Production of fat-1 transgenic rats using a post-natal female germline stem cell line

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ABSTRACT: Germline stem cell lines possess the abilities of self-renewal and differentiation, and have been established from both mouse and human ovaries. Here, we established a new female germline stem cell (FGSC) line from post-natal rats by immunomagnetic sorting for Fragilis, which showed a normal karyotype, high telomerase activity, and a consistent gene expression pattern of primordial germ cells after 1 year of culture. Using an in vitro differentiation system, the FGSC line could differentiate into oocytes. After liposome-based transfection with green fluorescent protein (GFP) or fat-1 vectors, the FGSCs were transplanted into the ovaries of infertile rats. The transplanted FGSCs underwent oogenesis, and the rats produced offspring carrying the GFP or fat-1 transgene after mating with wild-type male rats. The efficiency of gene transfer was 27.86–28.00%, and 2 months was needed to produce transgenic rats. These findings have implications in biomedical research and potential applications in biotechnology.

Key words: cell culture / cell proliferation / germ cells / animal model

Introduction

Germ cells are the founder cells of all sexually reproducing organisms. In mammals, the male can produce sperm throughout its entire lifetime. Thus, spermatogonial stem cells (SSCs) were recognized and confirmed very early (de Rooij and Kramer, 1968). At present, the techniques have greatly improved for isolation, purification and culture of SSCs in vitro and SSC transplantation in vivo. However, in most mammalian species, a long-persisting belief in reproductive and developmental biology is that germ line stem cells only exist in post-natal or adult testis of males, and production of ovarian oocytes is thought to cease before birth (Zuckerman, 1951; Borum, 1961; Peters, 1970; McLaren, 1984; Anderson and Hirshfield, 1992). In 2004, oogenesis in post-natal mammalian ovaries became a controversial issue, because of one line of evidence indicating an ovarian regenerative activity in juvenile and adult mice by estimation of follicle numbers and death (atresia) rates in mouse ovaries (Gosden, 2004; Johnson et al., 2004; Telfer et al., 2005; Egan et al., 2006). Recently, we demonstrated that female germ line stem cells (FGSCs) from neonatal and adult mouse ovaries can be successfully isolated and purified using immunomagnetic sorting for mouse vasa homolog (MVH), and also located these FGSCs on the cortical surface of ovaries immediately below the ovarian surface epithelium. Furthermore, a FGSC line was established from neonatal mice, which had been cultured for more than 15 months with a normal karyotype and high telomerase activity. Adult mouse FGSCs have also been cultured for more than 6 months. FGSCs in long-term culture maintain their capacity to produce normal oocytes and fertile offspring after transplantation into ovaries (Zou et al., 2009). To improve the purification efficiency, several germ cell-specific markers have been screened. The results have shown that the efficiency of FGSC purification was remarkably enhanced using the germ-line-specific protein Fragilis, compared with that using MVH (Zou et al., 2011). In addition, we have successfully generated transgenic or gene knockdown mice using FGSCs (Zhang et al., 2011). The efficiency of gene transfer or gene knockdown was 29–37%, and 2 months was needed to produce transgenic offspring. Pacchiarotti et al. (2010) and Hu et al. (2012) also demonstrated the existence of a population of germ line stem cells in post-natal mouse ovaries. Using fluorescence-activated cell sorting for MVH, White et al. (2012) successfully isolated and purified FGSCs from adult mice and reproductive-age women. However, the existence of a population of germ line stem cells in post-natal rat ovaries remains to be explored.

