Article

Production of transgenic mice by random recombination of targeted genes in female germline stem cells

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Oocyte production in most mammalian species is believed to cease before birth. However, this idea has been challenged with the finding that postnatal mouse ovaries possess mitotically active germ cells. A recent study showed that female germline stem cells (FGSCs) from adult mice were isolated, cultured long term and produced oocytes and progeny after transplantation into infertile mice. Here, we demonstrate the successful generation of transgenic or gene knock-down mice using FGSCs. The FGSCs from ovaries of 5-day-old and adult mice were isolated and either infected with recombinant viruses carrying green fluorescent protein, Oocyte-G1 or the mouse dynein axonemal intermediate chain 2 gene, or transfected with the Oocyte-G1 specific shRNA expression vector (pRS shOocyte-G1 vector), and then transplanted into infertile mice. Transplanted cells in the ovaries underwent oogenesis and produced heterozygous offspring after mating with wild-type male mice. The offspring were genetically characterized and the biological functions of the transferred or knock-down genes were investigated. Efficiency of gene-transfer or gene knock-down was 29%–37% and it took 2 months to produce transgenic offspring. Gene manipulation of FGSCs is a rapid and efficient method of animal transgenesis and may serve as a powerful tool for biomedical science and biotechnology.

Keywords: female germline stem cells, gene-transfer, knock-down, Oocyte-G1, mouse dynein axonemal intermediate chain 2 (Dnaic2)

Introduction

Stem cells are a unique cell population with self-renewal potential (Potten, 1992). They often have migratory activity and can be transplanted between animals (Brinster and Zimmermann, 1994; Clouthier et al., 1996). Transplanted stem cells can migrate to a specific position and regenerate self-renewing tissue. Germline cells are unique in that they transmit genetic information from parent to offspring (Meistrich and van Beek, 1993; De Rooij and Russell, 2000). Males retain germline stem cells for spermatogenesis throughout adult life and spermatogonial stem cells (SSCs) can be transfected to produce transgenic offspring via SSC transplantation (Kanatsu-Shinohara et al., 2005, 2006). Female germline stem cells (FGSCs) stop dividing after birth in most mammalian species (Zuckerman, 1951; Borum, 1961; Peters, 1970; McLaren, 1984; Anderson and Hirshfield, 1992). This theory has been challenged with findings from an in vivo mouse study suggesting that the female gonad may exhibit unexpected regenerative activity in adulthood (Johnson et al., 2004). Recently, we reported that an FGSC line from neonatal mice was established and cultured for more than 15 months with normal karyotype and high telomerase activity. FGSCs from adult mice were also isolated and cultured for more than 6 months (Zou et al., 2009). These FGSCs of long-term culture maintained their capacity to produce normal oocytes and fertile offspring after transplantation into ovaries (Zou et al., 2009). Pacchiarotti et al. (2010) also showed the existence of a population of germline stem cells in postnatal mouse ovaries.

Oocyte-G1 is a newly discovered member of the kinesin superfamily of proteins (KIFs). KIFs play a significant role in transport of various membranous organelles and protein complexes on microtubules (Hirokawa, 1998). A previous study showed that Oocyte-G1 encodes a 997-residue protein with an open reading frame (ORF) of 2994 bp. Northern blot analysis revealed the presence of ca. 3.6-kb Oocyte-G1 mRNA in the ovary, testis, lung, brain and kidney tissue (Zhang and Wu, 2009). However, the functions of Oocyte-G1 are not well understood.

In our earlier work, we cloned a novel gene, the mouse dynein axonemal intermediate chain 2 gene (Dnaic2), which has an 87% homology with human DNA2, a candidate gene for primary ciliary dyskinesia (PCD) (Guichard et al., 2001). Its ORF is 1872 bp encoding a polypeptide of 623 residues. Northern and western...
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blot analyses indicate that Dnaic2 produces ca. 3-kb mRNA that is translated into ca. 70 kDa protein. The mRNA is predominantly expressed in the mouse ovary, testis, and lung tissue (Yang and Wu, 2008). However, the functions of Dnaic2 in mice remain unknown.

Here, we generated transgenic green fluorescent protein (GFP), Dnaic2 and Oocyte-G1 mice and Oocyte-G1 knock-down (Kd-O) mice by viral or liposome-mediated transduction in short-term cultured FGSCs. Dnaic2 transgenic mice showed female subfertility and male infertility. Transgenic mice overexpressing Oocyte-G1 showed postnatal growth retardation. Kd-O mice died at 14–24 days with brain, ovary and testis abnormalities. The efficiency of the gene transfer was 29%–37% with transgenic mice produced after 2 months. FGSC-mediated transgenesis is a rapid, inexpensive and efficient new method. It has important implications for future gene therapies and biotechnology research, especially in the field of large animal mutagenesis.

