in vitro* evidence has indicated that no mitotically active female germline progenitors exist in postnatal mouse ovaries (6). However, these statements have been challenged by information that the female gonad-ovary has unexpected regenerative activity in adulthood (1,7–9). Additionally, FGSC lines from neonatal and adult mice have been established and cultured for periods *in vitro*, having characteristics of GSCs and ESCs (10,11). Furthermore, such long-term cultivated FGSCs were shown to preserve their abilities to produce normal oocytes and fertile offspring after transplantation into recipients' ovaries (11). Pacchiarotti *et al.* (2010) have shown the existence of populations of GSCs in postnatal mouse ovaries (12) and Parte *et al.* (2011) reported the presence of very small pluripotent stem-like cells, settling in ovarian surface epithelium (OSE) of adult rabbit, sheep, monkey and menopausal human (13). Meanwhile, the concept that adult ovaries might possess any type of stem cells remained drastically controversial (6,14,15). Efficiency of FGSCs isolation was still low, and mechanism of FGSC proliferation and differentiation remained unclear (16).

Accordingly, to clearly demonstrate existence of FGSCs, porcine ovary has been chosen for this piece of research. As the pig is a convenient domestic species for biological investigation and biomedical application, with characteristics of diversity, valuable products and relative short gestation period, pigs share anatomical metabolism and physiological characteristics with humans that make them a unique and viable model for biomedical research (17,18). Fortuitously, our data have suggested that FGSCs of porcine ovary mainly settle in theca lay-

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ers compared to early perspectives on FGSCs existing at in regions of ovarian surface epithelium (OSE) in series of vertebrate species, including human (11,19–22). Additionally, although thecal stem cells of mouse ovary have been identified recently (23), knowledge of their development, exhaustive morphological characteristics compared to male counterpart-Leydig cells (24,25) has rarely been manifested.

Consequently, to confirm the supposition and elucidate characteristic of porcine FGSCs, we detected pluripotent and GSC markers by utilization of immunofluorescence, qRT-PCR and western blotting in different samples, fragments of OSE layers, thecal layers and regions of the granulosa. Furthermore, results have also suggested that FGSCs dissected from thecal regions could form GSC-like colonies in serum- and feeder-free medium maintained up to 8 passages, *in vitro*. Simultaneously, these had characteristics similar to ESCs (26). These results demonstrate that porcine FGSCs mainly settle in regions of thecal layers; this provides a new resource for exploring development of oocytes and a potential for fertility treatment.

Materials and methods

Collection of porcine ovarian tissue

Juvenile Changbai porcine ovarian tissues were supplied from Yangling Hi-tech abbatoir. Guidelines for utilization of animals in research were followed according to standards of Chinese Association for Laboratory Animal Science.

Immunofluorescence and H&E staining

Ovaries from 4 to 6 month postnatal Changbai pigs were dissected and rinsed in PBS (pH 7.5); selected tissues were immediately fixed in 4% paraformaldehyde for 24 h. Residual segments including fragments of OSE layers, thecal layers and regions of granulosa were respectively prepared for qRT-PCR and western blotting. Segregation of fragments of OSE layers was cautiously performed using surgical scalpels. Whole follicles were isolated and dissected, fragments of thecal layers were easily and successfully removed by forceps simultaneously; remaining tissue, thus, was mainly composed of granulosa cells.

Two-micrometer thick paraffin sections were cut and left floating on water at 48 °C for stretching, then collected on coated poly-L-lysine slides. Slides were dried for 4–8 h in an oven at 60 °C, then deparaffinized in xylene 20 min (two changes, 10 min each) followed by absolute ethanol 9 min (three changes, 3 min each); subsequently, tissue sections were rehydrated in 96% ethanol (three changes,

3 min each), 70% ethanol (three changes, 3 min each) and finally brought to PBS. All tissue sections were washed in PBS, three times, 5 min each.

