Supplementary information, Data S1

Materials and Methods

Mouse spermatogonial culture

Mouse use and surgical procedures followed the NIH protocols and were approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences. F1 progenies of C57BL6 and DBA/2 mice were used for regular mSPG culture. The donors and recipients for spermatogonial transplantation experiments were CD1 and busulfan-treated B6/DBA hybrid mice, respectively. Before transplantation, the 2-week mSPG culture was infected with GFP lentivirus. Spermatogonial culture from CD1 GFP-transgenic mice was used in FGF2 knockdown followed by transplantation to test SSC activity.

Mouse spermatogonia isolation, enrichment and culture were performed following protocols developed by Kubota *et al* [1]. Briefly, testicular cells were isolated from the testes of the 5~6-dpp pup mice by two-step enzymatic digestion. In the first step, albuginea-removed testes were digested with 1 mg/ml type IV collogenase (Sigma) and 500 μg/ml DNase I (Sigma) for 5 min at 37 °C until the surface of the tubules becomes rough as a result of projected cells. In the second one, the tubules were digested with 0.25% trypsin (Invitrogen) and 500 μg/ml DNase I at 37 °C for 5 min into single cells. About 1×10^7 dissociated cells were resuspended in 5 ml of DPBS containing 1% FBS (Hyclone) and were overlaid on the top of 2 ml of 30% Percoll solution and centrifuged. The anti-Thy-1 antibody magnetic microbeads (Miltenyi Biotec) were used for MACS to enrich the Thy-1 positive cells from the percoll-fractioned cells. Spermatogonia were cultured in the serum-free MEMα (Invitrogen) supplemented with other components such as BSA, insulin, transferrin, free fatty acids, *etc* [1], and this medium is referred to as SSCM. The cell density is $5 \times 10^4/cm^2$ for high density culture and is $1 \times 10^4/cm^2$ for low-density culture, for which exogenous sFGF2 (5 ng/ml) was required. Spermatogonia were passaged every 4-5 day at the dilution of 1:2-1:4 depending on the size of the clumps and the growth of the somatic cells. The medium was changed every 2 days.

Immunocytochemistry and immunohistochemistry

For immunocytochemistry, cultured-mSPG cells were fixed by 4% paraformaldehyde for 15 min at room temperature (RT). Fixed cells were washed and blocked. Cells were incubated for 1 h with primary antibodies at the appropriate dilution (see Supplementary information, Table S5) in blocking buffers at RT. Cells were washed in blocking buffer and incubated for 1 h with fluorescence or HRP-labeled secondary antibodies at the appropriate dilution (see Supplementary information, Table S5) in blocking buffer at RT. The color development was conducted using DAB kit (China) according to the manufacturer's instruction. DAPI or hematoxylin was used to label nuclei. Samples were imaged with a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss) or light microscopy.

For immunofluorescence staining of frozen sections, pup testes and adult testes were fixed for 24 h in 10% formalin (PH 7.5), equilibrated in 30% sucrose (Sigma) for 24 h at 4 °C, and embedded in O.C.T (Sakura). The 9 μm sections were cut and mounted on APES-coated slides. Sections were incubated with the primary antibodies at the appropriate dilution (see Supplementary information, Table S5) in blocking buffer overnight at 4 °C. Then the sections were washed and incubated for 1 h with fluorescence-labeled secondary antibodies at the appropriate dilution (see Supplementary information, Table S5) in blocking buffer at RT. DAPI was used to label nuclei. Samples were imaged on a Zeiss Lsm 510 Meta confocal microscope (Carl Zeiss).

Western blotting

For western Blotting, the same number of cells was lysed with 2× SDS-Page loading buffer, and then were resolved by SDS-PAGE (10%) and transferred to nitrocellulose membranes. Blots were blocked for 2 h with blocking buffer, and incubated with primary antibodies at the appropriate dilution (see Supplementary information, Table S5) in blocking buffer overnight at 4 °C. Blots were washed in TBST and incubated for 2 h with HRP-conjugated secondary antibodies at the appropriate dilution (see Supplementary information, Table S5) in blocking buffer at RT.

