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Production of offspring from a germline stem cell line derived from neonatal ovaries

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The idea that females of most mammalian species have lost the capacity for oocyte production at birth[1–5] has been challenged recently by the finding that juvenile and adult mouse ovaries possess mitotically active germ cells[6]. However, the existence of female germline stem cells (FGSCs) in postnatal mammalian ovaries still remains a controversial issue among reproductive biologists and stem cell researchers[6–10]. We have now established a neonatal mouse FGSC line, with normal karyotype and high telomerase activity, by immunomagnetic isolation and culture for more than 15 months. FGSCs from adult mice were isolated and cultured for more than 6 months. These FGSCs were infected with GFP virus and transplanted into ovaries of infertile mice. Transplanted cells underwent oogenesis and the mice produced offspring that had the *GFP* transgene. These findings contribute to basic research into oogenesis and stem cell self-renewal and open up new possibilities for use of FGSCs in biotechnology and medicine.

In most mammalian species the production of ovarian oocytes is thought to cease before birth[1–5]. However, this belief has been challenged by recent research indicating that the female gonad may have regenerative activity in juvenile and adult mice *in vivo*[6]. Subsequently, it has been suggested that both bone marrow and peripheral blood may serve as a source of cells responsible for this regenerative capacity[11]. Other studies have shown that bone marrow cells, or other normally circulating cells, are not involved in the formation of mature, ovulated oocytes[7], and that instead of FGSCs, putative thecal stem cells could be isolated from newborn mouse ovaries[9]. Therefore, the presence of FGSCs in mammalian ovaries after birth is still highly controversial[6–10,12].

To identify and confirm the presence of FGSCs in mouse ovaries[6], we located ovarian cells positive for mouse vasa homologue (MVH) protein, which is expressed exclusively in germ cells, using immunohistochemistry. Next, we assessed the proliferative potential of these MVH-positive cells. Adult and 5-day-old female mice were injected with 5′-bromodeoxyuridine (BrdU) and ovaries were collected 1 h later for dual immunofluorescence analysis of BrdU incorporation and expression of MVH. The results showed the presence of BrdU–MVH double-positive cells in the ovarian surface epithelium, suggesting that they might be FGSCs. Morphological and histological analysis of these BrdU–MVH double-positive cells was performed (Supplementary Information, Fig. S1).

To create mouse FGSC lines, FGSCs were isolated from the ovaries of 5-day-old or adult C57BL/6×CD-1 F1 hybrid mice. Large round or ovoid cells were obtained from 9–12 neonatal (200–300 cells) or 6–8 adult mice (50–100 cells) by two-step enzymatic digestion and immunomagnetic isolation of MVH-positive (MVH^+) cells[13,14]. Most of the isolated cells were morphologically similar to freshly isolated type A spermatogonia (SSCs)[15], showing large cell bodies with little cytoplasm, spherical nuclei with slight staining, a large ratio of nuclear plasma and nuclear diameter of 12–20 μm (Figs 1a, 2a; Supplementary Information, Fig. S2d). To determine whether the MVH^+ cells contained FGSCs, cell proliferation analysis was carried out by adding BrdU to the culture medium. The results show that the isolated MVH^+ cells consisted of BrdU-positive cells (Figs 1b–d, 2b–d), suggesting that the cells might be FGSCs. BrdU-negative cells were derived from oocytes that had already entered prophase I (Supplementary Information, Fig. S2c). For confirmation, dual immunofluorescence analysis of BrdU incorporation and expression of MVH was performed on isolated cells cultured for more than 14 days with 2 passages. Almost all of these cells were positive for BrdU and MVH (Figs 1e, 2i–m), as oocytes degenerate during culture. After 7 or 8 passages, the isolated cells increased in number, forming clusters (Fig. 1f). Immunofluorescence analysis of BrdU incorporation and DAPI was performed on these FGSCs to confirm that they were undergoing mitosis (Fig. 2e–h). As culturing continued, the clustered cells proliferated and formed compact clusters of cells with blurred cell boundaries. However, these clusters of cells did not resemble embryonic stem (ES) cell colonies. At the time of writing, the FGSCs from neonatal mice (nFGSCs) had been in culture for more than 15 months, had been passaged more than 68 times (5–8 days per passage) and maintained a characteristic morphology similar to that of freshly isolated FGSCs (Fig. 1g). FGSCs from adult mice (aFGSCs) were cultured for more than 6 months and passaged more than 25 times. The proliferation patterns of these FGSCs

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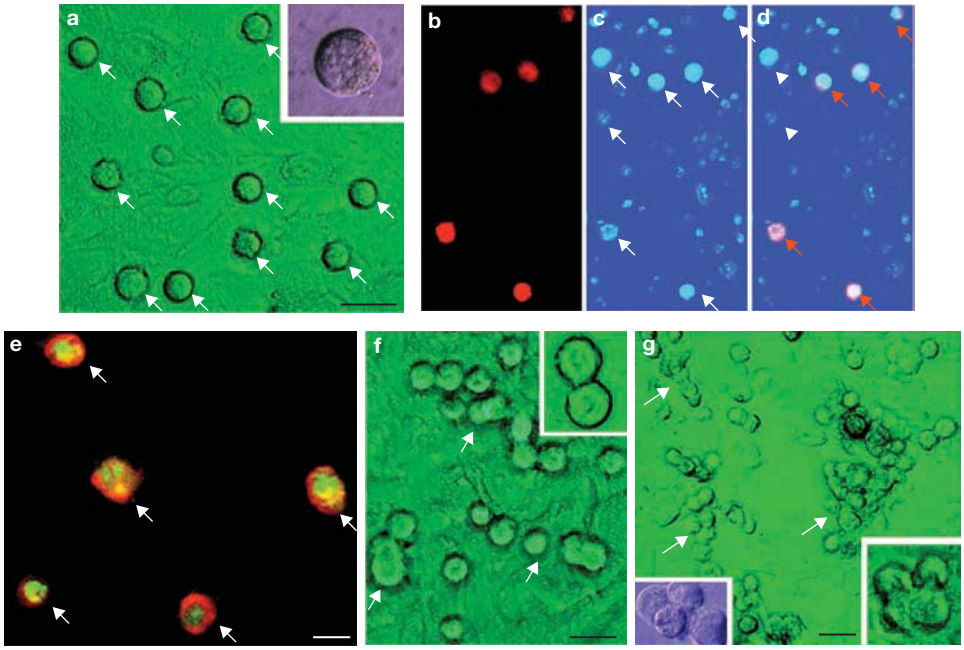

Figure 1 Establishment of nFGSC or aFGSC culture. (**a**) An example of the morphology of nFGSCs that were cultured on mitomycin C-treated STO cells for 24 h after purification by immunomagnetic isolation of MVH$^+$ cells. The inset shows the morphology of a freshly isolated nFGSC. (**b**) Representative view of BrdU immunofluorescence in isolated and cultured MVH$^+$ cells. Red, BrdU-positive cells. (**c**) DAPI immunofluorescence. Arrows indicate MVH$^+$ cells. (**d**) Merger for BrdU and DAPI immunofluorescence. Red arrows indicate BrdU positive cells; white arrowheads indicate BrdU negative cells. (**e**) An example of dual immunofluorescence for BrdU (green) and MVH (red) in nFGSCs (arrows) that were isolated and cultured for more than 14 days. (**f**) Representative morphology of aFGSCs or aFGSC clusters (arrows) after 7 weeks of culture. The inset shows an aFGSC undergoing mitosis. (**g**) Representative morphology of nFGSC clusters (arrows) after more than one year of culture. The insets show nFGSCs undergoing mitosis (right) and a small nFGSC cluster (left). Scale bars, 25 μm (**a**, **f**, **g**), 10 μm (**e**).

are shown in Supplementary Information, Fig. S2f. After cryopreservation and thawing, FGSCs were used successfully to re-establish cell cultures.

To characterize the nFGSC line and long-term cultured aFGSCs, we tested for the expression of Dazl (a germ cell-specific RNA-binding protein)[16], Oct4 (a germ cell-specific transcriptional factor)[17], MVH, Blimp-1 (a known transcriptional repressor)[18], Fragilis (Ifitm3, interferon-induced transmembrane protein 3; refs 19, 20), Stella (an important maternal factor)[21], c-kit (stem cell factor receptor)[22], Figla (meiosis-specific marker)[23], Sox-2 (a key transcription factor in regulating stemness related to pluripotency)[24], Rex-1 (zfp42, zinc-finger protein 42; ref. 25), Nanog (a pluripotency sustaining factor)[26], Scp (synaptonemal complex protein)[27] 1, 2, 3 and ZP3 (zona pellucida protein 3). Immunocytochemical analysis and RT–PCR showed that the cells expressed Oct4, MVH, Dazl, Blimp-1, Fragilis, Stella and Rex-1. Dual immunofluorescence analysis of BrdU incorporation and expression of MVH was also performed to determine the quality of the nFGSC line and aFGSCs. Most FGSCs were positive for BrdU and MVH (Fig. 3c; Supplementary Information, Fig. S2g). In contrast, the cells did not express c-kit, Figla, Sox-2, Nanog, Scp1-3 or ZP3 (Fig. 3a–c, h; Supplementary Information, Figs S2a, b, e, g, S3, S4). These results show that the cell lines were probably derived from germline stem cells and had undifferentiated FGSC phenotypes. The cells also showed stem cell characteristics, including high telomerase activity (Fig. 3i). Only germline stem cells express telomerase activity as it is progressively lost during germ cell differentiation[28]. Cytogenetic analysis by DAPI immunofluorescence showed that the cells had a normal karyotype (40, XX) in 65–80% (for nFGSCs) of metaphase spreads (Fig. 3f, g). In addition, the FGSC was positive for alkaline phosphatase staining ($n = 4$ experiments). However, the intensity seemed weaker than that of ES cells (Fig. 3d, e). To determine the imprinting pattern of the FGSCs, differentially methylated regions (DMRs) of two maternally imprinted regions (Igf2r and Peg 10 regions) and two paternally imprinted regions (H19 and Rasgrf1 regions) were examined by combined bisulphite restriction analysis (COBRA). The results indicate that the maternally imprinted region was partially methylated and the paternally imprinted regions were demethylated in FGSCs, showing a female imprinting pattern (Supplementary Information, Fig. S5). Collectively, the results suggest that cultured FGSCs are chromosomally normal and maintain characteristics of germline stem cells.