Although different antigens may behave differently under similar treatment conditions, the following protocol was found to be acceptable for most tested in this study. Steps for antigen retrieval were as follows: after rinsing in PBS, sections were soaked in retrieval solution, pH 6.0 (10 mM sodium citrate, 0.05% Tween 20), and heated for 10 min in a microwave oven, once solution had reached micro-boiling, or temperature had reached 98 °C. We then removed the vessel and kept it at room temperature. After three washes in PBST (PBS contained 0.25% Triton X-100) for 5 min each tissue sections were covered in 1% BSA for 30 min at 37 °C in a humidity chamber (27). Thereafter, we incubated sections in primary antibodies specific to OCT4 (1:500; Chemicon, Temecula, CA, USA), C-MYC (1:200; Chemicon), NANOG (1:200; Chemicon), CD49f (1:500; Chemicon), SOX2 (1:200; Chemicon), CD133 (1:500; Chemicon), SSEA1 (1:200; Chemicon), SSEA3 (1:200; Chemicon), SSEA4 (1:200; Chemicon), LIN28 (1:400; Chemicon), TERT (1:200; Chemicon), VASA (1:200; Abcam, Cambridge, MA, USA), C-KIT (1:200; Abcam), NANOS2 (1:200; Abcam), at 4 °C overnight in a humidity chamber. After three washes in PBST, 5 min each, samples were incubated in secondary antibodies R594, R488, M594, M488 (1:500; Chemicon) 1 h room temperature, then rinsed three times in PBST, 3 min each. Nuclei were counterstained with DAPI (Sigma, 5 µg/ml, St. Louis, MO, USA); slides were retained in tap water until mounting. Simultaneously, treatment of control groups was identical as for experimental groups except for replacement of primary antibody incubation with PBS. Finally, samples were viewed under the fluorescence microscope. Tissue section staining of each sample was repeated four times to confirm results, and murine, bovine and caprine ovaries (mouse, cow and goat) were stained by the same process.

Rehydrated tissue sections were rinsed in distilled water for 5 min and slides were dipped in haematoxylin and agitated for 30 s. They were then rinsed in water for 1 min and stained in 1% eosin solution for 10–30 s with agitation. Sections were then dehydrated through two changes of 95% ethanol and two changes of absolute ethanol for 30 s each. Finally, ethanol was extracted by two changes of xylene, and one or two drops of mounting medium (natural resin) were added; specimens were then covered with coverslips.

Isolation and culture of FGSCs

To minimize potential contamination resulting from transportation from the abattoir, samples were gently

washed at least 10 times in PBS, with dissolved 300 mg/ml penicillin and 75 mg/ml streptomycin. Target cells were mechanically segregated using a surgical needle, horizontally placed and repetitively scraped between surface of OSE and medulla (Fig. S1); scratched regions were filtered through a 200 mesh filter then dissociated cells were cultured in 0.1% gelatin-coated 60 mm plates. DMEM/F12 (Hyclone, Logan, UT, USA) used was comprised of 20% knock-out serum replacement (KSR, Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine, 1 mM non-essential acids (Invitrogen), 0.1 mM β -mercaptoethanol (Sigma), 20 ng/ml EGF (epidermal growth factor, Millipore, Billerica, CA, USA), 5 μ g/ml insulin, 20 μ g/ml transferrin (ITS, Invitrogen) and 1000 U/ml leukaemia inhibitory factor (LIF, Millipore). Cells were cultured at 38 °C in 5% CO₂, with fresh medium replaced every 2–3 days, then they were dissociated with 0.05% (w/v) trypsin at 70–80% confluence.

RT-PCR and qRT-PCR

Total RNA was extracted from different regions of ovaries including fragments of OSE layers, thecal layers and regions of granulosa, using Trizol reagent (Tiangen Beijing, China). Single-strand cDNAs were prepared from 2 μ g RNA, using a reverse transcription Kit (Fermentas, Riga, Latvia) and specific gene expression was analysed. Primers employed are listed in Table 1, all markers of pluripotent stem cells and GSCs (28–30). PCR conditions were: initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s; annealing temperature used was in accordance with properties of different primers for 30 s and 72 °C for 35 s, with final extension at 72 °C for 10 min. PCR products were separated by 1.5% agarose gel electrophoresis (30).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was set up in 25 μ l reaction mix-

tures containing 12.5 μ l SYBR® PremixExTaq™ (BI-OER Co. Ltd., Hangzhou, China), 0.5 μ l sense primer, 0.5 μ l antisense primer, 11 μ l distilled water and 0.5 μ l template. Reaction conditions were as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, and 58 °C for 20 s. All expression levels were normalized to β -actin in each well (31). Expression was quantified as ratio of mRNA levels among fragments of OSE layers, thecal layers and regions of granulosa. qRT-PCR primers including VASA, Dazl, Oct4, Sox2, c-Myc, Klf4, Nanog, c-Kit, CD90 are listed in Table 1.

