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Supplemental Information

FGF2 Has Distinct Molecular Functions from GDNF in the Mouse Germline Niche

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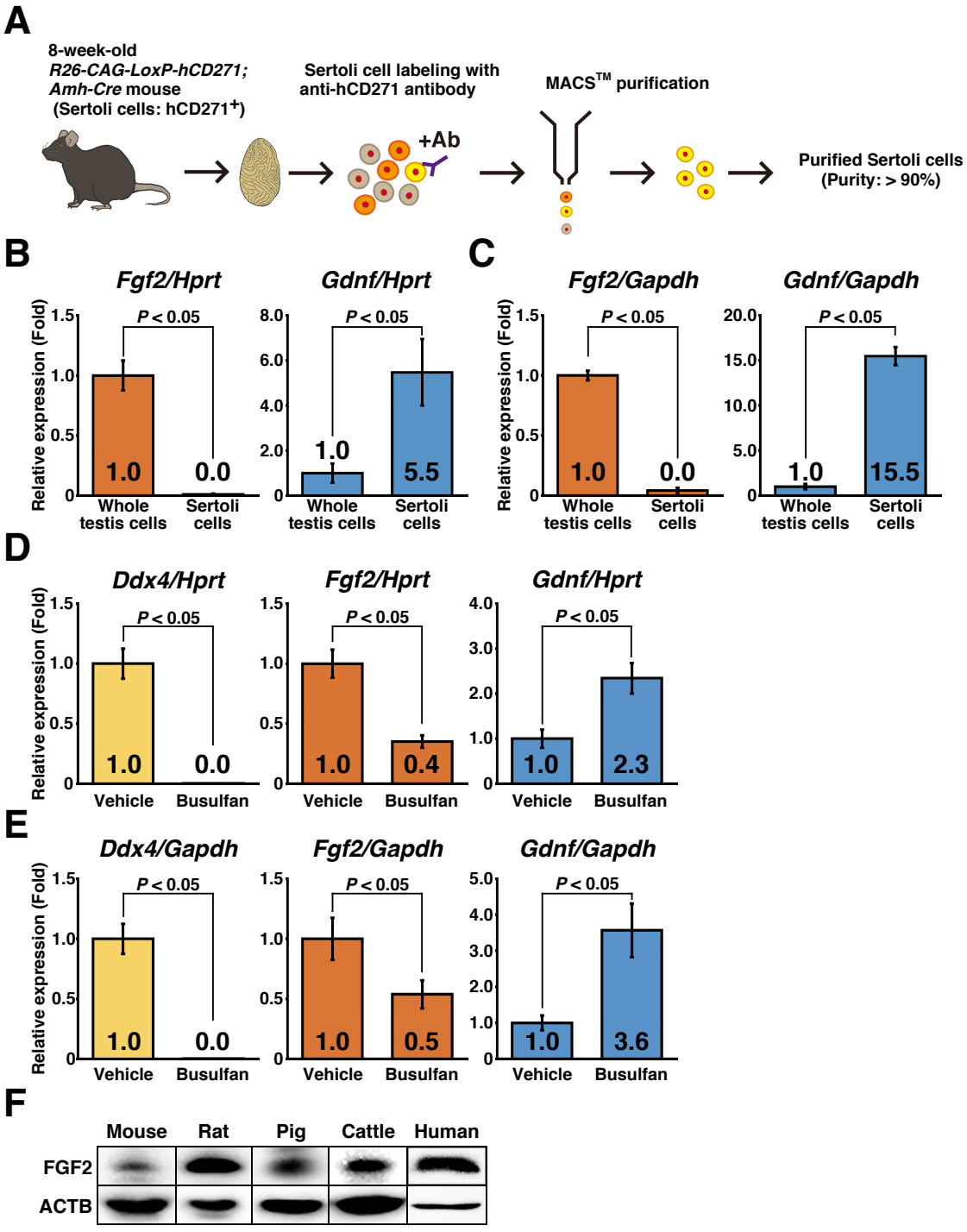

