Isolation and characterization of string-forming female germline stem cells from ovaries of neonatal mice

Running title: Stringing stem cells of mouse ovary

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ABSTRACT

Germline stem cells are essential in the generation of both male and female gametes. In mammals, the male testis produces sperm throughout the entire lifetime, facilitated by testicular germline stem cells. Oocyte renewal ceases in postnatal or adult life in mammalian females, suggesting that germline stem cells are absent from the mammalian ovary. However, studies in mice, rats, and humans have recently provided evidence for ovarian female germline stem cells (FGSCs). A better understanding of the role of FGSCs in ovaries could help improve fertility treatments. Here, we developed a rapid and efficient method for isolating FGSCs from ovaries of neonatal mice. Notably, our FGSC isolation method could efficiently isolate on average 15 cell “strings” per ovary from mice at 1–3 days postpartum (dpp). FGSCs isolated from neonatal mice displayed the string-forming cell configuration at mitosis, i.e. a “stringing” FGSC (sFGSC) phenotype, and a disperse phenotype in postnatal mice. We also found that sFGSCs undergo vigorous mitosis especially at 1–3 dpp. After cell division, the sFGSC membranes tended to be connected to form sFGSCs. Moreover, F-actin filaments exhibited a cell-cortex distribution in sFGSCs, and E-cadherin converged in cell–cell connection regions, resulting in the string-forming morphology. Our new method provides a platform for isolating FGSCs from the neonatal ovary, and our findings indicate that FGCS exhibit string-forming features in neonatal mice. The sFGSCs represent a valuable resource for analysis of ovary function and an in vitro model for future clinical use to address ovarian dysfunction.

Keywords: Stem cells, germline, ovary, mammals

INTRODUCTION

In mammals, the male testis can produce sperm throughout entire lifetime. Male germline stem cells, spermatogonial stem cells (SSCs), provide a renewal pool to produce sperms (1,2). However, it is traditionally believed that oocytes cannot be renewed in postnatal or adult life in mammalian females (3-7), which probably implies no germ stem cells in mammalian ovary. Recent data in mice, rats and humans provide evidences of female germline stem cells (FGSC) in ovary (8-14). Millions of women suffer from endocrine dysfunction
and aged fertility-failure (15), and young women are also rendered sterile by some diseases causing oocyte loss, such as polycystic ovary syndrome (16,17). Understanding of the FGSCs brings light to treatments of these diseases. In addition, it will also help in extending of reproductive age of females.

Primordial follicle renewal was observed in the postnatal mouse ovary, suggesting existence of germ stem cells in ovary (8). FGSCs were successfully isolated from neonatal and adult mouse ovaries by DDX4 antibody-based magnetic-activated cell sorting (MACS) (11,13). The cells can proliferate in vitro for months, and viable offsprings were obtained through transplantation of GFP-expressing FGSCs in ovaries (11). Human FGSCs were also isolated from reproductive-age women through DDX4 antibody-based fluorescence-activated cell sorting (FACS) (12). GFP-expressing human FGSCs were injected into adult ovarian cortical tissue biopsies of human, and the ovarian tissue grafts were then xenografted into NOD-SCID female mice. GFP-positive oocytes can be detected in the tissue grafts, indicating their differentiation into oocytes (12). In addition to mice and human, FGSCs from neonatal rats of 5-day-old were also isolated by MACS and characterized (10). The rat FGSCs exert similar features of mice cells in both proliferation and differentiation. In addition, the neonatal FGSCs of both mice and rats were successfully used to generate transgenic or gene knockdown animals (10,11,18).

Stably proliferating FGSCs can convert into female embryonic stem-like cells (fESLCs) using embryonic stem cell medium, which exhibited similar gene expression and differentiation potential to embryonic stem cells (19). Comparison of gene expression profiles among FGSC, primordial germ cells (PGCs) and spermatogonial stem cells (SSCs) revealed a similar pattern, but with distinct gene sets especially in stem cell markers (20,21). Lineage-specific enhancers with germline stem cell features were also detected through comparison between ESCs and FGSCs. Their DNA methylation determined FGSC unipotency by suppressing the somatic program (9). Although some of FGSCs or SSCs revealed a stringing growth pattern (21), the characterization of the stringing growth or sFGSCs remains to be further studied.
Antibody against C-terminal of Mvh (known as Ddx4 in human) was first used for mice FGSC isolation through MACS (11). In the subsequent studies, antibody against Fragilis (known as Ifitm3, a membrane protein), was used to isolate FGSCs from mice and rats through MACS (10,13). Coupled with Mvh antibody, the FACS method was used for FGSC isolation from humans and mice (12). FACS method was also used to isolate Oct4+ ovarian germline stem from Oct4-GFP transgenic mice (14). These isolation methods employed slightly different features of the cells, thus FGSCs isolated revealed distinct characters.