During physiological examination of these cells, nFGSCs that were passaged more than 45 times were infected with the MSCV–PGK–GFP virus. The aFGSCs were passaged more than 15 times and were also infected with the GFP virus. After infection and culture for one week, FGSCs were transplanted into ovaries of recipient females sterilized by pre-treatment with cyclophosphamide and busulphan to destroy the existing pre- and post-meiotic germ cell pools[6,11,29]. Two months after FGSC transplantation, ovaries were collected and the presence of oocytes was determined by their morphological appearance and expression of GFP. Histological evaluation showed that ovaries injected with FGSCs had many oocytes at all stages of development, including GFP-positive oocytes (Fig. 4c–i). However, 2 months after sterilization, immature oocytes or follicles were not found in control ovaries, which did not receive FGSC transplantation. Furthermore, the control ovaries consisted of stromal, interstitial cells and a few atretic follicles (Fig. 4a, b) indicating that chemotherapy can destroy oocytes or ovarian follicles. These results suggest that oocytes can be regenerated in sterile recipient females by transplantation of FGSCs.

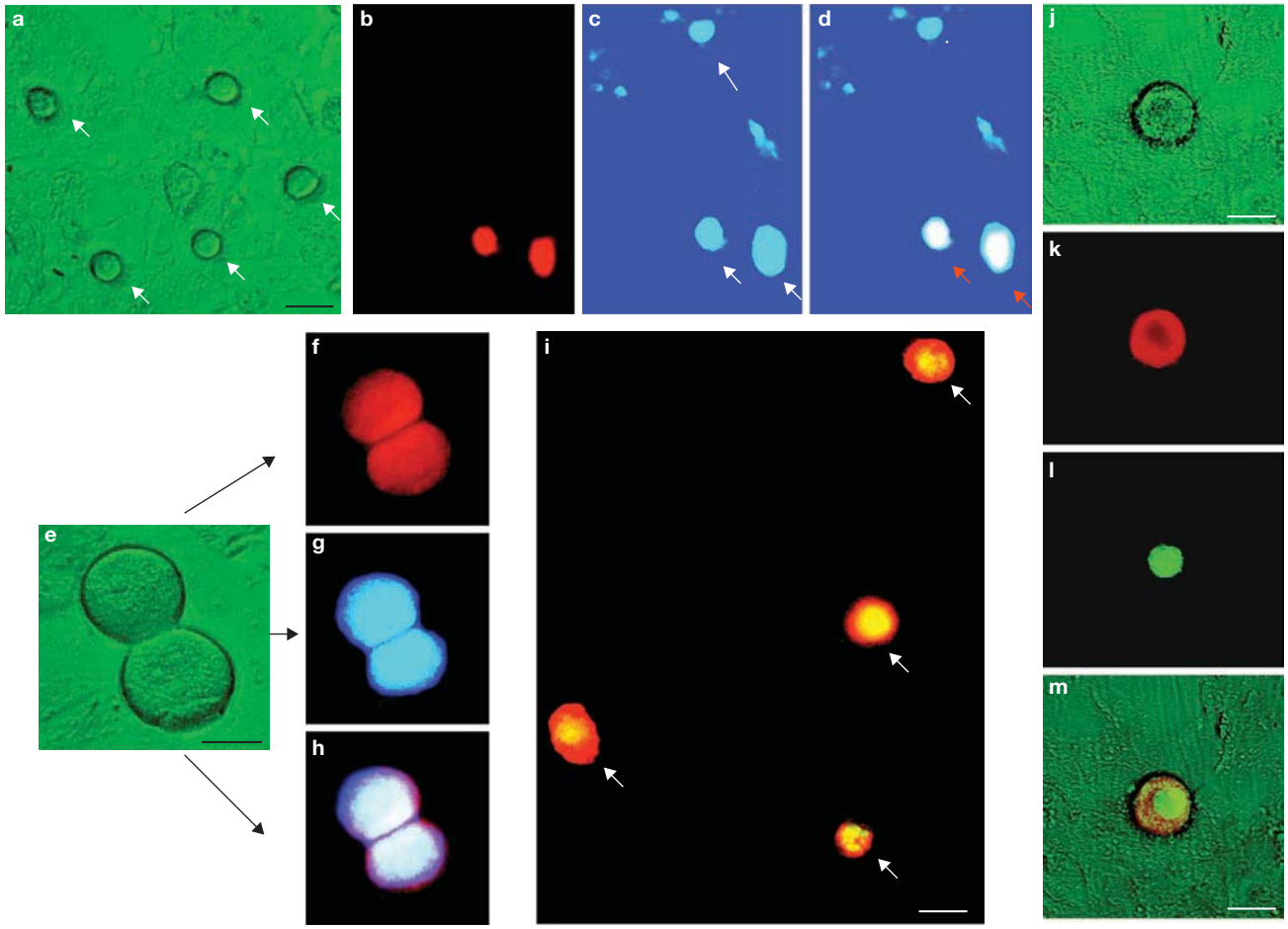

Figure 2 Isolation and culture of aFGSCs. (**a**) Morphology of FGSCs (arrows) cultured on mitomycin C-treated STO cells for 24 h after purification by immunomagnetic isolation of MVH$^+$ cells. (**b**) Representative view of BrdU immunofluorescence in isolated and cultured MVH$^+$ cells. Red, BrdU-positive cells. (**c**) DAPI immunofluorescence. Arrows indicate MVH$^+$ cells. (**d**) Merger for BrdU and DAPI immunofluorescence. Red arrows indicate BrdU-positive cells. (**e**) FGSC undergoing mitosis. (**f**, **g**) BrdU (**f**) and DAPI (**g**) immunofluorescence in the mitotic FGSC. **(h)** Merger for BrdU and DAPI immunofluorescence. (**i**) Dual immunofluorescence for BrdU (green) and MVH (red) in FGSCs (arrows) isolated and cultured for more than 14 days. (**j**) Morphology of FGSC after fixation with 4% paraformaldehyde and immunocytochemistry. (**k**) MVH immunofluorescence in the FGSC. **(l)** BrdU immunofluorescence in the FGSC. (**m**) Merger for **j**, **k** and **l**. Scale bars, 25 μm (**a**), 10 μm (**e**, **i**, **j**, **m**).

We examined whether the nFGSC line or long-term cultured aFGSCs could restore fertility when transplanted into infertile recipients. Female mice sterilized by chemotherapy were used as recipients ($n = 20$). Control females ($n = 7$) were given an intraperitoneal injection of dimethyl sulphoxide (DMSO) and had normal fertility. After 75–110 days post-transplantation, 82% (18/22) of nFGSC recipients produced offspring by natural mating with a wild-type C57BL/6 male. For aFGSCs, 80% (12/15) of recipients produced offspring (Fig. 5b). The progeny had no abnormalities and were fertile. The offspring were examined for the presence of *GFP* transgenes in their genomes using PCR techniques (Fig. 5d). Southern blot analysis using a *GFP*-specific probe (Fig. 5a) and live-imaging were also used (Fig. 5c, e). For nFGSCs, 29 of the 108 F1 offspring analysed were heterozygous for the *GFP* transgene. Twenty-four of the 85 F1 progeny from aFGSCs were GFP transgenic mice. F2 GFP transgenic progeny were also produced after the mating of F1 GFP transgenic mice, indicating that the transgene can be transmitted by the germ line. As a control, GFP retrovirus particles (~10^6 cfu ml^{-1}; 6 μl) were injected directly into ovaries of 6-week-old mice ($n = 14$); however, we failed to obtain transgenic offspring. One explanation for the inability of the virus to infect FGSCs and generate transgenic offspring is that somatic cells shield stem cells from the virus present in ovaries. The cultured cells were also injected into the subcutis of BALB/c nude mice to rule out that they were FGSCs or, for example, embryonic germ cells, and to investigate whether these cells are endowed with pluri/multipotency. After 4–8 weeks of subcutaneous injection, no teratoma formation was observed in the nude mice. These results indicate that the FGSC lines developed during this study can be used not only to produce offspring from previously sterile recipients, but also to generate transgenic progeny.