Figure S1, related to Figures 1 and 4. FGF2 expression in the testis. (A) Flow chart of Sertoli cell purification from an 8-week-old mouse testis. (B and C) qRT-PCR analysis of *Fgf2* expression in Sertoli cells. Whole testis cells before MACS™ purification and purified testis cells were compared. After normalization to *Hprt* (B) or *Gapdh* (C) expression, values of whole testis cells were set to 1.0 (n = 3 experiments using individual mice). Results were presented as means of values ± standard error of the mean. (D and E) qRT-PCR analysis of *Fgf2* expression in germ cell-depleted testes. Busulfan- and vehicle-treated testes were compared. After

normalization to *Hprt* (D) or *Gapdh* (E) expression, values of vehicle-treated testes were set to 1.0 (Vehicle, n = 5 testes from individual mice; Busulfan, n = 6 testes from individual mice). Results were presented as means of values ± standard error of the mean. (F) Western blot analysis of FGF2 in testes of mammals including mice, rats, pigs, cattle, and humans. ACTB was analyzed as a loading control. Typical blots from multiple experiments using independent samples are shown. $P < 0.05$ was considered as statistically significant (Student's *t*-test, B to E).

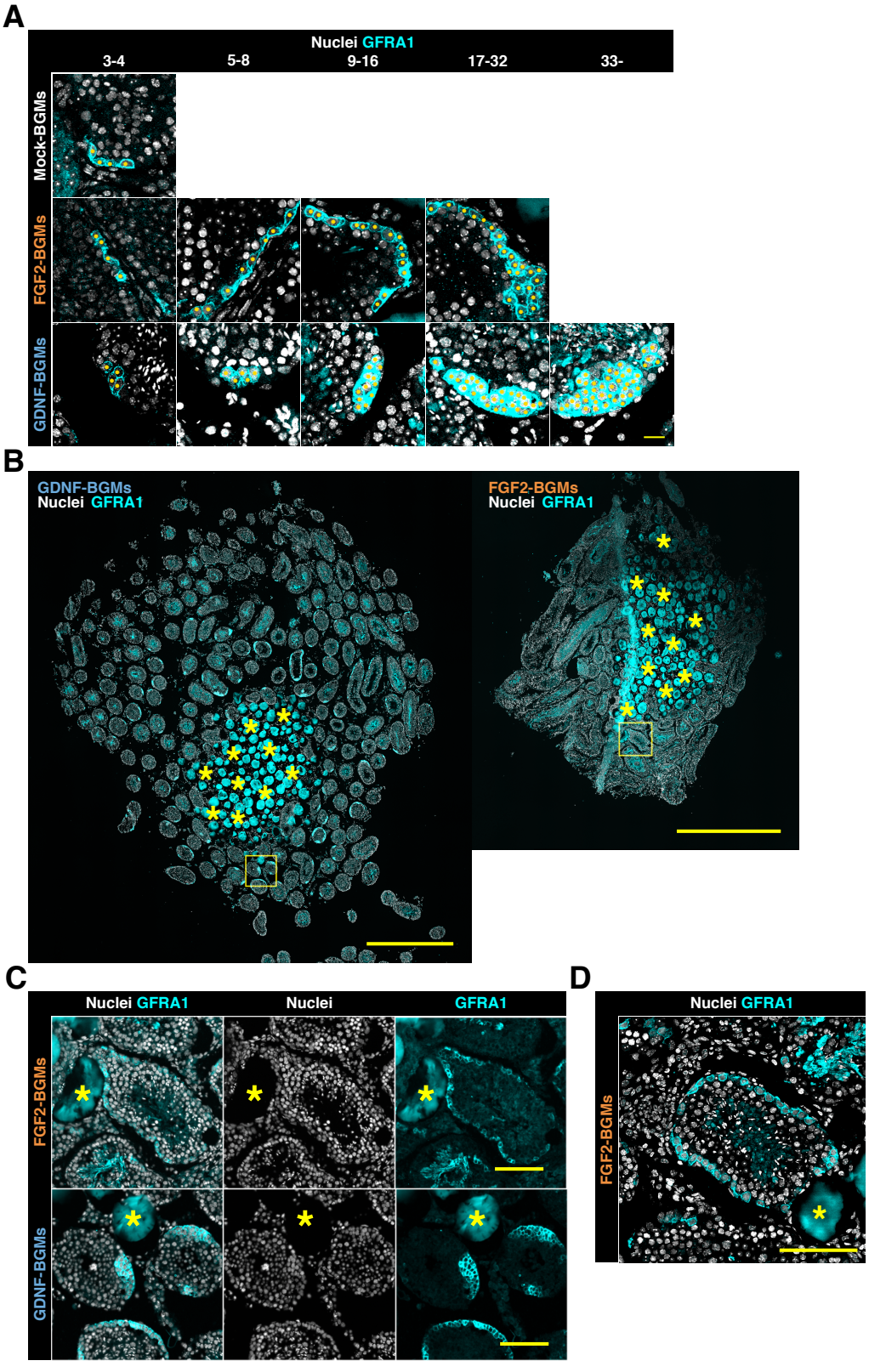
A
Nuclei GFRA1
3-4
5-8
9-16
17-32
33-
Mock-BGMs
FGF2-BGMs
GDNF-BGMs
B
GDNF-BGMs
Nuclei GFRA1
FGF2-BGMs
Nuclei GFRA1
C
Nuclei GFRA1
Nuclei
GFRA1
FGF2-BGMs
GDNF-BGMs
D
Nuclei GFRA1
FGF2-BGMs