Results

Isolation and short-term culture of FGSCs

To create a quick, inexpensive and efficient method of generating transgenic animals, we first isolated and then short-term cultured FGSCs from ovaries of neonatal and adult mice. For isolation of FGSCs in vitro, a two-step enzymatic digestion was used (see the section ‘Materials and methods’) and approximately 1000 large round or ovoid cells were obtained from the ovaries of 6–10 C57BL/6 × CD-1 F1 neonatal mice. Using the same protocol, about 300 cells were obtained from three to five adult mice. Some of the isolated cells displayed morphology similar to freshly isolated type A spermatogonia (Figure 1A and B). After 2–3 days of culture, the isolated cells expanded and were undergoing mitosis (Figure 1E and F). To determine whether the isolated cells contained FGSCs, dual immunofluorescent analysis of 5'-bromodeoxyuridine (BrdU) incorporation and expression of mouse vasa homolog (MVH) was performed. Assay for BrdU incorporation was carried out to assess cell proliferation. The results showed that more than 50% of these round or ovoid cells were BrdU-MVH double-positive cells (Figure 1C and D). This suggested that the double-positive cells may be FGSCs.

Production of GFP transgenes

FGSCs cultured for 3–5 days were infected with murine stem cell virus-phosphoglycerate kinase-GFP (MSCV-PGK-GFP) virus particles (see the section ‘Materials and methods’, ca. 10^8 cfu/ml). After 48 h, the FGSCs were transplanted into ovaries of recipients sterilized by chemotherapy (pretreated with cyclophosphamide and busulfan, see the section ‘Materials and methods’; Shiromizu et al., 1984; Johnson et al., 2004; Johnson et al., 2005). After 2 months of sterilization treatment, healthy oocytes or follicles were not found in the recipient ovaries (Supplemental Figure S1C), indicating that chemotherapy essentially destroyed all oocytes. After FGSC transplantation for 2 months, the recipient ovaries were collected, and the presence of oocytes was evaluated by morphological appearance and expression of GFP. The results showed that ovaries of mice receiving FGSCs after chemotherapy possessed a number of oocytes, including oocytes from large healthy antral follicles and GFP-positive oocytes (Supplemental Figure S1A, B, and D). This indicates that retroviral vectors can be used to deliver genes into short-term cultured FGSCs and that the FGSCs can subsequently generate oocytes in vivo.

To examine whether the short-term cultured FGSCs could restore fertility and generate transgenic offspring when transplanted into infertile recipients, we used mice sterilized by chemotherapy (pretreated with cyclophosphamide and busulfan) as recipients for transplantation. To confirm recipient infertility, eight of the recipients were mated with wild-type (WT) C57BL/6 males and none were fertile. Female control mice

![Figure 1](https://academic.oup.com/jmcb/article-abstract/3/2/132/905775/13320479)
(n = 6) were given an intraperitoneal injection of a vehicle (dimethyl sulfoxide, DMSO) and had normal fertility. The FGSCs infected by MSCV-PGK-GFP virus (see the section ‘Materials and methods’) were transplanted into ovaries of sterilized recipients. No information is available on how many days are required for differentiation of FGSCs into mature oocytes in mice. In preliminary experiments, we determined the time that the recipients began to produce offspring after FGSC transplantation by natural mating with WT C57BL/6 males was on day 25, 35, 45 or 55 of transplantation. Supplemental Table S1 shows that offspring, including transgenic progeny, from recipients were obtained as early as 57 days after transplantation. Based on the information above, we carried out the following experiments. From 57 to 100 days transplantation, 81.82% (18/22) of recipients produced offspring for FGSCs from neonatal mice and 80% (12/15) for FGSCs from adult mice (Figure 2B). There were no abnormalities in the progeny, and they were all fertile. The offspring were also examined for the presence of GFP transgenes in their genomes by PCR (further confirmation of successful transgenes was obtained by sequencing the PCR products; data not shown), southern blot analysis using a GFP-specific probe (Figure 2A), and live imaging (The eXplore Optix, GE Company) (Figure 2C–E). For FGSCs of neonatal mice, 48 F1 mice (30%) were heterozygous for the transgenes from screenings of 160 offspring. For FGSCs of adult mice, 29.17% (21/72) of mice were heterozygous. The transgene was transmitted via the germline because 61 F2 GFP transgenic progeny (88.41%) were produced from 69 F1 transgenes. For control 1, the oocytes from adult mice (see the section ‘Materials and methods’) infected by MSCV-PGK-GFP virus (see the section ‘Materials and methods’) were transplanted into ovaries of sterilized recipients (n = 11). The recipient mice were infertile. For control 2, washing buffer (see the section ‘Materials and methods’) was injected into ovaries of adult mice, and no transgenic offspring were obtained. The results indicate that the FGSCs from both neonatal and adult mice can be used to generate transgenic offspring at high efficiency.

Production of Oocyte-G1 and Dnaic2 transgenes
To further confirm that the short-term cultured FGSCs can be used to generate transgenic mice and study the roles of Oocyte-G1 and Dnaic2 in mouse development, we produced transgenic mice overexpressing Oocyte-G1 (Tg-O) or Dnaic2