The fat-1 gene of Caenorhabditis elegans encodes an n − 3 fatty acid desaturase that converts n − 6 to n − 3 fatty acids. Meat products normally contain large amounts of n − 6 fatty acids and small amounts
of n — 3 fatty acids, but only balanced ratio of n — 6 fatty acids and n — 3 fatty acids are beneficial to human health (Hibbeln et al., 2006). Diets with a ratio of n — 6/n — 3 fatty acids, and especially those with a deficiency of n — 3 fatty acids, may result in an increased risk of many diseases, such as coronary artery diseases, diabetes, cancer, obesity, hypertension and psychiatric disorders (Leaf and Weber, 1987; Simopoulos, 2000; Kang, 2005). Moreover, mammals cannot convert n — 6 fatty acids into n — 3 fatty acids because of the absence of the n — 3 fatty acid desaturase gene (Simopoulos, 1998). After the first fat-1 transgenic mice were produced, transgenic pigs and cattle have also been generated to satisfy the need for n — 3 polyunsaturated fatty acids in a healthy diet (Lai et al., 2006; Guo et al., 2011). Furthermore, numerous studies of fat-1 transgenic mouse models have shown that n — 3 fatty acids are involved in many biological processes such as cell proliferation, growth, apoptosis and signaling transduction, which can profoundly affect the physiological activity and pathological processes of the body through different mechanisms (Xia et al., 2006; Schmocker et al., 2007; Das and Puskas, 2009; He et al., 2009; Mayer et al., 2009; Smith et al., 2010; Bousquet et al., 2011). Considering the key roles of rat models in pathology and biomedicine, we introduced the fat-1 gene into a new research model, which is more relevant to humans than mouse models, and established fat-1 transgenic rats for the first time. Furthermore, we demonstrated that FGSCs are an effective tool for animal gene manipulation.

Materials and Methods

Animals

Six-week-old female SD rats were sterilized by intraperitoneal injection of busulfan (20 or 40 mg/kg body weight) and cyclophosphamide (100, 120 or 140 mg/kg body weight) that were dissolved in dimethyl sulfoxide (DMSO), and then used as recipients to explore the optimal dose (Supplementary data, Table SI). Controls were treated with DMSO alone. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Shanghai, and were performed in accordance with the National Research Council Guide for Care and Use of Laboratory Animals.

Isolation and culture of rat FGSCs

Twenty ovaries were harvested from 5-day-old female rats. FGSCs were isolated using a two-step enzymatic digestion method that has been described previously (Zou et al., 2009). After digestion, the ovaries were placed in Hank’s balanced salt solution (HBSS) without calcium or magnesium and containing collagenase IV (1 mg/ml; Sigma), followed by incubation at 37°C with gentle agitation for 25 min. Then, the ovarian tissues were centrifuged, 5 min × 300 g, and washed twice. The ovarian tissues were placed in HBSS containing 1 mM EDTA and 0.05% trypsin at 37°C with gentle agitation for 5 — 10 min. After the ovarian tissues were digested and most of the cells were dispersed, 10% fetal bovine serum (FBS) was added to stop the digestion. After centrifugation for 5 min at 300g, the supernatant was removed and the pellet was resuspended with HBSS. FGSCs were obtained by immunomagnetic sorting of Fragilis + cells. The Fragilis + cells were separated by goat anti-rabbit IgG microbeads (Dynal Biotech) precoated with a rabbit anti-Fragilis polyclonal antibody (AbI5592; Abcam) as described previously (Zou et al., 2011). The cells isolated by immunomagnetic sorting were cultured on mitotically inactivated STO mouse embryonic fibroblast feeder cells (ATCC) in minimum essential medium-a (Life Technologies) supplemented with 10% FBS, 1 mM non-essential amino acids, 2 mM L-glutamine (Sigma), 0.1 mM β-mercaptoethanol (Sigma), 15 mg/L penicillin, 10 ng/ml recombinant mouse leukemia inhibitory factor (LIF; Santa Cruz Biotechnology), 10 ng/ml mouse epidermal growth factor (EGF) (Sigma), 40 ng/ml human glial cell line-derived neurotrophic factor (R&D systems) and 2 ng/ml human basic fibroblast growth factor (bFGF; BD Biosciences Clontech). The medium was changed every 2 days, and the cells were passaged at a 1:1 — 3 split ratio every 6 — 7 days by enzymatic dissociation with 0.1% trypsin. The STO cells were pretreated with mitomycin C (10 µg/ml Sigma) for 2 — 3 h, washed in phosphate-buffered saline (PBS), and then plated in 0.1% (w/v) gelatin-coated 48-well plates. All cultures were carried out in a humidified environment at 37°C with 5% CO2 in air.

