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Stage-specific mice ovarian somatic cell is involved in primordial folliculogenesis

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1.ABSTRACT

Although recent studies have shown that female germ cells can be produced from stem-cell lines in mice, whether these germ cells can interact with ovarian somatic cells (OSCs) to form primordial follicles (PFs) is still unclear. Here, we found after the PF pool is established, Irx3 and FoxL2 which were extensively expressed in the OSCs of the perinatal mouse decreased. Additionally, during primordial folliculogenesis, down-regulation of FoxL2 prevents OSCs invading germ cell cysts. Therefore, we investigated whether OSCs at different stages of folliculogenesis were able to reconstitute PFs with fetal ovarian germ cells *in vitro*. In this system, neither OSCs at the post-PF pool-establishment stage nor OSCs from normal or doxorubicin-treated adult mice were able to reconstitute PFs. Mice eliminated most of pre-existing PFs failed to exhibit any spontaneous regeneration of PFs. Our results suggest that OSCs can only support physiological primordial folliculogenesis during the prenatal period. OSCs lose the ability to form PFs after the establishment of the PF pool may be another reason why the PF pool is non-renewable in most adult mammals.

2.INTRODUCTION

Mammalian primordial folliculogenesis is a precisely regulated process that includes two critical events: the oogonium enters meiosis and becomes an oocyte, and then pre-granulosa cells interacts with the oocyte to form a primordial follicle (PF). In mice, oogenesis starts 10.5 days post-coitum (dpc) when the primordial germ cells (PGCs) arrive at the genital ridge, then PGCs undergo mitosis and meiosis to form oocytes occurs, and the oocyte begins to arrest in the meiotic diplotene stage at approximate 17.5 dpc. Then, invasion of the ovarian germ cells (OGCs) cysts by ovarian somatic cells (OSCs) leads to formation of the PF, which consists of a diplotene quiescent oocyte surrounded by a single layer of flattened somatic cells (1). By 3 or 4 days post-parturition (dpp), the PF pool is established, and no more PFs are made (2).

During the process of primordial folliculogenesis, bidirectional communication between the oocyte and its surrounding somatic (pre-granulosa) cells is important. Follicular somatic cells are considered to be

active regulators of folliculogenesis, not mere passive participants (3), and the primary function of granulosa cells is to form the microenvironment or "niche" in which germ cells survive and mature (4-5). OSCs are of the same lineage as Sertoli cells in the testis, deriving from mesonephros and coelomic epithelium that disaggregates and enters the urogenital ridge (6). As birth approaches, OSCs invade the cysts of germ cells so that each oocyte is surrounded by a single layer of flattened pre-granulosa cells, forming the PF. Using an *in vitro* PF reconstitution system, we previously found that this synchronized OSC/OGC interaction is the basis of folliculogenesis and suggested that OSCs of an appropriate developmental stage are crucial for this process (7). However, little is known about the developmental and differentiation characteristics of fetal OSCs; *e.g*., the mechanism by which OSCs are directed to participate in the primordial folliculogenesis in a timely manner is poorly understood.

Recent studies have suggested that several transcription factors expressed in OSCs, such as Iroquois-related homeobox 3 (Irx3) (8) and Forkhead box L2 (FoxL2) (9), are important for sex determination and follicle growth. In vertebrates, Irx3 activity is important in spinal cord development (10); in mice, the Irx3 signal is restricted to OSCs and corresponds precisely to a special period of ovarian development that ends abruptly at birth (8). FoxL2 is expressed in OSCs throughout the entire process of folliculogenesis (9, 11). In mice homozygous for a FoxL2 mutant, PFs can be observed in the ovaries, but ovarian granulosa cell differentiation is blocked at the squamous-to-cuboidal transition, and no advanced follicles are present (12). The appearance of these Irx3 and FoxL2 in OSCs after the establishment of sexual dimorphism and their involvement in the entire process of primordial folliculogenesis suggest that pre-granulosa cells may be pre-determined at the initial stage of primordial folliculogenesis.

Whether PFs can regenerate in adult mammalian ovaries has been a topic of heated debate (13-14), and recent mouse and human studies have cast further doubt on this issue (15-20). However, some studies have revealed that embryonic stem cells (21) and some somatic stem cells (22) can differentiate into female germ cells *in vitro*, giving new hope to millions of infertile women. However, most of the oocyte-like cells produced in these latter studies were dysfunctional and unable to complete their development; it appears that oocytes cannot survive outside of the follicular environment, including OSCs and OGCs. Therefore, two highly vexing questions remain: Can the spontaneous process of primordial folliculogenesis be renewed after the establishment of the PF pool? And do OSCs in postnatal ovaries retain the ability to form PFs?

Here we used histological and molecular approaches to examine the process of primordial folliculogenesis in mice. As a result, we discovered there was a cluster of unique OSCs participated in this process. These OSCs gradually lost the ability to package oocytes to form follicles after the establishment of the PF pool. In addition, doxorubicin treatment of adult female mice did not confer upon their OSCs the ability to reconstitute follicles *in vitro*. Our results indicate that stage-specific OSCs are involved in primordial folliculogenesis in the mouse ovary.