Differential adherence selection was successfully used to enrich SSCs from postnatal testis (22-24). As looser adherence of male germline stem cells compared to other somatic cells during culture (23,24), we adopted the strategy of differential adherence selection to enrich female germ stem cells from neonatal ovary. After 2-step digestions by collagenase IV and trypsin, dispersed ovary cells were selected by multiple rounds of differential adherence selections. Final detached cells were cultured for 3-5 times of passaging, and the FGSCs were further characterized. We found the stringing FGSCs (sFGSCs) from primary to over 8 generations of culture. In addition, we tested mitotic kinetics and cell strings forming abilities of cultured sFGSCs. Membrane connection through E-cadherin and F-actin cytoskeleton of cell cortex in sFGSCs were also analyzed, which revealed tight connections between cells in the sFGSCs. Our work demonstrated that sFGSCs exist in neonatal ovary, especially in 1-3 dpp (days post partus) of mice. Besides providing an alternative strategy for sFGSC isolation, which is much easier and costs less than the FACS and MACS, the sFGSCs are valuable cell sources for further analysis of ovary functions and in vitro models for future clinic use of treating ovarian dysfunction.

RESULTS

A methodological system of stringing FGSC isolation from neonatal ovaries through differential adherence selection

In previous studies of ovary germline stem cells in mice and human, antibodies against Mvh and Fragilis were used to isolate the stem cells through MACS (11,13) and
FACS (12). We adopted differential adherence selection to enrich germline stem cells from postnatal ovaries without any antibody, and to select mitotic cells from the enriched cells through multiple passaging. To determine enrichment efficiency, primary cells from ovaries of 1, 3, 6 and 14 dpp mice were cultured in Alpha-MEM containing EGF, bFGF, LIF, GDNF and N2 supplements after two-step enzymatic digestions using type IV collagenase and trypsin. The cells were transferred to gelatin-coated wells for adherence selection as shown in schematic diagram (Fig. 1A). After multiple rounds of differential adherence selection, detached ovary cells were obviously enriched (Fig. 1B). Over 26,000 detached cells with a diameter of 10-20 µm can obtained from thirty ovaries of 1 dpp mice, and the cell numbers declined significantly with age (Fig. 1C), indicating that 1 and 3 dpp ovaries were optimal for the cell isolation. Interestingly, we observed cell strings when cultured on STO feeder cells in first week of the culture (Fig. 1D). Diameter of the cells in strings was between 10-20 µm, which are consistent with those of previously reported FGSCs (11). The cell strings consisted of two cells on the first day of culture, and four and eight cells in strings can also observed in subsequent culture.

To detect marker gene expressions of germline and pluripotency in attached cells, final detached primary cells and these detached primary cells cultured for 1 week and passaged once, RT-PCR showed that the well-attached cells were somatic cells with scarce expression of germline genes, final detached ovary cells expressed germline markers (Mvh and Fragilis) and telomerase, while the passaged cells did not express differentiation marker c-kit but express germline markers, telomerase and pluripotency factors (Fig. 1E), suggested a feature of the FGSCs.

To further investigate morphology of FGSC strings in continuous passaging, the cell strings with 2, 4, >4 cells cultured on STO monolayer were still detected in following passaging at least 2 months (Fig. 2A-C). In order to observe cell connection of strings, especially the connection region of adjacent cells, scanning electron microscopy (SEM) was also performed. SEM showed that the cells in strings had an obvious connection and 1/4~1/3 of membrane region were connected together (Fig. 2D).

To test whether numbers of cell strings are
associated with age, the FGSCs from 1, 3, 6 and 14 dpp mice were cultured for 3 days and the cell strings were counted. As difficulty to determine the number of freshly isolated germline stem cells, we counted the strings as mitotic units to predict isolation efficiency of the FGSCs. Mice of 1 and 3 dpp had an average of 14 and 15 strings per ovary respectively, while the numbers per ovary of 6 and 14 dpp declined significantly to less than 6 strings (Fig. 2E). These results showed that neonatal mouse had more abundant stringing FGSCs than old one. Compared to disperse FGSCs in later stages of postnatal mice observed in our and other studies (11), thus we call the stringing FGSCs from neonatal mice as sFGSCs.

Characterization of the sFGSCs

We further characterized the expressions of germline marker genes Mvh, c-kit, Dazl, Dppa3, Prdm1 and Fragilis, and pluripotency marker genes Oct4, Sox2, Nanog, Tert in the sFGSCs cultured for 2 months. RT-PCR showed that the stringing cells expressed Mvh, Dazl, Dppa3, Prdm1, Fragilis, Oct4 and Tert, while oocyte differentiation marker c-kit, and pluripotent markers Sox2 and Nanog expression were not detected (Fig. 3A), which were consistent with those of the FGSCs through MACS isolation (11). Immunofluorescence analysis of the sFGSCs demonstrated that the expression of Mvh was in the cytoplasm (Fig. 3B), while Oct4 protein was restricted in the nuclei of the sFGSCs (Fig. 3C), which were consistent with previous reports (11). Chromosome analysis showed that the sFGSCs had a normal female karyotype (40, XX) after 2 months’ in vitro culture (Fig. 3D). These results suggested that these sFGSCs had typical characteristics of female germline stem cells.