Although the general characteristics of FGSCs were similar to those of SSCs, several differences were noted. For example, the number of FGSCs per ovary was much smaller than the number of SSCs per testis. During the initial phase of culture, growth of FGSCs was slower than that of SSCs. In contrast to SSCs, which formed clumps, FGSCs formed compact clusters of cells when they proliferated. Moreover, SSCs showed a complete androgenic imprinting pattern, whereas FGSCs showed a female pattern. As with the study of SSCs, this work has important implications for gene therapy and animal mutagenesis. Using FGSCs, we are generating Dnaic2 (dynein axonemal intermediate chain 2, ref. 30) transgenic mice in addition to production of GFP transgenic mice. Our findings have implications for clinical and animal biotechnological applications related

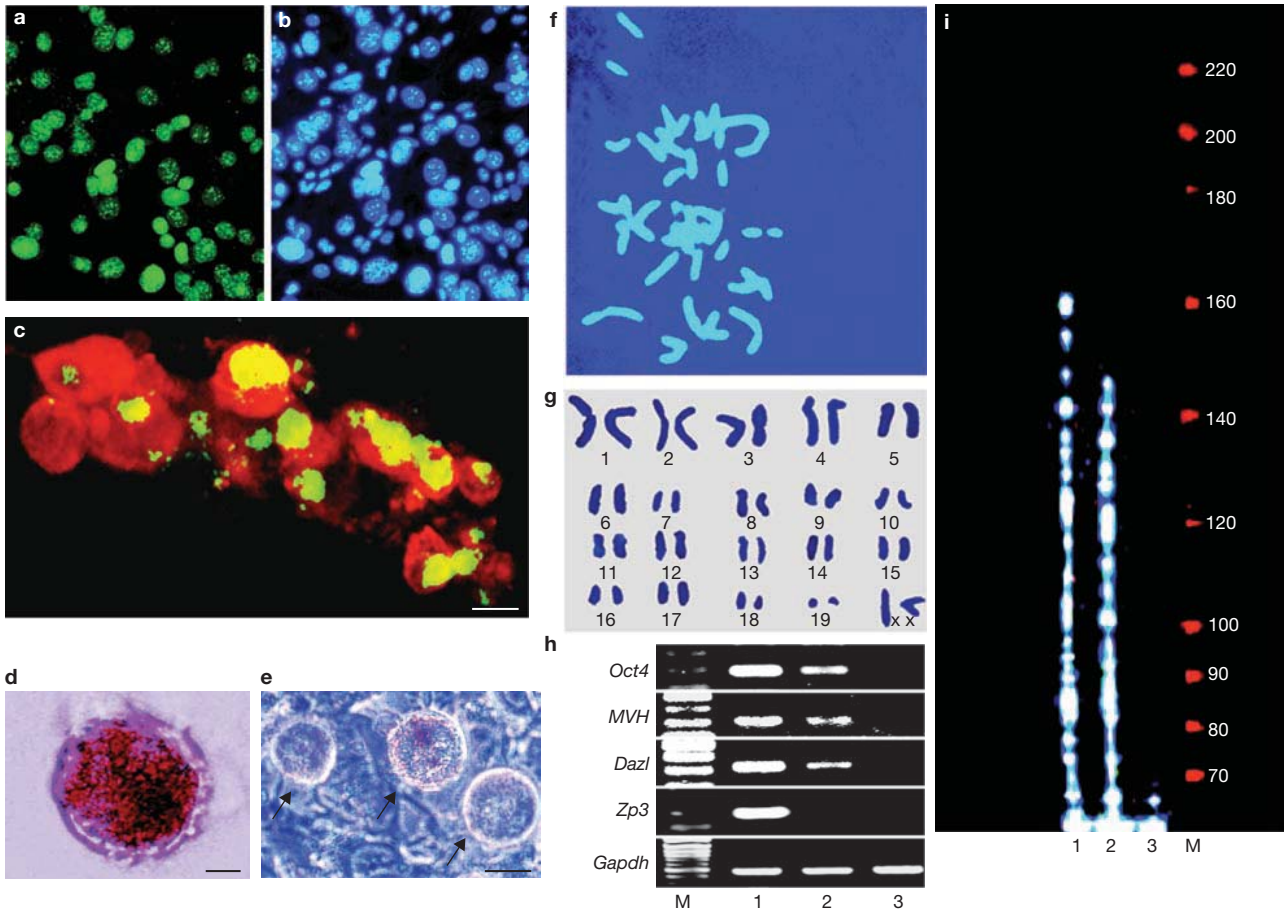

Figure 3 Characteristics of the nFGSC line. (**a**, **b**) An example of Oct4 expression in cells of the nFGSC line showing immunofluorescence of Oct4 (**a**) and DAPI (**b**). Note that nFGSCs were cultured for 1 day after passage without the formation of compact clusters of nFGSC. (**c**) Dual immunofluorescence for MVH and BrdU in long-term cultured nFGSCs in cluster. Red, MVH immunofluorescence; green, BrdU immunofluorescence. (**d**, **e**) Alkaline phosphatase staining. FGSCs (arrows, **e**) are weakly positive, whereas ES cells (**d**) are strongly positive for alkaline phosphatase. (**f**, **g**) Cytogenetic analysis by DAPI staining showed that these FGSCs had a normal karyotype (40, XX). (**h**) RT-PCR analysis of transcription factors and genes. M, 100 bp DNA marker; lane 1, ovary; lane 2, nFGSCs; lane 3, STO (negative control). The sizes of the resolved DNA fragments are (in bp): *Oct4*, 198; *MVH*, 213; *Dazl*, 328; *Zp3*, 183; *Gapdh*, 458. (**i**) TRAP assay showing that the cells had high telomerase activity. Lane 1, F9 cells (ATCC, positive control); lane 2, FGSCs; lane 3, STO (negative control); M, MRK400 Genescan ROX ladder. The data shown are representative of four experiments. Scale bars, 50 μm (**d**), 10 μm (**c**, **e**).

to the generation of new oocytes. They are also crucial to the future use of stem cells in regenerative medicine, anti-ageing medicine.

METHODS

Animals. C57BL/6 wild-type and C57BL/6×CD-1 F1 hybrid mice were used in this study. Six-week-old C57BL/6×CD-1 F1 hybrid females used as recipients were sterilized by intraperitoneal injection of busulphan (30 mg kg^{-1}, resuspended in DMSO) and cyclophosphamide (120 mg kg^{-1}). Controls were injected with DMSO. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Shanghai, and were conducted in accordance with the National Research Council Guide for Care and Use of Laboratory Animals.

Isolation of FGSCs. Between 12 and 24 ovaries from 5-day-old mice or adult mice were collected for each experiment. FGSCs were isolated using the two-step enzymatic digestion method described previously[31–34]. Dissected ovaries were placed in Hank's balanced salt solution without calcium or magnesium (HBSS) and containing collagenase (1 mg ml^{-1}, Type IV; Sigma), then incubated at 37 °C with gentle agitation for 15 min. The ovarian tissue was then washed 2 to 4 times in HBSS, placed in HBSS containing 1 mM EDTA and 0.05% trypsin, and incubated at 37 °C for 10 min. When most of the cells were dispersed, trypsin was neutralized by adding 10% fetal bovine serum (FBS). The suspension was centrifuged at 300*g* min^{-1} for 5 min and the supernatant was carefully removed from the pellet. The pellet was resuspended and clumps of cells were removed by passing the suspension through a 70-μm nylon cell strainer. These cells enriched for FGSCs were prepared by immunomagnetic isolation of MVH^+ cells. Goat anti-rabbit IgG microbeads (Miltenyi Biotec) were pre-coated with rabbit polyclonal MVH antibody (Abcam). MVH^+ cells were separated by magnetic separation, according to the manufacturer's instructions. For additional information, see Supplementary Information Methods and Fig. S6.

Culture of FGSCs. The isolated cells were cultured at a final concentration of $4–6 \times 10^2$ cells ml^{-1}. The FGSC culture system uses mitotically inactivated STO cell feeders (derived from mouse SIM embryonic fibroblasts, strain SIM, 5×10^4 cells/cm^2, ATCC). STO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high-glucose (Life Technologies), 1% non-essential amino acids (Life Technologies), 10% FBS (Life Technologies), glutamine (2 mM, Sigma), penicillin (30 mg l^{-1}, Sigma) and streptomycin (75 mg l^{-1}, Sigma) . The STO cells were first treated with mitomycin C (10 μg ml^{-1}, Sigma) for 2–3 h, then washed in PBS and plated on 0.2% (w/w) gelatin-coated wells of a 24-well plate. The culture medium for FGSCs consisted of minimum essential medium α medium (MEM-α), 10% FBS, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol (Sigma), 10 ng ml^{-1} LIF (Santa Cruz Biotechnology), 20 μg ml^{-1} transferrin, 5 μg ml^{-1} insulin (Sigma), 60 μM putrescine (Sigma), 10 ng ml^{-1} EGF (mouse epidermal growth factor; Sigma), 40 ng ml^{-1} human GDNF (glial cell line-derived neurotrophic factor; R&D systems), 1 ng ml^{-1} human bFGF (basic fibroblast growth factor; BD Biosciences