3.MATERIALS AND METHODS

3.1. Mice

Adult KunMing white and C57BL/6 mice were purchased from the Laboratory Animal Centre of the Institute of Genetics at Beijing and housed in the Animal Care Facility of China Agricultural University with free access to water and food under a 12/12-h light/dark cycle. Adult female and male mice were mated at a 1:1 ratio during the late afternoon, and the female mice were checked for the presence of a vaginal plug the next morning. The noon of the day when a vaginal plug was found was defined as 0.5 dpc. Term labour usually occurred between 19.5 and 20.5 dpc.

3.2. Immunohistochemistry

Fetal and neonatal ovaries were collected at 15.5 and 17.5 dpc and at 1 and 7 dpp, respectively, and immunostained to detect FoxL2 and Irx3 expression as described previously (23). Ovarian tissue was fixed in cold 4% paraformaldehyde for 4 h, embedded in paraffin, and sectioned at 5 μm on a microtome. After dewaxing, rehydration, and high-temperature antigen retrieval with 0.01% sodium citrate buffer (pH 6.0), the sections were immunostained with rabbit anti-FOXL2 antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit anti-Irx3 antiserum (1:400; gift of J.S. Jorgensen). Subsequently, they were incubated with biotinylated goat anti-rabbit IgG (1:200; Zhongshan Company, Beijing, China) and ABC complex (Zhongshan Company). Peroxidase activity was detected using diaminobenzidine (Zhongshan Company) for 1 min. Finally, the sections were counterstained with hematoxylin. Non-immune rabbit serum was used as a control.

3.3 Plasmid DNA transfection of shRNA

The specific FoxL2 shRNA plasmid for RNAi-mediated knockdown of FoxL2 gene expression, a control shRNA plasmid containing a scrambled shRNA sequence that does not lead to the specific degradation of mRNA, and Plasmid Transfection Reagent were purchased from Sigma–Aldrich (St. Louis, MO, USA). Testing in a variety of cell lines according to the instructions provided by Sigma–Aldrich confirmed the high transfection efficiency of the FoxL2 shRNA plasmid. For fetal mouse ovaries, optimum efficiency was obtained when 1 μg of shRNA plasmid DNA was co-transfected with 1 μl of shRNA Plasmid Transfection Reagent for 10 h.

Fetal mouse ovaries (17.5 dpc) were transfected for 10 h with FoxL2 or control shRNA in 24-well plates. Then, 2 vol of Dulbecco's Modified Eagle: Nutrient Mixture F-12 Medium (DMEM-F12; Gibco BRL, Carlsbad, CA, USA) medium were added to each well, and the cells were cultured for 7 days. Finally, the ovaries were fixed, serially sectioned, stained, and counted. The total RNA was extracted for real-time PCR analysis, and protein

expression was analyzed by Western blotting of cell lysates.

3.4.Separation of OGCs and OSCs and reconstitution of PFs

PFs were reconstituted as described previously (7). In brief, fetal or postnatal ovaries in pre-chilled phosphate-buffered saline (PBS) (pH 7.2) were identified by their morphology under a stereomicroscope and isolated with a pair of fine needles. A single-cell suspension was achieved by digestion with trypsin (0.125%; Amresco, Solon, OH, USA) at room temperature, and the OGCs and OSCs were separated according to a previously described protocol (24). The ratio of OGCs and OSCs in 17.5-dpc ovaries was determined, and this ratio was maintained when the 17.5-dpc OGCs were mixed with OSCs at different developmental stages in 100 μl DMEM/F12 serum-free medium containing 3 mg/ml bovine serum albumin, 0.3 mg/ml glutamine, 5 mg/ml insulin, 5 mg/ml transferrin, 30 nM sodium selenite, and 0.25 mg/ml fetuin. The total cell number was kept constant for all groups.

To produce cell aggregates for histologic analysis, the cell suspension was cultured in agar (2%) at 37°C under a humidified 5%-CO_2 atmosphere (25). The developmental status was examined under an inverted microscope every 24 h and recorded. Cultures were terminated at 5 days, and the cell aggregates were collected for histologic and molecular analysis.

3.5. PF counts

Numbers of PFs were determined as previously described (13-14, 26). Briefly, the ovarian tissue was fixed, paraffin-embedded, and cut into serial 5-μm sections on a microtome. After dewaxing and rehydration, the sections were stained with hematoxylin. The number of PFs in every fifth section was determined by counting under a microscope, and the sum of these numbers was multiplied by five to yield the total number of PFs in each mouse ovary.

3.6. RT– and real-time PCR

Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, washed in 75% ethanol, and then dissolved in water. RT of the RNA was carried out using oligo (dT) with Moloney Murine Leukaemia Virus Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions.

In RT–PCR, the DNA was amplified using specific primers (see Table 1 for sequences) in a thermal cycler set for an initial incubation at 94°C for 5 min; 26–32 cycles of denaturation at 94°C for 30 s, annealing for 30 s (see Table 1 for annealing temperatures), and extension at 72°C for 30 s; and a final extension at 72°C for 5 min. The number of cycles was determined by pre-testing a range of cycles for which the product expression was linear. β-actin (NM_007393), a "housekeeping" gene, served as an internal standard. The PCR products were separated by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining. Relative intensities were quantified using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Inc., Bethesda, MD, USA).

