LETTER

doi:10.1038/nature09850

In vitro production of functional sperm in cultured neonatal mouse testes

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Spermatogenesis is one of the most complex and longest processes of sequential cell proliferation and differentiation in the body, taking more than a month from spermatogonial stem cells, through meiosis, to sperm formation[1,2]. The whole process, therefore, has never been reproduced *in vitro* in mammals[3–5], nor in any other species with a very few exceptions in some particular types of fish[6,7]. Here we show that neonatal mouse testes which contain only gonocytes or primitive spermatogonia as germ cells can produce spermatids and sperm *in vitro* with serum-free culture media. Spermatogenesis was maintained over 2 months in tissue fragments positioned at the gas–liquid interphase. The obtained spermatids and sperm resulted in healthy and reproductively competent offspring through microinsemination. In addition, neonatal testis tissues were cryopreserved and, after thawing, showed complete spermatogenesis *in vitro*. Our organ culture method could be applicable through further refinements to a variety of mammalian species, which will serve as a platform for future clinical application as well as mechanistic understanding of spermatogenesis.

Studies on *in vitro* spermatogenesis date back to organ culture experiments about a century ago[8]. In 1937, it was reported that spermatogenesis proceeded up to the pachytene stage of meiosis in testis tissues of newborn mouse placed on a clot[9]. In the 1960s, organ culture methods had advanced and various conditions were extensively examined. However, it was not possible to promote spermatogenesis beyond the pachytene stage[10,11]. Thereafter, cell culture methods, instead of organ culture, were used with new concepts and devices, including immortalized germ cell lines[12], the production of Sertoli cell lines as feeder cells[13], bicameral chamber methods[14], etc[3,4]. Despite such endeavours, progress has been limited, and it is still impossible to produce fertility-proven haploid cells from spermatogonial stem cells *in vitro*[3–5].

At the outset of our research on *in vitro* spermatogenesis, we decided to re-evaluate organ culture methods first. According to the standard gas–liquid interphase method[15], testis tissue fragments, 1–3 mm in diameter, were placed on an agarose gel half-soaked in medium (Fig. 1a). To make evaluation simple and easy, we exploited two lines of transgenic mice: *Gsg2-GFP*[16] (*Gsg2* is also known as *Haspin*) and *Acr-GFP*[17,18], where the *GFP* gene is under control of the *Gsg2* and *Acr* promoters, respectively. These marker green fluorescent proteins (GFPs) specific for meiosis and haploid cells were extremely useful for monitoring the progress of spermatogenesis *in vitro* (Supplementary Fig. 1). Then, we devised a grading system for the extension of GFP expression to quantify the progress of spermatogenesis in each tissue (Supplementary Fig. 2).

In our previous experiments, the optimal temperature for organ culture was 34 °C, and the most effective medium was αMEM (or RPMI) + 10% FBS[19] (Supplementary Fig. 3). Among others, FBS was indispensible to induce spermatogenesis in the organ culture experiments (Fig. 1b). Under these conditions, we found that round spermatids, haploid cells, were produced in an organ culture experiment using 7.5–10.5 days postpartum (dpp) pup mouse testis tissues[19]. However, we were not able to identify any elongating spermatids or sperm. In addition, as meiosis starts around 7 dpp in mice[20], the testis tissues may have included some spermatocytes from the beginning. Therefore, the study is inconclusive regarding whether or not haploid cells were produced from spermatogonia.