Division kinetics of neonatal sFGSCs

To determine the mitotic ability of the sFGSCs, we observed cell division of a single FGSC in situ under inverted microscopy. Continuous images of a single FGSC from 1-cell to 8-cell string were taken at different time points (0, 7 h 38 m, 24 h 5 m, 47 h 54 m and 58 h 13 m) (Fig. 4A), indicated that 3 times of cell divisions occurred during the culture.

To determine doubling time of the sFGSCs of different age, cells in strings from 1, 3, 6 and 14 dpp ovaries were counted every 12 hrs during culture of 60 hrs. Proliferation curves showed that sFGSCs from both 1 and
3 dpp ovaries had a higher proliferation rate, in comparison with 6 and 14 dpp ovaries (Fig. 4B). Accordingly, we can determine doubling time of the sFGSCs, which was approximately 22 hrs for sFGSCs from 1 and 3 dpp ovaries.

The cell division was further confirmed by observation of dividing cells using immunofluorescence analysis of microtubule marker Tubb3 (Tubulin, beta 3 class III). The microtubules with typical Tubb3 signals exerted allocation type of early telophase phase in the FGSC with a diameter of ~10 µm, and the cell division can be detected in the cultures of both 1 and 5 days (Figure 4C). Moreover, the division ability of the sFGSCs was significantly inhibited by mitomycin C (10 μg /ml) (Fig. 4D), a mitotic antagonistic agent which inhibits DNA synthesis when cell division.

In addition, N2 supplement (human transferrin, insulin, progesterone and putrescine) can significantly promote proliferation of the sFGSCs (Fig. 4E). These results suggested that the sFGSCs can undergo vigorous mitosis especially at the neonatal mice of 1~3 dpp.

To investigate connection features of sFGSC membrane, we used a fluorescent lipophilic cationic indocarbocyanine dye, DiIC$_{18}$(3), which is highly fluorescent and quite photostable when incorporated into membrane (25). Membrane probing revealed obvious membrane connections of adjacent cells in sFGSCs, and central cells in strings even had a square-like shape, indicating a force to form membrane connection (Fig. 5A). In addition, local separation of membranes between cells in some 2-cell strings can observed in sFGSCs during cell culture (Fig. 5B). These results indicated that sFGSCs membranes trend to connect together after cell divisions.

**Cytoskeleton converges in cell cortex adjacent to plasma membrane in sFGSCs**

To explore morphological dynamics of plasma membrane of sFGSCs, we first used microtubule marker Tubb3 to immunostain the microtubule networks. Immunofluorescence of microtubule marker Tubb3 showed that microtubule network was mainly distributed in the cell cortex of sFGSCs (Fig. 6A). Further Phalloidin staining of F-Actin of sFGSCs was conducted to detect F-Actin filaments distribution in sFGSCs. After staining,
obvious signals can be observed in the cell cortex mostly adjacent to plasma membrane of sFGSCs, in comparison with a typical cytoplasmic distribution of F-Actin filament in control STO cells (Fig. 6B). These data suggested that cell-cortex convergence of cytoskeleton filaments could facilitate morphological structure of the stringing FGSCs.

**E-cadherin mediates cell-cell contact in sFGSCs**

Previous studies showed that F-actin interacted with E-cadherin through α-catenin and β-catenin, and E-cadherin is a trans-membrane protein and mainly expressed in epithelial cells, facilitating cell–cell contact (26). To investigate whether E-cadherin play a role in maintaining cell-cell membrane contact of sFGSCs, we used immunofluorescence to detect E-cadherin distribution in single cell and strings of 2-cells and 4-cells. E-cadherin signals were converged in cell-cell connection regions of sFGSCs, while the signals were weak in the membrane of single FGSC (Fig. 7A). A similar E-cadherin location pattern was also observed in cell-cell connection regions of FGSCs purified by MACS method (Fig. 7A). To test whether the string formation was affected when E-cadherin expression was knockdown by shRNA (a confirmed target sequence of E-cadherin from previous study (27)), we counted numbers of FGSC strings at day 4 after shRNA lentivirus infection. E-cadherin knockdown significantly inhibited the strings numbers of FGSCs, compared to shRNA control and mock treated cells (Fig. 7B; Fig. S1). Thus, E-cadherin probably participates in formation of adherens junction between the stem cells, leading to the stringing FGSCs (Fig. 7C). The results demonstrated that E-cadherin acts as mediator together with F-actin for cell-cell connection and facilitate morphological structure of the stringing FGSCs.

