GSK3 inhibitor-BIO regulates proliferation of female germline stem cells from the postnatal mouse ovary

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Abstract

Objective: It is widely believed that in most female mammalian neonates, all germ cells enter meiosis to form the primary oocyte at the end of foetal development, and as a result, the postnatal mammalian ovary harbours only a limited supply of oocytes that cannot be regenerated. However, this idea has been challenged by the discovery of the existence of female germline stem cells (FGSCs) in postnatal mammalian ovaries.

Materials and Methods: We have isolated ovarian GSCs from neonatal and adult mouse ovaries and expanded them in the same culture conditions as embryonic stem cells (ESCs).

Results: LIF and BIO were beneficial for formation of FGSC colonies. BIO promoted proliferation of FGSCs through activation of β -catenin and up-regulation of E-cadherin. The FGSCs formed compact round colonies with unclear borders, maintained ESC characteristics and alkaline phosphatase (AP) activity, expressing germ-cell markers-Vasa, and stem-cell markers: Oct4, Klf4, C-myc, Nanog, CD49f, Sox2, CD133, SSEA1 and SSEA4. These cells had the ability to form embryoid bodies (EBs), which expressed specific markers for all three germ layers. Then we induced EBs to differentiate into neurons, cardiomyocytes, pancreatic cells and germ cells, which showed the expression of specific markers, β -III-tubulin, cardiac α -actin, Pdx1 and Zps respectively.

Discussion and Conclusion: This study reveals the existence of FGSCs in postnatal mouse ovary with multipotent characteristics. BIO played an important role in regulation of proliferation and maintenance of the FGSCs. This could help provide a better understanding of causes of ovarian infertility, prevention and potential treatment of infertility.

Introduction

Germ cells are a unique cell population in that they alone transmit genetic material to offspring. Although germ cells are highly specialized populations for generation of gametes, several lines of evidence suggest their multipotentiality (1,2). Scientists have already discovered that male germline stem cells (mGSCs), also called spermatogonial stem cells (SSCs), exist in adult testis for spermatogenesis throughout life and can be transfected to produce transgenic offspring *via* SSC transplantation (3,4).

In contrast, few studies have shown that female germline stem cells (FGSCs) exist in the ovary, or indeed stop dividing, after birth in most mammalian species (5). Recently, some studies have shown that the female gonad-ovary has unexpected regenerative activity in adulthood (6,7). Additionally, FGSC lines from neonatal or adult mice, with the characteristics of germ cells and embryonic stem cells (ESCs), have been established and cultured for a period *in vitro* (8,9). These FGSCs of long-term culture maintained their capacity to produce normal oocytes and fertile offspring after transplantation into ovaries (9). Pacchiarotti *et al.* also showed the existence of a population of germline stem cells in postnatal mouse ovaries (10). Recently, Parte *et al.* have reported that there are pluripotent, very small, embryonic-like stem cells in adult rabbit, sheep, monkey and menopausal human ovarian surface epithelium (OSE), which may undergo asymmetric cell division to maintain

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homeostasis and have the ability to differentiate into oocyte-like structures, *in vitro* (11).

However, the idea that adult ovaries possess any type of stem cell remains highly controversial. Additionally, the efficiency of isolation of FGSCs has still been low, and the mechanism of FGSCs activity has still been unclear (12). Previous studies have shown that the GSK3 inhibitor-BIO activates Wnt signalling and is known to sustain pluripotency of both human and mouse ESCs, by inhibiting GSK3 β (13,14). Whether BIO could regulate proliferation and differentiation of FGSCs has still been an issue.

In this study, we have investigated the existence of FGSCs in postnatal mouse ovary, optimized the culture system for FGSCs, and demonstrated that these cells share pluripotent ESC characteristics; thus, we propose that germline stem cells may provide a resource for study of development of oocytes, causes of ovarian infertility and prevention and potential treatment of infertility.