To quantify the steady-state mRNA levels of selected genes, real-time PCR was carried out using a QuantiTect SYBR Green PCR Kit (Qiagen, Inc., Valencia, CA, USA) on an ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). β-Actin was used as a control for normalization. The PCR primers for FoxL2 were 5′-GAGGCTCACTCTGTCCGGC-3′ (forward) and 5′-TCTGCCAGCCCTTCTTGTTCT-3′ (reverse), for Irx3 were 5′-ACGAGGAGGGCAATGCTTATG-3′ (forward) and 5′-CATCTCCAGCTCGCGTTTG-3′ (reverse) and for β-actin were 5′-CACTATTGGCAACGAGCGGT-3′ (forward) and 5′-GGATGCCACAGGATTCCAT-3′ (reverse).

3.7.Western blot analysis

In brief, the total protein from 20 ovaries was extracted in MEM-R medium (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. The sample was centrifuged at 14,000 × *g* for 15 min, and the protein concentration in the supernatant fraction was determined using a BCA procedure (Cell Chip; BJ Biotechnology Co., Ltd, Beijing, China). After each sample was heated to 100°C for 10 min and cooled on ice for 20 min, aliquots containing 25 mg of protein were subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The separated proteins were electrophoretically transferred to a Protran nitrocellulose membrane (Schleicher & Schuell Bioscience GmbH, Dassel, Germany). The membrane was blocked for 1 h with 5% non-fat dry milk in TBST buffer (20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.6) and incubated overnight at 4°C with rabbit anti-FoxL2 (38 kDa) antibody (1:200 dilution). After six 5-min washes in TBST, the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit antibody (diluted 1:5,000 in TBST). The membrane was washed six times in TBST, and the proteins on the membrane were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA). Levels of β-tubulin were also determined.

3.8. Statistical analysis

The data are given as mean percentages ± SEM for at least three independent experiments for each experimental group. Immunostaining and PF counting analyses were performed using groups of 4–6 ovaries. Data for PF numbers were statistically analyzed using analysis of variance (ANOVA) using SigmaPlot 10.0 software (Systat Software, Inc., Chicago, Illinois, USA). When a significant F ratio was defined by ANOVA, groups were compared using the Holm–Sidak test. Differences were considered significant at $P < 0.05$.

4.RESULTS

4.1.Expression of transcription factors FoxL2 and Irx3 during primordial folliculogenesis in mice OSCs

To characterize the pattern of FoxL2 and Irx3 expression in OSCs during primordial folliculogenesis, we separated OSCs from ovaries at different developmental

Table 1. Primers For RT-PCR

Genes	Primer sequence	Cycle number	Product size (bp)
SPO11	F:5′CAGAAGCGATTCACCAAA 3′	30	689
	R:5′CAGAAGGGAGGAGACCAA 3′		
SCP3	F:5′AAGATGGTGCCTGGTGG 3′	30	664
	R:5′CTTGCTGCTGAGTTTCC 3′		
SCP1	F:5′GGTTCCGTTCCATGTCGT 3′	30	695
	R:5′CTGGTTTCTTCCCGCTCA 3′		
DMC1	F:5′CCAGGAGCAACTATGACC 3′	30	245
	R:5′CCCTAATAAGCAGCAAGA 3′		
ZP1	F:5′CTGAGGATTGCCACGGATAA 3′	30	324
	R:5′GGAGTCAAGGAGCATGAAGGT 3′		
ZP2	F:5′GCACAGGATGGGTTTATGGA 3′	30	619
	R:5′GGTGGAAAGTAGTGCGGTAG 3′		
ZP3	F:5′ACGAAAGATGCCCTGGTGTA 3′	30	452
	R:5′GGGACTTGAAATGCCGAAAA 3′		
Figa	F:5′TACTCCACCACGGATGAC 3′	30	225
	R:5′CTCTGAGACCTTCGCTTC 3′		
Fshr	F:5′ACCACAAGCCAATACAAACTC 3′	30	787
	R:5′GCACCTCATAACAGCCAAAC 3′		
Cx43	F:5′AGGGAAGTACCCAACAGC 3′	30	247
	R:5′AAATGAAGAGCACCGACA 3′		
β-actin	F:5′TTGTTACCAACTGGGACG 3′	28	667
	R:5′GGCATAGAGGTCTTTACGG 3′		