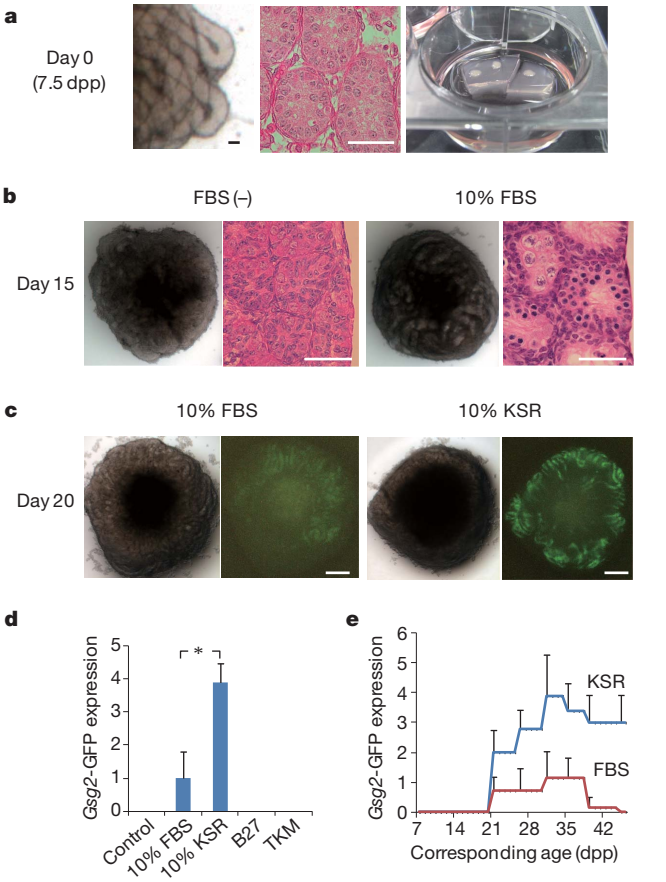

Figure 1 | Effect of FBS and serum replacement on pup testis tissues. **a**, Stereomicroscopic and histological views of testis tissue fragments of 7.5 dpp pups. The tissues were placed on agarose gel stands half-soaked in the medium. **b**, Stereomicroscopic and histological views of testis tissue fragments of 7.5 dpp cultured with αMEM medium without or with FBS. **c**, Testis tissue fragments of 7.5 dpp *Gsg2-GFP* transgenic mice were grown with RPMI supplemented with 10% FBS or 10% KSR. **d**, Five media, RPMI (control), RPMI + 10% FBS, RPMI + 10% KSR, RPMI + B27 and TKM, were compared on the basis of the extent of *Gsg2*-GFP expression scored using the grading scale on culture day 20 (means ± s.d., $n = 6–8$, $^*P < 0.0001$). **e**, The 10% KSR induced stronger GFP expression and maintained the expression for a longer period than 10% FBS (mean ± s.d.; $n = 8$ and 7 for KSR and FBS, respectively). Scale bars, 50 μm (**a**, **b**); 0.3 mm (**c**).

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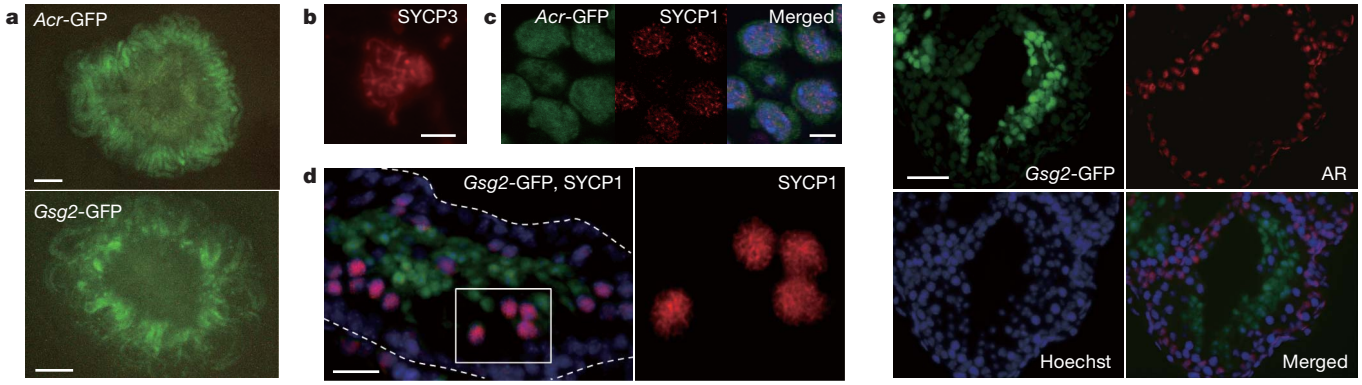

Figure 2 | Effect of KSR on neonatal testis tissues. **a**, 10% KSR induced the expression of both *Acr*-GFP and *Gsg2*-GFP in 2.5 and 0.5 dpp mouse testes, respectively. Pictures were taken on culture days 27 and 39, respectively. **b**, Immunostaining with anti-SYCP3 antibody. **c**, *Acr*-GFP-expressing cells (green) at the pachytene stage were also stained with SYCP1 (red). The merged picture includes Hoechst nuclear stain (blue). **d**, In a cryosection of a *Gsg2-GFP* testis tissue, SYCP1 was demonstrated in cells (red) outside the *Gsg2*-GFP-positive cells (green). Hoechst (blue). The boxed area is enlarged in the right panel. **e**, *Gsg2*-GFP-expressing testis tissue, originating from 2.5 dpp mice and cultured for 21 days, was cryosectioned and stained with antibodies against GFP, AR, and counterstained with Hoechst dye. Scale bars, 0.5 mm (**a**); 5 μm (**b**, **c**) and 30 μm (**d**, **e**).