Figure S2, related to Figure 1. GFRA1 expression in biodegradable gelatin microsphere (BGM)-treated mouse testes. Immunofluorescence image of BGM-transplanted testes at 10 days after transplantation. GFRA1 signals and nuclei are visualized by cyan and grey, respectively. (A) Typical images of large $GFRA1^+$ clusters classified by the number of $GFRA1^+$ spermatogonia. Yellow points indicate the spermatogonia forming cluster. (B) Overview of the testis at 10 days after transplantation of GDNF-BGMs (left) or FGF2-BGMs (right). Large $GFRA1^+$ clusters were often found around transplanted BGMs (marked with yellow asterisks). High power magnification images of regions indicated by yellow squares are shown in (C). (C) High power magnification views of large spermatogonial clusters observed in (B). Asterisks mark transplanted BGMs. (D) Most extreme case of FGF2-BGM treatment. The entire circumference of the seminiferous tubule was surrounded by $GFRA1^+$ spermatogonia. Asterisk indicates a transplanted FGF2-BGM. Bars = 20 μm (A), 1 mm (B), and 100 μm (C and D).

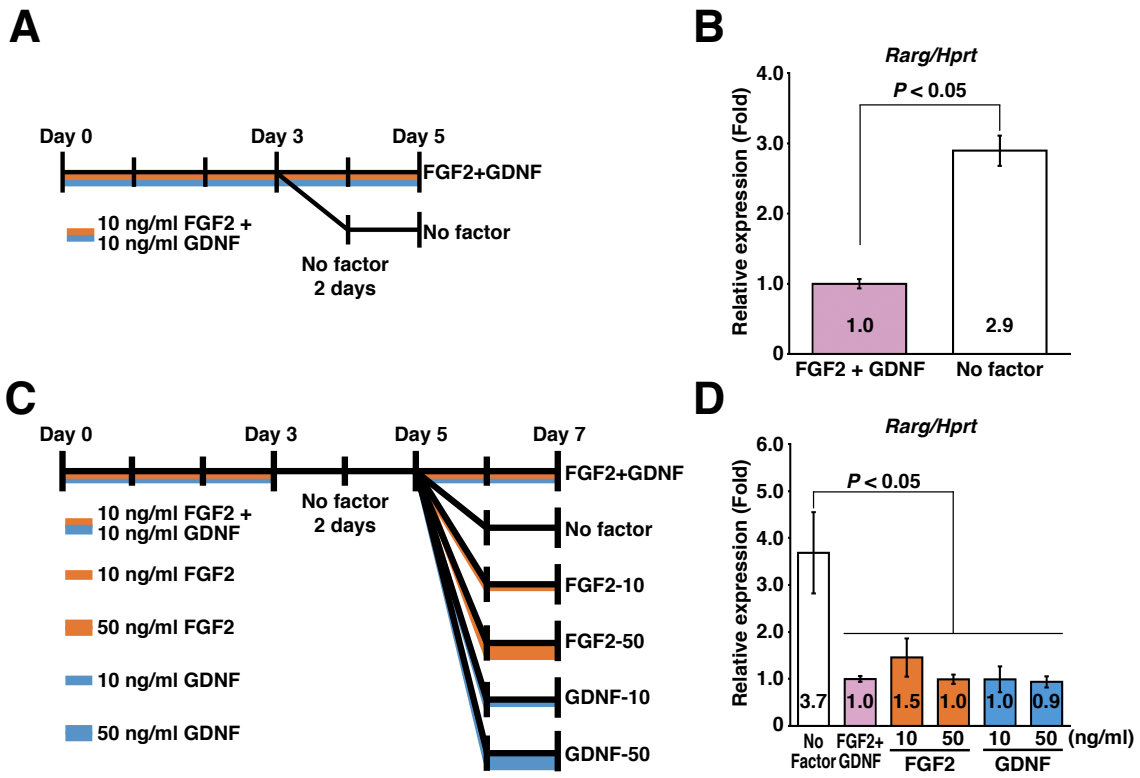

Figure S3, related to Figure 2. Both FGF2 and GDNF suppress *Rarg* expression in germline stem (GS) cells. GS cells were cultured on laminin-coated dishes under the conditions shown in (A) and (C), and then analyzed for *Rarg* expression by qRT-PCR. (A) GS cell culture conditions to assess the effects of FGF2 and GDNF. (B) qRT-PCR results of experiments shown in (A). After normalization to *Hprt* expression, the value of FGF2 + GDNF was set to 1.0 (n = 8 independent cultures for each group). Results were presented as means of values ± standard error of the mean. $P < 0.05$ was considered as statistically significant (Student's *t*-test). (C) GS cell culture conditions to assess the concentrations of FGF2 and GDNF. (D) qRT-PCR results of experiments shown in (C). After normalization to *Hprt* expression, the value of FGF2 + GDNF was set to 1.0 (n = 6 independent cultures for each group). Results were presented as means of values ± standard error of the mean. $P < 0.05$ was considered as statistically significant (one-way analysis of variance followed by Tukey's honest significant difference test).