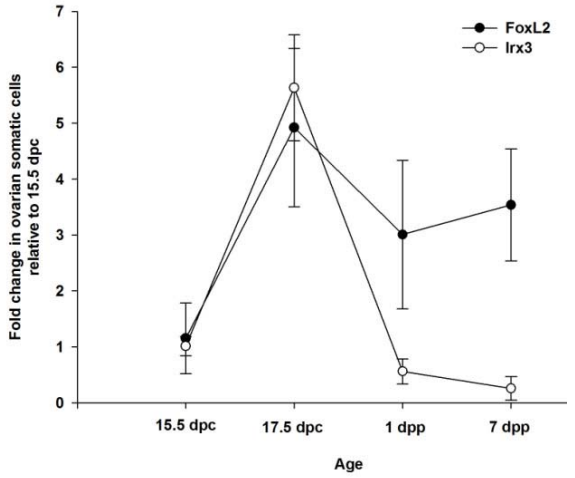

Figure 1. FoxL2 and Irx3 expressed in ovarian somatic cells during primordial folliculogenesis. A time course analysis of FoxL2 and Irx3 expression in mice ovarian somatic cells (OSCs) was performed using real-time PCR. The expression values of FoxL2 and Irx3 are reported relative to the value at respective 15.5 days post-coitus (dpc) as this time point was set to a value of one. The expression of FoxL2 and Irx3 peaked at 17.5 dpc, and rapidly decreased after birth [1 day post-parturition (dpp)]. After the establishment of primordial follicle (PF) pool (7dpp), the expression level of FoxL2 is comparable to that of 1 dpp, however, Irx3 is almost undetectable. Error bars represent the standard error of at least three different experiments.

stage and performed real-time PCR analysis. The results of FoxL2 and Irx3 from the PCR analysis were compared to respective 15.5 dpc as this time point was set to a value of one. Results indicated that both FoxL2 and Irx3 message in OSCs was low initially and then increased to peak levels at 17.5 dpc. After birth (1 dpp), the expression level of these transcription factors rapidly diminished. By postnatal day 7 (7 dpp), FoxL2 was similar to that present at 1dpp, but Irx3 was hardly detected (Figure 1). We further investigated the status of OSCs in the process of primordial folliculogenesis by using immunostaining to locate the transcription factors FoxL2 and Irx3. At 15.5 (Figure 2, A-A') and 17.5 dpc (Figure 2, B-B'), respectively, FoxL2 and Irx3 were expressed at high levels in most of the somatic cell streams surrounding germ cell cysts in the ovarian cortex, where the cluster of specific OSCs was probably preparing to incorporate with germ cells to form PFs. At 1 dpp (Figure 2, C-C'), when a large number of PFs began to form, FoxL2 and Irx3 were still expressed primarily in the nuclear of OSCs around the germ cell cysts; the number of FoxL2- and Irx3-positive somatic cells in the ovarian cortex was considerably lower than that in the fetal ovarian cortex, and interestingly, some FoxL2 and Irx3 expression appeared in the cytoplasm of oocytes. At 7 dpp (Figure 2, D-D'), when the PF pool was complete, Irx3 expression was restricted to the PFs, whereas FoxL2 was expressed in almost all granulosa cells of the growing follicles. These factors were almost undetectable in other cells of the ovarian cortex.

4.2.Down-regulation of FoxL2 expression moderately inhibits primordial folliculogenesis

To verify that the perinatal OSCs yielding positive immunostaining were participants in PF formation, we knocked down FoxL2 gene expression in the fetal ovary using a short hairpin RNA (shRNA) to generate small interfering RNAs (siRNAs). Fetal ovaries (17.5 dpc) were transfected *in vitro* with shRNA for 10 h and then cultured in basic medium for 7 days to reach an age which was comparable with that of 5-dpp ovaries *in vivo*. Real-time PCR and Western blot analysis showed an obvious decrease in FoxL2 gene and protein expression resulting from the plasmid-mediated delivery of shRNA (Figure 3A, B)., In ovaries treated with FoxL2-shRNA, the OSCs did not effectively invade germ cell nests to form PFs (shRNA, Figure 3C; control, Figure 3D). By counting the number of germ cells, we found that the total number of germ cells did not change in the FoxL2-deficient ovaries (control, 2298 ± 413; shRNA, 2307 ± 491; Figure 3E); however, the number of uncovered germ cells in cysts significantly increased (control, 110 ± 47; shRNA, 566 ± 99; P □ 0.05; Figure 3E).

4.3.Stage-specific OSCs are crucial for PF reconstitution *in vitro*

To further investigate the variation of OSCs during primordial folliculogenesis, we used an *in vitro* PF reconstitution culture model to study the capability of somatic cells at different developmental stages to package oocytes into PFs. First, we separated OGCs and OSCs from each other and then used reverse transcription (RT)–PCR to confirm their purity (data not show). In the absence of OGCs, 1-dpp OSCs did not form aggregates after 5 days in culture (Figure 4A). When the 1-dpp OSCs were mixed with 17.5-dpc OGCs, they interacted to reconstitute PFs after 5 days in culture (Figure 4B–D), as did the control cells (17.5-dpc OSCs+OGCs) (Figure 4E). When 1-dpp OSCs and 17.5-dpc OGCs were co-cultured *in vitro* for 72 h, multiple cell aggregates formed. After 5 days in culture, typical PFs consisting of an oocyte arrested in germinal vesicle stage surrounded by a single layer of flattened somatic cells were observed (Figure 4F).

