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. 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 days postpartum. After cell division, the sFGSC membranes tended to be connected to form sFGSCs. Moreover, F-actin filaments exhibited a cell–cell 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 FGSCs 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.

In mammals, the male testis can produce sperm throughout an entire lifetime. Male germline stem cells, spermatogonial stem cells (SSCs), provide a renewal pool to produce sperm (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 evidence of female germline stem cells (FGSC) in the ovary (8-14). Millions of women suffer from endocrine dysfunction and age-related 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 treatment of these diseases. In addition, it will also help in extending the reproductive age of females.

Primordial follicle renewal was observed in the postnatal mouse ovary, suggesting existence of germ stem cells in the 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 offspring was obtained through transplantation of GFP-expressing FGSCs in ovaries (11). Human FGSCs were also isolated from reproductive-age women through DDX4 antibody-based FACS (12). GFP-expressing human FGSCs were injected into adult ovarian cortical tissue biopsies of humans, 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 humans, FGSCs from neonatal rats 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 using embryonic stem cell medium, which exhibited gene expression and differentiation potential similar to those of embryonic stem cells (19). Comparison of gene expression profiles among FGSCs, primordial germ cells (PGCs), and 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 embryonic stem cells (ESCs) and FGSCs. Their DNA methylation determined FGSC unipotency by suppressing the somatic program (9). Although some FGSCs or SSCs revealed a stringing growth pattern (21), the characterization of the stringing growth or sFGSCs remains to be further studied.

Antibody against the C terminus of Mvh (known as Ddx4 in humans) was first used for mouse FGSC isolation through...
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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). A FACS method was also used to isolate Oct4+ ovarian germine stem cells from Oct4-GFP transgenic mice (14). These isolation methods employed slightly different features of the cells; thus, the FGSCs isolated revealed distinct characteristics.

Differential adherence selection was successfully used to enrich SSCs from postnatal testis (22–24). As there was looser adherence of male germine stem cells compared with other somatic cells during culture (23, 24), we adopted the strategy of differential adherence selection to enrich female germ stem cells from the 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 passages, and the FGSCs were further characterized. We found the stringing FGSCs (sFGSCs) from primary to more than eight generations of culture. In addition, we tested mitotic kinetics and cell string-forming abilities of cultured sFGSCs. Membrane connection through E-cadherin and F-actin cytoskeleton of the cell cortex in sFGSCs was also analyzed, which revealed tight connections between cells in the sFGSCs. Our work demonstrated that sFGSCs exist in neonatal ovary, especially in 1–3-day postpartum (dpp) mice. Besides providing an alternative strategy for sFGSC isolation, which is much easier and costs less than 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 germine stem cells in mice and humans, 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 germine 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 minimum essential medium α-modification (α-MEM) containing EGF, human basic fibroblast growth factor (bFGF), LIF, human glial cell line-derived neurotrophic factor (GDNF), and N2 supplements after 2-step enzymatic digestions using type IV collagenase and trypsin. The cells were transferred to gelatin-coated wells for adherence selection, as shown in a 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 be obtained from 30 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 the first week of the culture (Fig. 1D). The diameter of the cells in strings was between 10 and 20 μm, which is consistent with the diameters 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, the final detached ovary cells expressed germline markers (Mvh and Fragilis) and telomerase, and the passaged cells did not express differentiation marker c-kit but express germline markers, telomerase, and pluripotency factors (Fig. 1E), suggesting a feature of the FGSCs.

To further investigate the morphology of FGSC strings in continuous passaging, the cell strings with 2, 4, and >4 cells cultured on STO monolayer were still detected after passaging for at least 2 months (Fig. 2, A–C). To observe cell connection of strings, especially the connection region of adjacent cells, scanning electron microscopy was also performed. Scanning electron microscopy showed that the cells in strings had an obvious connection, and one-quarter to one-third of the membrane regions 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 it was difficult 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, whereas the numbers per ovary of 6 and 14 dpp declined significantly to <6 strings (Fig. 2E). These results showed that the neonatal mouse had more abundant stringing FGSCs than the old one. As opposed to the disperse FGSCs in later stages of postnatal mice observed in our study and other studies (11), we refer to 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, and Tert in the sFGSCs cultured for 2 months. RT-PCR showed that the stringing cells expressed Mvh, Dazl, Dppa3, Prdm1, Fragilis, Oct4, and Tert, whereas expression of oocyte differentiation marker c-kit and pluripotent markers Sox2 and Nanog was not detected (Fig. 3A), which was consistent with findings for the FGSCs through MACS isolation (11).