Materials and methods

Isolation and culture of female germline stem cells

To prepare the ovarian cells, ovaries of 1- and 8-week-old female mice were collected and minced after removal of adherent tissue. Specimens were digested in 1 mg/ml collagenase (Sigma Chemical Co., St. Louis, MO, USA) at 37 °C for 15 min with shaking at intervals. After being centrifuged at 300 g/min for 5 min, they were incubated in dissociation solution that consisted of a mixture of 0.2% (v/w) trypsin (Invitrogen, Carlsbad, CA, USA) and 1.4 mg/ml DNase (Invitrogen), for 10 min at 37 °C. Dissociated cells were cultured on mitomycin C (Sigma)-treated mouse embryonic fibroblasts (MEFs) in 0.1% gelatin-coated tissue culture plates in H-DMEM (Invitrogen), supplemented with 15% foetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mM L-glutamine (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1 mM β -mercaptoethanol (Sigma), 100 mg/ml penicillin/streptomycin and 1000 U/ml leukaemia inhibitory factor (LIF; Millipore, Billerica, MA, CA, USA). Cells were cultured at 37 °C in 5% CO₂ in air. Cultured cells were further dissociated in 0.05% (w/v) trypsin in combination with mechanical pipetting when reaching 70–80% confluence. Culture medium was refreshed every 2–3 days.

For the factorial design experiment, freshly isolated cells were plated on gelatin-coated plates or MEF feeders in standard GSCs medium without growth factors or with combination of LIF (1000 U/ml; Millipore), EGF (epidermal growth factor, 10 ng/ml; Millipore), bFGF (basic fibroblast growth factor, 10 ng/ml; Millipore), GDNF

(Glial cell line-derived neurotrophic factor, 10 ng/ml; Peprotech, US) or PDGF (platelet-derived growth factor, 50 ng/ml; Sigma), GSK3 inhibitor (BIO, 1–5 μ M; Sigma), ERK inhibitor (PD 98059, 10 μ M; Beyotime, Haimen, Jiangsu, China). On alternate days, half the medium was replaced by fresh medium supplemented with the same combination of growth factors. After 7 days, cultures were terminated and digital images were taken. The entire factorial design experiment was repeated at least twice.

Characterization of female germline stem cells

RT-PCR. Total RNA was extracted with Trizol reagent (Tiangen, Beijing, China) from ESCs or treated EBs. Single-strand cDNAs were prepared from 2 μ g RNA using a reverse transcription kit (Fermentas, Europe) and specific gene expressions were analysed. RT-PCR primers used for amplifying markers of stem cells, three germ layers or germ cells (16,17), are listed in Table 1. PCR conditions were: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s; annealing temperature used was in accordance with the primer sequence for 30 s and 72 °C for 35 s, with final extension at 72 °C for 10 min. PCR products were separated using 2% agarose gel electrophoresis. DNA markers were used to confirm size of the resultant fragments.

Immunofluorescence analysis. Cells were fixed in 4% paraformaldehyde and treated with 0.1% Triton X-100 for 10 min at room temperature. After being blocked with 10% FBS for 30 min, cells were incubated in primary antibodies against Oct4 (1:500; Chemicon, Temecula, CA, USA), 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), SSEA4 (1:200; Chemicon), Lin28 (1:400; Chemicon), Tert (1:200; Chemicon), PCNA (1:200; Millipore), β -catenin (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), Active- β -catenin (1:200; Millipore), E-cadherin (1:100; Millipore), β -III-tubulin (1:500; Santa Cruz Biotechnology Inc.), Pdx1 (1:200; Abcam, Cambridge, MA, USA), CT3 (1:1,000; DSHB, US), cardiac α -actin (1:500; Sigma), Vasa (1:200; Abcam), Stra8 (1:500; Abcam), Zp3 (1:100; Santa Cruz Biotechnology Inc.) and Figla (1:100; Santa Cruz Biotechnology Inc.), respectively, overnight at 4 °C. After being washed three times in PBS, specimens were incubated in appropriate secondary antibodies for 1 h at room temperature in the dark. Untreated ESCs and ovarian cells were used as negative control and positive control respectively. Images were captured using a Leica fluorescence microscope.