Figure 2 GFP offspring generated from the transplantation of FGSCs. (A) The MSCV-PGK-GFP vector carrying PGK. Arrow, self-inactivating mutation; LTR, long terminal repeat. (B) Offspring from a C57BL/6 × CD-1 F1 recipient mouse transplanted with GFP-transfected FGSCs. (C) The offspring were screened by live imaging (The eXplore Optix, GE Company) for the identification of GFP offspring. Left, middle: GFP-positive offspring. Right: GFP-negative offspring. (D) PCR analysis of offspring. Amplification via 32 cycles of PCR was performed using Taq polymerase (Promega) with primer set (5’-CGAGGGAATACGACGAGCCGG-3’ and 5’-ACGAACTCAGCGAGCCCCCATAG-3’) specific for the GFP gene. The PCR product was also sequenced for further confirmation of successful gene transgenesis. M, 100-bp DNA ladder; lane 1, positive control (DNA from MSCV-PGK-GFP plasmid); lanes 2, 5, transgenic mice; lanes 3, 4, 6, WT mice. (E) Southern blot analysis of tail DNA with a probe, a 366-bp-long PCR amplified fragment from the MSCV-PGK-GFP plasmid using primers for the GFP gene (Figure 2A). Genomic DNA was digested with EcoRI. Size markers are indicated to the right of the Southern blot. Lanes 1–3, 6, transgenic mice; lanes 4, 5, 7, WT mice.
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Figure 3 Production of Oocyte-G1 transgenic mice. (A) Diagram of the Oocyte-G1 transgenic strategy. Arrow, self-inactivating mutation; Neo, the neomycin phosphotransferase gene; Oocyte-G1, targeting gene (see Supplemental online material); LTR, long terminal repeat. (B) PCR analysis of gene transgenesis in offspring. PCR was performed using primer set (5′-GGTGGAGGGGCTATCGGTATGAGA-3′ and 5′-ATCCGTACGACAGCCGCTC-3′) specific for Neo gene. The PCR product was sequenced for confirmation of the presence of Neo gene. M, 100-bp DNA ladder; lanes 3, 5, 8, transgenic mice; lanes 2, 4, 6, 7, 9, WT mice. (C) PCR analysis of offspring with primer set (5′-GCAGAGTTTTCAGACCGCTC-3′ and 5′-CATCTCGGTGTTTTCCACCTC-3′) specific for Oocyte-G1. The PCR product was also sequenced for further confirmation of successful gene transgenesis. M, 100-bp DNA ladder; lanes 1, 3, 4, 6, transgenic mice; lanes 2, 5, 7, WT mice. (D) Southern blot analysis of offspring with a probe, a 755-bp-long PCR amplified fragment from the pFB-Neo-Oocyte-G1 plasmid using specific primers (5′-GCAGAGTTTTCAGACCGCTC-3′ and 5′-CATCTCGGTGTTTTCCACCTC-3′) for Oocyte-G1 (see Supplemental online material). Tail DNA was digested with EcoRI. Size markers are indicated to the right of the southern blot. Lanes 1, 2, 5, WT mice; lanes 3, 6, transgenic line 1; lane 4, transgenic line 2. (E) Western blot analysis of Oocyte-G1 in testicular protein obtained from Tg-O or WT mice. Lane 1, WT; lane 2, Tg-O. (F) Western blots were quantified by densitometry and the data were expressed as a ratio of Oocyte-G1:β-tubulin (mean ± SEM); qRT-PCR analysis of Oocyte-G1 mRNA expression in Tg-O and WT testes. The result showed that Tg-O mice overexpressed Oocyte-G1 in the testes. 1, western blot analysis; 2, qRT-PCR. (G) Tg-O (above) and WT (bottom) mice. (H) Macroscopic appearance of Tg-O (left) and WT (right) testes at 8 weeks of age.

(Tg-D) by random mutagenesis using the same approach described above. The result was that 85.71% (6/7) (for FGSCs from neonatal mice) of recipients produced offspring by natural mating with WT C57BL/6 males for Oocyte-G1, and 90.91% (10/11) (6/7 for FGSCs from adult mice, 4/4 from neonatal mice) for Dnaic 2 (Figure 3G and Supplemental Figure S4A). The offspring were examined by PCR for the presence of a neo gene or transfer gene, Oocyte-G1 or Dnaic2, in their genomes (PCR products were sequenced for further confirmation of successful gene transgenes; data not shown). Southern blot analysis was also carried out using an Oocyte-G1- or Dnaic2-specific probe (Figure 3A–D, Supplemental Figure S2A–D) and two transgenic lines (1 and 2) for Oocyte-G1 or three transgenic lines (1, 2 and 3) for Dnaic2, were identified (Figure 3D and Supplemental Figure S2D). For Oocyte-G1, 34 F1 mice (31.48%) were heterozygous for the transgenes from screenings of 108 offspring. For Dnaic2, 36.59% (45/123) of mice were heterozygous. To confirm overexpression of Oocyte-G1 or Dnaic2 mRNA, and protein in the testes of transgenic mice, quantitative RT-PCR (qRT-PCR) and western blot analysis were performed. The results indicate that transgenic mice overexpressed Oocyte-G1 or Dnaic2 in the testis (Figure 3E and F, Supplemental Figure S2E and F). For all subsequent analyses, we used the Tg-O line 2 with higher copies and the greater majority (>85%) of the phenotype of Tg-O mice. Postnatal growth retardation was observed in both the male and female offspring of the Tg-O mice (Figure 3G) with lower body weight than controls at 8 weeks of age (24.80 ± 0.61 g for Tg-O mice; 34.55 ± 0.89 g for controls; P < 0.05). When 8 weeks old, Tg-O mice were mated with WT mice, and were found to be infertile. However, Tg-O mice produced normal numbers of offspring (8.50 ± 0.62) when Tg-O mice at 12-18 weeks of age were mated with WT mice. Furthermore, testis weights and sizes in 8-week-old Tg-O mice were much smaller than that in WTs (Figure 3H; 60.23 ± 2.87 mg for Tg-O mice; 112.48 ± 2.93 mg for WTs; P < 0.05). We then conducted histological analyses of ovaries or testes from the Tg-O mice. Cross section of 6–8-week-old Tg-O ovaries showed secondary, primary and primordial follicles. No mature follicles were observed in these sections, suggesting delay of female sexual maturity (Supplemental Figure S3A and B). For Tg-O testes, some
spermatocytes were present in the seminiferous tubules of Tg-O mice at 8 weeks of age. No sperm was observed in seminiferous tubules at 12 weeks of age. A number of sperm were found in some seminiferous tubules at 16 weeks of age. Many sperm were observed in seminiferous tubules (arrows). Scale bars, 50 μm.