For further improvements of the culture media, we have tested different kinds of factor reported to be effective for promoting spermatogenesis or in the development of immature testes. However, none of these factors were able to promote *Acr*-GFP or *Gsg2*-GFP expression in our pilot studies (Supplementary Fig. 4). These results raised the possibility that FBS contains factors which suppress the progress of spermatogenesis, thus preventing further refinements of the culture conditions. To overcome such a possible limitation of FBS, we performed culture experiments using KSR[21,22] and B27 (ref. 23) as serum-replacement or serum-free TKM medium[24]. Whereas B27 and TKM did not induce GFP expression, KSR induced the expressions of both *Acr*- and *Gsg2*-GFP. Surprisingly, the level of GFP expression induced by KSR was stronger than that induced by FBS in every experiment (Fig. 1c, d). In addition, KSR significantly extended the duration of GFP expression in culture (Fig. 1e). KSR is commonly used in culture media for embryonic stem cells to promote their proliferation while keeping them in an undifferentiated state[21]. However, as it has been rarely used for organ culture experiments, the present results were unexpected.

Thus, we used KSR on more immature testes of neonates, 0.5–2.5 dpp, and found the expression of both *Acr*-GFP and *Gsg2*-GFP (Fig. 2a). The effect of KSR was evident compared to that of FBS (Supplementary Fig. 5a, b). In order to confirm that such GFP expression reflected genuine meiosis, we examined the expression of meiotic marker proteins, SYCP1 and SYCP3 (ref. 5), by immunochemistry. The GFP-expressing cultured tissues were dissociated and stained with SYCP3, showing representative chromosomal spreading in some cells (Fig. 2b). When GFP and SYCP1 were costained in *Acr*-GFP-expressing cultured tissues, they were colocalized in the pachytene stage of spermatocytes (Fig. 2c). With *Gsg2-GFP* testis tissues, it was shown that SYCP1 also stained pachytene-stage spermatocytes located just on the outer side of *Gsg2*-GFP-positive cells in the seminiferous tubules, confirming that *Gsg2*-GFP-expressing cells were finishing meiosis (Fig. 2d). These results demonstrated that authentic meiosis progressed in the testis tissues cultured with KSR. The somatic cells, Sertoli and peritubular myoid cells, expressed androgen receptors (AR), a mediator of testosterone effects that is essential for spermatogenesis[25], demonstrating their maturity to support spermatogenesis under the culture conditions (Fig. 2e).

Then we set experiments to find haploid cells in the cultured tissues. First, we found many spermatids in step 2–8 (refs 2, 18) in cultured samples of the *Acr-GFP* testis after the mechanical dissociation of cells into suspension in six out of the seven tissues examined, cultured for 23–50 days (Fig. 3a and Supplementary Fig. 6). In addition, we observed flagellated sperm in 5 out of the 11 samples examined, which were cultured for 27–45 days (Fig. 3b, c). These findings were also supported