**DISCUSSION**

Physiological functions of the ovary decline as aging-dependent decrease of follicle numbers and disruption of endocrine in women. Discovery of ovarian germline stem cells provides a deep understanding of follicle renewal and endocrine functions of adult ovary during the reproductive lifespan. It has also brought the prospects of new strategies to promote ovarian regeneration, delay menopause and cure ovarian
dysfunction. For example, human OSCs have been clinically used in ovarian therapies (28-30). However, to better understand ovarian physiology and develop efficient strategies for treatment of ovarian dysfunction through FGSCs, isolation, characterization and differentiation mechanisms of FGSCs, need further to be explored. In the present study, we have developed a rapid and efficient strategy for sFGSC isolation from ovary of neonatal mice through a strategy of differential adherence selection. Strikingly, we found that the isolated sFGSCs revealed a string of cell configuration as mitosis, i.e. stringing FGSCs, compared to disperse FGSCs in later stages of postnatal mice observed in our and other studies (11). The system can obtain a high efficient isolation with an average of 14-15 cell strings per ovary from mice of 1-3 dpp. Furthermore, these stringing FGSCs can undergo vigorous mitosis especially at the age of 1-3 dpp. After cell division, the membrane of sFGSCs trends to be connected together to form FGSC strings. Cell-cortex convergence of cytoskeleton filaments could facilitate morphological structure of the stringing FGSCs. Thus, the system provides a new paradigm of sFGSCs isolation from neonatal ovary.

Previous isolation methods of FGSCs adopt strategies of FACS (12,31) and MACS (10,11,13), which may interrupt physiological status of cell membrane during these treatments. Stringing FGSCs probably reflect real features of FGSCs, as our isolation procedures do not contain any mediators of membrane binding, such as antibodies and magnetic beads. Thus, natural characters of the membrane of FGSCs are retained. The differential adherence selection method presents a non-antibody based and fast performing way to isolate germ stem cells, and can also avoid FGSCs viability loss, which is often accompanied in antibody-based isolation process. Through multiple rounds of differential adherence selection, pure cell population of sFGSCs can be obtained as continued subculture, due to proliferation inability of differentiated oocytes.

The string configuration of sFGSCs probably represents an intriguing feature of freshly isolated stem cells from neonatal ovary. PGCs can also form clusters similar to sFGSCs through aggregation during in vitro culture (32). However, both sFGSC
and PGCs are probably at different stages of female germ cells. FGSCs could arise near the boundary of the PGC and oogonia stages (33), while the PGCs originate from early embryo of E7.25, migrate and colonize the genital ridges at E10.5 (34,35). The FGSCs are incapable of development into teratomas in nude mice (11,12), and only have unipotency of differentiating into oocytes, with female imprinting patterns in Igf2r and Peg 10 regions (11). Moreover, FGSCs can be induced into female embryonic stem-like cells (fESLCs) using embryonic stem cell medium (19).

From our data, the isolated sFGSCs are similar to FGSCs purified by MACS. The sFGSCs have an average of 10-20 µm in diameter, and express germline markers Mvh, Fragilis, Dazl, Dppa3, Prdm1 and pluripotency markers Oct4 and Tert, while Nanog, Sox2 and oocyte-differentiation markers c-kit were not detected, which are consistent with previous reports (11). In addition, a similar E-cadherin location pattern was observed in cell-cell connection regions of FGSCs purified by both differential adherence selection and MACS methods, suggesting that E-cadherin probably participates in formation of adherens junction in the stringing FGSCs. However, sFGSCs have a cell-doubling time of approximately 22 hours and mitotic ability can be observed in culture of 12 hours, which is faster than MACS-isolated cells, ~24 hours (13,18). MACS method needs multiple steps, long-time exposure to non-culture condition, which could affect cell viability, leading to the subsequent differences. During isolation of sFGSCs, the cells are maintained in culture medium through the whole isolation process, which contributes to obtaining of stem cells of high quality. This system is really appropriate for clinic use in humans.

Treatment of mitotic antagonistic agent mitomycin C, which inhibits DNA synthesis (36,37), can lead to significant decrease of sFGSCs formation, implying an association between the strings and cell division. In addition, strings formation is also associated with age. Neonatal ovary has more abundant sFGSCs than old one, as sFGSCs numbers decline dramatically after 6 dpp. The sFGSCs exhibit a special cell-cortex distribution of F-actin filaments. The F-actin cytoskeleton in cell-cortex probably facilitates maintaining of stringing morphology of sFGSCs. As F-actin and its
interacting proteins, cadherin and catenin, can form a zonula adherens junction (38). The interaction between extracellular domains of E-cadherin molecules on neighboring two cells is responsible for the junction (26,39). Consistent with the facts, Convergent distribution of E-cadherin in the stringing FGSCs facilitates the string formation through its interaction with F-actin, a similar feature as adherens junction. E-cadherin play a similar role in ES cell contact (40). The distribution in contact region between germline stem cells and the apical hub cells was also observed in male Drosophila (41). Cell connection of sFGSCs may also contribute to maintaining of self-renewal and inhibiting of cell differentiation, which need further investigation.

Here, we provided a rapid and efficient method to isolate sFGSCs from neonatal ovary through differential adherence selection, and morphology and molecular evidences of in vitro sFGSCs division. We characterized stringing FGSCs formation during in vitro culture. The system is also promising in further understanding of follicle renewal and endocrine functions of adult ovary.