Figure 4 Developmental retardation of the male sex organ (testis) in the Tg-O mice. (A–F) Morphometric analysis of WT (A, C, E) and Tg-O (B, D, F) testes. (A) Histological section of WT testis at 8 weeks of age. Many sperm were found in seminiferous tubules (arrow). (B) Morphology of Tg-O testis at 8 weeks of age. No sperm was observed in seminiferous tubules (arrows). (C and D) Morphology of WT (C) and Tg-O (D) testes at 12 weeks of age. A number of sperm were found in seminiferous tubules of (C) and (D) (arrows). (E and F) Morphology of WT (E) and Tg-O (F) testes at 16 weeks of age. Many sperm were observed in seminiferous tubules (arrows). Scale bars, 50 μm.

A number of sperm were observed in 12- or 16-week-old Tg-O testes (Figure 4C–F) and mature follicles were observed in 10–12-week-old Tg-O ovaries (Supplemental Figure S3C and D). There were no obvious structural changes in the lungs, kidneys and brains of the Tg-O mice (data not shown).

During Tg-D analysis, we used Tg-D line 3 with the highest copies and the majority (>70%) of the phenotype of Tg-D mice. Unlike Tg-O mice, adult Tg-D mice were normal in body weight and size (Supplemental Figure S4A). However, Tg-D female mice were mated and compared with matings between WT mice. The litter sizes were smaller in the Tg-D group (3.50 ± 0.65 offspring per litter) than in the WT group (8.75 ± 0.85 offspring per litter), and some Tg-D females never became pregnant. The results suggest that Tg-D female mice were subfertile or infertile. When the Tg-D males were mated with WT females, none of them became pregnant (intercrosses of Tg-D males and WT females over a 5 month period). The results indicated that Tg-D males were sterile. These findings are in agreement with observations from others (Casey and Hackett, 2000; Guichard et al., 2001; Ibañez-Tallon et al., 2002). Cross sections of Tg-D adult ovaries showed that the number of healthy antral follicles per cross section was significantly lower in Tg-D ovaries (0.40 ± 0.24) than in WTs (2.80 ± 0.37) (Supplemental Figure S4D and E). Abnormalities were also found in Tg-D fallopian tubes (Supplemental Figure S4B and C). In the testes of male Tg-D mice, sperm were absent in some seminiferous tubules (Supplemental Figure S4F and G). On further analysis, 92.6% of the sperm collected from the epididymes of WT mice appeared normal by light microscopic examination. However, a high percentage (48.4%) of sperm from Tg-D mice were abnormal, including sperm heads touching the flagella, sperm bending over, and/or looping in the sperm midpiece area (Figure 5A–F). Electron microscopy of sperm showed that Tg-D mice have axonemes with missing dynein arms (Supplemental Figure S5A–D), absence of central-pair structures (Supplemental Figure S5C), and/or pairs of outer doublet microtubules (Supplemental Figure S5D). Moreover, immunofluorescent analysis showed that Dnaic2 protein was highly expressed in the sperm midpiece and membrane of sperm head (Supplemental Figure S5G and H), suggesting that the role of Dnaic2 is critical for proper sperm morphology and function.

Production of Kd-O mice
To confirm the role of Oocyte-G1 in mouse development and determine whether the short-term cultured FGSCs can be used to generate mutagenic mice without using virus infection, we produced Kd-O mice by targeting Oocyte-G1 in the FGSCs with liposome-mediated transfection (see the section ‘Materials and methods’). The results showed that 100% (6/6) (3/3 for FGSCs from adult mice, 3/3 from neonatal mice) of recipients produced offspring by natural mating with WT CD-1 males (Figure 6H). The progeny were tested for the presence of Puro gene and SV40 early promoter in their genomes by PCR (PCR products were sequenced; data not shown) (Figure 6A–C). Southern blot analysis was also carried out using an SV40 early promoter-specific probe (Figure 6D). Thirty-five F1 mice (30.97%) were Puro- and SV40-positive progeny from screenings of 113 offspring. We verified shRNA mediated Kd-O by western blot analysis on testis, lung and kidney tissue. The results demonstrated a significant decrease in Oocyte-G1 expression levels between Kd-O mice and WTs (Figure 6E and F). Furthermore, we found that Kd-O mice were born alive but died 14–24 days after birth. Much smaller body weight and size were observed in Kd-O mice (6.57 ± 0.38 g) as compared with the age-matched WTs (12.69 ± 2.22 g; P < 0.05; Figure 6G). Testis weights and sizes in Kd-O mice (10.57 ± 1.18 mg) were also significantly smaller than in the age-matched controls (22.18 ± 4.64 mg; P < 0.05; Figure 6H). We then carried out histological analyses of several organs from the Kd-O mice. Their brains showed abnormalities in the dentate gyrus of the hippocampus (Figure 7E–H). For
histological analyses of ovaries, cross sections of 18-day-old ovaries of Kd-O mice showed that most follicles were atretic and no large antral follicles were present (Figure 7A and B). Furthermore, histological analyses of testes from Kd-O mice at 18 days showed abnormal spermatogenesis or disorganization in the development of spermatogenesis (Figure 7C and D). For lungs and kidneys, there were no obvious structural changes in the Kd-O mice (Supplemental Figure S6A–D).