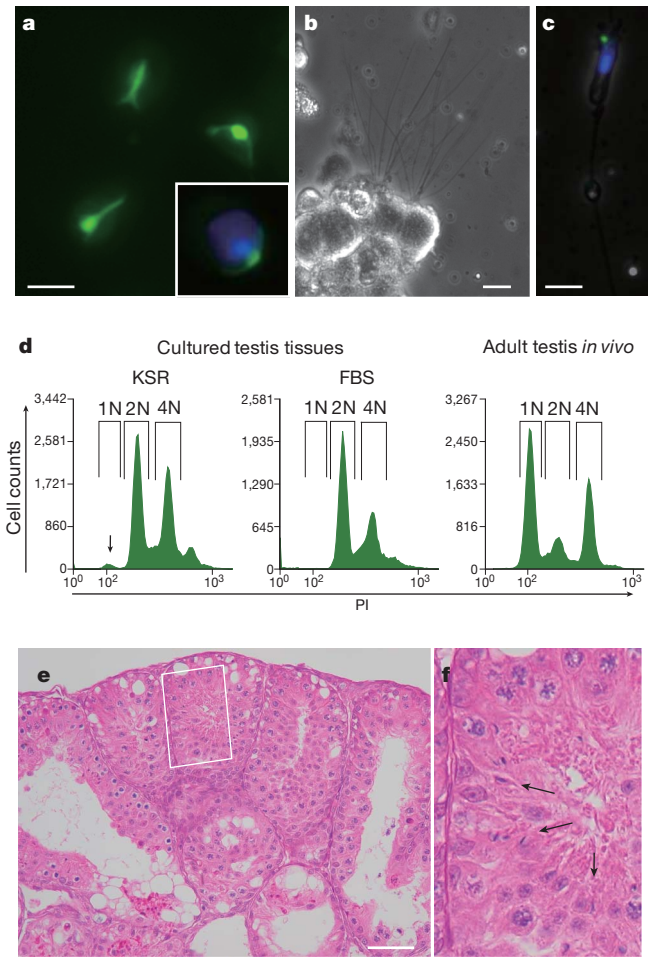

Figure 3 | Formation of spermatids. **a**, A GFP-positive acrosomal cap was observed, indicating the presence of step 6 spermatids, in samples of dissociated testis tissues of *Acr-GFP* mouse neonates cultured for 27 days. Inset shows Hoechst-stained *Acr-GFP* spermatid. **b**, In the same sample, flagellated cells were observed. **c**, Sperm with *Acr-GFP* acrosome was stained with Hoechst. **d**, *Gsg2-GFP* mouse testis tissues, 1.5 dpp, cultured for 30 days, were subjected to flow cytometry. Tissues cultured with KSR included cells of 1N (arrow, 1.71% of all cells). The adult testis served as the control. **e**, **f**, Thin section of the tissues of 2.5 dpp *Gsg2-GFP* mice, cultured for 38 days, showed well developed seminiferous tubules showing spermatogenesis. The white box in **e** is enlarged in **f**, showing sperm formation (arrows). Scale bars, 5 μm (**a**, **b**, **c**) and 50 μm (**e**).

by flow cytometric analysis of dissociated cells from cultured tissues, which identified cells showing 1N ploidy as a sign of spermatids, along with 2N and 4N cells (Fig. 3d and Supplementary Fig. 7). Meanwhile, histological examinations revealed the overall phenomena occurring in the cultured tissues (Supplementary Fig. 8). In the peripheral region of each tissue piece, spermatogenesis, up to elongating spermatid formation, was observed (Fig. 3e, f). In some experiments of extended culture period, *Gsg2*-GFP expression remained at its highest level until around a corresponding age of 30–40 dpp, and then gradually decreased, but lasted beyond 70 dpp. The formation of sperm was confirmed at both 38 and 60 days of culture in a single experiment (Supplementary Fig. 9). Our organ culture system, therefore, was able to induce and maintain spermatogenesis for more than 2 months.

Finally, we tested the fertility of spermatids and sperm produced *in vitro* by microinsemination. Round spermatids retrieved from tissues, originated from 3.5 dpp testes cultured for 23 days, were used for insemination with the round spermatid injection (ROSI) technique. Sperm retrieved from the tissues, originated from 2.5 dpp testes cultured for 42 days, were used for intracytoplasmic sperm injection (ICSI) (Fig. 4a, b). Using 23 and 35 oocytes for ROSI and ICSI, respectively, 7 and 5 live offspring were delivered (Fig. 4c, Supplementary Table 1) and weaned at 3 weeks (Fig. 4d). Although these experiments were small in scale and using only a single line of mice, the efficiencies of progeny production with the *in vitro*-produced gametes were comparable to that with *in vivo*-generated counterparts[26]. PCR analysis of their tail tip DNA identified 4 GFP-carrying offspring out of 12, compatible with cultured testis tissues being heterozygous for *Gsg2-GFP* (Fig. 4e). Their reproductive capacity was examined by brother–sister mating, demonstrating that all four males and eight females were fertile (Supplementary Table 2).