Liposomal transfection of rat FGSCs

At 1 day before transfection, rat FGSCs were seeded into four 35-mm culture dishes containing a mitotically inactivated STO feeder layer. Then, 1 µg MSCV-PGK-GFP vector (Zou et al., 2009; provided by II-Hoan Oh, Catholic University of Korea) or 1 µg pCAGGS vector (Kang et al., 2004) was transfected into ∼1 × 105 cells using PolyJetTM In Vitro DNA Transfection Reagent (SignaGen Laboratories) according to the manufacturer’s instructions. The cells were incubated in transfection medium for 12 — 18 h, and were then cultured in FGSC culture medium.

rat FGSC transplantation

Recipient rats were anaesthetized by intraperitoneal injection with 7% chloral hydrate. The transfected FGSCs were collected, washed twice in PBS, and then resuspended in PBS. A 10 µl cell suspension containing ∼1 × 106 cells were injected into each ovary through a glass pipette with a 45 µm tip (Wang et al., 2013). The supernatant from the second wash of the cells was collected as a washing buffer and injected into each ovary for the control.

In vitro differentiation

First, STO feeders were removed from FGSC cultures. To obtain STO-free FGSC cultures, the cells were plated in a culture dish precoated with 0.1% (w/v) gelatin to remove the STO cells by the differential adherence of STO and FGSCs. After 30 min, most STO cells had attached to the dish, the non-adherent cells were collected in culture medium without growth factors. Then, the cells were cultured in hanging drops at 20 µl per drop. After 2 days, the cells were transferred to Petri dishes to avoid attachment, and cultured for 1 day. Next, the cells were collected and resuspended in medium containing 12.5 ng/ml bFGF (PeproTech), 10 ng/ml bone morphogenetic protein (BMP)4 (PeproTech) and 2 µM RA (Sigma), and then co-cultured with mitomycin C-treated granulosa cells for 4 — 5 days. The medium was changed every 2 days. The granulosa cells were isolated from the ovaries of juvenile rats, treated with mitomycin C (10 µg/ml; Sigma) for 2 — 3 h, washed in PBS, and then plated in a culture dish precoated with 0.1% (w/v) gelatin. Last, the third stage, EGF (10 ng/ml), bFGF (12.5 ng/ml), transferrin (5 µg/ml), insulin (10 µg/ml), pregnant mare serum gonadotrophin (PMSG) (1 IU/ml), hCG (1 IU/ml), estrogen (1 ng/ml), progesterone (1 ng/ml) and the ovarian homogenate prepared from adolescent rat ovaries were added to the co-culture system, which was changed every 2 days. Every 6 days, the cells were transferred onto a freshly treated granulosa cell layer. This process was performed for 20 — 25 days.

Karyotypic analysis of FGSCs

On the third day after passing, cells cultured for 6 months were treated with colcemid at a final concentration of 0.1 µg/ml for 3 h. The cells were collected, resuspended in 5 ml of a pre-warmed hypotonic solution (75 mM KC1), and then incubated at 37°C for 15 min. Then, the cells were fixed in freshly prepared solution of methanol:acetic acid (3:1). Cell suspensions were smeared onto glass slides, air-dried, stained with 4′,6-diamidino-2-phenylindole
Telomeric repeat amplification protocol assay

Telomerase activity was assayed by telomeric repeat amplification protocol (TRAP) – PCR using a TRAPeze® Telomerase Detection Kit purchased from Chemicon (Millipore). All processes were performed according to the manufacturer’s instructions.

Combined bisulfite restriction analysis

Specific primers were designed to determine the methylation status of imprinted genes in FGSCs. The primers were 5′-TTTTTGGATTGGGATTTTGTGGG-3′ and 5′-ATCCCCCTTCTTCTTAAATACTC-3′ for H19; 5′-TTTTTGGATTGGGATTTTGTGGG-3′ and 5′-AACCCTAACTAAATTTATC-3′ for Peg10; 5′-AATGGGGTTAATTTTAGGTTTATGG-3′ and 5′-TTTCCCTTCTTCCACTTTTTAATATCTA-3′ for Rasgrf1. PCR products were examined by electrophoresis after digestion using restriction enzymes with a recognition sequence containing a CpG in the original unconverted DNA. The intensity of digested DNA bands was analyzed.

Gene expression analysis

The presence of each indicated mRNA was analyzed by conventional RT – PCR using the listed primers in Supplementary data, Table SII.

BrdU labeling

BrdU (50 μg/ml; Sigma) was added to FGSC cultures, followed by incubation for 5 h.