Figure 5 Morphological defects in sperm of Tg-D mice. (A) WT sperm. (B) Tg-D sperm bent over in the midpiece (white arrows) or formed loop (red arrow). (C) Tg-D sperm folded at the proximal midpiece. The sperm head is bent back on the tail (arrow). (D) Tg-D sperm formed loop (arrow). (E) Tg-D sperm head and neck attached to the midpiece (arrow). (F) Percentage of sperm with normal and abnormal morphology. (G) Immunofluorescence of Dnaic2 in WT sperm, showing Dnaic2 protein expressed in the sperm midpiece and membrane of sperm head (arrow). (H) Morphology of WT sperm after fixation with 4% paraformaldehyde and immunofluorescence.

Figure 6 Production of Kd-O mice. (A) Diagram of the Kd-O strategy. U6, U6 promoter; SV40 (Simian Virus), SV40 early promoter; Puro, puromycin-N-acetyl transferase gene; shOocyte-G1, the shRNA expression cassette consists of a 29-bp target Oocyte-G1 specific sequence (see the section ‘Materials and methods’), a 7-bp loop, and another 29-bp reverse complementary sequence. (B) PCR analysis of Kd-O offspring. PCR was performed using primer set (5′-GAGCGTCGAAGCGGGGGCTGTTC-3′ and 5′-CAGGTCGCGCGGTCCTTCGGGCACTC-3′) specific for Puro gene. The PCR product was sequenced for confirmation of the presence of Puro gene. M, 100-bp DNA ladder; lanes 1, 3, 7, Puro-positive mice; lanes 2, 4, 5, 6, WT mice. (C) PCR analysis of offspring with primer set (5′-CTGACCCTGTGGAATGTGT-3′ and 5′-GGGCTTGTACTCGGTCAT-3′) specific for SV40 (Figure 6A) early promoter. The PCR product was also sequenced for confirmation of the presence of SV40 early promoter. M, 100-bp DNA ladder; lane 1, positive control (DNA from pRS shOocyte-G1 plasmid); lanes 2, 4, SV40-positive mice; lane 3, WT mouse, lane 5, negative control. (D) Southern blot analysis of offspring with a probe, a 303-bp-long PCR amplified fragment using primers for SV40 early promoter (see above). Genomic DNA was digested with EcoRI. Size markers are shown on the right of the Southern blot. Lanes 1, 3, SV40-positive mice; lane 2, WT mouse. (E) Representative western blot analysis of Oocyte-G1 in testis, lung and kidney protein obtained from WT mouse (lane 1) or Kd-O (lane 2). (F) Western blots were quantified by densitometry and the data were expressed as a ratio of Oocyte-G1:β-tubulin (mean ± SEM). 1, western blot analysis of WT mouse; 2, western blot analysis of Kd-O. *P < 0.05. (G) Kd-O (right) and WT (left) testes from 18-day-old mice.
Figure 7 Phenotype of Kd-O mice. (A and B) Representative morphology of ovaries from WT (A) and Kd-O (B) mice at 18 days of age, showing that most follicles were atretic (white arrows) and large antral follicles were absent in Kd-O ovaries. Black arrows indicate large antral follicles. (C and D) Representative views of testis sections from WT (C) and Kd-O (D) mice at 18 days of age. There was active spermatogenesis in all seminiferous tubules (arrows) of WT mice. Abnormal spermatogenesis or disorganization in the development of spermatogenesis was observed in all seminiferous tubules of Kd-O mice. (E and F) Representative morphology of the hippocampus from WT (E) and Kd-O (F) mice at 18 days of age. An abnormality in the dentate gyrus of the hippocampus (arrow) was observed in brains of Kd-O mice (F). (G and H) Morphology of the dentate gyrus (higher power) from WT (G) and Kd-O (H) mice. A broken dentate gyrus (arrow) was found in brain of Kd-O mice (H). Scale bars, 50 μm (A, C, D), 25 μm (B, G, H), 200 μm (E, F).