Western blot analysis

Total cells and specific regions of ovary were prepared for protein extraction in SDS-PAGE sample loading buffer. After electrophoresis by SDS-PAGE, proteins were separated, then transferred to PVDF membranes and probed with β -actin (1:1000; Beyotime, Haimen, Jiangsu, China), PCNA (1:1000; Millipore), VASA (1:1000; Millipore), KLF4 (1:1000; Millipore) and DAZL (1:1000; Millipore). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG was used as secondary antibody (1:1000; Beyotime). Detection was performed using BM-chemiluminescence blotting substrate (Roche, Shanghai, China).

Immunofluorescence analysis of cultured FGSCs

Cells were fixed in 4% paraformaldehyde for 15 min, then treated with 0.1% Triton X-100 for 9 min at room temperature. After being blocked with 10% FBS for 30 min, cells were incubated in primary antibodies against OCT4 (1:500; Chemicon), OCT4A (1:100; Millipore), KLF4 (1:200; Chemicon), C-MYC (1:200; Chemicon), NANOG (1:200; Chemicon), CD49f (1:500; Chemicon), SOX2 (1:200; Chemicon), CD133 (1:500; Chemicon), SSEA1 (1:200; Chemicon), SSEA3 (1:200;

Table 1. Primers for RT-PCR and qRT-PCR

Gene	Sense primer	Anti-sense primer
Oct4	GCTGACAACAACGAGAATC	TTGCGAATAGTCACTGCTT
Sox2	GCCCAGGAGAACCCCAAGAT	GGGTGCCCTGCTGCGAGTA
Klf4	TGTCATCCTGCCTGCCG	CGGTAGTGCCTGGTCAGTTCAT
c-Myc	CTGGTGGGCGAGATCATCA	CACTGCCATGAATGATGTTCC
Nanog	GATTCTTCTACCAGTCCCAAAC	ATGCGTTCCACGATAGCC
c-Kit	TCCCAAACCTCAACACCGACAG	GTGTAAGTGCCTCCTTCAGTCCC
CD90	GATCCAGGACTGAGCTCTCGG	TCACGGGTGAGACTGAACATAC
Vasa	AGAGGACGAGGTGGTTAC	GCGATGTTGTTATTCACTGT
Dazl	CCTCCAACCATGATGAATCC	GGGCAAAATATCAGTCTCTG
Oct4A	GACACCTGGCTTCCGACTTC	GCTGAACACCTTCCCAAAGAG

Figure 1. Dual immunofluorescence images of porcine ovary. Ovary sections were analysed for expression patterns of GSCs and ESCs, from top panels to bottom, OCT4/CD49f, SOX2/NANOG, VASA/C-KIT, SSEA4/C-MYC, TERT/SSEA1, SSEA3/NANOS2, LIN28/CD133 and FITC/PE-labelled IgG as negative control. White boxes outline area magnified in panel and also demonstrate that some ESC- and GSC-like cells can be detected in the region of theca, but abutted upon granulosa layers. Scale bar = 100 μ m; F, Follicle; G, Granulosa; T, Theca.

Figure 2. H&E staining of porcine ovary structure. (a) Secondary follicle section is visible consisting of granulosa layers, basal lamina and thecal layer, bar = 50 μ m; (b) Magnified follicle section includes round cells, deeply stained (arrow), FGSC, bar = 25 μ m; (c) OSE layer with no follicles and primary follicle with oocytes are depicted in the cortex, bar = 50 μ m; (d) Intact follicle stripped as shown; (e) Top panel – magnified porcine ovary structure, bar = 50 μ m; G, granulosa cells; O, oocyte; OSE, ovary surface epithelium; P, primordial follicle; PF, primary follicle; T, theca; bottom panel depicts detailed regions of ovary, including A, antrum; B, blood vessel; F, follicle; G, granulosa cells; O, oocyte; OSE, ovary surface epithelium; P, primordial follicle; PF, primary follicle.