Immunonanlysis

Fixed ovarian tissues and cultured cells were assessed by immunohistochemistry or immunocytochemistry using antibodies against green fluorescent protein (GFP) (1:200; Chemicon, Temecula, CA, USA), DDX4/MVH (1:200; Abcam), BrdU (1:200; Lab Vision Corporation), Fragilis (1:200; Abcam), OCT4 (1:250 dilution; Chemicon), SCP3 (1:200; Novus Biologicals, Littleton, CO, USA) and ZP3 (1:200) and FITC-conjugated goat anti-rabbit IgG (1:400) or rabbit anti-mouse IgG (1:400) secondary antibodies.

Lipid testing

Both wild-type (WT) and fat-l transgenic rats were sacrificed at 8 weeks of age to test the function of fat-1. Samples were collected from the brain, heart, muscle, liver, kidney, lung and spleen. Lipids were extracted using a modified Folch method (Folch et al., 1957). After dissolving the lipids in hexane, the lipids were methylated with 2 N KOH/methanol. Then, all prepared samples were analyzed by gas chromatography. In addition, we chose fat-l transgenic rats and 15 WT animals at 18–26 months of age to test the function of fat-1. After dissolving the lipids in hexane, the lipids were methylated with 2 N KOH/methanol. Then, all prepared samples were analyzed by gas chromatography. In addition, we chose fat-l transgenic rats and 15 WT animals at 18–26 months of age to test the function of fat-1.

Results

Identification and localization of FGSCs in rat ovaries

To identify and locate FGSCs in Sprague–Dawley (SD) rat ovaries, we first tested the expression of DDX4 and Fragilis in the ovaries of 4–5-week-old rats by immunohistochemistry. The results showed that both DDX4 and Fragilis were expressed in the germ cells of rat ovaries. Furthermore, DDX4 was expressed at all stages of ovarian germ cells (Fig. 1A–D), but Fragilis was highly expressed in early stage germ cells, and there were no or weak signals of Fragilis in secondary oocytes and those at later stages (Fig. 1E–H). Interestingly, Fragilis was expressed exclusively in the membrane of ovarian germ cells (Fig. 1E–H). Next, we assessed the proliferative potential of DDX4- or Fragilis-positive cells. Oocytes from 5-day-old or 4 to 5-week-old rats were collected for dual immunofluorescence analysis of 5′-bromodeoxyuridine (BrdU) incorporation and expression of DDX4 or Fragilis at 3 h after BrdU injection. The results demonstrated the presence of DDX4–BrdU (Fig. 1I) and Fragilis–BrdU (Fig. 1J) double-positive cells on the cortical surface of rat ovaries, suggesting that these cells might be FGSCs.

Isolation, purification and culture of FGSCs

Based on our previous study (Zou et al., 2011) and the above results, we chose Fragilis-based immunomagnetic sorting for germ cell purification. To establish post-natal rat FGSC lines, FGSCs were isolated from the ovaries of 5-day-old rats. Using two-step enzymatic digestion and immunomagnetic isolation of Fragilis-positive (Fragilis+) cells (Zou et al., 2009; Zhang et al., 2011), 200–300 cells were obtained from 20 ovaries. The isolated cells were round with a high nuclear to cytoplasm ratio, and a size and morphology similar to those of mouse and freshly isolated type A spermatogonia (Fig. 2A). To confirm that the freshly isolated Fragilis+ cells were germ cells, we investigated DDX4 expression of Fragilis+ cells using immunofluorescence. The results showed that these Fragilis+ germ cells were immunomagnetically sorted cells were DDX4 positive (Fig. 2B). Because female germ cells include oocytes and FGSCs, and oocytes degenerate during culture (Zou et al., 2009), dual immunofluorescence analysis of BrdU incorporation and DDX4 expression was carried out after the cells had been cultured for two or three passages to further confirm that the isolated Fragilis+ cells included rat FGSCs. The results indicated that almost all Fragilis+ cells were positive for both BrdU and DDX4 (Fig. 2C), suggesting that these Fragilis+ cells were FGSCs. The above results confirmed that these cells might be FGSCs.
Characterization of rat FGSCs