Discussion

We previously showed that GFP transgenes were generated by random recombination in long-term cultured FGSCs (Zou et al., 2009). However, whether or not short-term cultured FGSCs can be transfected and produce transgenic offspring is still unknown. This is because the number of FGSCs in the ovary is very low, especially in the adult ovary. To create a rapid, inexpensive and efficient method of transgenesis, we first isolated and then short-term cultured FGSCs. The FGSCs were then injected with GFP virus and transplanted into ovaries of infertile mice. Transplanted cells underwent oogenesis and the mice produced offspring that had the GFP transgene. To exclude whether isolated and short-term cultured oocytes can be transfected and produce transgenic mice, we injected the oocytes with GFP virus and transplanted them into ovaries of sterile mice. No offspring were observed in the recipient mice. We also injected washing buffer into the ovaries of adult mice as a control, and no transgenic offspring were generated. Therefore, the results indicate that GFP transgenic mice were generated by viral infection of short-term culture FGSCs and also suggest that these FGSCs have a physiological role in the mouse ovary. Using the same method, Tg-D and Tg-O mice were generated. The phenotype of Tg-D mice is similar to that of PCD patients (Zariwala et al., 2007; Escudier et al., 2009). Some Tg-D female mice become subfertile, and all Tg-D male mice become sterile, suggesting that Dnaic2 is recessive gene placed on the X chromosome. The Tg-O mice showed postnatal growth retardation. However, there were no obvious functional changes in the lungs, kidneys and brains of the Tg-O mice. These differences are caused by two possible reasons: (1) there are other possible pathways that deactivate the Oocyte-G1 transgene in the lung, brain and kidney tissues; (2) Oocyte-G1 transgene may only be transmitted in the sexual organs. Further studies are needed to confirm this. In addition, we also produced Kd-O CD-1 mice by liposome-mediated transduction of the FGSCs, which differs from viral transduction in mechanism. Liposome-mediated transduction uses a DNA sample of the target gene coated with a special kind of lipid. When the coated DNA is mixed with the FGSCs, they engulf it through endocytosis. The DNA stays in the cytoplasm of the FGSC until the next FGSC division, at which time the FGSC nuclear membrane dissolves and the DNA has a chance to enter the nucleus, suggesting that liposome-mediated transduction is useful for proliferating cells. Viral transduction is when a viral RNA molecule is reverse transcribed and the cDNA of the target gene, flanked by the LTRs, is integrated into the FGSC DNA when the FGSCs are infected. The phenotype of Kd-O mice is partly similar to that of kinesin superfamily protein 2A (KIF2A) knock-out mice (Homma et al., 2003). We have demonstrated that transgenic or knock-down mice can be generated by not only viral but also liposome-mediated transduction of short-term cultured FGSCs, and that more than one strain of FGSCs can produce transgenic offspring.

Transgenic animals are currently produced using DNA microinjection of zygotes, microinjection of mutated embryonic stem (ES) cells into blastocysts, nuclear transfer of mutated somatic cells into oocytes (Zuckerman, 1991; Brinster et al., 1985; Nagy et al., 2003) or random mutagenesis of SSCs (Nagano et al., 2001). We have demonstrated a rapid, inexpensive and efficient new method for creating transgenic animals by random recombination of a functional gene in FGSCs or by knock-down targeting genes of FGSCs isolated from neonatal and adult mice. The use of preimplantation embryos, for direct DNA microinjection into fertilized eggs as well as ES cell work, can be prohibitively expensive.
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and difficult, particularly with larger animals (Jaenisch, 1988; Pursel et al., 1989; Wilmut et al., 1991; Hyttinen et al., 1994). These methods require expensive equipment and specialized training. Random mutagenesis of SSCs or FGSCs might provide an alternative approach. However, the method for transferring a gene in SSCs requires 4–5 months before transgenic mice are obtained (Nagano et al., 2001). Gene transfer in FGSCs requires less time and takes only 2 months to produce transgenic offspring. Furthermore, the gene-transfer efficiency (29%–37%) of FGSCs is much higher than that of SSCs (4.5%) (Nagano et al., 2001). Similarly with SSC-mediated transgenic techniques, derivation of our method may hopefully be adapted for various mammalian species, especially livestock. Apart from opening new avenues of basic research into oogenesis and self-renewal of stem cells, FGSC-mediated transgenesis has important implications for future gene therapies and biotechnology research.

Materials and methods

Animals

C57BL/6, CD-1, and C57BL/6 × CD-1 F1 hybrid mice were used in this study. Six-week-old C57BL/6 × CD-1 F1 hybrid and CD-1 females that were sterilized by intraperitoneal injection of busulfan (30 mg/kg; resuspended in DMSO) and cyclophosphamide (120 mg/kg) were used as recipients. Controls were injected with DMSO. All procedures for 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 FGSCs