Chemicon), SSEA4 (1:200; Chemicon), LIN28 (1:400; Chemicon), TERT (1:200; Chemicon), VASA (1:200; Abcam) and C-KIT (1:200; Chemicon) overnight at 4 °C. After being washed three times in PBS, they were incubated in appropriate secondary antibodies for 1 h at room temperature, in the dark. Negative controls were devoid of primary antibody incubation. Images were captured using a Leica fluorescence microscope.

Differentiation potential of female germline stem cells

For EB formation, 2–3 passage FGSCs were picked and dissociated with 0.05% trypsin (Invitrogen). Resuspended cells were plated on Petri dishes, 3×10^5 cells per 3.5 cm dish (30). After 3 days culture, cells aggregated and formed embryoid bodies (EBs) which were then transferred to 48-well culture plates (10–15 EBs per well) supplemented with differentiation medium, which was changed every 2 days. Seven days later, EBs and suspended cells were harvested and analysed.

Spontaneous differentiation medium comprised H-DMEM (Gibco, Carlsbad, CA, USA) supplemented with 15% foetal bovine serum (FBS; Hyclone), 2 mM L-

Figure 3. Molecular profiling of fragments of OSE layers, thecal layers and regions of granulosa. (a) Comparison of gene expression among OSE, theca and granulosa shown in different colours, by real-time PCR. mRNA levels normalized to β -actin and error bars show standard deviations; (b) Western blot analysis of translated protein PCNA, VASA, KLF4 and DAZL; (c) Oct4A isoform detected in OSE, thecal region and cultured FGSCs, but not expressed in granulosa.

glutamine (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1 mM β -mercaptoethanol (Sigma) (30).

Generation of RFP-expressing FGSCs

For cell tracking experiments, porcine FGSCs were transduced by lentivirus (Fig. S2) to obtain cells with RFP stable expression (porcine, RFP-sFGSCs). We are grateful for donation of pLenO-RTP lentivirus from Innovation Biotechnology Co., Ltd (Invabio, Shanghai, China). Transduction of FGSCs was performed using fresh viral supernatant facilitated by presence of polybrene (10 μ g/ml, Sigma-Aldrich, St. Louis, MO, USA). After 12 h, virus environment was removed and replaced with fresh FGSC culture medium. Porcine FGSCs with RFP expression were collected for kidney capsule transplantation.

Renal capsule transplantation

To achieve renal capsule transplantation easily, intact porcine follicle (diameter 6–8 mm) was dissociated (Fig. 2d) and utilized as a sack to aggregate FGSCs. Before transplantation, granulosa of follicles had been removed using vitreous Pasteur pipetting (Fig. S3). FGSCs (concentration 2×10^6) expressing RFP were collected and embedded in the follicular sack, which were then transplanted beneath left renal capsules of busulphan-treated female Kunming mice (32). After 4–

8 weeks transplantation, host mice were sacrificed and kidneys were retrieved, fixed and sectioned for further histological analysis (33).

Statistical analysis

All experiments were performed at least 3 times and data are presented as mean-SEM. Statistical comparisons were performed using Student's *t*-test. *P* value of less than 0.05 or 0.01 was considered statistically significant or clear statistical significant difference, respectively (34).

Results

Identification of porcine FGSCs by immunofluorescence and H&E staining

To test the hypothesis that porcine FGSCs may settle in thecal layers, ovaries of three different samples were collected, each undergoing routine immunofluorescence staining. Markers of pluripotent undifferentiated cells (OCT4, SSEA1, SSEA3, SSEA4, NANOG, KLF4, TERT, CD133, C-MYC and SOX2) had been utilized to identify FGSCs. Furthermore, cells positive for CD49f, C-KIT (CD117), VASA and NANOS2 could also be detected in the same regions (Fig. 1). Markers including those for pluripotent, proliferative and germline cells were also detected in similar region-theclal layers in dif-

Figure 4. Clonogenic proliferation of FGSCs collected from porcine ovary at different time and passages. Scale bar = 50 μ m.

ferent species ovaries (mouse, cow and goat) (Fig. S4). Additionally, HE sections manifested different layers including granulosa, basal membrane and thecal layers (Fig. 2a,b).