In contrast to the 1-dpp OSCs, co-culture of 7-dpp OSCs with 17.5-dpc OGCs (which coincided with the

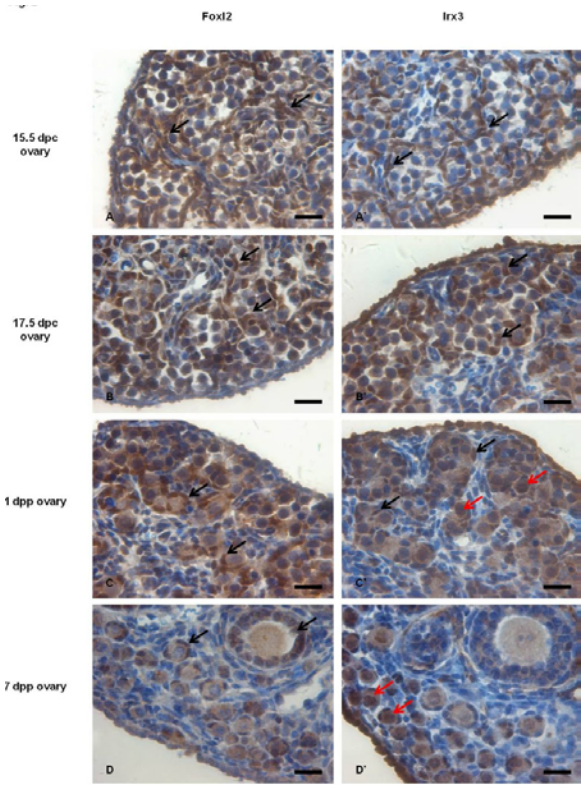

Figure 2. Immunohistochemical analysis of FoxL2 and Irx3 expression in paraffin sections of prenatal and neonatal mouse ovaries. At the early stage of folliculogenesis, both FoxL2 and Irx3 are expressed in OSCs at 15.5 (A, A') and 17.5 (B, B') dpc, particularly in those surrounding the germ cell cysts (black arrow). When lots of PFs form at 1 dpp, FoxL2 is expressed in ovarian germ cells (OGCs; C, black arrow), and Irx3 is expressed in both OGCs (C', red arrow) and OSCs (C', black arrow). After PF formation is complete (7 dpp), FoxL2 is expressed in granulosa cells of growth follicles (D, black arrow), and Irx3 is expressed in PFs (D', red arrow). Bar, 20 μm.

stage of primordial follicle pool established with 17.5 dpc OGCs) for 5 days did not yield any reconstituted PFs. The 7-dpp OSCs adhered to the bottom of culture dish, and after 5 days of culture, the OGCs were floating above the somatic cells. No aggregates of any kind were formed (Figure 4G).

To confirm the functional integrity of the *in vitro*-reconstituted follicles, we analyzed their expression profiles for several marker genes known to participate in oocyte and follicle growth, including zona pellucid (Zp1-3) (21), factor in the germline α (FIGα) (7), follicle-stimulating hormone receptor (Fshr) (27), and connexin 43 (Cx43) (28). All of these marker genes were expressed at comparable levels in the PFs from the co-culture of 1-dpp OSCs and 17.5-dpc OGCs, from the co-culture of 17.5-dpc OSCs and 17.5-dpc OGCs, and from normal 3-dpp ovaries. However, these genes were hardly detected in the co-cultured mixture of 7-dpp OSCs and 17.5-dpc OGCs (Figure 4H). In general, the RT–PCR results corresponded with the phenomena observed in culture.

4.4.Adult OSCs cannot form PFs when co-cultured with 17.5-dpc OGCs *in vitro*

To eliminate pre-existing oocytes, adult mice were treated with intraperitoneal injections of doxorubicin (DXR) (Sigma, 5 mg/kg) (14, 29). Then, OSCs from these mice, as well as from normal adult and vehicle-treated adult mice, were co-cultured with 17.5-dpc OGCs in our *in vitro* PF reconstitution model for 5 days. A few cell aggregates formed in each group, but none of the oocytes in these groups were properly packaged by OSCs to form new PFs (Figure 5).

4.5.No spontaneous primordial folliculogenesis occurs in adult mice

To determine whether adult ovaries can generate new PFs *in vivo*, we treated adult female C57BL/6 mice with doxorubicin (as described above) to eliminate the pre-existing PFs (29) and then evaluated the number of newly formed PFs in the ovaries after various lengths of time. A rapid and extensive loss of PFs occurred within 12 h of DXR treatment, as described previously (14), and the number of PFs did not change significantly during the next 5 days. After 2 months, none of the DXR-treated ovaries exhibited PF restoration compared with the vehicle-treated ovaries (Figure 6A).

We next isolated total RNA from DXR-treated adult ovaries, vehicle-treated and normal fetal, and used RT–PCR to analyze the expression of genes required for normal folliculogenesis (SPO11, SCP1, SPC3, and DMC1) (20). These transcripts were abundant in the fetal ovaries but were scarce or absent in ovaries from doxorubicin-treated and normal adults (Figure 6B). A similar experiment performed in female KunMing white mice yielded similar results, although the absolute PF numbers changed slightly (data not shown).