To profile the characteristics of the FGSC line, we found that the FGSCs (Fig. 3B) were positive for alkaline phosphatase staining, although the intensity appeared weaker than that of embryonic stem (ES) cells (Fig. 3A). Next, we assessed the expression of stem cell and germ cell marker genes: Nanog (a pluripotency sustaining factor; Chambers et al., 2003), Oct4 (a germ cell-specific transcriptional factor; Scholer et al., 1990), c-kit (a stem cell factor receptor; Hu and Colletti, 2008), Sox-2 (a key transcription factor related to pluripotency; Masui et al., 2007), Blimp-1 (a known transcriptional repressor and marker of early germ cells; Ohinata et al., 2005), Ddx4, Figla (a meiosis-specific marker; Joshi et al., 2007), Fragilis (Ifitm3, a marker of germ cells, especially early germ cells; Lange et al., 2003), Dazi (a marker of germ cells; Ruggiu et al., 1997), Scp (synaptonemal complex protein; Yuan et al., 2002) 1, 2 and 3 and Zp3 (zona pellucida protein 3). The results showed the expression of Oct4, Ddx4, Dazi, Blimp-1 and Fragilis, but no expression of Nanog, c-kit, Sox-2, Figla, Scp 1-3 or Zp3 in FGSCs (Fig. 3C–F and J), suggesting that the cells possessed stemness and the characteristics of germ cells. Moreover, the cells showed a high telomerase activity (Fig. 3G). Karyotyping by DAPI immunofluorescence was performed on the cells. Based on the analyzed metaphases, we observed a stable diploid karyotype (42, XX) in 65% of the total analyzed metaphase plates (Fig. 3H and I). In addition, we examined the differentially methylated regions of four imprinted genes, two maternally imprinted genes (Igf2r and Peg 10) and two paternally imprinted regions (H19 and Rasgrf1) by combined bisulfite restriction analysis. The results showed partial methylation on the maternally imprinted region and demethylation on paternally imprinted regions, suggesting a female imprinting pattern (Supplementary data, Fig. S1). Taken together, cultured rat FGSCs possessed the characteristics of germline stem cells and normal chromosomes.

In vitro differentiation of rat FGSCs

Subsequently, we tested the differentiation ability of cultured rat FGSCs in vitro. The differentiation process included three stages (see Materials and Methods). To determine the developmental stages of the cells during differentiation in vitro, RT–PCR was used to detect the expression
of Scp1, Scp2, Scp3 and Zp3. Scp1, Scp2, Scp3 was expressed in cells after differentiation for 15.5 days (Fig. 4A and Supplementary data, Fig. S2), suggesting that the cells could be committed to meiosis. Furthermore, we found that Zp3 was expressed in cells after 25.5 days of differentiation, indicating that the cells could form zona pellucida (Fig. 4B). Morphologically, we observed no significant changes of the cells that were differentiated in vitro for 3 days. However, as differentiation continued, the cells became enlarged. At 25.5 days of differentiation, the cells grew to 30–35 μm diameters, and started to form zona pellucida (Fig. 4C). After 29.5 days of differentiation, the cell diameters enlarged to 55–60 μm with 30% full formation of zona pellucida (including 5–10% germinal vesicle-stage oocytes; Fig. 4D). Formation of the zona pellucida in cells was confirmed by immunocytochemical analysis with an antibody against Zp3 (Fig. 4E and G). Zp3 expression in oocytes from the ovaries of adult rats was used as a positive control (Fig. 4F).

In vivo differentiation of rat FGSCs and production of transgenic rats with FGSCs