To clearly demonstrate whether FGSCs located in the OSE or not, antibodies OCT4A and VASA were utilized to detect their location. Compared to OSE, positive OCT4A and VASA cells were detected in thecae, as

Figure 5. Characterization of porcine FGSCs. Immunostaining of FGSC clusters with OCT4, SSEA4, C-KIT/C-MYC, KLF4/SOX2, NANOS2/CD49f, VASA/SSEA3; DAPI counterstain. Scale bar = 100 μm.

Figure 6. Differentiated potentiality of porcine FGSCs *in vitro*. FGSCs formed embryoid body (EB)-like structures; oil red-O positive cells can be detected, immunofluorescence stain of differentiation of FGSCs with PDX1(endoderm marker), desmin, cardiac α -actin (mesoderm marker) and NSE (ectoderm marker) positive neural-like cells. First panel scale bar = 200 μ m; second panel scale bar = 100 μ m; remaining scale bar = 200 μ m.

depicted in Figs S5,S6. Conversely, we were not able to find OCT4A or VASA-positive cells in OSE. We detected pluripotent marker-OCT4A, and germ cell marker-VASA specifically expressed in the theca by immunofluorescence staining and RT-PCR (Figs S7,S8,S9); these results suggest that FGSCs mainly settled in the thecal region.

Identification of porcine FGSCs by qRT-PCR and western blotting

Fragments of OSE layers, thecal layers and regions of granulosa were respectively mechanically dissected from ovaries; thus, expression of different genes could be detected in different segments. Newly synthesized cDNA from different areas was then detected by qRT-PCR and simultaneously, regions of granulosa were utilized as negative controls. Results manifested that expression level of GSCs markers, and pluripotent markers including Oct4, Sox2, Klf4, c-Myc, Nanog, c-Kit, CD90, Vasa and Dazl in thecal layers were distinctly higher than in OSE layers (Fig. 3a). To obtain more convincing evidence, western blotting was performed to detect expression of proteins of target genes of those segments, and similarly, granulosa was the control. Consequences indicated that both OSE and thecal layers expressed target proteins such as PCNA, VASA, KLF4 and DAZL (Fig. 3b); however, differences were not significant between segments of OSE and thecal layers. Nevertheless, these results do not contravene our hypothesis that FGSCs are mainly recruited in regions of thecal layers.

Enrichment and characterization of FGSCs in vitro

Cells from juvenile pig ovaries were mechanically dissociated and collected as described in the Materials and methods section and cultured in serum- and feeder-free medium for 7–14 days. They formed compact round colonies with unclear fringes, and maintained embryonic stem cell-like colonies up to passage 8; moreover, they preserved ESC and GSC characteristics (Fig. 4). These cells were positive for pluripotent and germline cell markers OCT4, SSEA4, C-KIT, C-MYC, KLF4, SOX2, NANOS2, CD49f, VASA and SSEA3 (Fig. 5). These results distinctly support our hypothesis that the region of the theca mainly recruits FGSCs for settlement.

Differentiation potential of porcine FGSCs in vitro and in vivo

To confirm the ability of FGSCs differentiation, their differentiated potential was examined *in vitro* and *in vivo*. After 3 days EB (embryoid body) formation, EBs

were transferred to attaching dishes using Pasteur pipettes; for 7–15 days they spontaneously differentiated into a variety of cell types, for example adipocyte-like cells, positive for oil red-O staining (Fig. 6). PDX1 (endoderm marker), desmin, cardiac α -actin (mesoderm marker) and NSE (ectodermal marker) positive cells were obtained and demonstrated by spontaneous differentiation assay.

As mentioned above, empty intact follicles were utilized as sacks, which embedded and aggregated FGSCs. After 2 months, four grafts grew larger and formed teratomas in the recipient's kidney; these contained endoderm (gut), mesoderm (muscle) and ectoderm (neural epithelium)-like structures (Fig. 7). FGSC-derived RFP positive cells could also be observed in the grafts; simultaneously, these cells expressed markers of germline cells (CD133, VASA, and DAZL) and pluripotency (OCT4) markers (Fig. 8).