We thought that the cryopreservation of testis tissues, if feasible, would extend our results to a variety of practical applications. Therefore, we froze neonatal testis tissues and placed them in liquid nitrogen for 4–25 days. After thawing, they were grown on agarose in the same manner as for non-frozen samples. GFP expression was observed in all four experiments using *Acr-GFP* mice, and in two experiments using *Gsg2-GFP* mice (Supplementary Fig. 10a). In three of the four experiments using *Acr-GFP* mice, GFP-expressing acrosomes were observed in mechanically dissociated samples (Supplementary Fig. 10b). On the histological examination of cultured *Gsg2*-GFP-expressing tissues, elongated spermatids were observed in one out of the five tissues examined (Supplementary Fig. 10c). These results demonstrated that testis tissue fragments can be cryopreserved and resume full spermatogenesis later *in vitro*.

KSR was vital for the success of the present experiments. Elucidating the mechanism of its action and identifying the critical factors are important for the further refinement of our culture conditions. We have tested several factors reportedly contained in KSR[22], and found that lipid-rich bovine serum albumin (AlbuMAX) was probably the most critical component regarding our present results, because the addition of AlbuMAX in place of KSR led to almost the same results (Supplementary Fig. 11a, c). In addition, it seemed that FBS does not contain factors which inhibit the progression of spermatogenesis, because medium containing both FBS and AlbuMAX was as effective in inducing spermatogenesis as that containing AlbuMAX alone (Supplementary Fig. 11b). Further studies to identify key molecule(s) in KSR and AlbuMAX are warranted.

We have demonstrated that the organ culture conditions, without a circulatory system as *in vivo*, can support the complete spermatogenesis of mice. Therefore, extending the present results to a wide range of species by refinements and the individualization of culture conditions to each of them seems promising. Such progress will contribute to the elucidation of the molecular mechanisms of spermatogenesis and development of new diagnostic and therapeutic techniques for male infertility.

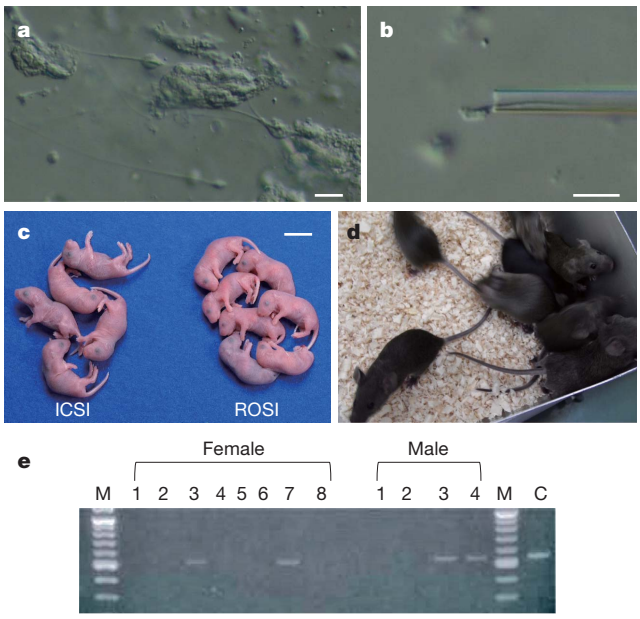

Figure 4 | Fertility of sperm and spermatids produced *in vitro*. **a**, In the microinsemination experiment, elongated spermatids and spermatozoa among round spermatids were observed. **b**, Sperm were retrieved from the testis tissues of 2.5 dpp mice after culturing for 42 days. **c**, Offspring were produced by ICSI and ROSI with sperm and round spermatids, respectively. **d**, A photo of offspring at 7 weeks. **e**, Tail tip DNA analysis by PCR for *GFP*. C, DNA sample taken from a *Gsg2-GFP* mouse tail tip as a positive control; M, marker. Scale bars, 10 μm (**a**, **b**) and 1 cm (**c**).