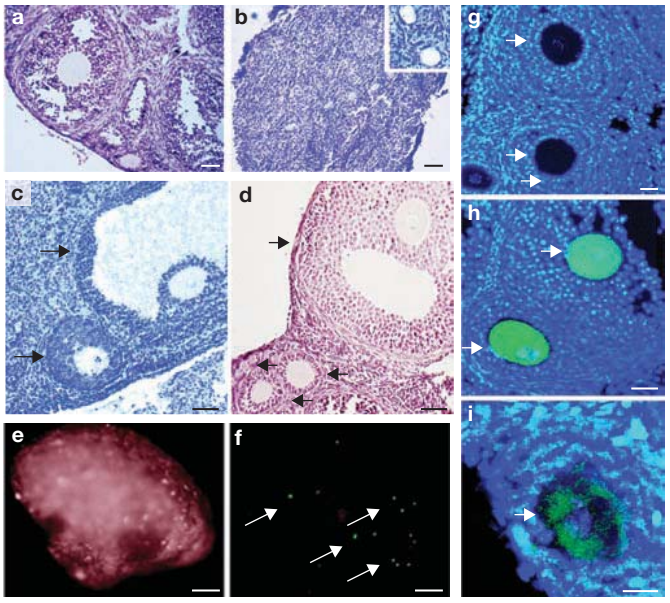

Figure 4 FGSC transplantation into recipient mice. (**a**–**d**) Representative morphology of ovaries from control females (**a**), recipients without FGSC transplantation (**b**) and recipients with FGSC transplantation (**c, d**). A large antral follicle is visible in **c** and primordial or primary follicles are visible in **d**. The inset in **b** shows atretic primordial or primary follicles. Arrows in **c** and **d** indicate follicles at various stages of maturational development. (**e**, **f**) Macroscopic appearance of a recipient ovary 2 months after transplantation with GFP-transfected FGSCs (**e**), showing fluorescence under UV light (**f**). Arrows in **f** denote oocyte-like cells. (**g**) Oocytes (arrows) in a wild-type ovary, showing a lack of GFP signal. (**h**, **i**) Follicles containing GFP-positive (green) oocytes in recipient ovaries 2 months after transplantation with GFP-transfected nFGSCs (**h**) or aFGSCs (**i**). Blue, DAPI immunofluorescence. Arrows in **h**, **i** indicate GFP-positive oocytes. Scale bars, 50 μm (**a**–**d**), 1 mm (**e**, **f**), 25 μm (**g**–**i**).

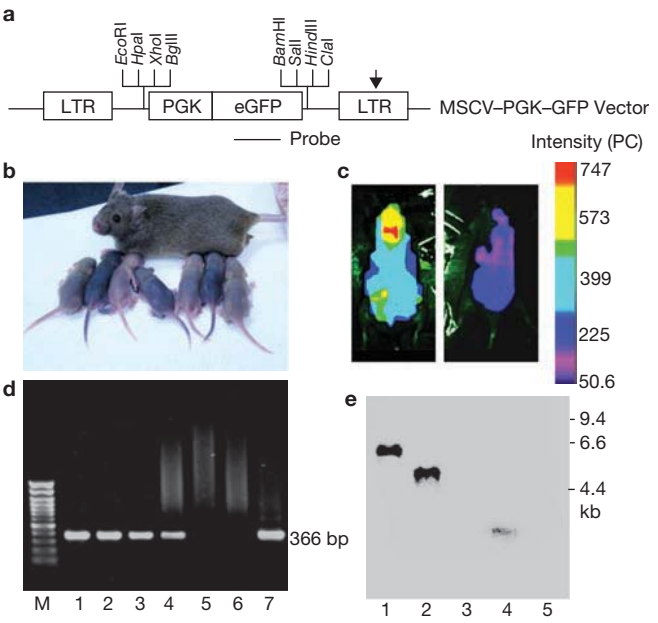

Figure 5 GFP offspring generated from the transplantation of nFGSC line or aFGSCs. (**a**) The MSCV–PGK–GFP vector carrying phosphoglycerate kinase (PGK). Arrow, self-inactivating mutation; eGFP, enhanced green fluorescent protein; LTR, Long terminal repeat. (**b**) An example of offspring from C57BL/6 × CD-1 F1 recipient mice transplanted with GFP-transfected nFGSCs that were passaged more than 45 times, or of aFGSCs that were cultured for more than 15 weeks. (**c**) The offspring were screened by live-imaging (the eXplore Optix, GE Company) for the identification of GFP offspring. GFP-positive (left) and GFP-negative (right) offspring are shown. (**d**) An example of PCR analysis of offspring. Amplification in 32 cycles of PCR was performed using Taq polymerase (Promega) with primer set (5′-CGACGGCAACTACAAGACCCG -3′ and 5′- ACGAACTCCAGCAGGACCATG -3′) specific for *GFP* gene. The PCR products were also sequenced for further confirmation of successful gene transfer. M, 100 bp DNA ladder; lanes 1–4, transgenic mouse; lanes 5, 6, wild-type mouse; lane 7, positive control (DNA from MSCV-PGK-GFP plasmid). (**e**) An example of Southern blotting of tail DNA with a probe, a 366 bp-long PCR amplified fragment from the MSCV–PGK–GFP plasmid using primers as described above for *GFP* gene (see Fig. 4a). Genomic DNA was digested with *EcoR* I. Size markers are indicated to the right of the Southern blot. Lanes 1, 2, 4, transgenic mice; lane 3, 5, wild-type mouse.

Clontech) and 15 mg l^{-1} penicillin. FGSCs were cultured on STO feeders in FGSC culture medium (500 μl per well). The medium was changed every 2–3 days and cells were subcultured using trypsin every 5–8 days at a 1:1–3 dilution. All cultures were maintained at 37 °C in a 5% CO_2 atmosphere.

FGSC cryopreservation and thawing. After detachment from the culture supports, FGSC suspensions were gently mixed with cryoprotective solution (FGSC culture medium with 10% DMSO) and stored in 2-ml freezing vials, which were placed in a plastic container and stored at 4 °C for 1 h, –20 °C for 2 h, overnight at –80 °C. The vials were then removed from the container and transferred to liquid nitrogen (–196 °C). After long-term cryopreservation, cells were rapidly thawed. Frozen-thawed cells were cultured using the FGSC culture system described above.

Retroviral infection of FGSC lines. HEK 293T cells (ATCC) were plated on 60-mm tissue culture dishes. One day later, the cells were transfected with 3 μg pVPack-GP (gag-pol-expressing vector), 3 μg pVpack-VSV-G (env-expressing vector), and 3 μg transfer plasmid, MSCV-PGK-GFP vector (provided by Il-Hoan Oh, Catholic University of Korea), using the ViraPack Transfection Kit (Stratagene). Retroviral supernatants were collected 48 h after transfection, and passed through a 0.45-μm filter. The collected retroviral supernatants were used immediately for transduction and titre determination. Titres were calculated by determining the number of GFP-positive cells as a percentage of visible NIH3T3 cells (ATCC) using fluorescence or light microscopy; the titre was about 10^6 cfu ml^{-1}. Retroviral supernatant (200 μl) and diethylaminoethyl-dextran (10 μg ml^{-1}) was added to each FGSC culture well. The 24-well plates were incubated at 37 °C for 3 h, and 200 μl FGSC culture medium was then added for 2 days.

BrdU labelling. BrdU (50 μg ml^{-1}; Sigma) was added to FGSC cultures for 5 h. The cultures were processed for immunofluorescence staining of BrdU as described below.

Preparation of ovarian tissue for analysis. Ovaries from recipient and control animals, or 5-day-old mice and adult mice, were fixed with 4% paraformaldehyde (4 °C, overnight), dehydrated through a graded series of ethanol, vitrificated in xylene, embedded in paraffin and 6-μm thick sections were mounted on slides. Before immunofluorescence staining, slides were de-waxed with xylene and a reverse graded series of ethanol. Slides used for histological analysis were stained with haematoxylin.

Karyotype analysis of FGSCs. After 3 days of FGSC passage, the cells that were cultured for more than 1 year were treated with FGSC culture medium containing colchicine (20 ng ml^{-1}) for 4 h, hypotonically treated with 40 mM KCl for 30 min, fixed in methanol-acetic acid (3:1), air-dried, stained with DAPI and viewed as described above.

Telomeric repeat amplification protocol (TRAP) assay. Telomerase activity was assayed using TRAPeze XL telomerase detection kit (Chemicon) following the manufacturer's instructions.

FGSC transplantation. The FGSC cultures were trypsinized, neutralized by 10% FBS, washed with PBS and resuspended in culture medium. Recipients were anaesthetized with sodium pentobarbitone injection (45 mg kg^{-1} body weight, intraperitoneal). A single-cell suspension of 6 μl, containing 1×10^4 cells,

was injected into each ovary of the recipients. Microinjection of each ovary was performed using a glass pipette with a 45-μm tip (Supplementary Information, Fig. S7). In addition, approximately 1×10^5 cells were subcutaneously injected into BALB/c nude mice.

Combined bisulphite restriction analysis (COBRA). The methylation status of the imprinted genes was determined by COBRA[35] using specific primers (outside, 5′-GGTTTTTTGGTTATTGAATTTTAAAAATTAG-3′ and 5′-AAAAACCATTCCCTAAAATATCACAAATACC-3′; inside, 5′-TTAGTGTGGTTTATTATAGGAAGGTATAGAAGT-3′ and 5′-TAAACCTAAAATACTCAAACTTTATCACAAC-3′ for H19; 5′-GTGTAGAATATGGGGTTGTTTTATATTG-3′ and 5′-ATAATACAACAACAACAATAACAATC-3′ for Rasgrf1; 5′-TTAGTGGGGTATTTTTATTTGTATGG-3′ and 5′-AAATATCCTAAAAATACAAACTACACAA-3′ for Igf2r; 5′-GTAAAGTGATTGGTTTTGTATTTTTAAGTG-3′ and 5′-TTAATTACTCTCCTACAACTTTCCAAATT-3′ for Peg 10). The PCR products were digested using restriction enzymes with a recognition sequence containing CpG in the original unconverted DNA. Intensity of digested DNA bands was quantified.

Immunofluorescence, TR-PCR, Southern blotting. See the Supplementary Information Methods and Table S1 for details).