Discussion

Several lines of evidence have suggested that proliferation and pluripotency activities of germline stem cells exist in the adult ovary (35); female germline stem cells have been identified and isolated from adult mouse ovaries (35) and from further animals of vertebrate and

Figure 7. Histology of teratomas formed after transplantation of FGSCs and differentiated cells into busulphan-treated Kunming mice. (a) FGSCs embedded in follicle transplanted into renal capsule (arrowhead); (b–d) Histological analysis of representative teratoma derived from FGSCs. Teratomas formed contain derivatives of all three embryonic germ layers (ectoderm, mesoderm and endoderm); (b) Neural epithelium-like structures at arrow (ectoderm); (c) Smooth muscle-like structures at arrow (mesoderm); (d) Gut epithelium-like structures at arrow (endoderm). All images obtained from 4% paraformaldehyde-fixed and paraffin-embedded teratoma sections stained with haematoxylin and eosin. (b–d) Scale bar = 50 μ m.

invertebrate species (36). Dissected FGSCs have been cultured *in vitro* retaining the capacity to differentiate into a variety of cell types in the presence of serum or feeder cells (37). White *et al.* (2012) used a fluorescence-activated cell sorting-based protocol to purify rare mitotically active (1), primitive germ cells from mouse ovaries and human ovarian cortical tissue. These results showed that such cultured cells could proliferate for months and spontaneously generate 35- to 50- μ m oocytes (determined by morphology), gene expression and

haploid status. These could produce oocytes *in vitro* which had the capacity for fertilization *in vivo*.

However, recent reports have suspected that there was no evidence concerning existence of FGSCs (mitotically active cells) *in vivo* and *in vitro* (6). Compared to this demonstration, FGSCs could be cultured and then de-differentiated into pluripotent ES-like cells *in vitro* in an appropriate microenvironment. Our results show that FGSCs might be reprogrammed into pluripotent stem-like cells similar to male germline stem cells *in vitro*

Figure 8. Differentiation potentiality of porcine FGSCs in the recipient mouse renal capsule. Segregated FGSCs transfected by lentivirus with RFP stable expression. Aggregated FGSCs with RFP expression transferred to empty intact follicles and whole sacks transplanted into the renal capsule. After 2 months, grafts grew larger and FGSC-derived RFP-positive cells could be observed in these grafts; these cells simultaneously expressed markers of germline cells and pluripotency (CD133, OCT4, DAZL and VASA as in Fig. 8).

(38,39)(Figs 4,5). Equally, recent studies have also demonstrated that potential somatic stem cells exist in the theca and OSE (40). Honda *et al.* (2007) isolated putative thecal stem cells (23), however, there were still questions to be answered concerning differences between thecal stem cells and FGSCs.

Furthermore, there was little information on porcine FGSCs, and efficiency of FGSC isolation was still low. We have shown existence of FGSCs in porcine ovary, detected by immunofluorescence staining (Fig. 1), and concrete location instructive to improve efficiency of FGSC segregation. Consequently, to distinguish where the FGSCs mainly settle, we dissociated three segments of ovary including fragments of OSE, thecal layers and regions of granulosa cells. FGSCs mainly assembled in the region of thecal layers (Fig. 2). According to previous reports, FGSCs should distribute in the region of OSE (13), however, qRT-PCR results supported our opinion by expression level of the same gene, remarkable between OSE and thecal layers. Simultaneously, high level expression of c-Kit in the theca may indicate recruitment of more PGCs; it also suggested that FGSCs mainly assembled in thecal layers and data of western blotting also supported this.

It has been reported that mammalian FGSCs developed colonies with morphology similar to ES cells after *in vitro* cultures in the presence of LIF, bFGF or EGF (10). Additionally, these cells were shown to share similar phenotypical characteristics to those of pluripotent ES cells (12,30). Therefore, dissected and cultivated FGSCs which formed clones were detected by immunofluorescence. Some markers such as pluripotency and germ cell-related markers OCT-4 (41), SSEA1 (42), VASA (43,44), CD133, α 6-integrin (CD49f), SOX2 and TERT (45) were identified in mice and human mGSCs and pluripotent ES cells (46). To obtain convincing results, these antibodies had been tested on different species tissue sections which we had chosen for experiments, on which they worked successfully. It has been reported that FGSCs differentiated into 3 germ layer types in spontaneous differentiation conditions and induction system *in vitro* and *in vivo* (47). Fortunately, FGSCs which were cultured in our serum- and feeder-free system appeared with some similar characteristics to those of pluripotent ES cells and GSCs (Figs 4,5). Although cultured FGSCs *in vitro* spontaneous differentiation could be detected (Fig. 6), FGSCs still did not differentiate to oocytes *in vitro*. The process of development and differentiation of xeno-FGSCs perhaps was limited by circumstances of the mouse renal capsule environment, the process might have been inhibited by the sack, perhaps blocking growth of vessels. Thus nutrition FGSCs need could not been transported to

