Testis tissue explantation cures spermatogenic failure in c-Kit ligand mutant mice

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Male infertility is most commonly caused by spermatogenic defects or insufficiencies, the majority of which are as yet cureless. Recently, we succeeded in cultivating mouse testicular tissues for producing fertile sperm from spermatogonial stem cells. Here, we show that one of the most severe types of spermatogenic defect mutant can be treated by the culture method without any genetic manipulations. The *Sl/Sl^d* mouse is used as a model of such male infertility. The testis of the *Sl/Sl^d* mouse has only primitive spermatogonia as germ cells, lacking any sign of spermatogenesis owing to mutations of the c-kit ligand (KITL) gene that cause the loss of membrane-bound-type KITL from the surface of Sertoli cells. To compensate for the deficit, we cultured testis tissues of *Sl/Sl^d* mice with a medium containing recombinant KITL and found that it induced the differentiation of spermatogonia up to the end of meiosis. We further discovered that colony stimulating factor-1 (CSF-1) enhances the effect of KITL and promotes spermatogenesis up to the production of sperm. Microinsemination of haploid cells resulted in delivery of healthy offspring. This study demonstrated that spermatogenic impairments can be treated in vitro with the supplementation of certain factors or substances that are insufficient in the original testes.

Spermatogenic impairments can be caused by malfunctions of either the germ cell itself or surrounding somatic cells, which collectively constitute the microenvironment for proper spermatogenesis. It is well known that the microenvironmental condition in the testis is under the body's systemic control, particularly through hormones from the pituitary. It is also well recognized that testicular somatic cells, Sertoli cells in particular, play a pivotal role in spermatogenesis (1, 2). Although it remains to be elucidated what molecules are essential constituents of that microenvironment and how they exert their role in spatial and temporal terms, it is a reasonable assumption that the spermatogenic impairments caused by microenvironmental factors are treatable by correcting the defect(s).

Recently, we succeeded in inducing complete spermatogenesis in mice using an organ culture method (3–5). In this culture system, germ cells developed from spermatogonial stem cells through mitotic differentiation of spermatogonia, meiosis in spermatocytes, and morphological transformation in haploid spermatids, to sperm. One of the greatest advantages of the in vitro system is that the culture medium can be modified freely to an extent that would be impossible to achieve in vivo. In the present study, we attempted to treat spermatogenic failure of a mutant mouse by culturing testis tissue fragments in media supplemented with combinations of growth factors.

Results

State of Germ Cells and Expression of *Kitl* in *Sl/Sl^d* Mouse Testis. For this experimental trial we chose the *Steel* (*Sl*) mutant mouse, which has long been used to study spermatogenesis, hematopoiesis, and proliferation and differentiation of melanocytes. *Sl* mutants are caused by defects in *Kitl* and now comprise more than 40 kindred mutants (Mouse Genome Database, The Jackson Laboratory). The original *Sl* mutant lacks a wide range of genomic regions, including the entire *Kitl* along with eight other genes (6). Another well-known mutant named *Sl^d* has a deletion in the domains of transmembrane and cytoplasmic *Kitl*. This partial deletion makes *Sl^d* unable to produce KITL in a membrane-bound form (mKITL) but allows it to produce the secretory form (sKITL), so this mutant is viable (Fig. 1*A*) (7). A combination of these two mutants, i.e., *Sl/Sl^d*, is therefore viable yet shows anemia, a white coat color, and spermatogenic defect (8–10).

In the testis, KITL is produced by Sertoli cells, and its receptor c-Kit is produced in differentiating spermatogonia and meiotic spermatocytes up to the pachytene stage (Fig. 1*A*) (11). Owing to the pivotal role of KITL in the development of germ cells as well as for spermatogenesis after birth, the testis of *Sl/Sl^d* is small at birth and remains underdeveloped in adulthood (Fig. 1*B*). In the testis, spermatogonia exist but are few and present regionally only in some seminiferous tubules (Fig. 1*C*) (12). These histological features remain the same even when the mouse grows, and no spermatogenesis develops (Fig. 1*D*). An immunohistochemical study with antibody to KITL demonstrated much weaker signals in *Sl/Sl^d* than in the wild-type mouse testis, corresponding to the lower production of sKITL and lack of mKITL (Fig. 1*E*).