Blots were stripped and re-probed for total AKT and total ERK and β-Actin. The proteins were detected using the Western Blotting Supersignal West Pico Chemiluminescent substrate (Thermo) and exposed to autoradiography film. The films were processed with Adobe Photoshop 7.0. Signal intensity measurements were made using commercial software (Quanity One, Biorad).

Flow cytometric analysis of immunostained cells

For flow cytometric analysis of intracellular molecules (such as PLZF), the germ cells on feeder layer were collected by gentle pipetting and digested into single cells, and then the cells were first fixed and permeablized before antibodies were added. Briefly, 1×10^6 cells were fixed in 1 ml of 4% PFA for 10 min at RT. Cells were collected by centrifugation at 600× *g* for 5 min, and then permeabilized using 1 ml of cold 0.1% Triton X-100 in PBS for 10 min on ice. The cells were then washed and with 0.5% BSA/PBS, and blocked with the same solution for 10 min at RT. Cells were incubated for 30 min with primary antibodies at the appropriate dilution (see Supplementary information, Table S5) in blocking buffer at RT. Cells were washed in blocking buffer and incubated for 30 min with fluorescence-labeled secondary antibodies at the appropriate dilution (see Supplementary information, Table S5) in blocking buffer at RT. For flow cytometric analysis of cell surface molecules, cells were treated with the same protocols written above except for cells permeabilization. The isotype IgG of the same sources as the primary antibodies was used as negative control. These cells were analyzed using FACS-Calibur system (BD Biosciences).

Characterization of cultured mSPG that contained mSSCs

As shown in Supplementary information, Figure S1, small clumps were usually observed 2 days after initial plating while big ones could be seen during subsequent passages as a result of vigorous cell proliferation and self-aggregation. As reported by others, these clumps had an irregular shape in contrast to the round tight ESC colonies. Most of the time we could maintain the mSPG culture in an exponentially proliferating

state for about one month, during which enough cells could be acquired for further experiments. The positive immunostaining of DAZL, a post migratory germ cell marker, and OCT4, a stem cell marker that is also expressed in spermatogonial cells, indicated that clumps are undifferentiated spermatogonia or SSCs free of somatic cell contamination. The positive immunostaining of PCNA, a proliferating cell marker, indicated that mSPG were undergoing active proliferation. At the end of the 1-month culture period, mSPG expressed several spermatogonial markers including CD9 [2] (a spermatogonium enriched cell surface marker) and GFRα1 [3] (the GDNF ligand binding subunit mostly expressed in SSCs) by flow cytometry. c-Kit, a differentiating spermatogonia marker, was weakly expressed. These results indicated that our cultured mSPG had maintained the undifferentiated state at the end of the 1-month culture period.

Spermatogonial transplantation basically followed the protocol by Ogawa *et al* [4]. Briefly, mice pre-treated with busulfan were used as recipients. SPG clumps were trypsinized into single cells, plated on MEF feeder layers in fresh medium. After 3 h incubation, spermatogonia were infected with Ubiquitin promoter-EGFP lentiviral particles (GENECHEM, China). 48 h later, SSCM was replaced and mSPG culture was maintained for 7 additional days before transplantation. The culture was digested into single cells with trypsin. Total cell concentration for transplantation was 1×10^7 cells/ml. Approximately 20 μl of the cell suspension were introduced into seminiferous tubules of the recipients through the efferent duct. Recipient testes were collected at 2 weeks and 2 months after transplantation and imaged using the fluorescent microscope. Appearance of individual fluorescent patches in recipient testis tubules represented colonization and spermatogenesis from donor-derived stem cells. One patch was defined as one colony when it could occupy the entire tubule and was at least 0.1 mm long [5]. Three months after transplantation, offspring derived from transplanted spermatogonia were identified as green mice under UV light.