them, although RFP marked FGSCs could spontaneously differentiate into many cell types including endoderm (gut), mesoderm (muscle), and ectoderm (neural epithelium) -like structures (Fig. 7). Moreover, some transplanted RFP positive FGSCs still expressed germ cell markers (Fig. 8). Thus, potential capacity of these cells' differentiation needs to be improved (13). We also found similar phenomena in mouse, cow and goat thecae analysed by immunofluorescence. These results showed that thecal cells formed FGSC-like cells similar to mouse FGSCs.

In conclusion, our data indicate that GSCs of porcine ovary were primarily located at the thecal region. Furthermore, we cultured such cells derived from thecae and successfully established FGSCs from juvenile ovaries. Cultured porcine FGSCs differentiated into many cell types including adipocyte-like cells, PDX1 positive (endoderm marker), desmin, cardiac α -actin (mesoderm marker) and NSE (ectoderm marker) cells. Evidence shows that these cells had similar characteristics to those of ESCs and SSCs. This work provides a new resource and *in vitro* model to study development of mammalian oocytes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. 12# syringe needle utilized as scraper and repetitively scratched to dissect cells around cortex of ovary.

Fig. S2. Lentivirus plasmid.

Fig. S3. (a) Intact follicle dissociated by scalpel; (b) Follicle by stereo microscopy; (c, d) Granulosa of this follicle had been removed by vitreous Pasteur pipette.

Fig. S4. All sections of different species (mouse, cow and goat) ovaries stained with LIN28, SOX2, VASA, SSEA4. Note specificity of expression; pluripotent characteristics of these cells were located in the region of theca, abutted upon granulosa layers, their location being similar to that of swine ovaries. Scale bar = 100 μ m.

Fig. S5. Immunofluorescence stain of juvenile porcine ovary sections. OCT4A positive cells detected in thecal regions, but negative in OSE. These results clearly illustrate that FGSCs were mainly located in the theca. As arrowhead depicted OCT4A-positive cell, oth-

ers might lose their stemness during OCT4A migrating progress from nucleus to cytoplasm, (arrow). F, follicle; G, granulosa cells; T, thecal region. Scale bar = 100 μ m.

Fig. S6. Immunofluorescence stain of juvenile porcine ovary sections. VASA-positive cells could be detected in the thecal region but were negative in OSE. Arrowhead depicts VASA-positive cell. F, follicle; G, granulosa cells; T, theca. Scale bar = 10 μ m.

Fig. S7. Porcine ovary section scanned by laser scanning confocal microscopy, stained with VASA and OCT4A antibodies. Positive VASA and OCT4A could be detected in single cell. Results clearly demonstrated location of antibodies in (white boxes outlined) simultaneously. Meanwhile, haematocytes could be detected yellow (arrowhead). A, antrum of follicle; G, granulosa; T, theca. Scale bar = 10 μ m, in magnified picture scale bar = 1 μ m.

Fig. S8. To design Oct4A primer, different mRNA of Oct4 including homo transcript variant 1, 2, 3 and *Sus scrofa* had been aligned by software ClustalX. Therefore, upstream primer only limited from 87 bp to 323 bp as depicted, because of high similarity of different sequences shown in that domain.

Fig. S9. Formed FGSC colonies were treated with collagenase IV, and digested into single cells. Then segregated cells were seeded on poly L-lysine coated slides, and stained with OCT4A. OCT4A-positive cells were found, some OCT4A proteins migrated from nucleus to cytoplasm simultaneously, meaning pluripotency of cells began to decrease.