**EXPERIMENTAL PROCEDURES**

**Animals**

Female ICR mice were used in this study. All animal experiments and methods were performed in accordance with the relevant approved guidelines and regulations, as well as under the approval of the Ethics Committee of Wuhan University.

**Isolation of FGSCs**

Thirty ovaries of 1, 3, 6 and 14 dpp mice were collected for each isolation experiment. Two-step enzymatic digestion method was used as described previously with slight modification (11). Dissected ovaries were placed in MEM containing Type IV collagenase (1 mg/ml; C5138, Sigma, St. Louis, MO, USA), then incubated at 37 °C for 45 min, with gentle shaking every 5 min. The samples were then washed 2 to 4 times in PBS, and incubated in HBSS containing 1 mM EDTA and 0.05 % trypsin at 37 °C for 10 min. Trypsin was neutralized by adding 10% fetal bovine serum (FBS), pipetted tissues up and down until dispersed. The suspension was centrifuged at 2000 rpm for 5 min, and the pellet was re-suspended in FGSC medium. The cell suspension was
transferred into 0.5% (w/w) gelatin (G9391, Sigma) -coated 48-well plates for differential adherence selection. The cells were cultured at 37 °C for 45 min at the first round of selection. After gentle shaking of the plates, cells suspension was transferred to a new coated well for second round of selection and cultured for 2-3 hrs. A third round of selection (repeat once) is needed until most detached cells are around 10-20 µm in diameter. Final detached cells were cultured in FGSC medium. After at least 3-5 times of passaging, the FGSCs were further characterized. MACS method using anti-MVH antibody (rabbit polyclonal anti-Mvh, ab13840, Abcam, Cambridge, MA, USA) was also used to isolate the MVH+ FGSCs from 3 dpp mice according to previous method (11,21).

**Culture of FGSCs**

Mitotically inactivated STO cells were used as feeders in FGSC culture system. The STO cells were treated with mitomycin C (10 µg/ml; M4287, Sigma) for 2-3 hrs, then washed in PBS and plated on 0.2 % (w/w) gelatin-coated 48-well plate. Medium of FGSCs was Minimum Essential Medium alpha modification (alpha-MEM; SH30265.01B, Hyclone, Logan, Utah, USA) with 15 % FBS (SH30070.02E, Hyclone), 1 mM sodium pyruvate (R25-000-CI, Corning, NY, USA), 1 mM non-essential amino acids (M7145, Sigma), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol (21985-023, Gibco, Waltham, MA, USA), 10³ units/ml LIF (ESG1106, Millipore, Darmstadt, Germany), 10 ng/ml mouse epidermal growth factor (mEGF; 315-09, Peprotech, Rocky Hill, NJ, USA), 40 ng/ml human glial cell line-derived neurotrophic factor (GDNF; 212-GD, R&D systems, Minneapolis, MN, USA), 1 ng/ml human basic fibroblast growth factor (bFGF, AF-100-18B, Peprotech), and 1X N2 supplements (AR009, R&D systems). The cells were cultured in the FGSCs medium at 37° C in 5 % CO₂. The medium was changed every 3 days. The cells were sub-cultured every 5-7 days at a 1:1-2 dilution.

**Immunofluorescence analysis**

FGSCs were collected and attached to PLL (poly-lysine) coated slides before immunofluorescence analysis. After fixation with 4 % paraformaldehyde (15 min, room temperature), the cells were permeabilized with 0.1% Triton X-100 and then incubated in blocking solution (10 % normal goat serum in PBS, 60 min, room temperature).
Primary antibodies, rabbit polyclonal anti-Mvh (1:200 dilution; ab13840, Abcam), rabbit polyclonal anti-Oct4 (1:250 dilution; 11263-1-AP, Proteintech, Rosemont, IL, USA), rabbit polyclonal anti-Tubb3 (1:200 dilution; AC008, ABclonal, Wuhan, China) and rabbit polyclonal anti-E-cadherin (1:100 dilution; 3195T, Cell Signaling Technology, Beverly, MA, USA) were used in different detection. FITC conjugated secondary antibody (goat anti-rabbit IgG, 1:200 dilution; SA00003-2, Proteintech) was used to detect the signals of Tubb3, Mvh and Oct4 respectively, Cy3 conjugated secondary antibody (goat anti-rabbit IgG, 1:200 dilution; SA00009-2, Proteintech) was used to detect the signals of E-cadherin. The nuclei were stained by Hoechst (C1026, Beyotime) before fluorescence microscopy. In Tubb3 immunostaining, the cells must be treated by microtubule stabilizing buffer (MTSB, 80mM PIPES, 1 mM MgCl and 5 mM EGTA) for 20 seconds prior to paraformaldehyde affixation as described previously (42). Fluorescent images were captured by confocal fluorescence microscopy (FV1000, Olympus). For F-Actin staining, phalloidin (23101, AAT Bioquest, Sunnyvale, CA, USA) was used according to manufacturer’s protocol. In brief, after paraformaldehyde affixation and 0.1% Triton X-100 treatment, the cells were incubated with phalloidin conjugate working solution at room temperature for 30 min. The nuclei were stained by Hoechst (C1026, Beyotime) before fluorescence microscopy. Images were captured by confocal fluorescence microscopy equipped with Z motor (FV1000, Olympus).