Endogenous FGF2 signaling blockade and rescue in mouse spermatogonia

sFGF2 signaling blockade in mSPG and its rescue by different proteins were conducted by culturing mSPG in different medium after a normal passage: normal SSCM (MEMα supplemented with 40 ng/ml GDNF, 5 ng/ml sFGF2 and many other components [1]); -F+S, exogenous FGF2-free normal medium supplemented with 10 μM FGFR specific inhibitor SU5402 (Calbiochem); -F+S+10G, -F+S medium supplemented with 400 ng/ml human GDNF (R&D Systems); -F+S+GR, -F+S medium supplemented with 400 ng/ml GFRα1 (R&D Systems); -F+S+E, -F+S medium supplemented with 10 ng/ml EGF; S, normal medium supplemented with 10 μM SU5402. The above rescue experiments were also conducted in the presence of ROCK inhibitor Y27632 (Calbiochem) which prevents apoptosis of spermatogonia. In order to exclude the toxicity of SU5402 and select a suitable SU5402 concentration, which completely block the possible sFGF2 signaling under the –F+S condition, we detected the effect of different concentrations of SU5402 on IVP mSPG proliferation by BrdU incorporation assay (Supplementary information, Figure S2A). About 10 times EC_{50} (10 μM) of SU5402 was chosen as the optimal dose for blocking endogenous sFGF2 signaling. The optimal concentration of Y27632 was also similarly determined from the dose response experiment (Supplementary information, Figure S2B). The EC_{50} of Y27632 was determined to be 4.3 μM. 10 μM of Y27632 was chosen as the optimal dose according to literature for inhibiting the apoptosis of spermatononia caused by sFGF2 signaling blockade. Fluorescent cells were analyzed using the FACS-Calibur system (BD Biosciences) and were imaged on a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss).

BrdU incorporation assay

To determine a suitable SU5402 concentration and analysis of endogenous FGF2 signaling blockade, 48 h after passage, mSPG cells were cultured respectively in –F+S condition with different SU5402 concentrations and in normal condition SSCM and in –F+S (10μM SU5402) for 24 h, and 10 μg/ml BrdU (Sigma) was added for another 24 h. To detect the effect of knockdown endogenous FGF2 on mSPG proliferation, BrdU

assays were performed 24 h after siRNA treatment. mSPG cells were exposed to BrdU for 1 h. The cultures were fixed in 4% paraformaldehyde for 15 min at RT, and washed with PBS. The cells were then treated with 1N HCl and 2N HCl for 10 min sequentially. After several washes, the cells were blocked and then were incubated with mouse anti-BrdU antibody at 4 °C overnight for immunocytochemistry and for 30 min for flow cytometry analysis. The FITC-conjugated goat anti-mouse IgG was used as the secondary antibody. The isotype IgG of the same source of primary antibody was used as the negative control. These cells were analyzed using FACS-Calibur system (BD Biosciences).

TUNEL assay

To determine the optimal concentration of Y27632 and and analysis of endogenous FGF2 signaling blockade, mSPG cells were cultured respectively in –F+S containing different concentrations of Y27632 and in normal condition SSCM and in –F+S (10μM SU5402) for 48 h. To determine the effect of knockdown endogenous FGF2 on mSPG survival, TUNEL assays were carried out 24 h after siRNA treatment. mSPG cells were fixed in 4% paraformaldehyde at room temperature for 15 min and incubated in permeabilization solution. The cells were labeled using the *in situ* cell death detection kit (Roche Applied Science, Germany) following the manufacturer's protocol, and were analyzed using the FACS-Calibur system (BD Biosciences) and were imaged with a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss).

Detection of endogenous FGF2 expression

For western blotting detection of FGF2 from cell lysates, spermatogonial clumps were collected by gentle pipetting and lysed with 2× SDS-PAGE loading buffer. MEFs, mESCs and hESCs were directly lysed with the same loading buffer after rinsing in PBS. MEF- and mSPG-conditioned media were collected after 5 days culture of corresponding cells in exogenous FGF2-free medium. Proteins of conditioned medium (CM) were concentrated using the Micron centrifugal filter device (Millipore). The proteins were

detected using the western blotting assays. For quantitation of sFGF2 by ELISA, 4×10^5 spermatogonia were seeded in a 6-well culture plate in SSCM without exogenous sFGF2. After 5 days, the mSPG-conditioned medium was collected. ELISA was performed using mouse FGF2 ELISA kit (CUSABIO, China) according to the manufacturer's protocol. The experiment was independently repeated six times.