Spermatogenesis of *Sl/Sl^d* Mice Induced by Recombinant KITL. First, testis tissue fragments of *Sl/Sl^d* mice, 4.5–11.5 d postpartum, were cultured for 43 d. Histological analysis showed that a few spermatogonia remained, but there was no sign of spermatogenesis. When recombinant KITL (rKITL) was added to the medium at 50 ng/mL, no difference in histological appearance was observed. However, when the concentration was increased to 100 ng/mL, spermatocytes and a few haploid round spermatids were observed (Fig. 2*A*). This indicates that a sufficient amount of rKITL added to the medium can substitute for the role of mKITL. When the concentration was raised to 500 ng/mL, the percentage of the seminiferous tubules counted that contained spermatocytes or round spermatids increased, even though it was not significant, from 3.1% to 4.0% and 0.1% to 0.4%, respectively (Fig. 2*B*). Then, we performed an immunohistological examination with antibodies to mouse vasa homolog (MVH, a marker for mitotic and meiotic germ cells) and synaptonemal complex protein 1 (SYCP1, a meiosis marker) to confirm the above results. The control samples cultured without rKITL showed the presence of MVH-positive spermatogonia but no

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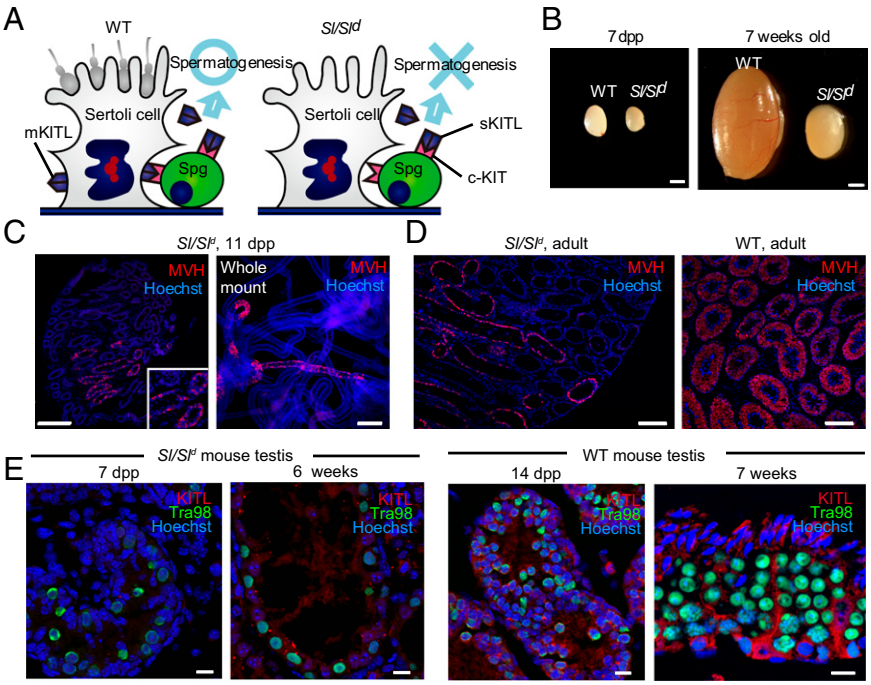