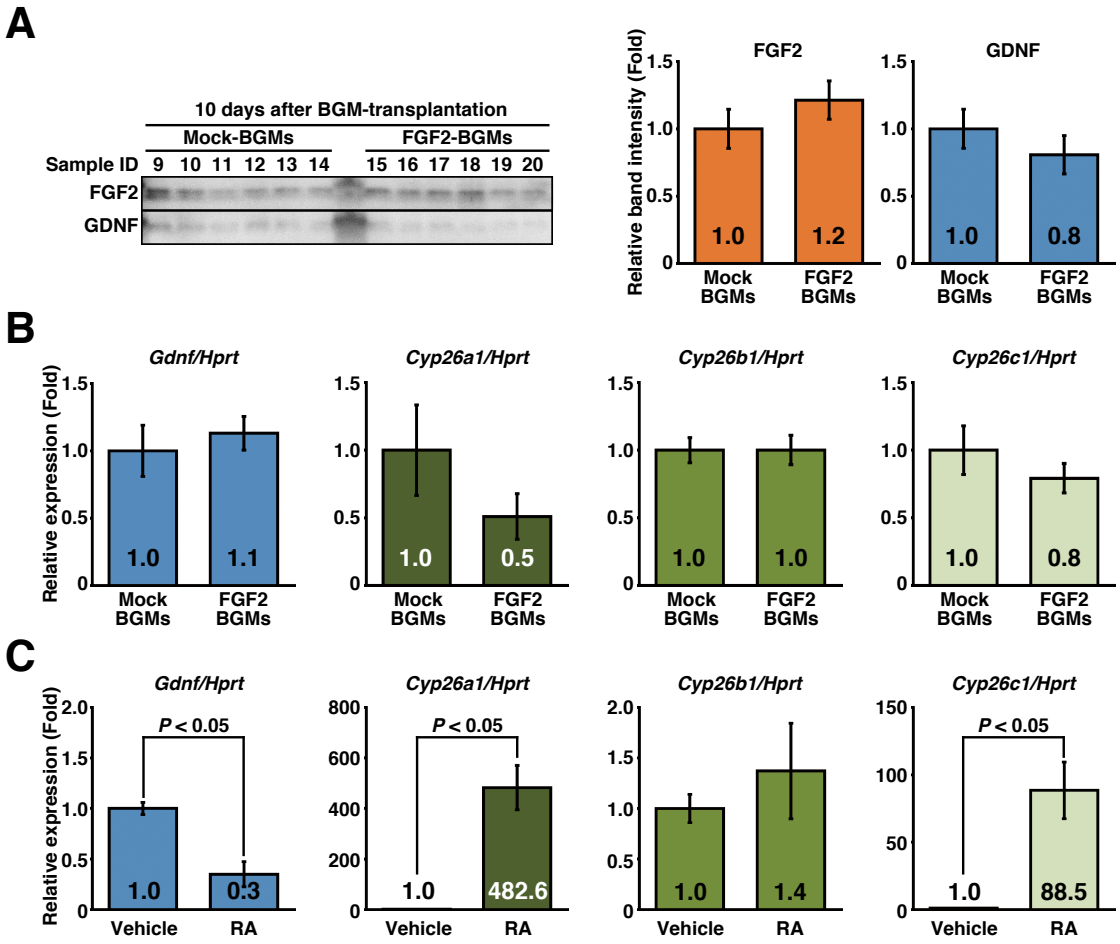

Figure S4, related to Figure 3. Functions of FGF2 and RA in the germline niche. (A) Western blot analyses of testes at 10 days after BGM treatment. Band intensities of Mock-BGM-treated testes were set to 1.0. (n = 6 testes for each condition). There was no significant difference in FGF2 levels between Mock-BGM- and FGF2-BGM-treated testes. Results were presented as means of values ± standard error of the mean. (B) qRT-PCR analysis of samples subjected to western blotting in (A). After normalization to *Hprt* expression, values of the Mock-BGM group were set to 1.0 (n = 6 testes for each condition). Results were presented as means of values ± standard error of the mean. (C) RA functions in the germline niche. At 4 weeks after busulfan treatment (44 mg/kg body weight), 7-week-old mice were further treated with RA (750 μg per mouse by intraperitoneal injection) or the vehicle. Testes were harvested at 11 hours after treatment and then subjected to qRT-PCR analysis. After normalization to *Hprt* expression, values of the vehicle group were set to 1.0 (n = 4 testes from individual mice for each condition). Results were presented as means of values ± standard error of the mean. $P < 0.05$ was considered as statistically significant (Student's *t*-test).