To determine the physiological function of FGSCs in vivo, cells that were passaged over 20 times were transfected with a MSCV-PGK-greens fluorescent protein (GFP) vector by liposomes. The transfected FGSCs were transplanted into the ovaries of infertile rats that were sterilized by busulfan (20 mg/kg) and cyclophosphamide (100 mg/kg) (see Supplementary data, Information and Table SI). For control females, the transfected FGSCs were replaced by DMSO. At 2 months after FGSC transplantation or DMSO injection, the ovaries were harvested and evaluated. Oocytes at different stages from rats that received FGSCs were confirmed by histology, and found to be GFP positive (Fig. 5B–D). In contrast, no healthy oocytes or follicles, only stromal and interstitial cells and a few atretic follicles, were found in control ovaries (Fig. 5A). Next, we examined whether FGSCs could restore the fertility of sterilized recipients after mating with WT males. At 60–150 days post-transplantation, 66.67% (4/6) of FGSC recipients produced offspring (Fig. 5E) with no abnormalities and were fertile. The offspring were examined for the presence of the GFP transgene in their genomes by PCR (Fig. 5G) and Southern blot analysis with a GFP-specific probe (Fig. 5H), and fluorescence was detected with live-imaging (The eXplore Optix, GE Company, Piscataway, NJ, USA) (Fig. 5F). A total of 39 out of the 140 F1 offspring (27.86%) carried the GFP transgene. F2 offspring were also produced after mating of the F1 transgenic rats, indicating that the transgene was transmitted through the germ line. Control females, which received an injection of washing buffer into their ovaries (see Materials and Methods), generated no

Figure 2 Establishment of FGSC cultures. (A) Representative image of FGSCs or female germ cells purified with the anti-Fragilis antibody and the associated pattern of female germ cells with immunomagnetic beads. (B) Merged image of DDX4 and DAPI immunofluorescence in isolated and cultured Fragilis⁺ cells. Arrows indicate DDX4-positive cells. (C) An example of dual immunofluorescence of DDX4 and BrdU in FGSCs that were cultured for two or three passages. (D) Proliferation pattern of FGSC cultures. The experiments were repeated three times. (E and F) Representative morphology of expanded FGSCs with spherical (E) (white arrows) or grape-like (F) (black arrows) clusters after 3 weeks of culture. (G) Representative image of FGSCs that were cultured for more than 1 year. Scale bars, 10 μm (A, C, E, F and G), 25 μm (B).
transgenic offspring. These results indicate that FGSCs can be used to generate transgenic offspring at a high efficiency.

To further confirm that the cultured FGSCs could be a new and effective tool to generate transgenic animals and establish research models in new species, we produced fat-1 transgenic rats using a pCAGGS vector (Fig. 6A) carrying the fat-1 gene cloned from C. elegans using the same approach described above. The results showed that 83.33% (5/6) of FGSC recipients produced offspring and 28.00% (32/114) of F1 rats were heterozygous for the transgene (Fig. 6B) after confirmation by RT–PCR (Fig. 6C) and Southern blot (Fig. 6D) analyses. All transgenic rats appeared to be normal and healthy. Next, we tested expression of the fat-1 gene in different tissues of F1 rats by RT–PCR, and found that all tissues expressed fat-1 (Fig. 6E). We also examined the fatty acid profiles in tissues of transgenic (Fig. 6G) and WT rats (Fig. 6F), and found that $n-3$ fatty acid levels were consistently raised in fat-1 transgenic rats, indicating that the transgene was functionally active in vivo.

After analysis of the $n-6:n-3$ fatty acid ratio in different tissues of fat-1 transgenic and WT rats, we found that the $n-6:n-3$ fatty acid ratio was greatly reduced in fat-1 transgenic rats. The old fat-1 transgenic rats and WT animals showed statistical differences in body weight, total cholesterol and the locomotor activity (Supplementary data, Table SIII).

**Discussion**

Rats have been used as an animal model in studies of physiology, toxicology, nutrition, behavior, immunology, endocrinology and neoplasia for over 100 years (Jacob, 1999), and are preferable over mice to physiologists and other biomedical researchers. However, stable ES cells were not available until 2008 (Li et al., 2008), which restricted the application of genetically modified rats, and most strains used to develop disease models were established by selective breeding.
In this study, we demonstrated the presence of germline stem cells in the post-natal rat ovary, and successfully established and characterized a rat FGSC line from 5-day-old rats. Furthermore, the rat FGSC line possessed a normal diploid karyotype and undifferentiated characteristics with a stable proliferation rate and high telomerase activity. To explore the ability of the rat FGSC line to differentiate into oocytes in vitro, we first removed the factors that support self-renewal of FGSCs, such as LIF and STO feeders, from the culture condition. Then, the FGSCs were cultured in hanging drops for 2 days (see Materials and Methods). This step helped the FGSCs to form intercellular contacts, and may initiate signaling events and spontaneous differentiation.