Fig. 1. State of spermatogonia and expression of the *KitL* in the testis of *Sl/Sl*^{d} mice. (*A*) A schematic presentation of the relationship between two types of KITL and c-Kit in the testis. Sertoli cells of the wild-type (WT) mouse produce both membrane-bound KITL (mKITL) and secretory KITL (sKITL). Spermatogonia (Spg) express the receptor c-Kit and receive a major signal from mKITL by which spermatogenesis proceeds. In the *Sl/Sl*^{d} mouse testis, only sKITL is produced, which is not sufficient to promote spermatogenesis. (*B*) The macroscopic appearance of WT and *Sl/Sl*^{d} mouse testes. (*C*) Immunostaining of a cryosection and whole-mount specimen of *Sl/Sl*^{d} mouse testes at 11.5 d postpartum (dpp) with antibody to MVH (red), counterstained with Hoechst (blue). Inset shows a magnified view of the same sample. (*D*) Immunostaining of cryosections of *Sl/Sl*^{d} (6-wk-old) and WT (7-wk-old) mouse testes with antibody to MVH (red), counterstained with Hoechst (blue). (*E*) Immunostaining of cryosections of *Sl/Sl*^{d} (left two) and WT (right two) mouse testes with antibodies to KITL (red) and TRA98 (green), counterstained with Hoechst (blue). (Scale bars: 1 mm in *B*; 200 μm in *C* and *D*; 10 μm in *E*.)

SYCP1-positive meiotic cells. Tissues cultured with rKITL contained an increased number of MVH-positive cells and some SYCP1-positive spermatocytes, mostly in the pachytene stage (Fig. 2*C*). However, we did not find elongating spermatids, let alone sperm, in those samples.

Cytokines Exhibit Synergistic Effect with KITL. Although increased doses of rKITL stimulated spermatogenesis in the testis tissues of the *Sl/Sl*^{d} mouse, the number of round spermatids produced remained few even with 500 ng/mL of rKITL, and there was no evidence of elongating spermatids. Previous studies reported that KITL has synergistic effects with other cytokines. The effect of KITL on hematopoietic cell lineages to promote their proliferation, differentiation, or survival can be augmented synergistically by combination with other cytokines such as colony stimulating factor-1, -2, or -3 (CSF-1, CSF-2, CSF-3), interleukin-3, and erythropoietin (EPO) (13–17). We added those cytokines to the medium to elucidate their effects on spermatogenesis. CSF-1 in combination with rKITL promoted spermatogenesis; among those counted, the percentage of seminiferous tubules containing spermatocytes or round spermatids increased to 11.2% and 2.1%, respectively, compared with 3.1% and 0.1% for those cultured with rKITL alone (Fig. 3 *A* and *B*). In addition, we found elongating spermatids in 0.7% of seminiferous tubules in histological sections (Fig. 3 *A* and *B*), although these histological data did not reveal significance. CSF-3 or EPO added in combination with rKITL did not show such an enhancing effect (Fig. 3*A*). CSF-2