Table 1. Primer sequences and PCR reaction conditions

Gene	Primer (5'→3')	T _m (°C)	Size (bp)
β-actin	Forward: GCGGCATCCACGAAACTAC Reverse: TGATCTCCTTCTGCATCCTGTCT	58	138
Oct4	Forward: CGCCCGCATACGAGTTCT Reverse: GCACCAGGGTCTCCGATTT	58	487
Nanog	Forward: GATTCTTCTACCAGTCCCAAAC Reverse: ATGCGTTCCACCAGATAGCC	54	376
C-myc	Forward: CTGGTGGGCGAGATCATCA Reverse: CACTGCCATGAATGATGTTCC	54	304
Klf4	Forward: CCAGGAGAACCCCAAGATGC Reverse: GGGTGGCCTGCTGCGAGTA	58	518
β-III-tubulin	Forward: CTTTGTGGCCAGATCTTTAGACC Reverse: CTCGTTGTCAATGCAATAGGTC	58	377
Nestin	Forward: GCCACAGTGCCCAAGTTCTA Reverse: ATCCTCCATTCTTGGTTCTTC	58	567
Gata4	Forward: TCCCTCTTCCCTCCTCAAATTC Reverse: TCAGCGTGTAAGGCATCTG	54	193
Brachury	Forward: AAGGTGGCTGTTGGGTAGGGAGT Reverse: ATTGGGCGAGTCTGGGTGGATGT	58	451
Pdx1	Forward: AACGCAGGAACCACGATGAGAGG Reverse: AAGGGGTCGCCCCGAGTAAGAATG	67	483
PCNA	Forward: AGTGGAGAACTTGGAATGGAA Reverse: GAGACAGTGGAGTGGCTTTTGT	58	154
CyclinA	Forward: TGGCTGTGAACCTACATTGA Reverse: ACAAACTCTGCTACTTCTGG	50	136
CyclinD1	Forward: TGAACCTACCTGGACCGCT Reverse: CAGGTTCCACTTGAGYTTGT	50	212
Dazl	Forward: ATGAAAGATAAAACCACCAACC Reverse: TGTTGACAGCCTGGTCCACTGA	58	391
Cyp26b1	Forward: CGGGAGAAGTATGGCAACG Reverse: TGGTGTTCGCCCAGTAGGA	58	104
Stra8	Forward: AGCAGCTTAGAGGAGGTCAAGA Reverse: TACTCGGAACCTCACTTTTGTC	58	111
Zp1	Forward: TGGGACCAGAAGGGAAGC Reverse: CTGAGGATTGCCACGGATAA	58	281
Zp2	Forward: CTGTCATCCTGAACTCGCTGT Reverse: ACAAAACCCGCACTGAACC	60	229
Zp3	Forward: GAGCTTTTCGGCATTTCAG Reverse: AGCTTATCGGGGATCTGGTT	58	150
Gdf9	Forward: TAGTCCACCCACACACCTGA Reverse: CCAGAAGCCTGAGAACCAAG	62	197
Figla	Forward: CTCTGCTGCCCCGTGGTCTT Reverse: CTGCTCTGTGGTAGAAACGGC	58	304
Vasa	Forward: AAAGTGCCCAAGTCTTGTGTGCTAC Reverse: ACTGGATTGGGAGCTTGTGAAG	58	392

BrdU incorporation assay. Proliferation of FGSCs was assayed by BrdU incorporation, performed similarly as in previous reports (15). First, FGSCs in absence or presence of BIO (2.5 μ M, 3 days) were treated with 30 μ g/ml BrdU (Sigma) for 5 h, then subjected to BrdU immunostaining. More specifically, cells were fixed in 4% PFA for 15 min at room temperature and washed three times, for 10 min each in PBS (pH 7.4) containing 0.1% Triton X-100. Cells were then washed three times in PBS (pH 7.4) alone. Anti-BrdU (1:100; Santa Cruz Biotechnology Inc.) diluted in

0.1 M PBS (pH 7.4) containing 5% normal goat serum was added and cells were incubated overnight at 4 °C. Cells were washed in PBS (pH 7.4) three times, and then incubated in secondary antibody (FITC-labelled, 1:500; Millipore) for 1 h at room temperature. Three more washes were carried out and cells were visualized using a Leica fluorescence microscope and were analysed for BrdU uptake. Levels of BrdU positive cells in absence or presence of BIO were determined by manual counting using the fluorescence microscope (16).

Growth curves. Cells were seeded in 48-well plates at 3×10^4 cells/well and their proliferative ability was assessed by construction of growth curves at intervals of 24 h. Cells were trypsinized and cell number was determined for 8 consecutive days ($n = 3$).