In vivo, oocytes grow and develop in primordial follicles surrounded and supported by granulosa cells. In an attempt to mimic this system in vitro, we isolated granulosa cells from juvenile rats. After mitomycin C treatment, the granulosa cells were co-cultured with FGSCs in medium containing retinoic acid (RA), bone morphogenetic factor (BMP)-4 and bFGF as the first inducer. RA is known to participate in meiosis (Bowles et al., 2006; Doyle et al., 2007) by regulating sex-specific timing of meiotic initiation (Koubova et al., 2006). Female germ cells exposed to high RA concentrations can enter the meiotic cycle (McLaren, 2003). BMP-4 is involved in commitment of the germline during embryogenesis, and promotes primordial germ cell differentiation. Exogenous factors in the culture medium, such as BMPs, can push germ cells into the meiotic process in vitro (Kee et al., 2006). Moreover, some reports have used fetal testicular extracts (Hua et al., 2011) or conditioned medium collected from testicular cell cultures (Lacham-Kaplan et al., 2006), which contain hormones [e.g. follicle-stimulating hormone and human chorionic gonadotrophin (hCG)] to differentiate human ES cells into oocytes. The testis is an abundant source of growth factors, such as BMP-4, stem cell factor, bFGF and growth differentiation factor 9 (Takabayashi et al., 2001; Creemers et al., 2002; Pellegrini et al., 2003; Huleihel and Lunenfeld, 2004), suggesting that the developing ovary may also contain various growth factors. Therefore, we generated ovarian homogenates from adolescent rat ovaries. Using the ovarian homogenate together with hormones (PMSG, hCG, estrogen and progesterone) and growth factors (EGF, bFGF, insulin and transferrin), we prepared an induction medium for further differentiation. After culturing in the induction medium for more than 15 days, we found that the differentiating cells became larger over time and expressed ZP3, suggesting that

**Figure 4** In vitro differentiation of FGSCs. (A) RT–PCR analysis of Scp3 expression at various time points (Days 10.5–23) during differentiation of FGSCs. M: 100 bp DNA marker; lanes 1–6: Days 10.5, 13, 15.5, 18, 20.5 and 23, respectively. (B) RT–PCR analysis of Zp3 expression. The analyses were carried out at various time points (Days 23–33) during the differentiation of FGSCs. M: 100 bp DNA marker; lanes 1–5: Days 23, 25.5, 28, 30.5 and 33, respectively. (C) Representative morphology of enlarged germ cells that were forming zona pellucida after differentiation for 25.5 days. Arrow indicates the germinal vesicle (GV). (D) Oocyte morphology with (arrow) or without the GV and with a fully formed zona pellucida after 33 days’ differentiation. (E and F) Immunofluorescence detection of Zp3 in oocytes. (E) Zp3 expression in an oocyte after differentiation of FGSCs for 30 days. (F) Zp3 expression in an oocyte from the ovary of an adult rat. (G) Negative control for Zp3 expression. Scale bars, 10 μm (C, E, F, G), 25 μm (D).
rat FGSCs can differentiate into oocytes in vitro. Oocyte maturation encompasses a number of complexities, including meiosis, cytoplasmic growth, organelle production and redistribution, RNA production and nuclear and cytoplasmic maturation, which cause difficulties for the differentiation of germ cells in vitro. It is a long-term goal to obtain functional female gametes by differentiation of ES cells, induced pluripotent stem cells, adult stem cells and FGSCs in vitro. Moreover, FGSCs may become an important research model in biomedicine because they possess reproductive characteristics.

To evaluate the physiological function of FGSCs, rat FGSCs were transfected with a GFP vector, and then transplanted into the ovaries of infertile rats. The results showed that the transplanted cells underwent...
oogenesis. Furthermore, long-term cultured FGSCs could restore fertility, including production of GFP transgenic offspring, when transplanted into infertile recipients. These findings indicate that rat FGSCs not only have a physiological role in the rat ovary but they may also serve as a powerful tool for genetic modification such as production of transgenic rats for pathology and biomedical research.

In addition to GFP transgenic rats, fat-1 transgenic rats were also generated in this study. These transgenic rats showed an increase in the ratio of \( n-3 \) to \( n-6 \) fatty acids in tissues. Furthermore, fat-1 transgenic rats were normal and healthy. These results are in line with previous findings (Kang et al., 2004). In the same genetic background, the old fat-1 transgenic rats appeared more healthy and vivacious than the normal rats.