Table S1, related to Figures 1, 3, S1, S2, and S4. Antibodies used in this study

Antibody	Company
Primary antibody	
Goat anti-rat GFRA1 antibody (AF560)	R&D Systems, Inc., Minneapolis, MN
Rabbit anti-BrdU antibody (NBP2-14890)	Novus Biologicals, Littleton, CO
Rabbit anti-PLZF antibody (sc-22839)	Santa Cruz Biotechnology, Dallas, TX
Rabbit anti- RARG monoclonal antibody (#8965)	Cell Signaling Technology, Danvers, MA
Rabbit anti-mouse GDNF antibody (sc-328)	Santa Cruz Biotechnology
Rabbit anti-mouse FGF2 antibody (sc-79)	Santa Cruz Biotechnology
Rat anti-human CD271 monoclonal antibody (Clone ME20.4-1.H4, 130-091-883)	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Mouse anti-ACTB monoclonal antibody (Clone 2F3, 013-24553)	Wako Pure Chemical Indutries, Osaka, Japan
Secondary antibody	
Alexa Fluor 488-conjugated donkey anti-rabbit IgG (A-21206)	Thermo Fischer Scientific, Waltham, MA
Alexa Fluor 555-conjugated donkey anti-rabbit IgG (A-21432)	Thermo Fischer Scientific
Alexa Fluor 647-conjugated donkey anti-goat IgG (A-21447)	Thermo Fischer Scientific
Horseradish peroxidase-conjugated goat anti-mouse IgG (#7076)	Cell Signaling Technology
Horseradish peroxidase-conjugated goat anti-rabbit IgG (#7074)	Cell Signaling Technology