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS

K.Z. and L.S. isolated and cultured FGSCs, and created the FGSC line; K.Z., Z.Yu, Y.Z. and R.H. characterized the nFGSC line and long-term cultured aFGSCs; Z.Yu, Z.Ya and Q.Y. examined the physiological function of FGSCs and the presence of GFP transgenes; H.L. and K.S. carried out COBRA; L.Z. and J.X. performed identification of FGSCs in ovaries; K.Z. participated in data analysis; J.W. planned and supervised the project, carried out FGSC transplantation, analysed data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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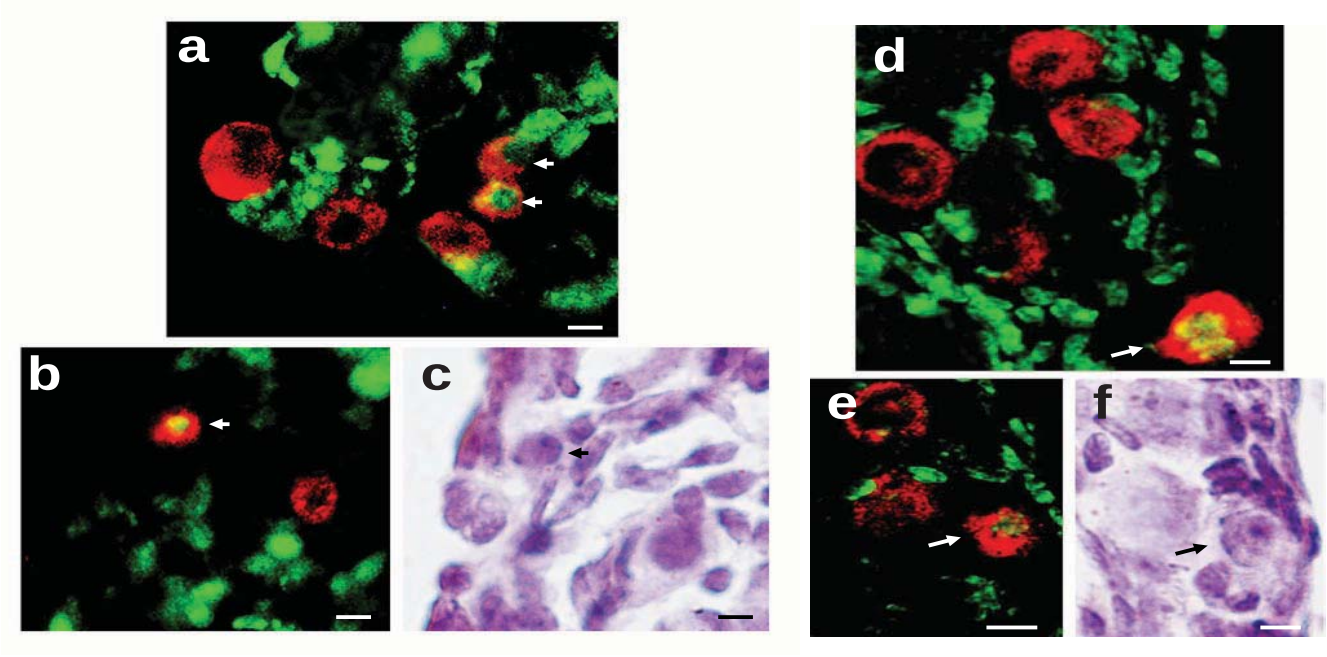

Figure S1 Putative FGSCs in day 5 and adult mouse ovaries. **(a)** Dual immunofluorescence for MVH (red) and BrdU (green) in day 5 ovary. Arrows indicate putative FGSCs in ovarian surface epithelium. **(b, c)** Dual immunofluorescence for MVH (red) and BrdU (green) (**b**) corresponding to histological analysis of the section (**c**). Arrow in (**b**) indicates putative FGSC corresponding to its morphology (arrow in **c**) in neonatal ovarian surface epithelium. **(d)** Dual immunofluorescence for MVH (red) and BrdU (green) in adult ovary. Arrow indicates putative FGSC undergoing mitosis in ovarian surface epithelium. **(e, f)** Dual immunofluorescence for MVH (red) and BrdU (green) **(e)** corresponding to histological analysis of the section **(f)**. Arrow in **(e)** denotes putative FGSC corresponding to its morphology (arrow in **f**) in adult ovarian surface epithelium. Scale bars, 10 μm.

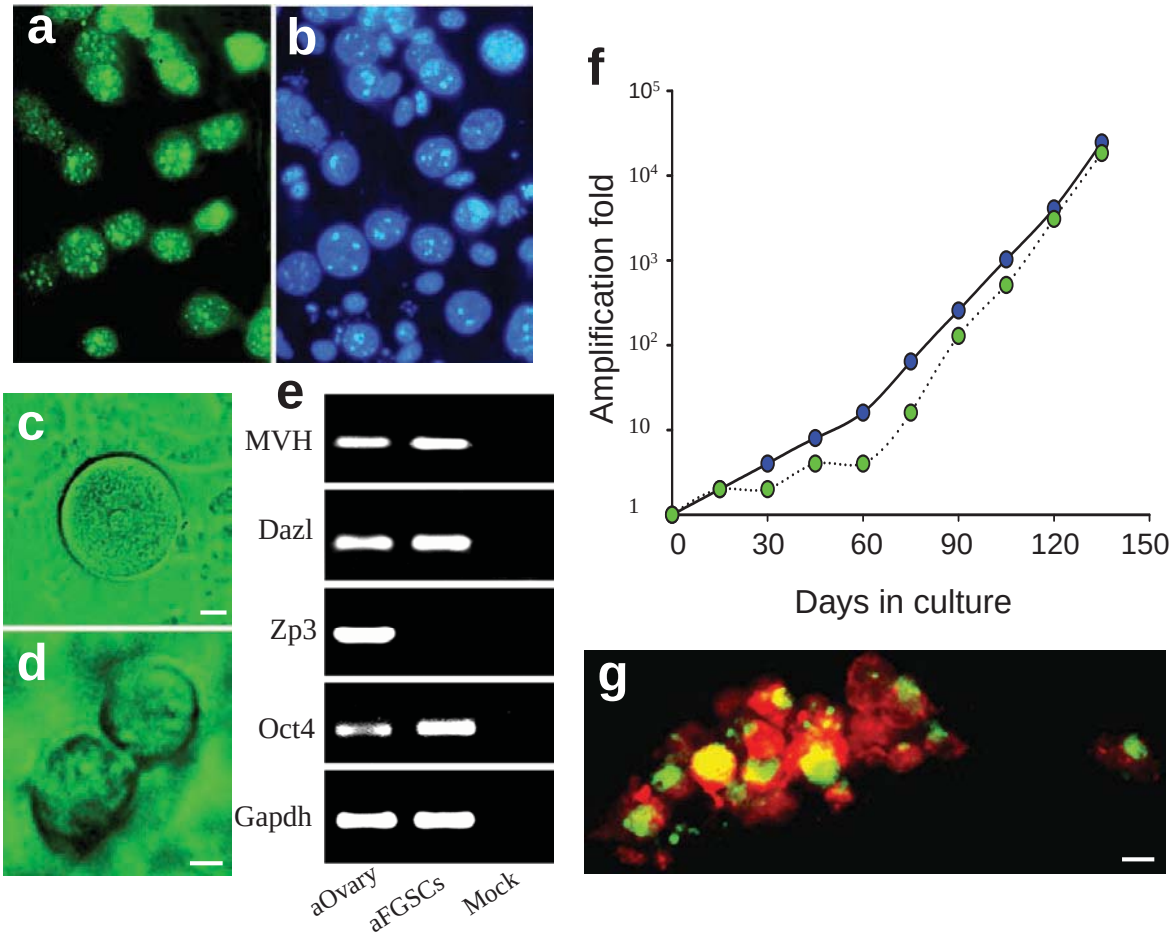

Figure S2 Characteristics of adult FGSCs (aFGSCs) and proliferation patterns of FGSC cultures. **(a, b)** An example of Oct4 expression in the aFGSCs. **(a)** Oct4 immunofluorescence. **(b)** DAPI immunofluorescence. **(c, d)** Comparison of the morphology between oocytes in prophase I of meiosis and FGSCs. **(c)** The morphology of oocyte in prophase I of meiosis. **(d)** The morphology of FGSCs. **(e)** RT-PCR analysis of transcription factors and genes for the cultured aFGSCs. The sizes of the resolved DNA fragments (in bp): Oct4, 198; MVH, 213; Dazl 328; Zp3, 183; Gapdh, 458. Ovary from adult mice (aOvary). Mock-transcribed aFGSC RNA samples (Mock). **(f)** Proliferation patterns of FGSC cultures. Note the slow growth of FGSCs during the initial phase of cultures. Blue: nFGSCs; Green: aFGSCs. **(g)** An example of dual immunofluorescence for MVH (red) and BrdU (green) in cultured aFGSCs. Scale bars, 5 μm.