Differentiation potential of female germline stem cells. For EB formation, colonies in 1–3 passage FGSCs cultures were picked and dissociated with 0.05% trypsin solution (Invitrogen). Resuspended cells were plated on Petri dishes at 3×10^5 cells per 3.5 cm dish. After 3 days culture, cells were seen to have aggregated and formed embryoid bodies (EBs). Resulting EBs were transferred into culture 48-well plates (10–15 EBs per well) and supplemented with differentiation medium, which was changed every 2 days to avoid its degradation. Seven days later, EBs and suspension cells were harvested and analysed.

Media

Media were as follows:

Spontaneous differentiation medium: H-DMEM (Invitrogen) supplemented with 15% foetal bovine serum (FBS; Hyclone), 2 mM L-glutamine (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1 mM β -mercaptoethanol (Sigma) and 100 mg/ml penicillin/streptomycin (Sigma) (17).

Germ-cell differentiation medium: spontaneous differentiation medium supplemented with 10% porcine follicular fluid (PFF) (18).

Cardiomyocyte differentiation medium: spontaneous differentiation medium supplemented with 0.75% DMSO and 0.1 μ M RA in mESC control medium (19).

Pancreatic cell differentiation medium: FGSCs-derived EBs were cultured in RPMI 1640/B27 medium (Invitrogen) supplemented with 1% BSA (Sigma), 100 mM nicotinamide (NIC; Sigma), 10 ng/ml exendin-4 (Sigma), 1 mM sodium pyruvate (Invitrogen), 2 mM glutamine (Invitrogen), 1 mM β -mercaptoethanol (Sigma), 100 mg/ml penicillin/streptomycin, 20 ng/ml EGF (Millipore) and 20 ng/ml bFGF (Millipore) for 7 days (20).

Results

Enrichment and characterization of FGSCs

Cells from neonate and adult mouse ovaries cultured for 1–3 passages formed compact round colonies with unclear borders (Fig. 1a–f); they maintained embryonic stem-cell characteristics – AP activity (Fig. 1g,h). Colony-forming efficiency of FGSCs of neonate mice (1-week old) per ovary was 0.20% compared to 0.08%

in adult ovaries (8-week old) (Fig. 1i); percentage of FGSC in 1-week-old mice was significantly higher than in 8-week old mice. However, pluripotentiality of FGSC between 1-week-old and 8-week-old mice was similar here. RT-PCR analysis showed that these cells were similar to mouse ESCs in expressing pluripotency markers Nanog, C-myc, Oct4 and Klf4 (Fig. 1j). FGSC colonies were also shown to be positive for characteristic surface markers of pluripotent ES cells and mGSCs, including Klf4, C-myc, Nanog, Oct4, Sox2, SSEA1, SSEA4, Lin28, Tert, CD133, CD49f, and germ-cell marker Vasa, as analysed by immunofluorescence staining (Fig. 2).

To further determine the effects of different commonly used growth factors in primary cultures of GSCs, a factorial design experiment was performed. For LIF, EGF, bFGF, GDNF and PDGF, effects were determined on number of FGSC colonies formed in culture. LIF had significant positive effects on number of colonies and the colonies were compact and round. On the other hand, cells grown in bFGF had almost no typical type of colony formation. Colonies were bigger when cultured on feeder layers than when cultured feeder-free. No significant effects were detected for bFGF, GDNF and PDGF on number of colonies obtained (data not shown). We then observed conformation of GSC colonies when FGSCs were cultured in standard mouse ES medium, human ES medium and medium supplemented with retinol (RE; Sigma). We found that mouse ES was the most efficient medium to obtain FGSC colonies, although both human ES medium and medium supplemented with RE were able to promote proliferation of FGSCs (Fig. 3a). GSK3 inhibitor-BIO significantly increased the number of GSC colonies and number of AP-positive colonies with combination of LIF, while ERK inhibitor-PD98059 had no detectable effects (Fig. 3b). BIO promoted colony formation in the presence of LIF, but not with bFGF, and effects of BIO are presented as a dose-dependent model (Fig. 3c,d).

GSK-3 inhibitor BIO promoted colony formation of FGSCs

FGSCs presented as typical colonies with long spindle cell morphology of mesenchymal-like cells, in the presence and absence of BIO, when cultured feeder-free. There were more colonies and they were significantly more dense in BIO specimens, than without BIO (Fig. 4a). Number of colonies was significantly increased (in the region of 3-fold, $P < 0.01$) in the presence of BIO (Fig. 3d).