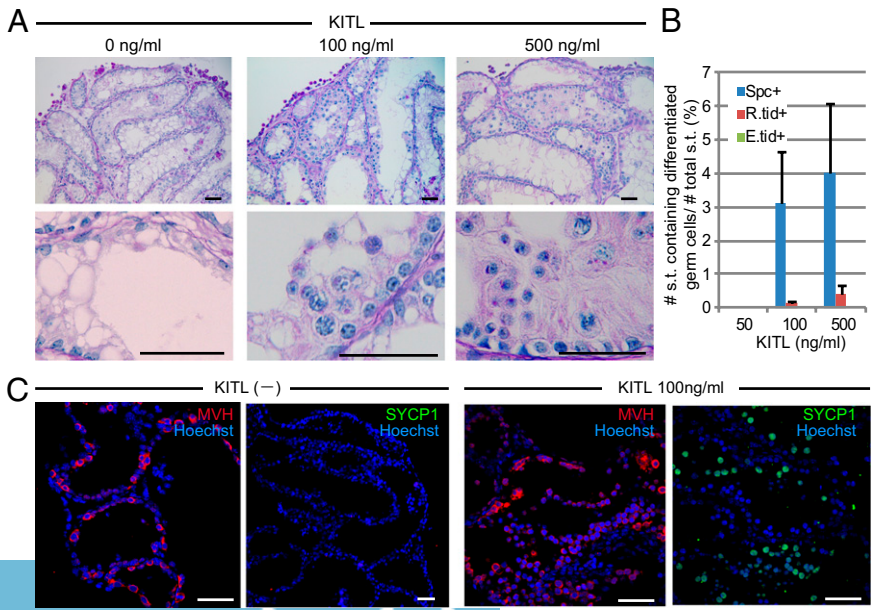

Fig. 2. Recombinant Kit ligand (rKITL) promotes differentiation of spermatogonia in testis tissues of a *Sl/Sl*^{d} mouse. (*A*) Histology of cultured testis tissues of the *Sl/Sl*^{d} mouse, stained with the periodic acid Schiff reaction. Testis tissue fragments of the mouse at 10.5 d postpartum were cultured with α–MEM+10% KnockOut Serum Replacement supplemented with rKITL, at 0, 100, and 500 ng/mL, for 43 d. (*B*) Concentration-dependent effect of rKITL on spermatogenesis. Serial sections stained with periodic acid Schiff were examined to count the number of seminiferous tubules (s.t.) containing spermatocytes (Spc), round spermatids (R. tid), and elongating spermatids (E. tid) to calculate their ratio among the total number of s.t. in each tissue (mean ± SE, n = 8–11). (*C*) Immunostaining of cryosections of cultured testis tissues with antibodies to MVH (red) and SYCP1 (green), counterstained with Hoechst (blue). Testis tissue fragments of *Sl/Sl*^{d} mice, 7.5–11.5 d postpartum, were cultured for 42 d. (Scale bars: 50 μm in *A* and *C*.)