FGSCs were isolated and cultured as previously described with modifications (Wu et al., 2008; Yuan et al., 2009; Zou et al., 2009). Briefly, ovaries from 5-day-old mice (n = 6–10) or adult mice (n = 3–5) were collected for each experiment. Dissected ovaries were placed in Hank’s balanced salt solution (HBSS) containing 1 mg/ml collagenase (Type IV, Sigma), then incubated at 37°C with gentle agitation for 15 min. After washing two to four times in HBSS, the ovarian tissue was placed in HBSS containing 0.02% EDTA and 0.05% trypsin, and incubated at 37°C with agitation for 10 min. Trypsin was neutralized by adding fetal bovine serum (FBS) when the cells were dispersed. The suspension was then centrifuged and the supernatant was carefully removed from the pellet. The pellet was resuspended and the isolated cells were cultured at a final concentration of 5–10 × 10^5 cells/ml. FGSCs were cultured on STO (SIM mouse embryo-derived thiguanine and ouabain resistant) feeders in 500 μl/well FGSC culture medium. The culture medium for FGSCs consisted of minimum essential medium alpha medium, 1 mM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 10 ng/ml mouse leukemia inhibitory factor (LIF; Santa Cruz, Inc.), 10 ng/ml mouse epidermal growth factor (Sigma-Aldrich), 40 ng/ml human glial cell line-derived neurotrophic factor (R&D Systems), 1 ng/ml human basic fibroblast growth factor (bFGF; BD Biosciences), 10% FBS, and 15 μg/ml penicillin. The medium was changed every 2 days. All cultures were maintained at 37°C in a 5% CO₂ atmosphere.

Construct

A 1.872-kb fragment containing the ORF of Dnaic2 or a 2.994 kb fragment containing ORF of Oocyte-G1 was subcloned into EcoRI-Xhol sites of the pFB-Neo retroviral vector (Stratagene) (pFB-Neo-Dnaic2, or pFB-Neo-Oocyte-G1), which was used to generate infectious virus particles that carry the Dnaic2 or Oocyte-G1 gene. The Oocyte-G1-specific shRNA expression vector, pRS-shOocyte-G1, was constructed by OriGene Technologies, Inc. The target sequence of shOocyte-G1 is ATCCAGAGACTCAAGTGCAAGATCGACCA.

Retroviral transfection of FGSCs

Human embryonic kidney 293T cells (ATCC) were cultured on 60-mm tissue culture dishes with culture medium. One day later, the cells were transfected with 3 μg env-expressing vector (pVpack-VSV-G), 3 μg gag-pol-expressing vector (pVpack-GP), and 3 μg transfer plasmid, MSCV-PGK-GFP or pFB-Neo-Dnaic or pFB-Neo-Oocyte-G1 vector, using the ViraPack Transfection Kit (Stratagene). After the cells were transfected for 48 h, the retroviral supernatants were collected and passed through a 0.45-μm filter. The collected retroviral supernatants were immediately used for titering and transduction. The titer was ca. 10^6 cfu/ml. For each FGSC culture well of 24-well plates, 200 μl retroviral supernatant and 10 μg/ml diethylaminoethyl-dextran was added. The FGSC cultures were incubated at 37°C for 3 h, and 200 μl of FGSC culture medium was then added for 2 days.

Transfection of FGSCs

After culture for 3–5 days with the same protocol as above, the FGSCs were transfected with pRS-shOocyte-G1 vector using TurboFectin 8.0 transfection reagent according to the manufacturer’s instructions (OriGene). In brief, the lipid–DNA complex was formed by adding 0.6 μl TurboFectin 8.0 and 0.2 μg pRS-shOocyte-G1 vector into 20 μl serum free culture medium and incubating at room temperature for 15–30 min. Spent medium was removed from the FGSC culture well (see above) and replaced with 500 μl of fresh FGSC culture medium. The lipid–DNA complex was added to the FGSCs for 2 days.

BrdU labeling

BrdU (50 μg/ml; Sigma-Aldrich) was added to FGSC cultures for 5 h. The cultures were prepared for immunofluorescent staining with BrdU as described below.

Preparation of ovary, testis, lung, brain, kidney and fallopian tube tissue

Ovaries from recipient and control animals or ovaries, testes, lung, brain, kidney and fallopian tube tissue from WT and transgenic mice were fixed with 4% paraformaldehyde and 0.2% glutaraldehyde (4°C, overnight), dehydrated through a graded series of ethanol, vitrified in xylene and embedded in paraffin. The sections (6-μm thick) were mounted on slides. Prior to immunofluorescence staining, slides were dewaxed with xylene and a reverse graded series of ethanol. Slides used for histological analysis were stained with hematoxylin.
**Immunofluorescence**

After equilibration in phosphate-buffered saline (PBS), deparaffinized sections were digested with 0.125% trypsin for 15 min at 37°C, and then washed twice with PBS. The sections were blocked for 10 min with blocking solution (10% normal goat serum in PBS) at room temperature then incubated overnight at 4°C with rabbit polyclonal anti-GFP (dilution 1:200; Chemicon). After washing in PBS, the sections 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 4',6-diamidino-2-phenylindole (DAPI, dilution 1:2000). The sections were then covered with mounting medium (glycerol diluted 3:1 in PBS). Representative sections were photographed with a Nikon Eclipse E600 microscope equipped with a Nikon Dxm 1200 digital camera, using fluorescein optics for FITC and ultraviolet optics for DAPI.

FGSC cultures or sperm were fixed with 4% paraformaldehyde for 15 min at room temperature. After fixation, the FGSCs or sperm were blocked in 10% normal goat serum in PBS for 60 min at room temperature, followed by rinsing and overnight incubation at 4°C with primary antibodies, rabbit polyclonal anti-MVH (1:200 dilution; Abcam), or mouse monoclonal anti-BrdU (1:200 dilution; Lab Vision Corporation) or rabbit polyclonal anti-Dnaic2 (1:50; Proteintech Group Inc.). After washing, FGSCs or sperm 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 washed, mounted in DAPI-containing medium, and viewed as described above.