Immunofluorescence analysis of the sFGSCs demonstrated that the expression of Mvh was in the cytoplasm (Fig. 3B), whereas Oct4 protein was restricted in the nuclei of the sFGSCs (Fig. 3C), which was consistent with previous reports (11). Chromosome analysis showed that the sFGSCs had a normal female karyotype (40, XX) after 2 months of 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 a 1-cell to an 8-cell string were taken at different time points (0, 7 h 38 min, 24 h 5 min, 47 h 54 min, and 58 h 13 min) (Fig. 4A), indicating that cell divisions occurred three times 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 h during a culture of 60 h. 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 ~22 h 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, β3 class III). The microtubules with typical Tubb3 signals indicated that the FGSCs with a diameter of ~10 μm were in the stage of early telophase, and the cell division can be detected in the cultures of both 1 and 5 days (Fig. 4C). Moreover, the division ability of the sFGSCs was significantly inhibited by mitomycin C (10 μg/ml) (Fig. 4D), a mitotic antagonistic agent that inhibits DNA synthesis during 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 in the neonatal mice of 1–3 dpp.

Connection features of the sFGSC membrane

To investigate connection features of the sFGSC membrane, we used a fluorescent lipophilic cationic indocarbocyanine dye, DilC1_16(3), which is highly fluorescent and quite photostable when incorporated into the 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 cul-
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**Figure 2. Morphology of FGSC strings.** A, morphology of FGSC strings in a 48-well plate containing 2, 4, or >4 cells cultured on STO monolayer for 3 days after the second passaging. Dashed oval highlights 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 nuclei per cell are presented in FGSC strings. Dotted lines highlight individual cells 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. Numbers are presented as mean ± S.E. (error bars). Three independent experiments are shown; n = 3 wells of cells/group. One-way ANOVA was performed. *, p < 0.05; **, p < 0.01.

Cytoskeleton converges in cell cortex adjacent to plasma membrane in sFGSCs

To explore morphological dynamics of the plasma membrane of sFGSCs, we first used microtubule marker Tubb3 to immunostain the microtubule networks. Immunofluorescence of microtubule marker Tubb3 showed that the 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 filament 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 plays a role in maintaining cell–cell membrane contact of sFGSCs, we used immunofluorescence to detect E-cadherin distribution in single cells and strings of two cells and four cells. E-cadherin signals converged in cell–cell connection regions of sFGSCs, whereas the signals were weak in the membrane of single FGSCs (Fig. 7A). A similar E-cadherin location pattern was also observed in cell–cell connection regions of FGSCs purified by the MACS method (Fig. 7A). To test whether the string formation was affected when E-cadherin expression was knocked down by shRNA (a confirmed target sequence of E-cadherin from a previous study (27)), we counted numbers of FGSC strings at day 4 after shRNA lentivirus infection. E-cadherin knockdown significantly inhibited the string numbers of FGSCs, compared with shRNA control and mock-treated cells (Fig. 7B and supplemental 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 facilitates the morphological structure of the stringing FGSCs.

Discussion

Physiological functions of the ovary decline with an aging-dependent decrease of follicle numbers and disruption of endocrine in women. The discovery of ovarian germline stem cells has provided a deep understanding of follicle renewal and endocrine functions of adult ovary during the reproductive lifespan. It has also brought the prospect of new strategies to promote ovarian regeneration, delay menopause, and cure ovarian dysfunction. For example, human oogonial stem cells have been clinically used in ovarian therapies (28–30). However, to better understand ovarian physiology and develop efficient strategies for the treatment of ovarian dysfunction through FGSCs, isolation, characterization, and differentiation mechanisms of FGSCs need to be further explored. In the present study, we have developed a rapid and efficient strategy for sFGSC isolation from ovaries 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 with disperse FGSCs in later stages of postnatal mice observed in our study and other studies (11). The system can obtain highly 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 tends 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 sFGSC isolation from the neonatal ovary.

Previous isolation methods of FGSCs adopt strategies of FACs (12, 31) and MACS (10, 11, 13), which may interrupt the physiological status of cell membrane during these treatments. Stringing FGSCs probably reflect real features of FGSCs, because our isolation procedures do not contain any mediators of membrane binding, such as antibodies and magnetic beads. Thus, natural characteristics 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 FSGC viability loss, which often accompanies antibody-based isolation processes. Through multiple rounds of differential adherence selection, a pure cell population of sFGSCs can be obtained after a continued subculture, due to the inability of differentiated oocytes to proliferate.

The string configuration of sFGSCs represents an intriguing feature of freshly isolated stem cells from the 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), whereas the PGCs originate from early embryos of embryonic day 7.25, migrate, and colonize the genital ridges at embryonic day 10.5 (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 Peg10 regions (11). Moreover, FGSCs can be induced into female embryonic stem–like cells using embryonic stem cell medium (19).