5.DISCUSSION

In the present study, we sought to determine whether stage-specific OSCs are responsible for PF formation and whether differentiated somatic cells retain the ability to cooperate with germ cells to form follicles. We found that OSCs highly expressing both Irx3 and FoxL2 at the early stage of primordial folliculogenesis and remained present throughout the entire period of PF formation, they appeared in most of OSCs around the germ cell cysts of the ovarian cortex. In postnatal mice ovaries (when the PF pool was complete), however, the OSCs which expressed Irx3 and FoxL2 was just detected in follicular somatic cells. We speculate that the Irx3/FoxL2-positive OSCs in the fetal ovarian cortex participate in primordial folliculogenesis and differentiate into granulosa cells. The ovarian cortex is an important source of pre-granulosa cells (2). Since the expression of Irx3 and FoxL2 faded in the somatic cells of the ovarian cortex after the establishment of the PF pool, the status change of the ovarian cortex suggests that the OSCs might involve in spontaneous primordial folliculogenesis are stage-specific.

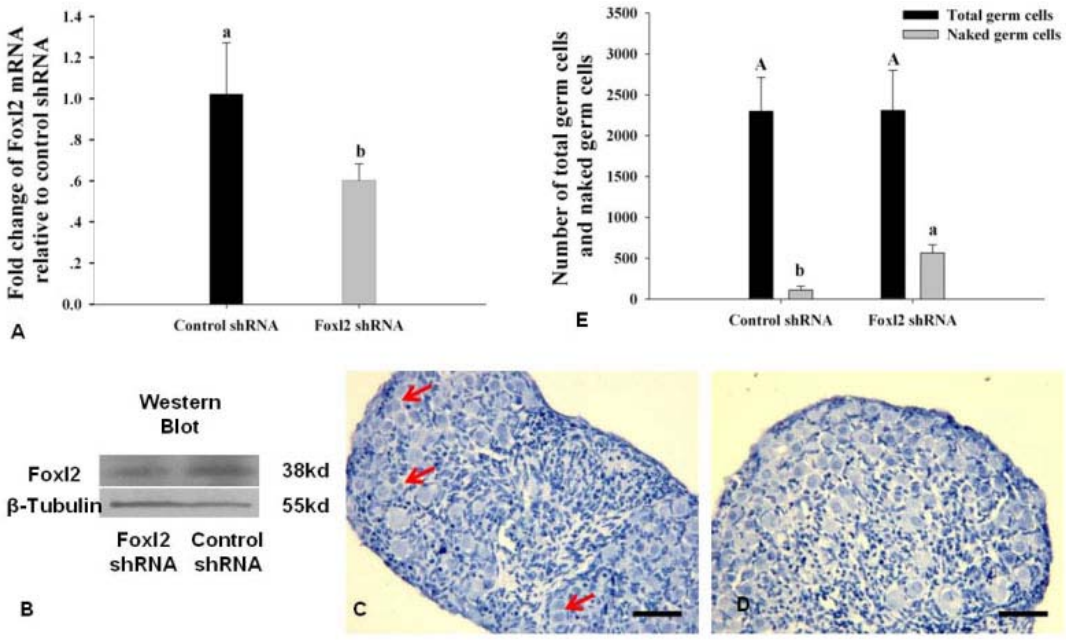

Figure 3. Down-regulation of FoxL2 expression reduces PF formation. After transfection with a control or FoxL2 shRNA plasmid for 10 h, 17.5-dpc ovaries were cultured for 7 days and then characterized. The mRNA expression (A) and the protein expression (B) of FoxL2 and control shRNA-transfected ovaries. Morphological analysis of FoxL2 (C) and control (D) shRNA-transfected ovaries. Red arrows noted the naked germ cells. Total numbers of PFs and naked germ cells (E). Real-time PCR and Western blotting analyses were performed at least three times with similar results. Numerical data shown are means from five ovaries, and statistically significant differences ($P < 0.05$) are denoted by different superscripts. Bar, 100 μm.