When cells were treated with BrdU, it was more likely to be found to incorporate in GSCs colonies,

Figure 1. The morphology and characterization of FGSCs. FGSC colonies from neonatal mouse ovaries cultured for 1 day (a), 2 days (b) and 3 days (c, d) formed compact, round colonies with unclear borders. FGSC colonies from adult mouse ovaries (e, f). FGSC colonies were positive for alkaline phosphatase (AP) staining (g, h). Colony-forming efficiency of neonatal mouse ovarian FGSCs was 0.20% compared to 0.08% of adult ovarian GSCs (i). (j) Expression of pluripotent markers expressed in ES cells (ES), feeder-FGSCs (GSC), feeder-free-GSCs (N-GSC) and MEF. (a–c, f, h) Scale bar = 200 μ m; (d) Scale bar = 800 μ m; (e, g) Scale bar = 50 μ m.

Figure 2. Effects of different factors on primary cell cultures were determined by numbers of FGSC colonies and numbers of AP-positive FGSC colonies. (a) Mouse ES medium was the most efficient medium to obtain GSC colonies, and both human ES medium and medium supplemented with RE promoted proliferation of GSCs. (b) BIO significantly increased numbers of GSC colonies and number of AP-positive GSC colonies with combination of LIF, while PD98059 had no detectable effects. (c) BIO promoted colony formation in the presence of LIF but not bFGF, and the effects of BIO are presented as a dose-dependent model (d).

Figure 3. The expression of pluripotent markers in FGSCs. Effects of different growth factors on primary cell cultures were determined by numbers of GSC colonies and numbers of AP-positive GSC colonies. FGSC colonies expressed markers characteristic of pluripotent ES cells and mGSCs including Klf4, C-myc, Nanog, Oct4, Sox2, SSEA1, SSEA4, Lin28, Tert, CD133, CD49f and germ-cell marker Vasa, as analysed by immunofluorescence staining. Nuclear staining with Hoechst33342 (blue) is also shown. Scale bar = 200 μ m.

including round flat monolayer cell colonies, with or without a clear boundary, and in cells of compact round colonies with a clear border (Fig. 4b). In total, control cells had lower levels of BrdU incorporation. The BrdU incorporation assay demonstrated that mitotic index in the presence of BIO was higher than without BIO (Fig. 4c). Percentages of PCNA positive cells also had an up-trend compared to that in the absence of BIO.

Expression of proliferation markers C-myc and PCNA were up-regulated in BIO medium compared to in the absence of BIO analysed by RT-PCR, and expression of cell cycle proteins cyclinA and cyclinD1 were increased in BIO treated cells (Fig. 4d).

Growth curves of GSCs cultured in absence and presence of BIO (2.5 μ M) are shown in Fig. 4e. Proliferation of total cells was not significantly different between presence of BIO and control specimens, but

when treated with BIO, numbers of GSCs increased clearly.

FGSCs in the presence of BIO seemed to find it easier to form colonies and to express C-myc, as shown by immunofluorescence staining. Meanwhile, β -catenin was activated when FGSCs were cultured in the presence of BIO. In control cells, β -catenin staining was weak and appeared to be located at cell membranes only. In contrast, BIO-stimulated cells showed some nuclear staining, suggesting that there was increased level of β -catenin. After treatment with BIO, FGSCs had more compact morphology and were aggregated, suggesting changes in cell adhesion (21). Therefore, we studied levels of E-cadherin upon BIO stimulation. As visualized by immunofluorescence, E-cadherin was expressed in BIO-treated FGSCs, while it was not expressed in untreated cells (Fig. 5).

Figure 4. GSK-3 inhibitor BIO promoted colony formation and proliferation of FGSCs. (a) FGSCs presented as typical colonies and long spindle cell morphology of mesenchymal-like cells, in the presence and absence of BIO when cultured feeder-free. There were more cell colonies and colonies were significantly more dense in BIO than without BIO. When cells were treated with BrdU, control cells had a lower level of BrdU incorporation. Percentage of PCNA positive cells also had an up-trend compared to that in the absence of BIO. (b) BrdU was more likely to incorporate into cells of compact round FGSC colonies with a clear border. (c) Percentage of BrdU-positive cells treated with or without BIO from (a). (d) expression of cell cycle proteins cyclinA and cyclin D1, and proliferation markers C-myc and PCNA were up-regulated in BIO medium compared to in the absence of BIO, analysed by RT-PCR. (e) Growth curves of FGSCs cultured in absence and presence of BIO (2.5 μ M). Scale bar = 200 μ m.