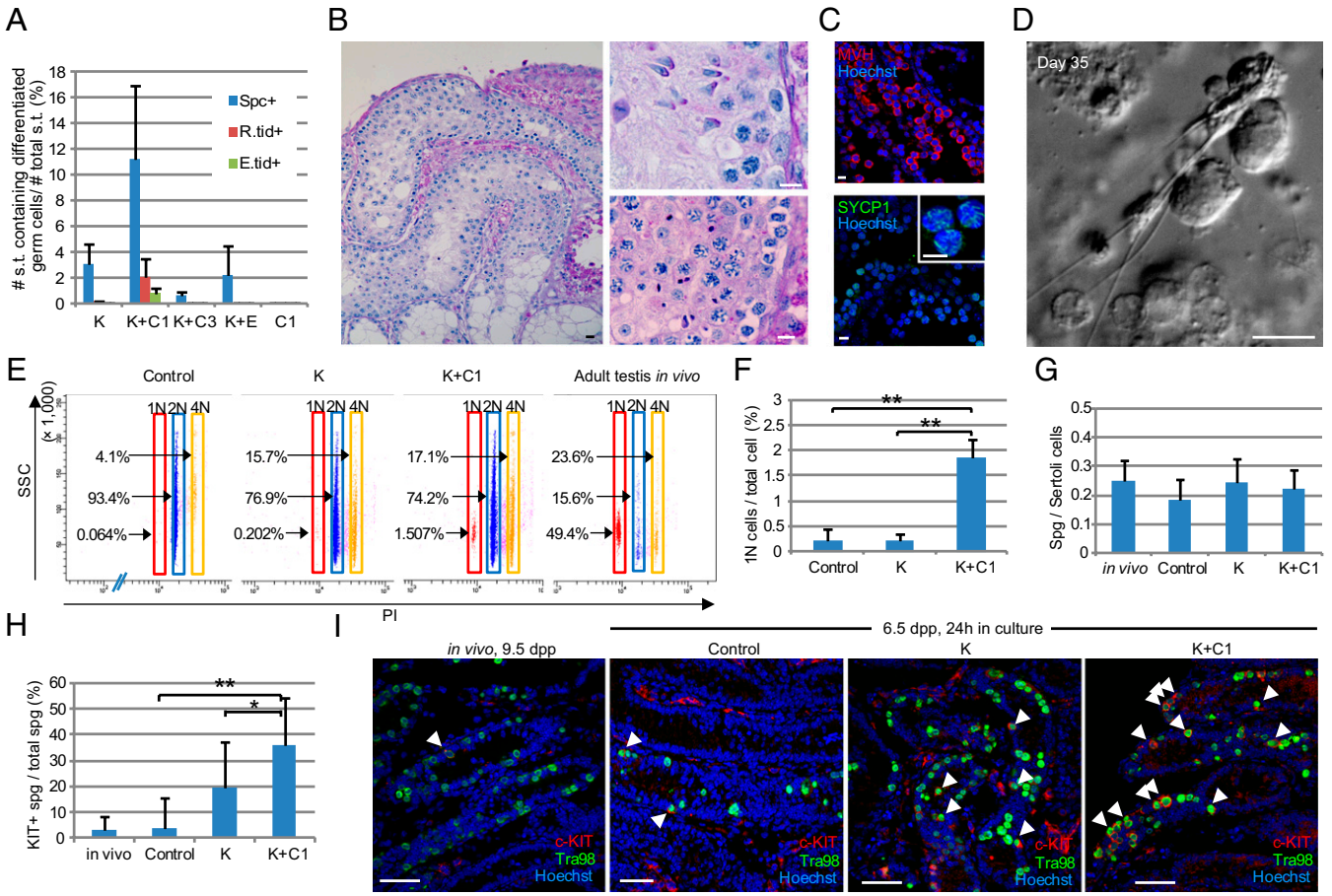

Fig. 3. Combinatory effect of rKITL and CSF-1. (*A*) The combinatory effect between rKITL and other cytokines on spermatogenesis is shown graphically. Testis tissue fragments of *Sl/Sl*^{d} mice, 4.5–10.5 d postpartum, were cultured for 42–45 d with cytokines of different combinations: rKITL, 100 ng/mL (K); CSF-1, 20 ng/mL (C1); CSF-3, 20 ng/mL (C3); and EPO, 20 ng/mL (E). Serial sections were made from each tissue and stained with periodic acid Schiff to count the number of seminiferous tubules (s.t.) containing spermatocytes (Spc), round spermatids (R. tid), and elongating spermatids (E. tid) (mean ± SE, n = 4–11). (*B*) Periodic acid Schiff staining histology of testis tissue of the *Sl/Sl*^{d} mouse, 10.5 dpp, cultured for 43 d with rKITL and CSF-1. (*C*) Immunostaining of a cryosection of *Sl/Sl*^{d} mouse testis, 7.5–11.5 dpp, cultured for 42 d with rKITL and CSF-1. MVH (red), SYCP1 (green), Hoechst (blue). Inset shows a magnified view of the same sample. (*D*) Flagellated sperm of the *Sl/Sl*^{d} mouse found in dissociated cultured tissue. (*E*) Flow cytometric analysis of DNA contents of cultured testis tissues at d 52. *Sl/Sl*^{d} mice, 4.5–8.5 dpp, were used. Culture media contained rKITL, rKITL+CSF-1, or nothing (control). (*F*) Proportion of haploid cells (1N) in cultured tissues, 4.5–8.5 dpp, analyzed by a flow cytometer. Data of three experiments were combined (mean ± SD). (*G*) Number of spermatogonia (Spg) per Sertoli cell in *Sl/Sl*^{d} mouse testis tissues, 6.5 dpp, at 24 h of culturing. Eight to fourteen seminiferous tubules were observed for counting in each group (mean ± SD). (*H*) Proportion of c-Kit-positive Spg among all spermatogonia (Tra98-positive) in cultured *Sl/Sl*^{d} mouse testis tissues in 24 h (mean ± SD). (*I*) Typical pictures of immunostaining, using 6.5-dpp *Sl/Sl*^{d} mouse testis tissues cultured for 24 h with cytokines, with antibodies to c-Kit (red), Tra98 (green), along with counterstaining with Hoechst (blue). Noncultured 9.5-dpp testis tissues of the *Sl/Sl*^{d} mouse were used as a control. Arrows indicate c-Kit–positive spermatogonia. (Scale bars: 10 μm in *B–D*; 50 μm in *I*.) *P < 0.05, **P < 0.001.

and interleukin-3 appeared to work negatively on the effect of rKITL, and no spermatogenesis was observed in those specimens.