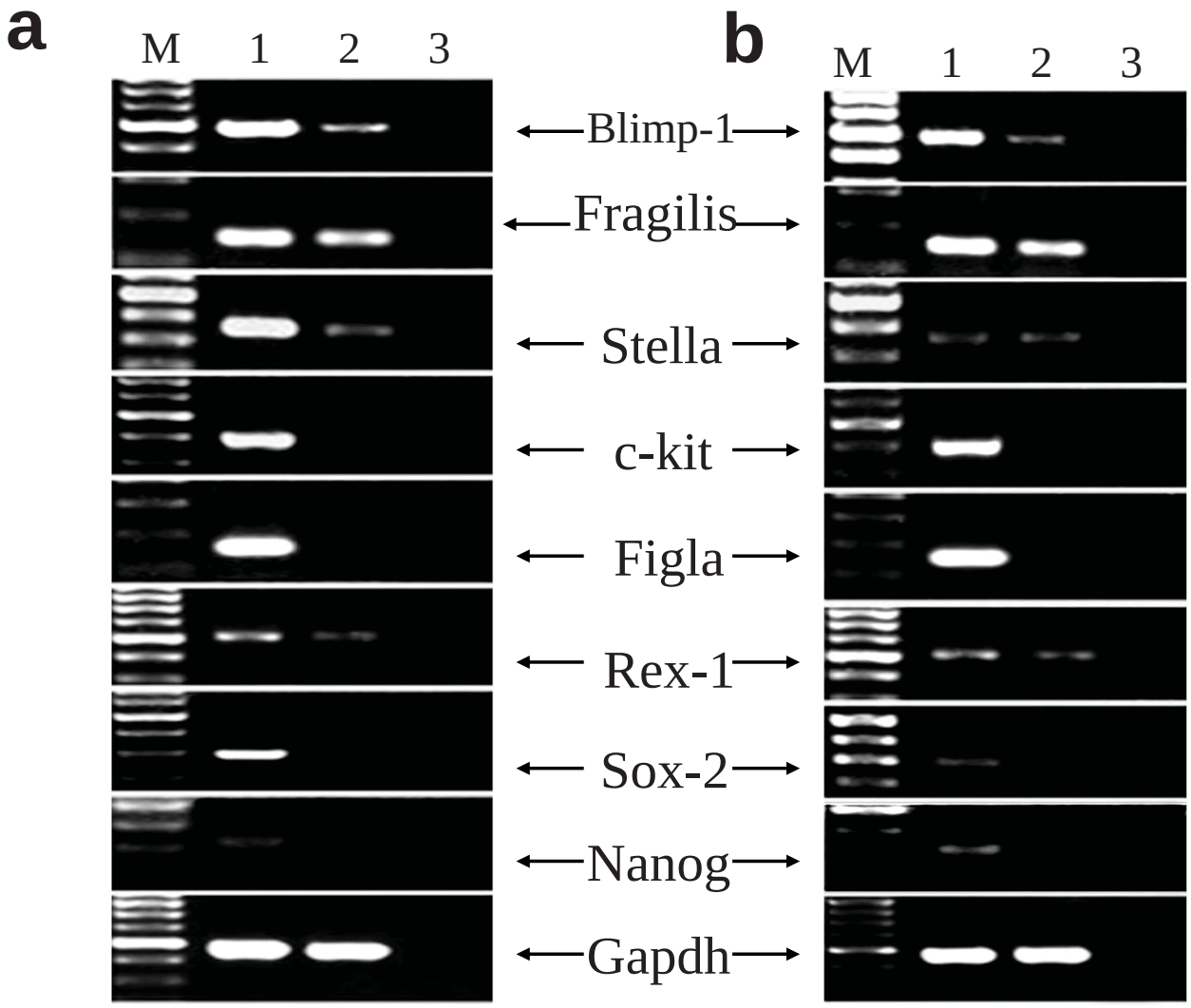

Figure S3 The gene expression profiles for neonatal FGSC (nFGSC) line and the cultured aFGSCs. **(a)** RT-PCR analysis for nFGSC line. M, 100bp DNA marker; 1ane 1, neonatal ovary; lane 2, nFGSCs; lane 3, mock-transcribed nFGSC RNA samples. **(b)** RT-PCR analysis for the cultured aFGSCs. M, 100bp DNA marker; lane 1, adult ovary; lane 2, aFGSCs; lane 3, mock-transcribed aFGSC RNA samples. The size of the resolved DNA fragments (in bp): Blimp-1, 483; Fragilis, 151; Stella, 354; c-kit, 401; Figla, 154; Sox-2, 297; Rex-1, 504; Nanog, 223.

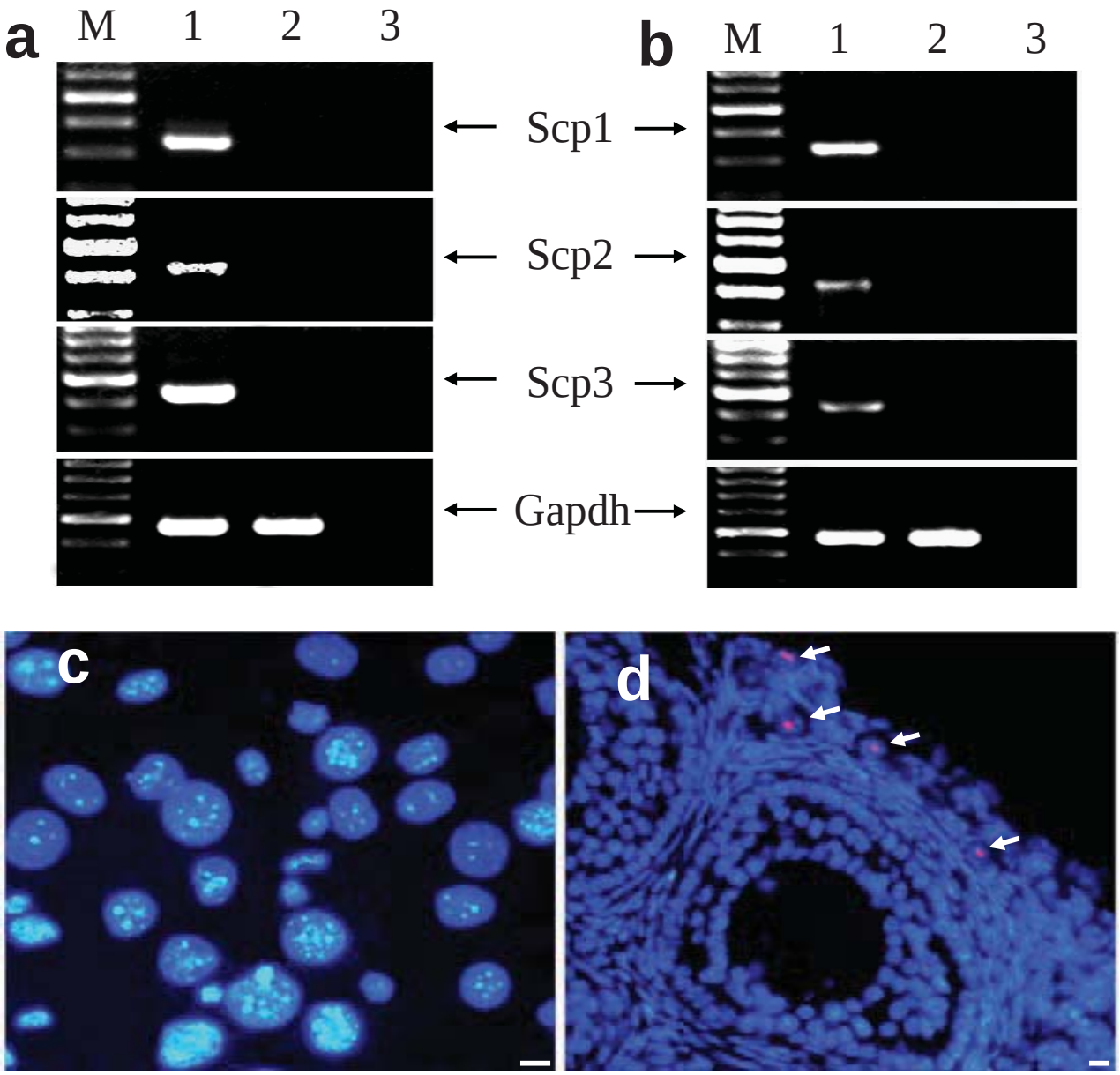

Figure S4 Expression of Scp1, Scp2 and Scp3 in nFGSC line and the cultured aFGSCs. **(a)** RT-PCR analysis for nFGSC line. M, 100bp DNA marker; lane 1, neonatal ovary; lane 2, nFGSCs; lane 3, mock-transcribed nFGSC RNA samples. **(b)** RT-PCR analysis for the cultured aFGSCs. M, 100bp DNA marker; lane 1, adult ovary; lane 2, aFGSCs; lane 3, mock-transcribed aFGSC RNA samples. The size of the resolved DNA fragments: Scp1, 342bp; Scp2, 428bp; Scp3, 437bp. **(c, d)** Immunofluorescence detection of SCP3 in the cultured aFGSCs. **(c)** Merger for SCP3 (red) and DAPI immunofluorescence (blue). The aFGSCs in the cultured cells show a lack of SCP3 signal. Note the aFGSCs were cultured for 1 day after passage without the formation of compact clusters of aFGSCs. **(d)** Merger for SCP3 (red) and DAPI immunofluorescence (blue). Arrows denote SCP3-positive oocytes in the juvenile mouse ovary. Scale bars, 5 μm (**c**), 10 μm (**d**).