Cell membrane staining

For membrane staining, the cells were incubated with the recommended working solution of membrane probe DiIC$_{18}$(3) (C1036, Beyotime, Haimen, China) of 10 μM at 37 ℃ for 5-10 min, and then rinsed by PBS for 3 times. After attaching to PLL-coated slide, the cells were fixed by paraformaldehyde, stained by Hoechst (C1026, Beyotime), and mounted for viewing with confocal fluorescence microscopy (FV1000, Olympus).

Mytomycin C treatment of FGSCs

Freshly isolated FSGCs were incubated with mitomycin C (10 µg/ml) in FGSC medium at 37 ℃ for 1 h. After rinsed three times by
alpha-MEM, the treated cells were cultured in normal FGSC system for further investigation.

**Lentivirus generation and infection**

The short hairpin RNA (shRNA) targeting E-cadherin mRNA in mouse or the control shRNA was cloned into the pLvx-shRNA vector using BamHI and EcoRI sites. Target sequence of E-cadherin shRNA was 5'-GGAGATGCAGAATAATTAT-3', and randomly disrupted sequence 5'-GATTAATAGGATTGCAAA-3' was used as control shRNA (27). Primer sequences were listed in Table S2. Virus generation and infection was performed as described previously (43). In brief, lentivirus vectors were co-transfected with the lentiviral packaging vectors pRSV-Rev, pMD2.G and pCMV-VSV-G into HEK293T cells using LipofectamineTM2000. The collected supernatants were filtered through a 0.45-μm filter after transfection for 48 h. Virus purified by Lentivirus Purification Miniprep Kit (V1170-02, Biomiga, Shanghai, China) was used to infect cells.

**Western blot analysis**

Western blot was performed as described previously (43). In brief, protein extracts from cells were separated in SDS-polyacrylamide gels and then transferred onto 0.45 μm membranes. The primary antibodies (anti-E-cadherin, Cell Signaling Technology; anti-GAPDH, CW0100M, CWBIO, Peking, China) were incubated with the membranes overnight at 4°C. The membranes were washed in TBST (20 mM Tris-HCl pH7.5, 150 mM NaCl, 0.1% Tween 20) several times, incubated with the indicated HRP-conjugated secondary antibody for 1 h at room temperature and then washed in TBST several times. A Super Signal Chemiluminescent Substrate system (34080, Pierce, Rockford, USA) was used to detect the signals.

**Scanning electron microscopy**

Scanning electron microscopy was used to observe morphology of cell strings. After attaching to PLL-coated slide, the cells were washed with PBS and fixed in 2.5% glutaraldehyde. Then, the cells were re-fixed with 1% osmium tetroxide for 30 min, rinsed 3 times with PBS, and dehydrated through a graded series of alcohol (50%, 70%, 80%, 90% and 100%). Finally, the cells were transferred into isoamyl acetate solution for 30 min, critical-point dried, coated with gold, and mounted for viewing.
with SEM (S-3400N, Hitachi, Tokyo, Japan).

RT-PCR
RT-PCR was performed as described previously (44). Briefly, total RNAs of ovary and cells were isolated using TRIzol reagent (15596026, Thermo Fisher, Massachusetts, USA). Extracted RNAs were treated with DNase treatments (M610A, Promega, Madison, WI, USA). The first strands of cDNAs were synthesized following the protocol (M170A, Promega). Primer sequences and PCR conditions are listed in Table S1.

Karyotype analysis
After cultured for 2 months, the cells were treated with colchicine (20 ng/ml) for 4 hrs, hypotonically treated with 40 mM KCl for 30 min, fixed in methanol-acetic acid (3:1) and air-dried. The slides were stained by Hoechst (C1026, Beyotime). Karyotypes were analyzed after captured under confocal fluorescence microscopy (FV1000, Olympus).

Statistical analysis
One-way or two-way ANOVA was used to determine significant differences between control and experimental groups. In all analysis, data are presented as Mean ± SEM from at least 3 independent experiments. Statistical analysis was conducted using SAS software (ver. 9.2).

ACKNOWLEDGMENTS
We thank professor Ji Wu of Shanghai Jiao Tong University for providing instruction of FGSC culture and critical reading of the manuscript. This work was supported by the National Natural Science Foundation of China.

CONFLICTS OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
R.Z. and H.C. conceived and designed research. J.L. and R.Z. analyzed and interpreted the data and wrote the paper. J.L., Y.X. and D.S. carried out the experimental work. J.L. and P.Z.
analyzed the data. All authors read and approved the final manuscript.