Differentiation potential of FGSCs *in vitro*

FGSCs had the ability to form EBs after 3 days in suspension culture (Fig. 6a) and expressed specific markers of all three germ layers after 7 days spontaneous differentiation. RT-PCR analysis showed GSC-EBs expressed β -III-tubulin, Nestin, Gata4, Brachury and Pdx1. Primary ovarian cells were employed as the negative control (Fig. 6c).

To confirm potentiality for differentiation *in vitro*, 3-day suspension EBs were induced by germ-cell differentiation medium, neural cell differentiation medium, cardiomyocyte differentiation medium and pancreatic cell differentiation medium. EB-like clusters derived from FGSCs were attached and cultured for 7 days. Induced GSC-EBs showed expression of neuron-specific marker β -III-tubulin (Fig. 6e). Neural-like cells were observed when cultures were induced with neural cell differentiation medium. Additionally, cardiomyocyte specific markers CT3 (Fig. 6g), cardiac α -actin (Fig. 6i) and pancreatic cell-specific marker Pdx1 (Fig. 6k) positive cells were obtained after induction.

After treating EB-like clusters with PFF, morphological changes were observed 4 days after induction, characterized by formation of round cells suspended in the media (Fig. 7a). Furthermore, 10 days after induction, round cells differentiated into oocyte-like cells in expand-

ing size, and some clusters gradually formed cystic structures only loosely attached to the plate (Fig. 7c). The putative oocyte-like cells derived from GSCs, however, had no zona pellucida (Zp) and approximately ranged from 20 to 50 μ m in diameter, never reaching normal oocyte size. Immunofluorescence analysis demonstrated that some cells treated with PFF were positive for germ-cell specific markers Vasa, Stra8, Zp3 and Figla. At mRNA levels, germ-cell markers Vasa, Dazl and oocyte markers Figla and Zps, were up-regulated after PFF induction, while stem-cell marker Oct4 was down-regulated. Expressions of Stra8, Cyp26b1 and Gdf9 were not clearly different between induced cells and controls (Fig. 7t).

Discussion

FGSCs can be cultured over long time periods, and retain the capacity to differentiate into multiple cell types in the presence of serum or feeder cells, *in vitro* (9,10,22). It has been reported that *in vitro* cultures of mammalian FGSCs in the presence of LIF, bFGF or GDNF, have developed the morphology of colonies similar to ES cells (10,23) and additionally, these cells shared similar phenotypic characteristics to those of pluripotent ES cells (1,10). Pluripotency-related markers Oct-4 (24), SSEA1 (25), CD133 (26), α 6-integrin (CD49f), Sox2 and Tert have been identified in mice

Figure 5. GSK-3 inhibitor BIO activated Wnt signalling pathways in FGSCs. Immunofluorescence staining analysis indicated that expression of β -catenin and C-myc were up-regulated in BIO medium compared to in the absence of BIO. Activated- β -catenin and E-cadherin were expressed in BIO-treated GSCs, which was not observed in control cells. Scale bar = 200 μ m.

Figure 6. The differentiation potential of FGSCs. Ovarian GSCs formed EBs after 3 days of suspension culture (a, b). (c) RT-PCR analysis showed GSC-EBs expressed β -III-tubulin, Nestin, Gata4, Brachury and Pdx1; primary ovarian cells were employed as negative control. Induced GSC-EBs showed expression of neuron-specific marker β -III-tubulin (e), cardiomyocyte-specific markers CT3 (g) and cardiac α -actin (i), pancreatic cell-specific marker Pdx1 (k) respectively. d, f, h, g were Hoechst 33342 stained nucleus of e, g, i and k respectively. (a) Scale bar = 100 μ m; (b) Scale bar = 50 μ m; (d-k) Scale bar = 200 μ m.

and human mGSCs and pluripotent ES cells (1,27,28). Our cultured FGSCs shared some similar characteristics to pluripotent ES cells, mGSCs, primordial germ cells

(PGCs) and GSCs, as analysed by immunofluorescence staining (1,10,27). Additionally, we found that younger mice had higher percentages of FGSC in their ovaries.