RNA interference of FGF2 by shRNA

The FGF2 shRNA was introduced to the cells with the lentivirus that was produced from the 293T cells using the transfer vector plasmid pLL3.7, and the packaging plasmids pRRE, pREV, and pLVSV-G [6-8]. All plasmids were purified with Qiagen Endo-Free Maxiprep Kits. The FGF2 shRNA sequence follows a previous report [9]. Scrambled sequences were used as negative controls. The sense and antisense oligos (Sense: 5'-TAAACGACTGGGCAGTATATTCAAGAGATATACTGCCCAGTTCGTTTCAGT GCTTTTTTC-3'; Antisense: 5'-TCGAGAAAAAAGCACTGAAACGAACTGGGCAGTATATCTCTTGAATATAC TGCCCAGTTCGTTTCAGTGCA-3'; Scrambled sense: 5'-TGC AAGAAGCAATCGGCGGATTCATATTCAAGAGATATGAATCCGCCGATTGCTT CTTGCTTTTTTC-3'; Scrambled antisense: 5'-TCGAGAAAAAAGCAAGAAGCAATCGGCGGATTCATATCTCTTGAATATGA ATCCGCCGA TTGCTTCTTGC A-3') were annealed, phosphorylated with T4 polynucleotide kinase, and inserted into the lentiviral transfer vector pLL3.7 using restriction sites XhoI and HpaI. The construct was confirmed by sequencing. To produce shRNA lentivirus particles, 293T cells (5×10^6 cells/10-cm dish) were cotransfected with plasmids pLL3.7-shRNA/scrambled, pLVSV-G , pRRE , and pREV (11.5 μg, 4 μg ,7.5 μg, 3 μg per dish, respectively) by Lipofectamin 2000 transfection reagent (Invitrogen), viral particle-containing supernatants were harvested twice every 24 h. These supernatants were filtered through 0.45-μm pore size PVDF filters (Millipore) and concentrated by ultra centrifugation (20 000× *g* for 2.5 h). Viral particles preparations

were tittered on 293T cells for GFP expression by flow cytometry. The titer ranged from 1×10^6 to 6×10^6 IU/ml.

RNA interference of FGF2 by synthetic siRNA

The siRNA of FGF2 and scrambled control were synthesized according to published sequences [9]. Spermatogonial culture was digested into single cells (1×10^6 cells), which were transfected with 90 pmole of siRNA using Lipofactamine 2000 (Invitrogen) according to the manufacturer's protocol. Proteins were obtained from cells 24 h after transfection and determined by western blotting. 1×10^5 cells 24 h after siRNA treatment were transplanted into each of the testes of recipient mouse. Colony numbers were normalized for initial cultured cell numbers (1×10^5). 9 recipients for control group and 7 recipients for siRNA group were used for 3 independent experiments. The comparison was performed using the *t*-test. Colonies formed by transplanted SSCs were counted 1 month after transplantation.

Preparation of MEF-conditioned medium

To prepare MEF-conditioned medium (MCM), mitomycin C-inactivated MEF cells were cultured in SSC medium in the presence or absence of sFGF2 (5 ng/ml) for 5 days with medium changed every day. The medium was collected and pooled and was named 5F-MCM or 0F-MCM. These two types of MCM were then supplemented with additional FGF2 to make the theoretical final concentration to be 10 ng/ml, resulting in 5F-MCM-5F and 0F-MCM-10F, respectively. Similarly, 5F-MCM-SU5402 was prepared from 5F-MCM by supplementing 10 μM SU5402 before use. To culture spermatogonia on laminin-coated dishes, spermatogonial clumps cultured on MEFs were collected by gentle pipetting, dispersed into single cells by trypsin digestion and plated on laminin (Sigma) coated dishes in three types of MCM supplemented with 5% FBS.