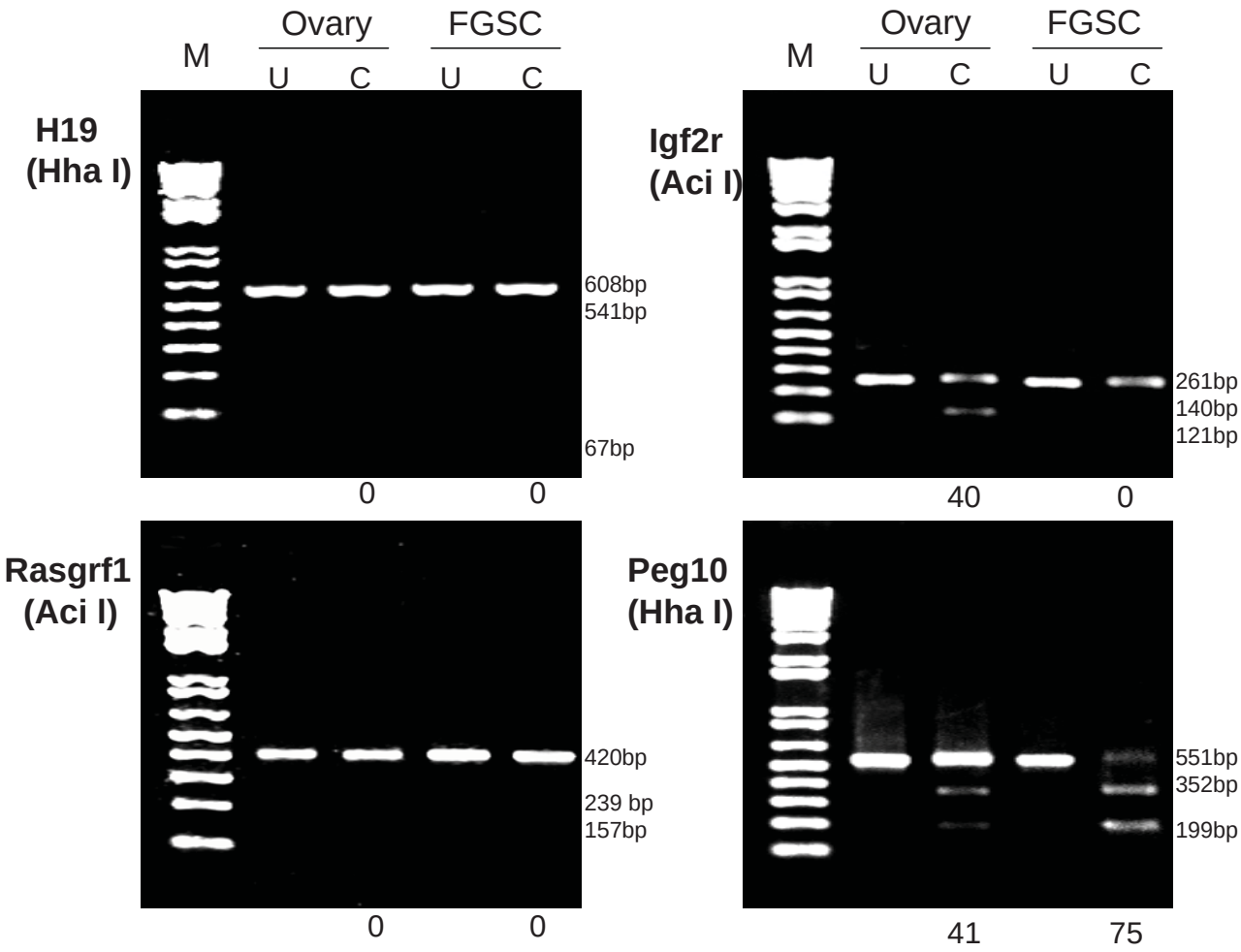

Figure S5 Combined bisulfite restriction analysis (COBRA) of imprinted genes. DNA was isolated from the cultured aFGSCs or the juvenile mouse ovaries. The percentage of DNA methylation is indicated below each lane. Restriction enzymes used to cleave each locus are shown in parentheses. M, 1kb plus DNA ladder; u, uncleaved; c, cleaved.

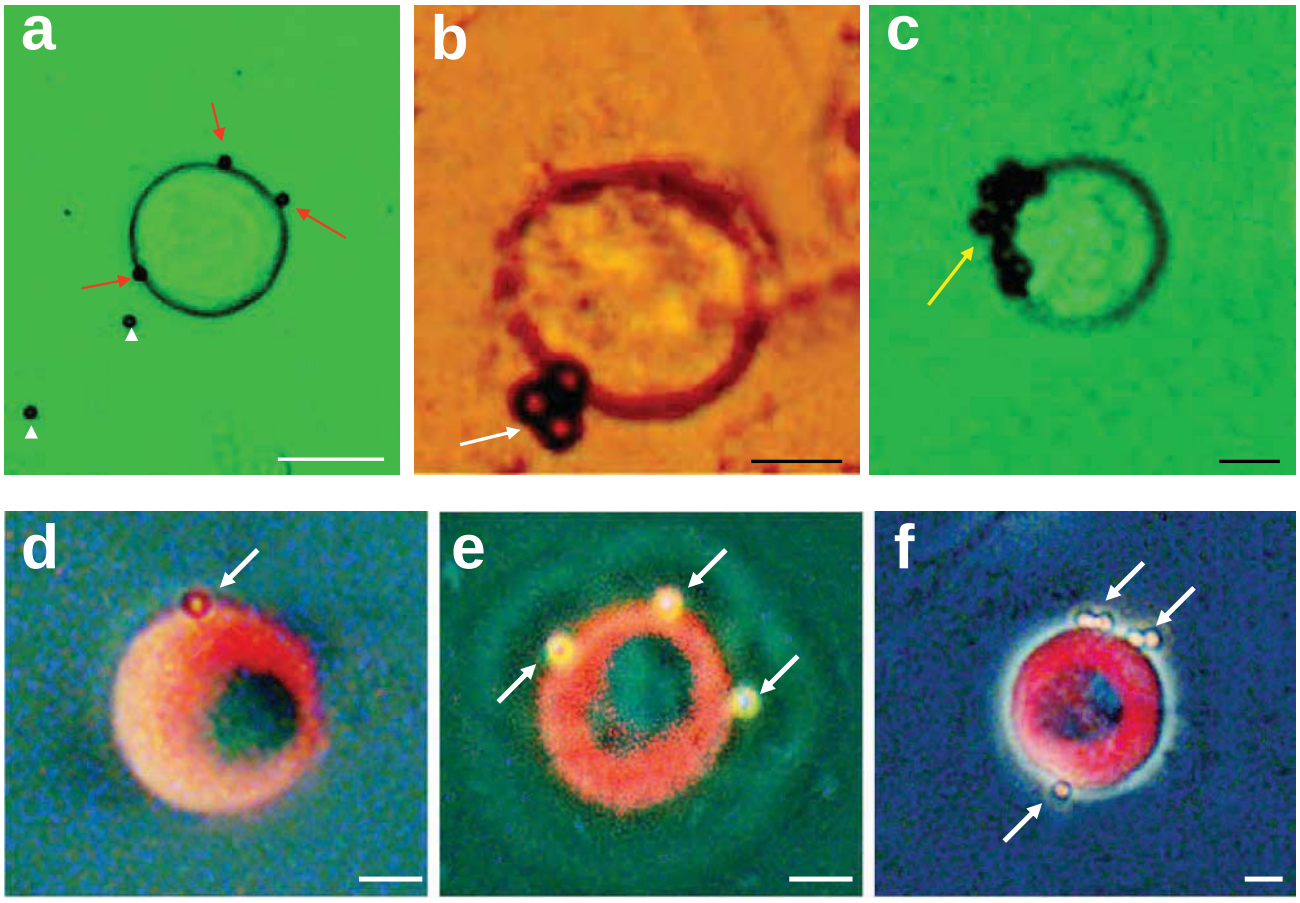

Figure S6 FGSCs or female germ cells after immunomagnetic isolation using a MVH antibody and Immunofluorescence detection of MVH in isolated FGSCs or female germ cells. **(a, b)** Examples for FGSCs or female germ cells isolated with MVH antibody and associated pattern of female germ cells with immunomagnetic beads. **(c)** An example of the associated pattern of the beads with the cells with increasing concentration. Associated sites of the cells with beads were not significantly increased when concentration of beads was increased. Arrows indicate associated beads. Arrowheads are free beads. Scale bars, 10 μm (**a**), 5 μm (**b, c**). **(d, e, f)** Merger for MVH immunofluorescence and the morphology of FGSCs or female germ cells after fixation with 4% paraformaldehyde and immunocytochemistry. FGSCs or female germ cells are still associated with the magnetic beads (arrows). Scale bars, 5 μm.