To confirm the combined effect of CSF-1 and rKITL, we performed immunohistological studies and found many MVH-positive germ cells in the seminiferous tubules of *Sl/Sl*^{d} testis tissues cultured with these two cytokines (Fig. 3*C*). Antibody to SYCP1 demonstrated pachytene-stage spermatocytes, verifying the progress of meiosis (Fig. 3*C*). In addition, when cultured tissues were mechanically dissociated, several flagellated sperm were observed (Fig. 3*D*). However, when tissues were cultured in medium supplemented with CSF-1 alone, without rKITL, no progression of spermatogenesis was observed (Fig. 3*A*). This suggests that CSF-1 alone has only a weak, if any, effect on spermatogenesis.

CSF-1 Enhances Effect of KITL to Promote Spermatogenesis. To confirm the effect of CSF-1, we applied flow cytometric analysis to measure the proportion of haploid cells produced in the cultured tissues of the *Sl/Sl*^{d} mouse testis. In control samples cultured with no cytokines, 2N cells dominated at 93.4%, with 4N cells at 4.1%. However, in tissues cultured with rKITL or rKITL+CSF-1, 4N cells increased to 15.7% and 17.1%, respectively, values closer to those of the adult testis, 23.6% (Fig. 3*E*), suggesting the emergence of meiotic cells. These results indicate that either rKITL alone or rKITL+CSF-1 promoted the differentiation of spermatogonia to become spermatocytes and enter meiosis. However, the data from three cytometry experiments showed that tissues cultured with rKITL alone contained 0.2% 1N cells, similar to the control sample without cytokines (Fig. 3*F*). Thus, rKITL alone may be sufficient to induce meiosis but may be insufficient to produce haploid cells. Tissues cultured with rKITL+CSF-1 contained an increased number of 1N cells, 1.9% of all cells analyzed (Fig. 3*F*). This result indicates that CSF-1 in combination with KITL promoted spermatogenesis, which resulted in haploid cell production.

The receptor of CSF-1 is reportedly produced in spermatogonia in the immature testis (18, 19). Our own immunohistochemical studies demonstrated its predominant expression in spermatogonia, both undifferentiated and differentiating, in the 18-d-old mouse testis (Fig. S1). We examined whether rKITL or rKITL+CSF-1 would affect the number of spermatogonia, both total [anti-Tra98 antibody (Tra98)-positive cells/Sertoli cells] and differentiating (c-Kit–positive cells/Tra98-positive cells), by counting them early in 24-h culture. Regarding the total number of spermatogonia, neither rKITL nor rKITL+CSF-1 showed any influence (Fig. 3*G*). As for differentiating spermatogonia, however, rKITL significantly increased their number among all spermatogonia. This effect was enhanced when rKITL+CSF-1 was applied (Fig. 3 *H* and *I*).

Next, we examined whether rKITL+CSF-1 can also affect the testis of wild-type mice to promote spermatogenesis. We tested this possibility using *Acr*-GFP transgenic mice whose germ cells express GFP when they become midpachytene spermatocytes. Testis tissues of the pup mice at 3.5 d postpartum were cultured in the media for 31 d, and numbers of GFP-positive cells, corresponding to cells differentiated beyond the midpachytene stage, were counted with a flow cytometer. The GFP-positive cells in tissues cultured in control medium, with no cytokines added, constituted 0.5% of all cells counted. The tissues cultured with rKITL or rKITL+CSF-1 contained 2.9% and 3.3% GFP-positive cells, respectively (Fig. S2). The effect of rKITL, therefore, was not unique to the Sl/Sl^d mutant but is also seen in normal testes. However, the effect of CSF-1 was not apparent in this experiment.

Production of Offspring. Finally, we tested the fertility of the haploid cells of the Sl/Sl^d mouse produced in vitro. The testis tissues of Sl/Sl^d mice, at 6.5–9.5 d postpartum, were cultured to obtain haploid cells for microinsemination, either round spermatid injection (ROSI) or intracytoplasmic sperm injection (ICSI) (Table S1). Using the round spermatids obtained from the tissues cultured for 49 d, 27 oocytes of B6D2F1 mice were inseminated. Out of 17 two-cell embryos transferred to the uterus, one female offspring was delivered (Fig. 4*A*). This individual grew normally with a typical coat color of heterozygous *Sl* mutants (a white midline on the underside) (Fig. 4*B*). PCR analysis of genomic DNA revealed the genotype to be $Sl^d/+$ (Fig. 4*C* and Fig. S3). When mated with a wild-type male, the F1 mouse gave birth to offspring, proving its reproductive competency (Fig. 4*D*). Thus, we succeeded in producing fertility-competent haploid cells from spermatogonia of Sl/Sl^d mutant mice through culturing their testis tissue in medium supplemented with the combination of rKITL and CSF-1.