Table S2, related to Figures 2, 3, S1, S3, and S4. Primers for qRT-PCR

Gene	Forward primer	Reverse primer
Cyp26a1	TGACCCGCAATCTCTTCTCT	GAGGAGCTCTGTTGACGATTG
Cyp26b1	TGGTCACTGGTTGCTACAGG	TGGGCAGGTAGCTCTCAAGT
Cyp26c1	CAAAATCCAGCAGGAGCTGT	AACCGTCCAGTTCAAAGGTG
Ddx4	CAGGCAATGGTGACACTTACC	ATGGAGTCCTCATCCTCTGG
Fgf2	CCAACCGGTACCTTGCTATG	TATGGCCTTCTGTCCAGGTC
Gapdh	AACTTTGGCATTGTGGAAGG	CACATTGGGGGTAGGAACAC
Gdnf	GCCACTTGGAGTTAATGTCC	CTTCGAGAAGCCTCTTACCG
Hprt	GCTGGTGAAAAGGACCTCT	CACAGGACTAGAACACCTGC
Rarg	AGGTCACCAGAAATCGATGC	CTGGCAGAGTGAGGGAAAAG

Supplemental experimental procedures

Animals, treatments, and testis tissues

Male C57BL/6NCrSlc (B6) mice were purchased from Japan SLC (Shizuoka, Japan). Seven-week-old mice were used as recipients of BGM transplantation. After the operation, recipients were administered with BrdU via water (0.5 mg/ml) *ad libitum* until sacrifice. Three-week-old mice were treated with a single intraperitoneal injection of 44 mg/kg body weight busulfan (Sigma-Aldrich, St Louis, MO) to deplete germ cells and then used as recipients of BGM transplantation and RA treatment as described below. For gene expression analysis of germ cell-depleted mice, 4-week-old mice were treated with busulfan (44 mg/kg body weight) and then subjected to qRT-PCR analysis at 8 weeks of age. For RA treatment, all-trans RA (Sigma-Aldrich) was dissolved in a 10% dimethylsulfoxide-sesame oil (Nacalai Tesque, Inc., Kyoto, Japan) solution and then injected intraperitoneally into busulfan-treated 7-week-old mice at 750 μg per mouse. Testes were harvested at 11 hours after treatment. 8-week-old male BN/SsNSlc rats were olso purchased from Japan SLC. Pig and cattle testes were collected at the time of castration to improve meat quality and growth rates at The Nagano Prefectural Livestock Experimental Station. Castrations were conducted at 5 months (cattle) and 8 weeks (pigs) of age. The institutional animal care and use committee of Shinshu University approved all animal experimentation protocols (Approval No. 260013 and No. 280120). Human testis tissues without pathological lesions were obtained and subjected to the experiments in accordance with the institutional ethics review board of Shinshu University to use human-derived material (Test No. 3039), and the institutional ethics review board of Nagano Red Cross Hospital to use human-derived material (Nagano-Byo-Ki Approval No. 25). Written consent was obtained following the committee-approved protocol before tissue collection.

Transplantation of biodegradable gelatin microspheres (BGMs)

Growth factor-adsorbed BGMs were transplanted into the testicular interstitium of recipient mice using a syringe with a 25 G needle (Terumo corporation, Tokyo, Japan). Male C57BL6/NCrSlc mice at 7 weeks of age (Japan SLC, Hamamatsu, Japan) with or without 44 mg/kg body weight busulfan (Sigma, St. Louis, MA) treatment at 3 weeks of age were used as recipients for BGM injection. For experiments shown in Figure 1, mice were administrated BrdU via water (0.5 mg/ml, Sigma) *ad libitum* until sacrifice. At 1 or 10 days after the procedure, mice were sacrificed and testes were harvested for analyses.

Enrichment of Sertoli cells

Sertoli cells were enriched from *R26-CAG-LoxP-hCD271; Amh-Cre* mice with a B6 background at Tokushima University under approval of the institutional animal care and use committee of Tokushima University (experimental number 14,108) (Kuroki et al., 2015). In brief, after removing the tunica albuginea, testis tissue from 8-week-old adult mice was digested with collagenase followed by trypsin-EDTA (Sigma) digestion. Resultant testis cells were further treated with collagenase, hyaluronidase, and a trypsin inhibitor. Single cell suspensions were subjected to MACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) using an anti-human CD271 antibody (Miltenyi Biotec GmbH) (See also Table S1).