From our data, the isolated sFGSCs are similar to FGSCs purified by MACS. The sFGSCs are an average of 10–20 μm in diameter and express germline markers Mvh, Fragilis, Dazl, Dppa3, and Prdm1 and pluripotency markers Oct4 and Tert, whereas Nanog, Sox2, and oocyte-differentiation marker c-kit were not detected, which is 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 ∼22 h, and mitotic ability can be observed in culture of 12 h, which is faster than MACS-isolated cells (∼24 h) (13, 18). The MACS method requires multiple steps and long-term exposure to non-culture conditions, which could affect cell viability, leading to subsequent differences. During isolation of sFGSCs, the cells are maintained in culture medium through the whole isolation process, which contributes to the ability to obtain stem cells of high quality. This system is appropriate for clinical use in humans.

Treatment with the mitotic antagonistic agent mitomycin C, which inhibits DNA synthesis (36, 37), can lead to a significant decrease in sFGSC formation, implying an association between the strings and cell division. In addition, string formation is also associated with age. The neonatal ovary has more abundant sFGSCs than the older ovary, as sFGSC numbers decline dramatically after 6 dpp. The sFGSCs exhibit a special cell–cortex distribution of F-actin filaments. The F-actin cytoskeleton in the cell cortex probably facilitates the maintenance of the 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 two neighboring cells is responsible for the junction (26, 39). Consistent with these facts, convergent distribution of E-cadherin in the stringing FGSCs facilitates the string formation through its interaction with F-actin, a feature similar to the adherens junction. E-cadherin plays a similar role in embryonic stem cell contact (40). The distribution in the

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**Figure 3. Characterization of FGSCs.**

A, RT-PCR analysis of germline and pluripotency markers. FGSCs were isolated from ovaries of 3-dpp mice and cultured for 2 months. Ovary cDNA was used as positive control. No RT, PCR without reverse transcription. Primer sequences and PCR conditions are listed in supplementary 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 were omission of the primary antibody. Scale bar, 10 μm. D, karyotype analysis of FGSCs cultured for 2 months. Chromosomes were stained by Hoechst and excited by blue light (green). Normal female karyotype (40, XX) was presented in FGSCs.
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 maintenance of self-renewal and inhibition of cell differentiation, which require further investigation.

Here, we provided a rapid and efficient method to isolate sFGSCs from the neonatal ovary through differential adherence as well as morphology and molecular evidence of in vitro sFGSC division. We characterized stringing FGSC formation during in vitro culture. The system is also promising with regard to 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. A 2-step enzymatic digestion method was used as described previously with slight modifica-

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Figure 4. Division kinetics of FGSCs. A, continuous images of FGSCs from single-cell to 8-cell string through three cell divisions taken at different time points (0, 7 h 38 min, 24 h 5 min, 47 h 54 min, and 58 h 13 min) under an inverted microscope. 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 h, and 30 ovaries were used in each group. Cell numbers of 1, 3, and 6 dpp were statistically compared with those of 14 dpp. Cell numbers are presented as mean ± S.E. (error bars). Shown are 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 mice using anti-Tubb3 (green). FGSCs were cultured for 1 day (top) and 5 days (bottom). Signals of Tubb3 showed that FGSCs were at early telophase of the 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 with normal groups without mitomycin C. String numbers are presented as mean ± S.E. Three independent experiments are shown; n = 3 wells of cells/group. Two-way ANOVA was performed. **, p < 0.01. E, effect of N2 supplement removal on FGSC string formation. Complete FGSC medium, FGSC medium without N2 supplement, and α-MEM with 15% FBS were used in different groups. Numbers of strings were calculated at culture day 2. N2 removal significantly inhibited FGSC string numbers compared with the complete FGSC medium group, whereas formation was nearly absent in the α-MEM + FBS group. String numbers are shown as mean ± S.E. Three independent experiments are shown; n = 3 wells of cells/group. Two-way ANOVA was performed. *, p < 0.05; ***, p < 0.01.
tion (11). Dissected ovaries were placed in MEM containing Type IV collagenase (1 mg/ml; C5138, Sigma) and then incubated at 37 °C for 45 min, with gentle shaking every 5 min. The samples were then washed 2–4 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% FBS, and tissues were pipetted up and down until dispersed. The suspension was centrifuged at 2000 rpm for 5 min, and the pellet was resuspended 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, the cell suspension was transferred to a new coated well for second round of selection and cultured for 2–3 h. A third round of selection (repeated once) was needed until most detached cells were around 10–20 μm in diameter.