METHODS SUMMARY

Acr-GFP and *Gsg2-GFP* transgenic mice were mated with female mice of ICR, C57BL/6, or ICR × C57BL/6F1 to produce pups. The pups were used for the culture experiments at 0.5 to 11.5 dpp. The testis tissues were place on agarose gel half-soaked in the medium. The cultured tissues were observed every 3–7 days under a stereomicroscope equipped with an excitation light for GFP to score the extent of GFP expression in each tissue. They were also processed for histological and immunohistological examinations. For the observation of *Acr-GFP* acrosomes, cultured tissues were mechanically dissociated using needles to release cells into PBS. For cryopreservation, fragments of testis tissues were immersed in cryoprotectants for several hours or overnight at 4 °C, and then placed at −70 °C overnight before being stored in liquid nitrogen. On the initiation of culture, tissues were thawed at room temperature, soaked briefly in the medium, and then placed on agarose for culture.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 8 June 2010; accepted 17 January 2011.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank S. Yoshida and G. Yoshizaki for comments and pre-submission review. We thank A. Tanaka and Y. W. Zheng for their technical help in flow cytometric analysis. T.O. is grateful to his mentor R. L. Brinster for his advice on devising the experimental strategies and for his encouragements. We would like to thank A. Steinberger and the late E. Steinberger whose work in the study of *in vitro* spermatogenesis became the ground work for our present study. This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas, ''Regulatory Mechanism of Gamete Stem Cells'' (#20116005); a Grant-in-Aid for Scientific Research (C) (#21592080) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; a grant from the Yokohama Foundation for Advancement of Medical Science; and a grant for Research and Development Project II (No.S2116) of Yokohama City University, Japan (to T.O.).

Author Contributions T.S. performed the experiments, interpreted the results, and prepared the manuscript. K.K. performed all culture experiments. A.G. contributed to the culture experiments. K.I. and N.O. performed microinsemination experiments. A.O. performed microinsemination experiments and discussed the results. Y.K. supervised the project and discussed the results. T.O. designed and performed the experiments and wrote the manuscript.

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METHODS

Animals. *Acr-GFP* and *Gsg2-GFP* transgenic mice were provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Female mice of ICR, C57BL/6 (CLEA Japan), or ICR × C57BL/6F1 were mated with a sire of the transgenic mice to produce pups. The pups were used for the culture experiments at 0.5 to 11.5 days post-partum (dpp). All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation (Animal Research Center of Yokohama City University, Yokohama, Japan).

Culture media and reagents. The culture media used were α-Minimum Essential Medium (α-MEM) (Invitrogen), Roswell Park Memorial Institute 1640 (RPMI) (Invitrogen), DMEM (Dulbecco's modified Eagle's medium) (Invitrogen), and F-12 nutrient mixture (Ham) (Invitrogen). Serum and serum replacements used were fetal bovine serum (FBS) for embryonic stem cell (Gibco Invitrogen), KnockOut Serum Replacement (KSR) (Invitrogen), B-27 supplement (Gibco Invitrogen), and AlbuMAX (Invitrogen). Factors below were added to the culture media as indicated in the text. Hepatocyte growth factor (HGF) (5 ng ml^{-1}) (R&D Systems), Activin A (100 ng ml^{-1}) (Sigma-Aldrich), follicle stimulating hormone (FSH) from human pituitary (200 ng ml^{-1}) (Sigma-Aldrich), testosterone (1 μM) (Wako Pure Chemical Industries), recombinant human BMP-4 (20 ng ml^{-1}) (R&D Systems), recombinant human BMP-7 (20 ng ml^{-1}) (R&D Systems), and bovine pituitary extract (50 μg ml^{-1}) (Invitrogen).

Culture method. The testes of the neonatal or pup mice were decapsulated and gently separated by forceps into 1 to 8 pieces of 1–3 mm in diameter. The tissue fragments were then positioned on stands made of agarose gel placed in culture plate wells. To make the agarose gel stand, agarose-1 (Dojindo Molecular Technologies) was heated to dissolve it in distilled water (1.5% (w/v)) and then poured into a 10-cm dish. After cooling, the gels were cut into hexahedrons of about 10 × 10 × 5 mm in size. They were then soaked in the culture medium for more than 24 h to replace the water in them with the medium. Three to four pieces of the agarose gels were placed in the wells of a 6-well plate (Sumitomo Bakelite). Each gel was loaded with one to three testis tissue fragments. The amount of medium was adjusted so it would come up to half to four fifth of the height of the agarose gel. Medium change was performed once a week. The culture incubator was supplied with 5% carbon dioxide in air and maintained at 34 °C.