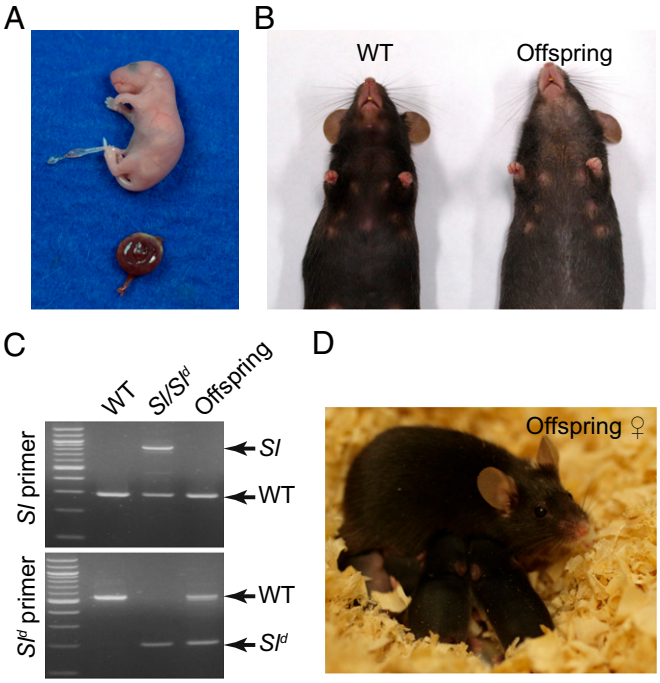

Fig. 4. Progeny production. (*A*) Offspring obtained by the round spermatid injection technique using round spermatids derived from Sl/Sl^d mouse testis tissue cultured with recombinant Kit ligand and colony stimulating factor-1 for 49 d. (*B*) The offspring showed a white midline on the abdomen as a typical feature of heterozygous *Sl* mutation. (*C*) Genotyping of the offspring with PCR. The *Sl* primer set amplified the genomic sequence of 294 bp [wild-type (WT)] but not 839 bp (*Sl*), whereas the Sl^d primer set amplified both 540 bp (WT) and 206 bp (Sl^d), indicating the offspring to be $Sl^d/+$. The leftmost lane is a 100-bp ladder marker. (*D*) The offspring delivered her progeny by natural mating. The picture shows the offspring suckling her pups.

Discussion

Several therapeutic experiments have been reported involving spermatogenic defects using the Sl/Sl^d mouse as a model animal. They can be categorized into three strategies. The first is germ cell transplantation. The germ cells of Sl/Sl^d mice were transplanted into the testes of *W* mice, which lack spermatogenesis but provide normal microenvironmental conditions. This gave rise to highly efficient spermatogenesis in the host testes and resulted in progeny produced by natural mating of the recipient male to females (20) (Fig. S4*A*). In our previous study, we modified this transplantation system by excising the testes from the host mouse and transplanting Sl/Sl^d testis cells into the freed host testes, followed by culturing the testis tissues. This in vitro transplantation system also succeeded in inducing complete spermatogenesis of Sl/Sl^d germ cells (5) (Fig. S4*B*). However, the transplantation of germ cells requires a source of host testis, which is a critical hurdle that makes this strategy unrealistic for clinical application. The second strategy is the replacement of defective Sertoli cells with those of wild-type (21) (Fig. S4*C*). Although it is a logical strategy, the efficiency of the replacement is very low. In addition, this strategy also requires a source of donor Sertoli cells, which is again impractical in clinical settings. The third strategy is viral transduction of Sertoli cells to correct the defect (Fig. S4*D*). Two studies using adenovirus and lentivirus succeeded in making Sertoli cells of Sl/Sl^d mice produce mKITL, thereby inducing spermatogenesis in their testes (22, 23). This strategy appears more realistic for clinical application than the first two. However, it may be difficult to rule out the possible transduction of germ cells completely, which is critically important for clinical application in terms of safety and ethics. The present study proposes an alternate strategy for the treatment of spermatogenic failure (Fig. S4*E*). This strategy can overcome the weakness of previous ones: efficiency, source of materials, and safety issues. Although testis biopsy is necessary, its invasiveness is minimal compared with other strategies, making our strategy more realistic for clinical applications.