**Figure 6** Production of fat-1 transgenic rats. (A) Diagram of the fat-1 transgenic strategy. The pCAGGS vector carried the fat-1 gene. (B) An example of offspring from recipient rats transplanted with fat-1-transfected FGSCs. (C) PCR analysis of offspring. Gene amplification by 32 cycles of PCR was carried out using Taq polymerase (Promega) with a primer set (5’TATGGTCGCTCATTTACGC-3’ and 5’TGACGACGGCATAT-3’) specific for the fat-1 gene. PCR products were sequenced for further confirmation of successful gene transfer. M: DNA marker III; lane 1: positive control (pCAGGS plasmid); lanes 2 and 4: transgenic rats; lane 3: WT rat. (D) An example of Southern blotting of tail DNA with a probe, a 412 bp PCR-amplified fragment from the pCAGGS plasmid using the primers (5’TCTCTGTTCGTTTCTGCG-3’ and 5’TTGACACCGTCGTTGCT-3’) specific for the fat-1 gene. Genomic DNA was digested with EcoRI. Size markers are indicated to the right of the Southern blot. Lanes 1 and 3: transgenic rats; lane 2: WT rat. (E) RT–PCR analysis of different tissues from fat-1 transgenic rats. PCR amplification and primers specific for fat-1 gene were as described above. The fat-1 gene was expressed in almost all tissues of transgenic rats. Arrows indicate the detected fat-1 transcripts. The size of the resolved DNA fragments (in bp) were fat-1, 412; G3pdh, 438. M: 100 bp DNA marker; lanes 1–8: brain, heart, liver, spleen, kidney, lung, muscles and WT muscles, respectively. (F and G) Partial gas chromatograph traces showing the polyunsaturated fatty acid profiles of total lipids extracted from skeletal muscles of a WT rat (F) and fat-1 transgenic rat (G). Both WT and transgenic rats were 8-week-old females that were fed on the same diet. Note that the levels of \( n-3 \) polyunsaturated fatty acids (18:2n-6, 20:4n-6 and 22:4n-6) were lower, while \( n-3 \) fatty acids (18:3n-3, 20:5n-3 and 22:6n-3) were abundant in the transgenic rat compared with that in the WT rat with very little \( n-3 \) fatty acid. The experiments were repeated three times.
suggesting that fat-1 gene could benefit the body in many different ways. That may explain why the fat-1 transgenic rat models have stronger resistance and resilience than the other animal models (Hudert et al., 2006; Xia et al., 2006; Das, 2008; Bazan, 2009; Mayer et al., 2009; Bellenger et al., 2011).

After the first fat-1 transgenic mouse was produced in 2004, many fat-1 transgenic animals have been generated using other species, such as pigs and cattle, which can supply large amounts of n − 3 fatty acids for a healthy diet (Lai et al., 2006; Guo et al., 2011; Ren et al., 2011). Moreover, fat-1 transgenic mice have been used to backcross with mouse disease models and many new research models have been generated as a result (Smith et al., 2010; Bousquet et al., 2011; Lebbadi et al., 2011). These research models can test the effects of n − 3 fatty acids on the pathogenesis and treatment of diseases, and are also ideal models to study the mechanisms of such diseases (White et al., 2010; Bellenger et al., 2011; Song et al., 2011). By introducing the fat-1 gene into disease model rats through backcrossing fat-1 transgenic rats with disease model rats, new rat disease models will be generated for research. Such model rats will possess advantages over mouse models, as mentioned above, to study the function of n − 3 fatty acids in physiological activity and pathological processes, and to delineate proper treatments for diseases.

Supplementary data
Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors’ roles
L.Z. conducted all the major experiments, data analysis and wrote the manuscript; L.W. was responsible for establishment of the infertile rat model and fat-1 vector preparation; J.X.K. was responsible for fat-1 vector preparation; W.X. was responsible for analysis of Zp3 expression; X.L. conducted transgenic rat screening with Southern blot. C.W. was responsible for histological analysis of ovarian tissue; B.X. conducted FGSC transplantation. J.W. initiated and supervised the entire project, conducted FGSC transplantation.

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Conflict of interest
None declared.

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