Microarray analysis of genes regulated by sFGF2

Normal mSPG culture was pipetted and digested into single cells and plated onto two 3.5-cm culture dishes with MEF as feeder cells. After a 3 day incubation period, medium

from one plate was removed and replaced with normal medium, thus serving as the control, while medium from the second plate was removed and replaced with medium devoid of exogenous FGF2 and containing FGFR specific inhibitor SU5402, thus serving as the treated sample. The culture was terminated 12 h later and RNA was isolated and purified with the QIGEN RNeasy mini RNA kit. Four independent experiments were conducted. Gene expression changes were examined using the Agilent Whole Mouse Genome Microarray Kit which is a dual color labeling microarray system (part number G4122F) based on 60-mer probes that can identify 41 000 genes (22505 Unigene Accession Numbers, UANs). The service was provided by Shanghai Bio Company Ltd. (China). The RNA quality of each sample was assured by using the Agilent 2100 Bioanalyzer. Differential gene screening was carried out with the RankProd R package [10]. The up- and down-regulated gene sets were submitted to the DAVID Functional Annotation Clustering (DFAC) web tool [11] for the identification of enriched functional annotation terms (FATs). DFAC not only identifies enriched FATS but also groups them into clusters and ranks them by their enrichment scores. FATs from the top 10 clusters with $P < 0.05$ were recorded along with their corresponding genes.

For comparing genes regulated by sFGF2 and GDNF, we downloaded microarray data generated by Oatley *et al*. from the NCBI GEO database [30]. We regarded the triplicate data sets from the 3 time points upon GDNF supplementation and the normal culture as treated groups under four different experimental conditions and the data sets from the GDNF withdrawn samples as the untreated group under each condition. Genes regulated by GDNF and enriched FATS were acquired using RankProd and DFAC software as was done for sFGF2. The similarity index for overlapped genes or enriched FATs was defined as the percentage of the elements in the intersecting set of the two sets to be compared over elements in their union set.

Examination of sFGF2-regulated gene expression by real-time PCR

Spermatogonia treatment and RNA isolation followed the same procedure as for microarray assays. 1 μg of total RNA was used to synthesize the first-strand cDNA with

random primers using the Superscript III kit (Promega). Primers were designed such that genomic fragments containing introns would not be amplified. PCR reactions were carried out using the ABI PRISM 7000 Sequence Detection System and analyzed using the comparative C_t method ($\Delta\Delta C_t$) with 18S RNA as the internal control. The primer sequences are listed in Supplementary information, Table S6.

Statistics

All experiments were independently repeated at least three times. All quantification data are presented as mean ± SEM. All statistical tests were performed using SPSS 13.0 software (SPSS Inc, Chicago, IL.). Significant differences between means for single comparisons were determined using the *t*-test. Multiple comparison analyses were performed using an univariate ANOVA followed by Tukey HSD test.

References

1 Kubota H, Brinster RL. Culture of rodent spermatogonial stem cells, male germline stem cells of the postnatal animal. *Methods Cell Biol* 2008; **86**:59-84.

2 Kanatsu-Shinohara M, Toyokuni S, Shinohara T. CD9 is a surface marker on mouse and rat male germline stem cells. *Biol Reprod* 2004; **70**:70-75.

3 Nakagawa T, Sharma M, Nabeshima Y, Braun RE, Yoshida S. Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. *Science* 2010; **328**:62-67.

4 Ogawa T, Arechaga JM, Avarbock MR, Brinster RL. Transplantation of testis germinal cells into mouse seminiferous tubules. *Int J Dev Biol* 1997; **41**:111-122.

5 Nagano M, Avarbock MR, Brinster RL. Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. *Biol Reprod* 1999; **60**:1429-1436.

6 Fleury S, Simeoni E, Zuppinger C, *et al.* Multiply attenuated, self-inactivating lentiviral vectors efficiently deliver and express genes for extended periods of time in adult rat cardiomyocytes *in vivo*. *Circulation* 2003; **107**:2375-2382.

7 Rubinson DA, Dillon CP, Kwiatkowski AV*, et al.* A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* 2003; **33**:401-406.

8 Villani GR, Follenzi A, Vanacore B*, et al.* Correction of mucopolysaccharidosis type IIIb fibroblasts by lentiviral vector-mediated gene transfer. *Biochem J* 2002; **364**:747-753.

9 Izikki M, Guignabert C, Fadel E*, et al.* Endothelial-derived FGF2 contributes to the progression of pulmonary hypertension in humans and rodents. *J Clin Invest* 2009; **119**:512-523.

10 Hong F, Breitling R, McEntee CW*, et al.* RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis. *Bioinformatics* 2006; **22**:2825-2827.

11 Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; **4**:44-57.