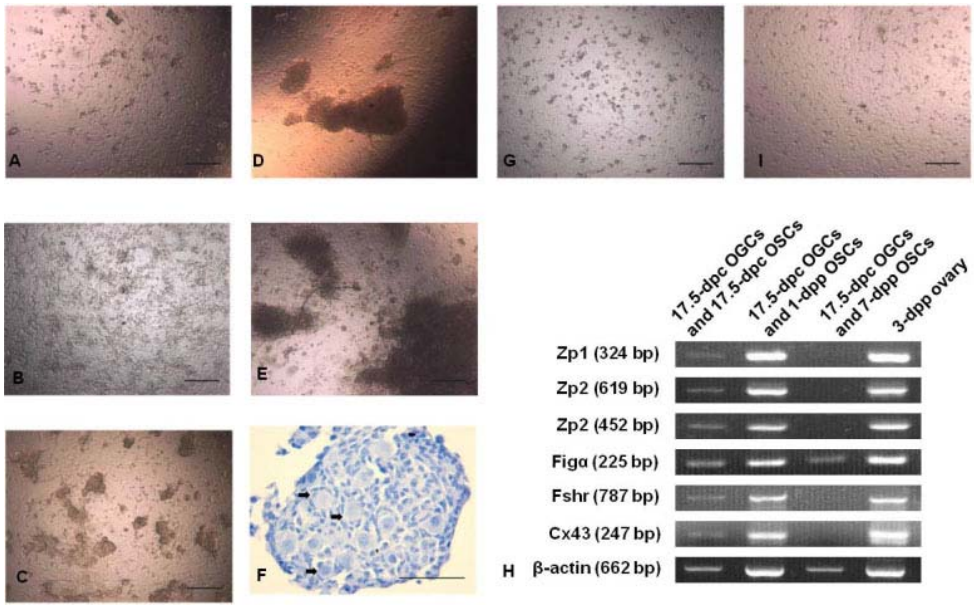

Figure 4. Reciprocal PF reconstitution from OSCs of various ages in culture.Only 1-dpp OSCs cultured for 5 days (A). Gross morphology of aggregates and reconstituted PFs formed after 1-dpp OSCs and 17.5-dpc OGCs were mixed and cultured for 24 h (B), 72 h (C), and 5 d (D). The control was that 17.5-dpc OSCs and 17.5-dpc OGCs were co-cultured for 5 days (E). Hematoxylin-stained cell aggregates formed after 5 d in culture (D) present a typical PF structure in which a central oocyte is arrested in the germinal vesicle stage and is surrounded by a single layer of flattened granulosa cells (F, black arrowhead). The status of 7-dpp OSCs and 17.5-dpc OGSs (G), 7-dpp OSCs only, underwent 5 d *in vitro* culture. Reverse transcription (RT)–PCR analysis of ZP1-3, Figα, Fshr, and Cx43 mRNA expression in different experimental groups after 5 days of culture and in 3-dpp neonatal mouse ovaries (H). Bar, 100 μm.

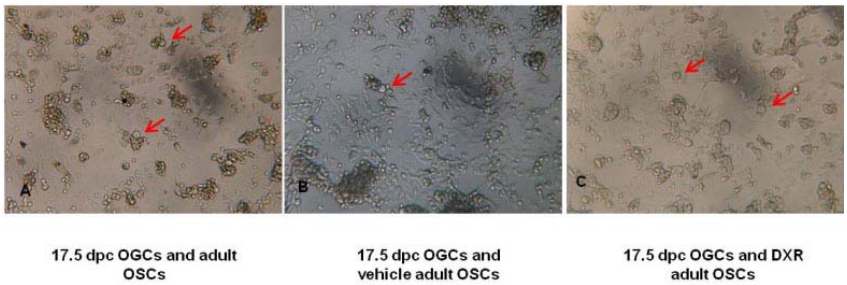

Figure 5. OSCs in ovaries from doxorubicin-treated adults fail to form PFs. OSCs from normal (A), vehicle-treated (B), and doxorubicin (DXR)-treated (C) adult mice were cultured with 17.5-dpc OGCs. Accurate oocyte packaging by the adult OSCs (red arrows) was not observed for any of the experimental groups. Bar, 100 μm.

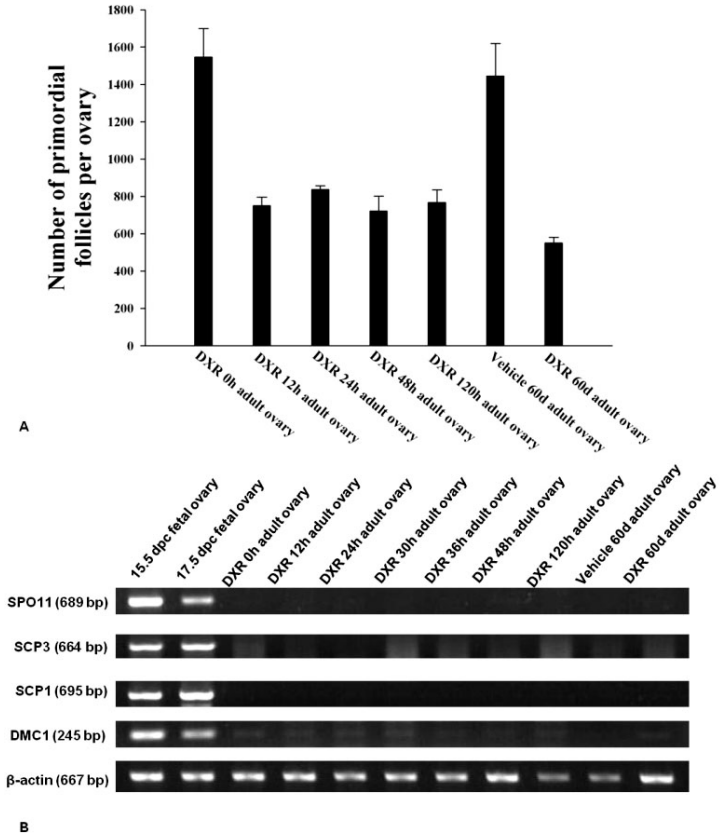

Figure 6. PFs in ovaries from doxorubicin-treated adult mice. Adult female C57BL/6 mice were injected with doxorubicin (5 mg/kg), and their ovaries were removed and analyzed after the indicated lengths of time. Numbers of PFs in the ovaries (A). Values shown represent means ± SEM for four mice per treatment group. RT–PCR analysis of SPO11, SCP3, SCP1, and DMC1 mRNA expression in the ovaries and in normal fetal mouse ovaries (B).

