



Autologous transplantation of spermatogonial stem cells restores fertility in congenitally infertile mice

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The blood–testis barrier (BTB) is thought to be indispensable for spermatogenesis because it creates a special environment for meiosis and protects haploid cells from the immune system. The BTB divides the seminiferous tubules into the adluminal and basal compartments. Spermatogonial stem cells (SSCs) have a unique ability to transmigrate from the adluminal compartment to the basal compartment through the BTB upon transplantation into the seminiferous tubule. Here, we analyzed the role of *Cldn11*, a major component of the BTB, in spermatogenesis using spermatogonial transplantation. *Cldn11*-deficient mice are infertile due to the cessation of spermatogenesis at the spermatocyte stage. *Cldn11*-deficient SSCs failed to colonize wild-type testes efficiently, and *Cldn11*-deficient SSCs that underwent double depletion of *Cldn3* and *Cldn5* showed minimal colonization, suggesting that claudins on SSCs are necessary for transmigration. However, *Cldn11*-deficient Sertoli cells increased SSC homing efficiency by >3-fold, suggesting that CLDN11 in Sertoli cells inhibits transmigration of SSCs through the BTB. In contrast to endogenous SSCs in intact *Cldn11*-deficient testes, those from WT or *Cldn11*-deficient testes regenerated sperm in *Cldn11*-deficient testes. The success of this autologous transplantation appears to depend on removal of endogenous germ cells for recipient preparation, which reprogrammed claudin expression patterns in Sertoli cells. Consistent with this idea, *in vivo* depletion of *Cldn3/5* regenerated endogenous spermatogenesis in *Cldn11*-deficient mice. Thus, coordinated claudin expression in both SSCs and Sertoli cells expression is necessary for SSC homing and regeneration of spermatogenesis, and autologous stem cell transplantation can rescue congenital defects of a self-renewing tissue.

claudin | Sertoli cell | spermatogenesis

Spermatogenesis is maintained by close interactions between germ cells and somatic cells. Any defects in this interaction result in defective spermatogenesis, leading to infertility. Spermatogonial stem cells (SSCs) undergo self-renewal divisions (1, 2) and can recolonize empty seminiferous tubules and regenerate spermatogenesis (3). SSCs from a congenitally defective microenvironment can rescue SSC defects caused by *Kit* mutations such that normal offspring are born (4). However, spermatogenic defects can occur due to a number of mutations, most of which cannot be explained at the molecular level. This is particularly true in clinical cases, in which many responsible genes have not yet been identified.

The blood–testis barrier (BTB) forms between the Sertoli cells at ~12–14 d postpartum (dpp) in mice, when they stop mitotic proliferation (5, 6). The BTB divides the seminiferous tubules into adluminal and basal compartments (7). After mitotic division of SSCs, a clone of an interconnected spermatocyte transmigrates through the BTB by continuous dynamic restructuring, and haploid cells eventually develop in the adluminal compartment (8). These spermatocytes are temporarily enclosed in an intermediate compartment and transported into the adluminal side. It is considered that the integrity of the BTB is essential for normal spermatogenesis because it creates a special environment

for meiosis and also protects haploid germ cells from the immune system (5). Thus, the BTB is unique among blood–tissue barriers in the body in terms of its cell biology and immunological aspects, and understanding the molecular mechanism underlying the germ cell–Sertoli cell interaction has important implications for our understanding of infertility.

Research over the last decade has revealed the molecular structure of the BTB. Although the tight junctions of the BTB are formed between Sertoli cells, the functional BTB is composed of the Sertoli cell tight junctions, a physiological permeability barrier, and an immunological barrier (5, 9). Several tight junction proteins (TJPs) are identified, and the phenotypes of knockout (KO) mice for these components vary from normal, as seen in *F11r* KO mice, to slowly degenerative, as seen in *Ocln* KO mice (10), to sterile in *Cldn11* KO mice (11). All animals with BTB defects are infertile because these defects likely cause immunological or other types of damages to the meiotic and postmeiotic cells (5, 9). Although the BTB is formed between Sertoli cells, spermatogonia and spermatocytes also express several TJPs (12). However, the roles of these TJPs are unknown because germ cells do not form tight junction by themselves. Because germ cells also express TJPs, defective spermatogenesis in TJP KO mice may be a result of defects in both the germ cells and Sertoli cells.

Here, we used spermatogonial transplantation to analyze the role of TJPs in *Cldn11* KO mice, which completely lack the BTB.