**ABBREVIATIONS** sFGSCs, stringing female germline stem cells; FGSCs, female germline stem cells; dpp, days post partus; SSC, spermatogonial stem cells; MACS, magnetic-activated cell sorting; FACS, fluorescence-activated cell sorting; fESLCs, female embryonic stem-like cells; PGC, primordial germ cells; SEM, scanning electron microscopy; PLL, poly-lysine; FBS, fetal bovine serum; mEGF, mouse epidermal growth factor; GDNF, glial cell line-derived neurotrophic factor; bFGF, basic fibroblast growth factor.

**REFERENCES**


**Figure legends**

**Figure 1. Stem cell isolation from neonatal ovaries through differential adherence selection.**

(A) Schematic diagram of differential adherence selection procedures for isolating female germline stem cells. After 2-step digestions by collagenase IV and trypsin, dispersed ovary cells were cultured and selected by 3-5 rounds of differential adherence selections in gelatin coated wells. Final detached cells were cultured in STO feeder condition with alpha-MEM medium containing bFGF, EGF, Lif, N2 Supplement, GDNF and NEAA. After 4 times of subculture, the FGSCs were further characterized.

(B) Morphology of final detached ovary cells from 1 dpp, 3 dpp, 6 dpp and 14 dpp ovaries. Scale bar, 40 μm.

(C) Numbers of final detached cells with diameters of 10–20 μm from 1 dpp, 3 dpp, 6 dpp
and 14 dpp ovaries. Thirty ovaries were used in each group. Values of cell numbers are presented as mean ± SEM. Three independent experiments, n = 3 wells of cells/group. One-way ANOVA was performed followed by Tukey’s multiple comparison test. *p < 0.05, **p < 0.01.

(D) Typical morphology of strings composed of 2, 3 and 4 cells cultured in 1 week. Scale bar, 10 μm.

(E) Detection of marker genes expressions of germline and pluripotency in attached cells, final detached primary cells and primary FGSCs cultured for 1 week and passaged once by RT-PCR. Lane 1, attached cells; Lane 2, final detached ovary cells; Lane 3, female germline stem cells cultured for 1 week; No RT, PCR without reverse transcription. Final detached ovary cells expressed germline markers (Mvh, Fragilis), FGSCs did not express differentiation marker c-kit but express germline markers, while the well-attached cells were somatic cells which showed no germline-specific gene expression. Primer sequences and PCR conditions are listed in Table S1.

Figure 2. Morphology of FGSC strings.

(A) Morphology of FGSC strings in a 48-well containing 2, 4, >4 cells cultured on STO monolayer for 3 days after second passaging. Dashed ovals highlight strings of cells. Scale bar, 10 μm.

(B) Representative morphology of single cell, 2-cell and 4-cell FGSC strings formed during culture. Cell numbers in strings are consistent with mitosis division. Scale bar, 10 μm.

(C) Hematoxylin-eosin staining of single cell, 2-cell and 4-cell FGSC strings. Obvious individual nucleus per cell were presented in FGSC strings. Dotted lines highlighted individual cell in strings. Scale bar, 10 μm.

(D) Scanning electron microscopy of representative FGSCs of 2-cell and 4-cell strings. Dotted lines highlight individual cell in strings. Scale bar, 10 μm.

(E) Numbers of FGSC strings per ovary of 1, 3, 6 and 14 dpp mice. FGSC numbers decline after 6 dpp. Strings numbers are presented as mean ± SEM. Three independent experiments, n = 3 wells of cells/group. One-way ANOVA was performed. *p < 0.05, **p < 0.01.
Figure 3. Characterization of FGSCs.

(A) RT-PCR analysis of germline and pluripotency markers. FSGCs were isolated from ovary of 3 dpp and cultured for 2 months. Ovary cDNA were used as positive control; No RT, PCR without reverse transcription. Primer sequences and PCR conditions were listed in Table S1.

(B) Immunofluorescence of Mvh protein (green) in 2-cell and 4-cell FGSC strings. Nuclei were stained with Hoechst (blue). Negative controls (N.C.) were omission of the primary antibody. Scale bar, 10 µm.

(C) Immunofluorescence of Oct4 protein (green) in 2-cell and 4-cell FGSC string. Nuclei were stained with Hoechst (blue). Negative controls (N.C.) were omission of the primary antibody. Scale bar, 10 µm.

(D) Karyotype analysis of FGSCs cultured for 2 months. Chromosomes were stained by Hoechst (green). Normal female karyotype (40, XX) was presented in FGSCs.

Figure 4. Division kinetics of FGSCs.

(A) Continuous images of FGSCs from 1 cell to 8-cell string through 3 times of cell divisions taken at different time points (0, 7 h 38 m, 24 h 5 m, 47 h 54 m and 58 h 13 m) under an inverted microscopy. Dotted lines highlighted individual cell in strings. Scale bar, 10 µm.

(B) Proliferation curves of FGSCs from 1, 3, 6 and 14 dpp ovaries. Numbers of cells in strings were calculated every 12 hours, and thirty ovaries were used in each group. Cells numbers are presented as mean ± SEM. Three independent experiments, n = 3 wells of cells/group. Two-way ANOVA was performed. *p < 0.05, **p < 0.01.