Figure 7. FGSCs differentiated into germ-like cells. After treating clusters with porcine follicular fluid (PFF), morphological changes were observed at 4 days of induction, characterized by formation of round cells suspended in the media (a). Furthermore, round cells and follicular-like cells appeared at 10 days after induction (b, c). (d) Clusters differentiated 10 days without PFF were employed as negative control. Immunofluorescence analysis demonstrated that some cells were positive for germ cell-specific markers Vasa (g), Stra8 (k, s), Zp3 (o) and Figla (p). e, i, m, r were light microscopy of g, k, o and s respectively. f, j, n were Hoechst 33342-stained nuclei of g, k and o respectively. (t) RT-PCR analysis showed that expressions of germ-cell markers Vasa, Dazl and oocyte markers Figla and Zps were up-regulated in PFF induction, while stem-cell marker Oct4 was down-regulated. (a, d) Scale bar = 100 μ m; (b, c, r, s) Scale bar = 50 μ m; (e–q) Scale bar = 200 μ m.

Niches and microenvironments play an important role in the fate and behaviour of stem cells (29). In the present study, mouse FGSCs were cultured with up to four passages, over 3 weeks, still maintained partially homogenous characteristics to mouse ESCs, indicating that our culture system is able to simulate the stem cell niche of ovary and maintain proliferation and differentiation of FGSCs *in vitro*. Co-culture of adult ovarian cells with somatic fibroblasts can produce colony-forming cells having ESC-like activity, which may provide an alternative for establishing autologous stem cells from adults that can be obtained without genetic manipulation (30). Previous studies have shown that there may exist very small embryonic-like cells (VSELs) in many adult tissues such as the heart and brain, as well as in umbilical cord blood and peripheral blood in adult human bone marrow, lung and heart. These VSELs express several markers of pluripotent stem cells (PSCs), such as Oct-4, Nanog and SSEA-1 (31). Cultured FGSCs then de-differentiated into pluripotent ES-like cells *in vitro* in appropriate microenvironments (29,30). Our results show that LIF and BIO promoted formation of FGSC colonies and AP-positive FGSC colonies; this demonstrated that FGSCs could be reprogrammed into pluripotent stem cells similar to male germline stem cells *in vitro* (1,27). It is possible, how-

ever, that there are two different lineages in the adult ovary, one pluripotent and the other pre-committed towards the germ-cell line.

In this study, our results have demonstrated that FGSCs could successfully be cultured in BIO medium and that BIO up-regulated expression of proliferation markers including C-myc and PCNA, compared to controls. Moreover, several cell cycle regulators, known to regulate cell cycle progression during G₁/S transition, were also detected. Both cyclin D1 and cyclin A were up-regulated in BIO, which indicated that both were involved in regulating cell cycle progression from G₁ to S phase, as induced by BIO. Myc is activated through various mitogenic signals such as Wnt, Shh and EGF (via the MAPK/ERK pathway). Myc activation results in many biological effects including driving cell proliferation (by up-regulating cyclins), regulating cell population growth (by up-regulating ribosomal RNA and proteins), apoptosis, differentiation and self-renewal of stem cells. Myc is a strong proto-oncogene and its overexpression stimulates gene amplification (32). Proliferating cell nuclear antigen (PCNA) is a distinct marker for cell proliferation (33,34), which here was up-regulated in BIO medium. These results demonstrate that BIO may regulate C-myc, which results in up-regulation of PCNA, cyclinA and cyclin D1 to maintain self-renewal of FGSCs.