Significance

Stem cell transplantation is widely used to rescue defects in stem cell-derived cells. However, it is generally impossible to rescue tissue dysfunction caused by defective microenvironment. In this study, we report that autologous spermatogonial stem cell (SSC) transplantation rescues congenital male infertility caused by *Cldn11* deficiency. *Cldn11*-deficient mice lack spermatogenesis due to defects in the blood–testis barrier. However, WT or *Cldn11*-deficient SSC transplantation allowed development of fertile sperm from the donor cells in chemically castrated *Cldn11*-deficient mice. Because *in vivo* depletion of *Cldn3* or *Cldn5* restored endogenous spermatogenesis, complete spermatogenesis may be inhibited by the imbalance of claudin expression caused by *Cldn11* deficiency. Our result suggests that some forms of male infertility can be rescued by autologous SSC transplantation.

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The authors declare no competing interest.

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SSCs have the unique ability to transmigrate through the BTB, and SSCs regenerated from the transplanted SSCs can complete normal spermatogenesis (3). Therefore, this technique has been used to analyze the germ cell–Sertoli cell interaction. Of the several TJP-related KO mice, *Cldn11* KO mice show the most prominent effects, because spermatogenesis in *Cldn11* KO mice does not proceed beyond the spermatocyte stage (11, 13). In our attempt to analyze germ cell–Sertoli cell interaction using this model, we found that autologous SSC transplantation restores fertility.

Results

Immunohistochemical Analysis of *Cldn11* KO Mice. *Cldn11* KO testes were significantly smaller than wild-type (WT) mouse testes when the testes were collected from 11-wk-old mice (Fig. 1A and B). Although germ cells were found in the seminiferous tubules (Fig. 1C, *SI Appendix*, Fig. S1A), no spermatozoa were found in the epididymis (*SI Appendix*, Fig. S1B). Close examination by lectin immunostaining showed a lack of peanut agglutinin (PNA)-expressing haploid cells in the mutant testes (Fig.