Final detached cells were cultured in FGSC medium. After at least 3–5 passages, the FGSCs were further characterized. A MACS method using anti-MVH antibody (rabbit polyclonal anti-Mvh; ab13840, Abcam, Cambridge, MA) was also used to isolate the MVH+ FGSCs from 3-dpp mice according to the previous method (11, 21).

**Culture of FGSCs**

Mitotically inactivated STO cells were used as feeders in the FGSC culture system. The STO cells were treated with mitomycin C (10 μg/ml; M4287, Sigma) for 2–3 h and then washed in PBS and plated on a 0.2% (w/v) gelatin-coated 48-well plate. Medium of FGSCs was 15% FBS (SH30070.02E, Hyclone), 1 mM sodium pyruvate (R25-000-Cl, Corning, Inc.), 1 mM non-essential amino acids (M7145, Sigma), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol (21985-023, Gibco), 10³ units/ml LIF (ESG1106, Millipore, Darmstadt, Germany), 10 ng/ml mouse EGF (315-09, Peprotech, Rocky Hill, NJ), 40 ng/ml GDNF (212-GD, R&D Systems, Minneapolis, MN), 1 ng/ml bFGF (AF-100-18B, Peprotech), and 1 × N2 supplements (AR009, R&D Systems). The cells were cultured in FGSC medium at 37 °C in 5% CO₂. The medium was changed every 3 days. The cells were subcultured every 5–7 days at a 1:1–2 dilution.

**Immunofluorescence analysis**

FGSCs were collected and attached to polylysine (PLL)-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, Cambridge, MA) rabbit polyclonal anti-Oct4 (1:250 dilution; 11263-1-AP, Proteintech, Rosemont, IL), 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) 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 with Hoechst (C1026, Beyotime) before fluo-
rescence microscopy. In Tubb3 immunostaining, the cells must be treated by microtubule-stabilizing buffer (80 mM PIPES, 1 mM MgCl₂, and 5 mM EGTA) for 20 s before paraformaldehyde affixation, as described previously (42). Fluorescent images were captured by a confocal fluorescence microscope (FV1000, Olympus, Tokyo, Japan) equipped with a Z motor.

**F-actin staining by phalloidin**

For F-actin staining, phalloidin (23101, AAT Bioquest, Sunnyvale, CA) was used according to the 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 a confocal fluorescence microscope equipped with a Z motor (FV1000, Olympus).

**Cell membrane staining**

For membrane staining, the cells were incubated with the recommended working solution of membrane probe DiIC₁₈(3) (C1036, Beyotime, Haimen, China) of 10 μM at 37 °C for 5–10 min and then rinsed by PBS three times. After attaching to a PLL-coated slide, the cells were fixed by paraformaldehyde, stained by Hoechst (C1026, Beyotime), and mounted for viewing with a confocal fluorescence microscope (FV1000, Olympus).

**Mytomycin C treatment of FGSCs**

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

**Lentivirus generation and infection**

The shRNA targeting E-cadherin mRNA in mice or the control shRNA was cloned into the pLvx-shRNA1 vector using BamHI and EcoRI sites. The target sequence of E-cadherin shRNA was 5'-GGAGATGCAGAATAATTAT-3', and a randomly disrupted sequence, 5'-GATTAATAGGATTGGAA-3', was used as control shRNA (27). Primer sequences are listed in supplemental Table S2. Virus generation and infection were 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 Lipofectamine™ 2000. The collected supernatants were filtered through a 0.45-μm filter after transfection for 48 h. Virus purified by the Lentivirus Purification Miniprep Kit (V1170-02, Biomek, Shanghai, China) was used to infect cells.

**Western blot analysis**

Western blotting 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, pH 7.5, 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 SuperSignal chemiluminescent substrate system (34080, Pierce) was used to detect the signals.

**Scanning electron microscopy**

Scanning electron microscopy was used to observe the morphology of cell strings. After attaching to a PLL-coated slide, the cells were washed with PBS and fixed in 2.5% glutaraldehyde.
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Then the cells were refixed with 1% osmium tetroxide for 30 min, rinsed three 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 a scanning electron microscope (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). Extracted RNAs were treated with DNase treatments (M610A, Promega, Madison, WI). The first strands of cDNAs were synthesized following the protocol (M170A, Promega). Primer sequences and PCR conditions are listed in supplemental Table S1.

Karyotype analysis

After they were cultured for 2 months, the cells were treated with colchicine (20 ng/ml) for 4 h, 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, Beyo-time). Karyotypes were analyzed after they were 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 analyses, data are presented as mean ± S.E. from at least three independent experiments. Statistical analysis was conducted using SAS software (version 9.2).

Author contributions—R. Z. and H. C. conceived and designed the 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.

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