To test this idea, we investigated whether the OSCs identified as expressing these transcription factors were participants in PF formation. Previous mouse studies have suggested that FoxL2 mutations cause abnormal sex determination, premature ovarian failure, and granulosa cell differentiation blockage, and PFs are still observed in FoxL2-mutant homozygotes (9, 30), but whether the process of PFs formation was influenced is still unclear. In the meanwhile, ovaries of Fused Toes mutant mice, which lack several genes, including Irx3, show abnormal primordial folliculogenesis and maintain cyst-like structures; furthermore, FoxL2 expression is not detectable in the granulosa cells from these ovaries (31). Therefore, we focused on the function of FoxL2 in the PF formation process. Down-regulation of OSC FoxL2 expression by RNAi reduced the efficiency of PF formation, indicating that the FoxL2 expressed in a specific cluster of fetal OSCs is involved in primordial folliculogenesis. FoxL2 might be useful as a pre-granulosa marker in future work.

The observed changes in FoxL2 and Irx3 expression before and after the PF pool establishment led us to speculate that the OSCs involved in spontaneous primordial folliculogenesis might be stage-specific. Therefore, using a mouse PF reconstitution culture model, we studied the

capability of somatic cells from ovaries at different developmental stages to form PFs when mixed with 17.5-dpc fetal mouse OGCs. When 1-dpp OSCs were co-cultured with 17.5-dpc OGCs, PFs were observed after 5 days in culture. These reconstituted PFs had no detectable morphological or molecular differences from PFs that were reconstituted from co-culture of 17.5-dpc OGCs and 17.5-dpc OSCs. However, when 7-dpp OSCs were co-cultured with 17.5-dpc OGCs, no PFs were reconstituted.

In the process of primordial folliculogenesis, OGCs and OSCs must be developmentally synchronous (7) so that signal transfer from the somatic cells to the germ cells can start the PF packaging process (32). We have observed that the number of OSCs is precisely controlled during primordial folliculogenesis and that a decrease in this number can cause primordial folliculogenesis to fail *in vitro* (unpublished results). Thus, the completion of the PF pool might mark the temporal border between OSCs that can package germ cells to form PFs and those that cannot.

Recent studies have suggested that PFs can renew in adult mouse ovaries (13-14) and that the injection of germ cells into adult mice can initiate PF reformation (33). Furthermore, some studies on OGCs have suggested that oogenesis may happen in ovaries of chemo-treated adult humans and mice (33-35). Thus far, oogenesis has not been replicated in ovaries of normal adult mammals, hinting that the elimination of germ cells by chemo-treatment might disrupt the balance between germ cells and somatic cells in the ovary, resulting in PF regeneration. However, in our *in vitro* mouse PF reconstitution model, we found that OSCs obtained from DXR-treated and normal adult mice did not retain the capability to reform PFs when mixed with germ cells from 17.5-dpc ovaries. In physiological conditions, the process of primordial folliculogenesis is about 10 days in mice(1). When almost half of pre-existing PFs were eliminated, PFs did not regain their previous numbers in adult mice ovaries. Some genes essential for folliculogenesis were abundantly expressed in the fetal ovaries, but they were only minimally expressed, if at all, in ovaries of normal and DXR-treated adults. Although folliculogenesis can be induced in ovaries of chemically treated adult mice by ovarian (33) or hematopoietic (14) stem cells, our results indicate that spontaneous folliculogenesis, such as the process in perinatal ovaries, does not occur in ovaries of normal or chemically treated adult mice, one of the reasons may be the OSCs in these ovaries have proliferated and differentiated to a state in which this function is disabled.

In summary, using cellular and molecular approaches, we have demonstrated that stage-specific OSCs are involved in primordial folliculogenesis in the mouse ovary. During PF pool establishment, OSCs gradually lose their ability to reform PFs. Furthermore, we have shown that spontaneous primordial folliculogenesis occurs rarely, if at all, in the ovaries of adult mice, due to their lack of functional OSCs.

6.ACKNOWLEDGEMENTS

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Abbreviations: OSCs: ovarian somatic cells; PFs: primordial follicles; dpc: days post-coitum; PGCs: primordial germ cells; OGCs: ovarian germ cells; dpp: days post-parturition; Irx3: Iroquois-related homeobox; FoxL2: Forkhead box L2; shRNA: short hairpin RNA; siRNAs: small interfering RNAs; ZP: zona pellucid; FIGα: factor in the germline α; Fshr: follicle-stimulating hormone receptor; Cx43: connexin 43; DXR: doxorubicin; RT-PCR: Reversed Transcript PCR.

Key words: Ovarian Somatic Cell, Pre-Granulosa Cell, Folliculogenesis, Primordial Follicle, FoxL2.

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