**Morphology and ultrastructure of sperm**

For light microscopy analysis of epididymal spermatozoa, cauda epididymes from Tg-D and WT mice were dissected and minced in PBS. Sperm were squeezed out with fine forceps and allowed to disperse in PBS at room temperature for 10 min. The sperm were observed under phase-contrast microscopy. Samples were fixed in 4% paraformaldehyde. Sperm were counted using a hematocytometer (four times for each sample).

The electron microscopy was performed according to the methods described by Wilton et al. (1985) with some modifications. Briefly, sperm were collected and fixed for 2 h in 2.5% glutaraldehyde fixing buffer, post-fixed with 1% OsO_4 for 2–3 h and then dehydrated through a graded series of alcohols. Finally, the sperm were left in acetone and embedded. Fifty to sixty nanometer thick sections were obtained by LKB-I ultramicrotome, post-stained with uranyl acetate and lead citrate, and then sixty nanometer thick sections were obtained by LKB-I ultramicrotome. Sections were then covered with mounting medium (glycerol 3:1 with PBS and resuspended in the culture medium. Recipients were anesthetized by an intraperitoneal injection of pentobarbital sodium (45 mg/kg body weight). Approximately 6 μl of a single-cell suspension, containing 2–5 × 10^3 cells, or 6 μl of the third washing buffer (see above) for control, was injected into each ovary of the recipients (pretreatment with cyclophosphamide and busulfan for 1 week). Microinjection of each ovary was performed as previously described (Zou et al., 2009).

**Collection, culture, GFP virus infection and transplantation of oocytes or oocyte–cumulus complexes**

Ovaries were collected from 6-week-old mice in Dulbecco’s PBS (DPBS; Gibco) supplemented with 3 mg/ml BSA (Sigma) and maintained at 37°C. The follicles from the collected ovaries were opened using two Omnican needles and oocyte–cumulus complexes (OCCs) and oocytes were flushed into the same solution as above (Wu et al., 2001). After washing three times in FGSC culture medium, the OCCs and oocytes were transferred to the same medium and cultured in the same conditions as that of FGSCs. The OCCs and oocytes were infected by MCV-PGK-GFP virus and transplanted into ovariies of sterilized recipients using the same protocols as those of FGSCs.

**Southern blot analysis**

Genomic DNA was extracted from tails of mice with a DNeasy Tissue Kit, following the manufacturer’s instructions (Qiagen, Valencia, CA, USA), and digested with EcoRI. Samples were loaded onto 0.7% agarose gel and run at 35 V for 10 h. After depurination, denaturation and neutralization as described by Wu et al. (2005), DNA was transferred to a nylon membrane (Amersham Pharmacia Biotech) using capillary action. Hybridization was performed with a probe (Figures 2A, 3A, and Supplemental Figure S2A). Prehybridization, hybridization and subsequent washings were carried out as previously described (Wu et al., 2005).

**Quantitative RT-PCR (qPCR)**

Total RNA was extracted from each sample using Trizol reagent (Qiagen) according to the manufacturer’s instructions. qPCR was performed and analyzed using SensiMix™ One-Step Kit (Quantace) according to the manufacturer’s instructions. Intron-spanning primers were designed by Primer premier 5.0 (Applied Biosystems) to amplify a 160-bp fragment of Dnaic2. Amplicons were measured by SYBR Green fluorescence in 10 μl reactions. Reactions were performed in triplicate. The amount of product was determined relative to an internal control gene, glyceraldehyde 3 phosphate dehydrogenase (Gapdh). The primers used for qPCR are as follows: 5’-AAGAACGTTGACCCGGGACACG-3’ (forward primer of Oocyte-G1), 5’-TGCGGAGGACGCTTCAAGCAGTTGAGCAGTTGACCCGGGACACG-3’ (reverse primer of Oocyte-G1), 5’-CCCGGACTGTTCTGAGGACAGGT-3’ (forward primer of Dnaic2), 5’-CCCGGACTGTTCTGAGGACAGGT-3’ (forward primer of Dnaic2), 5’-TGCGGAGGACGCTTCAAGCAGTTGAGCAGTTGACCCGGGACACG-3’ (forward primer of Gapdh), 5’-TGCGGAGGACGCTTCAAGCAGTTGAGCAGTTGACCCGGGACACG-3’ (reverse primer of Gapdh). Melting curves showed that PCR yielded a single product. Ct values for each sample were normalized against that of input DNA and percentage recovery was plotted. Results were analyzed with 2^(-ΔΔCt) methods.
Western blot analysis

Western blot methods used were as previously described (Wu et al., 2005; Shen et al., 2010; Sondarva et al., 2010). The membranes for western blot were exposed to anti-Oocyte-G (1:50, Proteintech Group Inc.), anti-DnaJC2 (1:50, Proteintech Group Inc.) and anti-β-tubulin (1:500, Santa Cruz Biotechnology).

Supplementary data

Supplementary data for this article are available online at http://jmb.oxfordjournals.org. Supplementary data including one table and six figures can be found online.

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References


De Rooij, D.G., and Russell, L.D. (2000). All you wanted to know about spermatogonia but were afraid to ask. J. Androl. 21, 776–798.