Wnt describes a large family of secreted glycoproteins, and there are extracellular Wnt-ligands, which interact with receptors of the frizzled family, involved in cell proliferation, differentiation, organogenesis and cell migration (35,36). Wnt-dependent genes include those encoding regulators of proliferation (cyclin D1, c-myc and *e2f1*), differentiation [mesendodermal markers T/Brachyury and Pitx2 (paired-like homeodomain transcription factor 2)], EMT (epithelial–mesenchymal transition) (Slug, Snail and vimentin) and these operate during development and in adult tissues (35,37–39). Upon Wnt signalling, β -catenin is stabilized, accumulating in the cytoplasm and eventually translocating to the nucleus, where it interacts with DNA-binding proteins of the T-cell Factor/Lymphocyte Enhancer binding Factor (Tcf/Lef) family. In the presence of β -catenin, Tcf/Lef behaves with transcriptional activity (40). However, BIO may inhibit glycogen synthase kinase 3β (GSK3 β) activity, and activate Wnt/ β -catenin signalling and Ca^{2+} signalling pathways (41,42). In this study, β -catenin was activated, and total β -catenin and E-cadherin were increased in BIO-treated cells. Accumulation of β -catenin is a clear hallmark of Wnt signalling (43). These results clearly demonstrate that BIO inhibited GSK3 β activity, and activated Wnt/ β -catenin signalling, and Ca^{2+} signalling pathways in FGSCs (21). Key downstream transcriptional regulators of the Wnt pathway influence transcription of target gene C-myc and cyclin D1, thus promoting proliferation of FGSCs.

The Wnt signalling pathway and its negative regulator kinase GSK3 β are also considered to be involved in maintenance of pluripotency. The function of GSK3 inhibitor-BIO in maintenance of pluripotent stem cells has been previously demonstrated (13). Our results show combined treatment of FGSCs with BIO and LIF resulted in a significantly higher level of colony formation over the of other groups. LIF and Wnt/ β -catenin pathway signalling have also been reported to have a synergistic effect and are successfully used in establishment of mouse ES cell lines (43,44).

Furthermore, our research found that GSK3 inhibitor-BIO helped to maintain the undifferentiated status of FGSCs by their undergoing mesenchymal to epithelial transition (MET). During MET, fibroblasts deviate from their mesenchymal origin and organize into compact, laterally connected cells. At the transcriptional level, c-Myc down-regulates TGF- β 1 and TGF- β receptor 2, suppressing EMT, and Klf4 induces typically epithelial genes, including that for E-cadherin (45). Moreover, induced pluripotent stem cells (iPSCs) are derived from mouse embryonic fibroblasts (MEF) by MET, at early stages of reprogramming (45–47). These results suggest the possibility that MET is associated with stem-cell

state. Our results demonstrate that combined treatment of LIF with BIO could significantly contribute to the establishment of FGSC colonies and maintain their undifferentiated status by a mechanism of β -catenin activation and E-cadherin up-regulation (44).

The FGSCs differentiated into three germ layer types in a spontaneous differentiation conditions and induction system (10,11,30), although self-renewal potentiality of these cells needs to be further investigated (11). Gong *et al.* (30) have demonstrated that ES-like cell lines were established, which expressed markers specific for ESC and formed EBs and teratomas. To our knowledge, this is the first study to obtain Kunbai mouse FGSCs lines with ES-like cell characteristics (1,9,10,30) and the characteristics of our cells were similar to mouse ESCs and mouse FGSCs (10,11,30), suggesting conserved pathways in mammalian germ cell development. Several lines of evidence have suggested that extensive proliferative activity and pluripotency of neonatal and adult germline stem cells exist in mouse ovary and testis (10,48–50). Most of the reports consider that the germline stem cell in ovaries exists in OSE (11). However, our results indicated that cells positive for both Vasa and c-kit, Oct4 or SSEA4 were localized around the follicles, which were internally and exteriorly examined by immunofluorescence analysis (data not shown). In mammalian ovaries, FGSCs originate from undifferentiated PGCs (7).

In our study, some oocyte-like cells were formed in PFF-treated FGSCs; these cells were positive for germ-cell markers including meiosis markers Dazl and Stra8, germ-cell marker Vasa and oocyte markers Figla and Zps. This was consistent with previous reports and demonstrated that oocyte-like cells may be derived from germline stem cells and further types of stem cell (6,50–56). Lee *et al.* demonstrated that autologous embryonic stem cells can be established by preantral follicle culture and oocyte parthenogenesis (57). However, efficiency of derivation of oocyte-like cells from stem cells was low and oocytes from stem cells were not truly functional (51–56).

In conclusion, we have successfully established FGSCs from mouse neonatal and adult ovaries. These cells have characteristics similar to ESCs, and have the ability to differentiate into oocyte-like cells *in vitro* following PFF treatment. This provides us with an unlimited resource and *in vitro* model to study development of the mammalian oocyte.

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