(C) Spindle immunofluorescence of mitotic FGSCs of 3 dpp using anti-Tubb3 (green). FGSCs were cultured for 1 day (upper panel) and 5 days (lower panel). Signals of Tubb3 showed that FGSCs were at early telophase of mitotic cycle. Nuclei were stained with Hoechst (blue). Negative controls (N.C.) were omission of the primary antibody. Scale bar, 10 µm.

(D) Mitotic inhibition effect of mitomycin C on string formation. FGSCs of 3 dpp were cultured for 4 days. Mitomycin C was added at a final concentration of 10 µg/ml. The treatment showed significantly decreased numbers of 2-cell, 4-cell and >4-cell strings compared to normal groups without mitomycin C. Strings numbers are presented as mean ±
SEM. Three independent experiments, n = 3 wells of cells/group. Strings numbers of 1, 3 and 6 dpp were statistically compared with those of 14 dpp. Two-way ANOVA was performed. **p < 0.01.

(E) Effect of N2 supplement removal on FGSC strings formation. Complete FGSC medium, FGSC medium without N2 supplement and alpha-MEM with 15% FBS were used in different groups. Numbers of strings were calculated at culture day 2. N2 removal inhibited significantly FGSC string numbers compared to complete FGSC medium group, while formation was nearly absent in alpha-MEM + FBS group. Strings numbers are shown as mean ± SEM. Three independent experiments, n = 3 wells of cells/group. Two-way ANOVA was performed. *p < 0.05, **p < 0.01.

**Figure 5. Cell membrane staining of FGSC strings.**

(A) Plasma membranes staining of 2, 4 and 6-cell strings by DiIC18(3). Membrane probe (red) showed cell connection between FGSCs. Nuclei were stained with Hoechst (blue). Scale bar, 10 µm.

(B) Local separation of membranes between cells. Membranes were stained by DiIC18(3) (red). Enlargement images showed partial separation of membrane in connection region. Dotted lines highlighted separated regions of membranes. Nuclei were stained with Hoechst (blue). Scale bar, 10 µm.

**Figure 6. Microtubule and F-Actin filament distribution in FGSCs.**

(A) Confocal z-series images of representative Tubb3 immunofluorescence (green) of 2-cell and 6-cell strings. Microtubule network was mainly distributed in the cell cortex of plasma membrane in FGSCs. Z motor scanning were utilized in fluorescence microscopy. Nuclei were stained with Hoechst (blue). Negative controls (N.C.) were omission of the primary antibody. Scale bar, 10 µm.

(B) Phalloidin staining of F-Actin cytoskeleton of FGSCs. F-Actin filaments (green) were observed in the cell cortex of plasma membrane in FGSCs. STO cells were utilized as controls. Z motor scanning were utilized in fluorescence microscopy. Negative controls (N.C.) were omission of the Phalloidin. Nuclei were stained with Hoechst (blue). Scale bar, 10 µm.
Figure 7. E-cadherin convergent distribution in connection regions in FGSC strings.

(A) E-cadherin antibody and Cy3 conjugated secondary antibody were used respectively. E-cadherin signals (red) were observed in cell membrane, mainly in the connection regions in FGSC strings (2-cell and 4-cell strings) from both differential adherence selection (upper panel) and MACS (lower panel) methods, while the signals were weak in the membrane of single cell. Negative controls (N.C.) were omission of the primary antibody. Nuclei were stained with Hoechst (blue). Images were taken under confocal microscopy. Scale bar, 10 μm.

(B) Inhibition effect of E-cadherin knockdown on string formation in sFGSCs isolated by differential adherence selection. Strings numbers of FGSCs from 3 dpp ovaries were analyzed at day 4 after lentivirus infection, shRNA control and mock treated FGSCs were used as controls. Strings numbers are shown as mean ± SEM. Three independent experiments, n = 3 wells of cells/group. Two-way ANOVA was performed. *p < 0.05, **p < 0.01.

(C) A working model of E-cadherin mediated cell-cell connection together with F-actin filaments in sFGSCs. E-cadherin is a trans-membrane protein. The interaction between extracellular domains of E-cadherin molecules on neighboring two cells is responsible for the junction. F-actin in cell-cortex interacts with the intracellular domain of E-cadherin through α-catenin and β-catenin. These molecules form a structure of adherens junction between the stem cells leading to the stringing FGSCs.
Figure 1
Figure 3
Figure 4

A

B

D

C

E

Figure 4

A

B

D

C

E
Figure 7
Isolation and characterization of string-forming female germline stem cells from ovaries of neonatal mice
Jing Liu, Dantong Shang, Yao Xiao, Pei Zhong, Hanhua Cheng and Rongjia Zhou
J. Biol. Chem. published online August 21, 2017

Access the most updated version of this article at doi: 10.1074/jbc.M117.799403

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