In this study, we succeeded in inducing the complete spermatogenesis of Sl/Sl^d mutant mice by culturing their testis tissues in culture media containing rKITL and CSF-1. Using the produced round spermatids for microinsemination, progeny of the Sl/Sl^d mouse were generated. This result was beyond our initial expectation and demonstrated that sKITL (rKITL), in place of mKITL, when added to culture medium, can exert an effect on spermatogenesis. We also found that CSF-1 enhances the effect of KITL on spermatogenesis, leading to the production of functional gametes in vitro. As for the mechanism of this effect of CSF-1, two possibilities can be considered. First, it might be a synergistic effect with KITL, as observed in hematopoietic cells. In fact, receptors of both cytokines, c-KIT and CSF-1R, are present on spermatogonia. Second, it is also possible that CSF-1 acts on interstitial cells, particularly macrophages, which also

express CSF-1R, inducing them to secrete certain factor(s) that enhance the effect of KITL. These two possibilities require elucidation in the future. Meanwhile, the physiological function of CSF-1 in the testis is not clear yet. The *op*/*op* mutant mouse, which has a mutation involving a single nucleotide insertion in the *Csf-1*, shows spermatogenic insufficiency involving significantly reduced sperm production. However, because this accompanies a decreased blood testosterone level, it is not clear whether spermatogenic insufficiency is a direct influence of the defect in CSF-1 (24). We continue working to elucidate the mechanism of CSF-1's effect on spermatogenesis, which should provide useful information to improve the culture conditions for more efficient promotion of spermatogenesis.

In most cases of human male infertility, spermatogenic impairments would not be caused by a single gene mutation or malfunction of a single protein, but rather by multiple factors including those of innate and acquired as well as genetic and environmental origins (25). Furthermore, it is now well known through practices of testicular sperm extraction that small foci of spermatogenesis are occasionally found even in the diminutive testes of males with azoospermic Kleinfelter syndrome of the nonmosaic type (26). This suggests that the testicular microenvironment differs from place to place in one testis. It is even possible that spermatogenic progression depends on the interplay between germ cells and their intimate microenvironment; thus, their mutual compatibility would be critical. This idea demands reconsideration of the simple notion that causes of spermatogenic impairment can be ascribed either to germ cells or the microenvironment. Our organ culture technique may become a unique and powerful method in the study of spermatogenesis to elucidate such delicate interactions between germ cells and the microenvironment. The extension of such understanding could help establish in vitro treatments for spermatogenic failure covering a variety of causes. The present study provides supporting evidence for the method's practicality.

Materials and Methods

Animals. WBB6F1-*Sl*/*Sl*^{d} mice were provided by Japan SLC. The pups were used for culture experiments at 4.5–9.5 d postpartum. For adult mouse experiments, 6-wk-old mice were used.

Culture Method. The testis tissues were placed on agarose gel half-soaked in the medium containing growth factors at certain concentrations. The cultured tissues were processed for histological and immunohistological examinations. For the evaluation of spermatogenesis they were scrutinized, and differentiated germ cells and Sertoli cells were counted for quantitative evaluation. Other cultured tissues were dissociated for analysis with a flow cytometer to measure haploid-cell production or simply dissociated in PBS to find spermatids and sperm under a microscope. Round spermatids or spermatozoa produced in vitro from *Sl*/*Sl*^{d} mouse testis tissues were collected and used for microinsemination experiments.

Additional materials and experimental procedures are provided in *SI Materials and Methods*.

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