Observations. The cultured tissues were observed every 3 to 7 days under a stereomicroscope equipped with an excitation light for GFP (Olympus SZX12; Olympus) to score the level of GFP expression of the tissues. For histological examination, the specimens were fixed with Bouin's fixative and embedded in paraffin. One section showing the largest cut surface was made for each specimen and stained with haematoxylin and eosin (H&E). For immunofluorescence staining, tissues fixed with 4% paraformaldehyde in PBS were cryo-embedded in OCT compound (Sakura Finetechnical) and cut into 7-μm-thick sections. The first antibodies to be used were rabbit anti-androgen receptor (AR) antibody (1:500; Santa Cruz Biotechnology), rabbit anti-SYCP1 antibody (1:600; Novus Biologicals), and rabbit anti-GFP Alexa Fluor 488 conjugate (1:50; Invitrogen). Alexa Fluor 546-conjugated goat anti-rabbit IgG (1:400; Invitrogen) was used as a second antibody for anti-AR and anti-SYCP1 antibodies. Nuclei were counterstained with Hoechst 33342 dye. Specimens were observed with a microscope (Nikon Eclipse TE200) or confocal laser microscope (Olympus FV-1000D). For the detection of SYCP3, cultured tissues were mechanically dissociated with fine forceps, then fixed with 4% paraformaldehyde in PBS, and stained with rabbit anti-SYCP3 antibody (1:400; Abcam) followed by Alexa Fluor 546-conjugated goat anti-rabbit IgG as a second antibody. For the observation of *Acr-GFP* acrosomes, cultured tissues were mechanically dissociated using needles to release cells into PBS. The cell suspension was observed with a microscope under GFP excitation light.

Flow cytometric analysis. The cultured testis tissue fragments were treated with 2 mg ml^{-1} of collagenase for 15 min, followed by 0.25% trypsin/1 mM EDTA digestion for 10 min at 37 °C to dissociate cells. After passing through a cell strainer with a 40 μm pore size (Becton Dickinson), the cells were suspended in PBS containing 3% fetal bovine serum (FBS) and subjected to flow cytometry to analyse GFP-expressing cells using a MoFlo sorter (Dako Cytomation). For DNA ploidy, the singly-dissociated cells were fixed in 1% paraformaldehyde at 4 °C for 15 min, followed by 70% ethanol at −25 °C for 12–24 h, and re-suspended and incubated in staining solution (0.1% Triton X-100 in PBS, 0.2 mg ml^{-1} RNase A, 0.02 mg ml^{-1} propidium iodide) at 37 °C for 15 min. The flow cytometric analysis was also performed with the MoFlo sorter.

Microinsemination. The cultured testes tissues were dissected out under a stereomicroscope. Round spermatids or spermatozoa were collected and injected into the ooplasm of wild-type matured oocytes of B6D2F1 using a Piezo-driven micromanipulator. For fertilization with round spermatids, oocytes were then activated by treatment with $SrCl_2$ in the presence of cytochalasin B to resume meiosis. After the formation of two female pronuclei, one was removed with a micro-pipette[27]. Fertilized oocytes were cultured for 24 h, and two-cell embryos were transferred into the oviducts of pseudopregnant ICR females. Live fetuses retrieved on day 19.5 were raised by lactating foster ICR dams.

PCR analysis. Genomic DNA was extracted from the mouse tail with a DNeasy Tissue kit (Qiagen). The DNA samples (10 ng) were added to a 20-μl reaction mixture containing 0.25 μM of each enhanced green fluorescent protein (*EGFP*)-specific primer and Premix Ex Taq (Takara Bio). *EGFP*-specific primers were 5′-TACGGCAAGCTGACCCTGAA-3′ and 5′-TGTGATCGCGCTTCTCGTTG-3′. The reaction profile was 30 cycles of denaturation at 95 °C for 60 s, annealing at 60 °C for 30 s, and extension at 72 °C for 60 s.

Cryopreservation of testis tissues. The fragments of testis tissues, prepared in exactly the same way as for culture, were immersed in TC-protector cell-freezing medium (BUF050, AbD Serotec) for several hours to overnight, and then frozen at −70 °C overnight before being placed in liquid nitrogen. The tissues were stored in liquid nitrogen for 4–25 days. On the initiation of culture, cryotubes were placed at room temperature to thaw the cryoprotectant solution and tissues were taken out to be soaked briefly in the culture medium to remove the cryoprotectants. Then, they were placed on agarose for culturing.

Statistical analysis. One-way analysis of variance (ANOVA) was used to compare differences between groups. Values with $P < 0.05$ were considered significant.

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