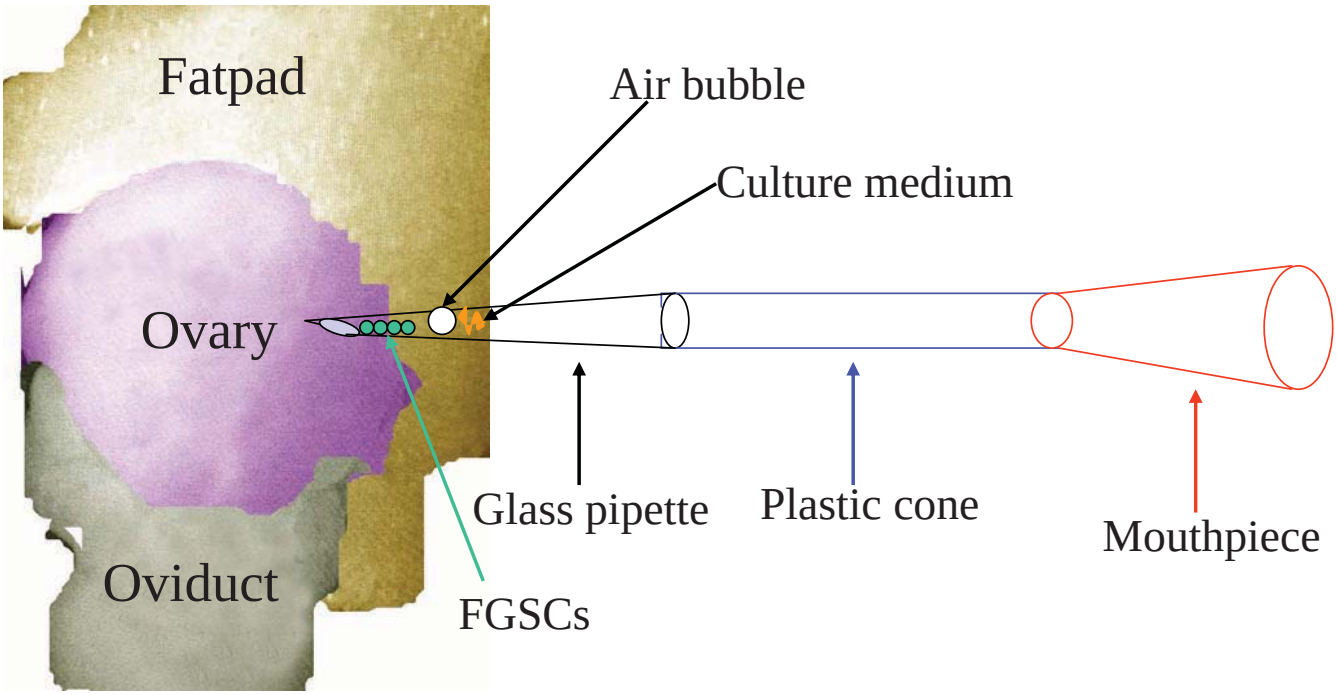

Figure S7 Microinjection of each ovary for FGSC transplantation using a glass pipette.

Supplementary Table 1 Details Regarding the RT-PCR Analysis of Mouse Cells and Tissues

Gene	Accession number	Product Size(bp)	Primer Sequence (5'-3')[1]	Region Amplified
Dazl	NM_010021	328	F: GTGTGTCGAAGGGCTATGGAT	420-747
			R: ACAGGCAGCTGATATCCAGTG	
Gapdh	NM_008084	458	F: GTCCCGTAGACAAAATGGTGA	37-494
			R: TGCATTGCTGACAATCTTGAG	
Mvh	NM_010029	213	F: GGAAACCAGCAGCAAGTGAT	520-732
			R: TGGAGTCCTCATCCTCTGG	
Oct4	NM_013633	198	F: AGCTGCTGAAGCAGAAGAGG	492-689
			R: GGTTCTCATTGTTGTCGGCT	
Zp3	M20026	183	F: CCGAGCTGTGCAATTCCCAGA	79-261
			R: AACCCTCTGAGCCAAGGGTGA	
Blimp-1	NM_007548	483	F: CGGAAAGCAACCCAAAGCAATAC	847-1329
			R: CCTCGGAACCATAGGAAACATTC	
C-kit	NM_001122733	401	F: CTGGTGGTTCAGAGTTCCATAGAC	1488-1888
			R: TCAACGACCTTCCCGAAGGCACCA	
Figla	NM_012013	154	F: CCAAAGAGCGTGAACGGATAA	251-404
			R: TCTTCCAGAACACAGCCGAGT	
Fragilis	NM_025378	151	F: GTTATCACCATTGTTAGTGTCATC	454-604
			R: AATGAGTGTTACACCTGCGTG	
Nanog	AK010332	223	F: CAGGAGTTTGAGGGTAGCTC	1915-2137

			R: CGGTTCATCATGGTACAGTC	
Rex-1	NM_009556	504	F: CACCATCCGGGATGAAAGTGAGAT	561-1064
			R: ACCAGAAAATGTCGCTTTAGTTTC	
Scp1	NM_011516	342	F: CCTCATCAATGGATAGTGGCAAATCC	2654-2995
			R: GAGTTGATAGCGATAAAGGAGTCTGT	
Scp2	NM_177191	428	F: ACACTCCAGATGGGCATGTT	1947-2374
			R: AAGGGCTTTTATCACAGATAGT	
Scp3	NM_011517	437	F: GAGCCGCTGAGCAAACATCTA	125-561
			R: ATATCCAGTTCCCACTGCTGC	
Sox-2	NM_011443	297	F: TAGAGCTAGACTCCGGGCGATGA	1768-2064
			R: TTGCCTTAAACAAGACCACGAAA	
Stella	AY082485	354	F: CCCAATGAAGGACCCTGAAAC	126-479
			R: AATGGCTCACTGTCCCGTTCA	

1 [1]F, Forward primer; R, reverse primer.

Supplementary Information

Methods

Additional information for immunomagnetic isolation of mouse vasa homologue (MVH) positive cells

MVH is a germline marker expressed in the testis and the ovary. Furthermore, MVH is expressed in spermatogenic cells from the spermatocyte stage to the round spermatid stage and in migratory primordial germ cells in the region of the gonadal ridge in both sexes. Unlike in the testis, Northern analysis of MVH in ovary showed alternative splicing[1]. A study from Toyooka et al. indicated that the strongest expression of MVH was detected in primordial oocytes of adult mouse and the expression appeared to decrease in amount as maturation proceeded[2]. Finally, anti-MVH staining disappeared completely in mature oocytes of antral follicles. However, Castrillon et al. reported that MVH is most abundant in mature oocytes. Though some studies indicated that MVH expressed in cytoplasm of germ cells[1, 2, 3]. Bioinformatics from website http://www.ch.embnet.org/cgi-bin/TMPRED_form-parser show that there are two strong transmembrane helices in MVH protein. Based upon the information above, we tried to enrich FGSCs by immunomagnetic isolation using MVH antibody. To show an associated pattern of immunomagnetic beads and germ cells, we used anti-rabbit IgG beads from Dynal Biotech with 2.8 μm particle size for isolation (see Supplementary Information, Fig. S6a-c). Further, these isolated cells were identified and confirmed as germ cells by their expression of MVH using immunocytochemistry (see Supplementary

Information, Fig. S6d-f). For culture, we used anti-rabbit IgG microbeads from Miltenyi Biotec for isolation.

Immunofluorescence

After equilibration in phosphate-buffered saline (PBS), slides were digested with 0.125% trypsin for 15 min at 37 °C, and then washed twice with PBS. The slides were blocked in 10% normal goat serum at room temperature for 10 min then incubated overnight at 4 °C with rabbit polyclonal anti-GFP (dilution 1:200; Chemicon, Temecula, CA, USA) or SCP3 (dilution 1:200; Novus Biologicals, Littleton, CO, USA). After washing in PBS, the slides were incubated at 37 °C for 30 min with fluorescein isothiocyanate (FITC) conjugated secondary antibody (goat anti-rabbit IgG, 1:400 dilution; Sino-American Biotechnology Co.), then incubated at 37 °C for 20 min with DAPI (dilution 1:2000). The slides were then covered with mounting medium (glycerol diluted 3:1 in PBS). Images were obtained by a Nikon Eclipse E600 microscope and a Nikon Dxm 1200 digital camera, using fluorescein optics for FITC and ultraviolet optics for DAPI.

FGSC cultures were fixed with 4% paraformaldehyde (15 min, room temperature). After fixation, the FGSCs were incubated in blocking solution (10% normal goat serum in PBS, 60 min, room temperature), followed by rinsing and overnight incubation at 4 °C with appropriate primary antibodies; rabbit polyclonal anti-MVH (1:200 dilution; Abcam), rabbit polyclonal anti-Oct4 (1:250 dilution; Chemicon), or mouse monoclonal anti-BrdU (1:200 dilution; Lab Vision Corporation). After rinsing, FGSCs were incubated in darkness with FITC conjugated secondary antibody (goat anti-rabbit IgG,

1:400 dilution, or rabbit anti-mouse IgG, 1:400 dilution), then rinsed, mounted in 4',6-diamidino-2-phenylindole (DAPI)-containing medium, and viewed as described above.

RT-PCR

Total RNA was extracted from each sample using Trizol reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Approximately 2 μg of RNA was reverse transcribed using 20 U of M-MuLV reverse transcriptase following the manufacturer's recommended protocol (MMLV First-strand cDNA Synthesis Kit; Qiagen). The reverse transcriptase was mixed with 0.5 μg Oligo$(dT)_{18}$ primer, 0.02 μM of each deoxynucleoside triphosphate (dNTP), 4 μl 5× Reaction Buffer and 20 U RNase Inhibitor to a total volume of 20 μl. After initial denaturation of the mRNA for 5 min at 70 °C, DNA complementary to the RNA (cDNA) first strand was synthesized for 60 min at 37 °C. The reaction was stopped by incubation at 70 °C for 10 min. Products were stored at –30 °C. One-tenth of the reverse transcription reaction was used for PCR. Amplification via 28–35 cycles of PCR was performed using Taq polymerase (Promega) with primer sets specific for each gene (see Supplementary Information, Table 1). The glyceraldehydes-3-phosphate dehydrogenase gene (GAPDH) was amplified for each sample, and used as a loading control. All PCR products were isolated, subcloned, and sequenced for confirmation of gene sequence.

Southern blotting

Genomic DNA was extracted from mouse tails with the DNeasy Tissue Kit, according to the manufacturer's instructions (Qiagen), and digested with EcoR I. Samples were loaded

onto 0.7% agarose gel, along with molecular weight markers, and run at 35 V for 10 h. After depurination, denaturation and neutralization as described by Wu et al.[4], DNA was transferred by capillary action to a nylon membrane (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). Hybridization was carried out with a probe (see Fig. 5a). Prehybridization, hybridization and subsequent washings were performed as previously described [4].

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