qRT-PCR

Total RNA was prepared using Sepasol-RNA I Super G (Nacalai Tesque, Inc.). To synthesize first-strand cDNA, RNA samples were subjected to RT reactions using ReverTra Ace with gDNA remover (TOYOBO, Osaka, Japan). Resultant cDNA samples were analyzed by qRT-PCR using SYBR Premix Ex Taq II with Thermal Cycler Dice® Real Time System TP800 (TAKARA BIO Inc., Shiga, Japan). The qRT-PCR conditions were 95°C for 1 minutes followed by 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by a melting curve program. Each qRT-PCR was run in duplicate. Ct values were calculated by second derivative maximum method on Thermal Cycler Dice® Real Time System Software Version 5.10 (TAKARA BIO Inc.). The standard curve method was applied to determine the absolute quantity. Standard cDNA stock for absolute quantitation was prepared from PCR product using FastGeneTM Gel/PCR Extraction Kit (Nippon Genetics Co. Ltd., Tokyo, Japan). For data evaluation, after normalization to *Hprt* or *Gapdh* expression, values of the control group were set to 1.0. Primers used in this study are listed in Table S2.

Immunofluorescence staining

Harvested testes were fixed with 4% paraformaldehyde in PBS (-) for 4 hours at 4°C. After overnight incubation in 30% sucrose/PBS (-) at 4°C, tissues were embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan) and processed for cryosectioning at 8 μm thickness. After permeabilization with 0.1% Triton-X 100 (Sigma) in ice-cold PBS (-) for 10 minutes followed by blocking with 10% donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), sections were incubated with a primary antibody overnight at 4°C. Sections were further incubated with a secondary antibody at room temperature for 1 hour. Hoechst 33342 (Sigma) was used for nuclear counterstaining. Images were captured by confocal laser scanning microscopy (FV1000-D; Olympus, Tokyo, Japan) or All-in-one fluorescence microscopy (BZ-X700, KEYENCE, Osaka, Japan). Collected images were manually (FV-1000D) or automatically (BZ-X700) joined to reconstruct an entire image of testicular cross sections. Only cross sections containing transplanted BGMs were analyzed. Antibodies used in this study are listed in Table S1.

Cell culture

GS cells, F-SPG, and G-SPG were originally established at T. Shinohara's laboratory and kindly provided. Culture medium used in this study was based on reports by Kanatsu-Shinohara et al (2003) for GS cells and Takashima et al. (2015) for F-SPG and G-SPG. GS cells were maintained by treatment with 10 ng/ml rat GDNF (Peprotech, London, UK) and 10 ng/ml human FGF2 (Peprotech). G-SPG were cultured with 10 ng/ml rat GDNF only, whereas F-SPG were cultured with 10 ng/ml human FGF2 only. All cell lines were maintained on laminin-coated dishes for more than 2 weeks and then subjected to qRT-PCR analysis. The concentrations of growth factors in experiments are indicated in the figures (Figures 2A, S3A, and S3C).

Western blotting

Collected testis samples were stored at -80°C and then lysed in lysis buffer consisting of 25 mM Tris-HCl (pH 8.0), 0.2% Triton X-100, 2.5 mM EDTA, 1 mM dithiothreitol (Sigma), and a protease inhibitor cocktail (Nacalai Tesque, Inc.). Protein concentrations were determined by BCA™ Protein Assay Kit-Reducing Agent Compatible (Thermo Fischer Scientific, Waltham, MA). After denaturation by mixing with sample buffer containing sodium dodecyl sulfate (SDS) and 2-mercaptoethanol followed by boiling, samples (60 μg protein/lane) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In case of FGF2 detection in human testes, samples were loaded at 100 μg protein/lane. Separated proteins were transferred onto polyvinylidene fluoride membranes (Amersham Hybond P 0.45: GE Healthcare, Buckinghamshire, UK), and then incubated with primary antibodies. After incubation with a horseradish peroxidase-conjugated secondary antibody, immunoreactive bands were detected using Lumi GLO reagent (Cell Signaling Technology, Inc., Danvers, MA) and a *LuminoGraph* I luminescence imager (ATTO CORPORATION, Tokyo, Japan). The signal intensities of specific bands were quantitatively analyzed by ImageJ (http://imagej.nih.gov/ij/index.html). Values of Mock-BGM samples were set to 1.0. The antibodies used are listed in Table S1.