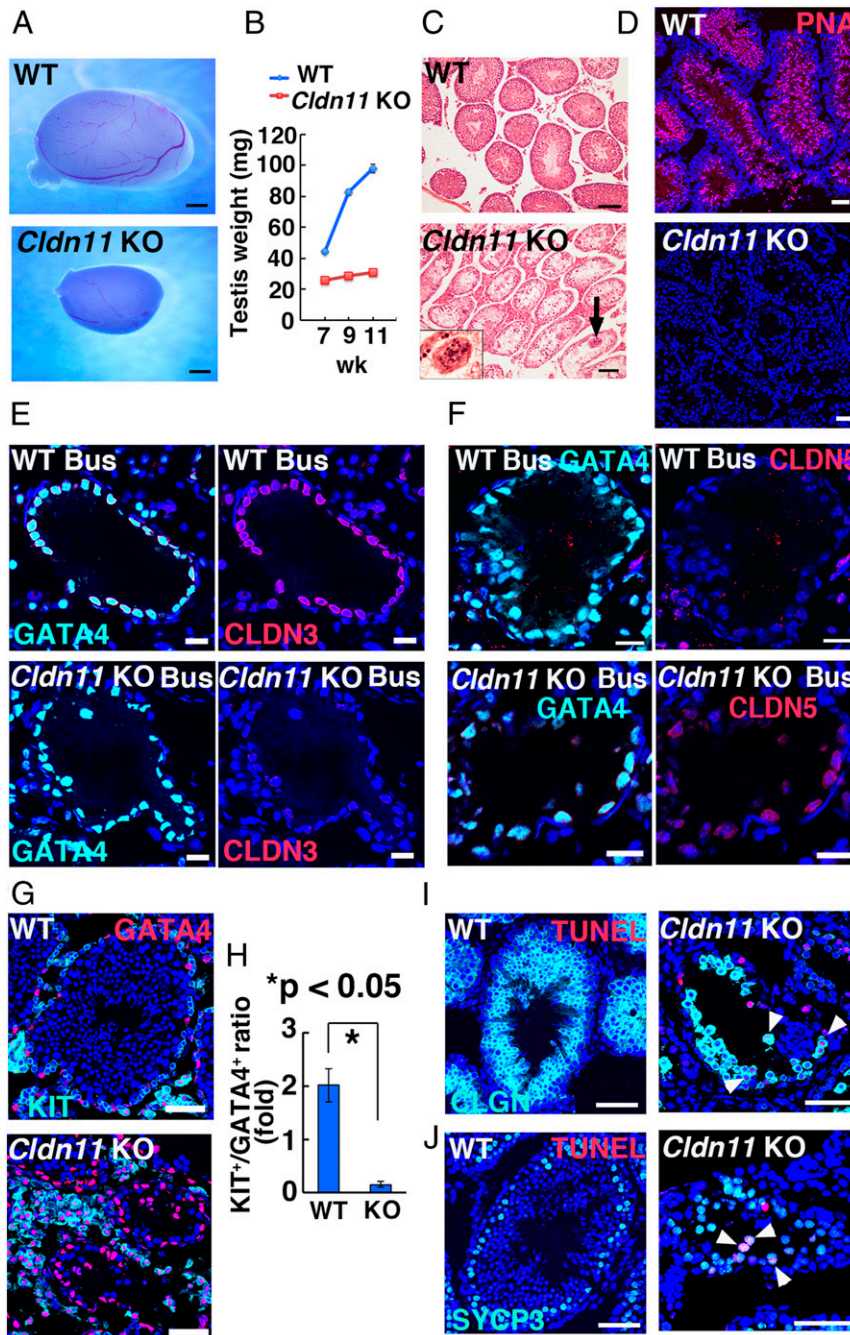


Fig. 1. Characterization of *Cldn11* KO mouse testes. (A and B) Appearance (A) and testis weight (B; $n = 4-8$) of *Cldn11* KO mouse testes. (C and D) Histological appearance of testis (C). Sertoli cell clusters (arrow) are shown in *Inset*. (D) Lectin (PNA) immunostaining. (E and F) Immunostaining of CLDN3 (E) and CLDN5 (F) in busulfan-treated *Cldn11* KO mouse testes. (G) Immunohistochemical analysis of KIT⁺ spermatogonia. (H) Quantification of KIT⁺ cells. At least 11 tubules were counted. (I and J) Immunohistochemical analysis of apoptotic CLGN⁺ (I) and SYCP⁺ (J) cells by TUNEL staining. Arrowheads indicate apoptotic cells. (Scale bars: A, 1 mm; C, 200 μ m; D, G, I, and J, 50 μ m; E and F, 20 μ m.) Stain: hematoxylin & eosin (C), Hoechst 33342 (D–G, I, and J).

1D). Interestingly, we occasionally found clusters of Sertoli cells in the tubule lumen (Fig. 1C, *Inset*) (14). Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining showed no evidence of increased apoptosis in these Sertoli cells (*SI Appendix, Fig. S1C*).

We also examined the impact of *Cldn11* deficiency on the distribution of other TJPs after busulfan treatment, which destroys germ cells. Busulfan treatment did not influence the functional BTB because biotin microinjected into the interstitial tissue did not penetrate into the adluminal compartment (*SI Appendix, Fig. S2A*). Immunohistochemical analysis not only confirmed the lack of CLDN11 (*SI Appendix, Fig. S1D*) but also showed reduced expression of CLDN3 and OCLN after busulfan treatment (Fig. 1E and *SI Appendix, Fig. S1E*). In contrast, CLDN5 is more widely expressed in Sertoli cells on the basement membrane (Fig. 1F). These results suggested that TJP expression patterns in the BTB were significantly influenced by germ cells and CLDN11 (*SI Appendix, Table S1*).

To examine the impact of *Cldn11* deficiency on the spermatogonial population, immunohistochemistry was carried out using antibodies against several spermatogonia markers. Although *Cldn11* KO testes contained a reduced number of CDH1⁺ undifferentiated spermatogonia, no statistically significant difference was found (*SI Appendix, Fig. S3A*). However, the number of KIT⁺ differentiating spermatogonia was significantly decreased (Fig. 1G and H). Nevertheless, the proportion of cells expressing MKI67 (proliferation marker) was comparable between the *Cldn11* KO and the control testes (*SI Appendix, Fig. S3A*). These results show that the loss of CLDN11 influences premeiotic germ cells that are outside of the BTB.

Because a significant number of germ cells undergo apoptosis during meiosis, *Cldn11* KO testes lack haploid cells. TUNEL staining was carried out and an analysis was performed to determine the number of apoptotic cells in WT mice. Quantification of TUNEL⁺ cells revealed that *Cldn11* KO testes contained a large number of apoptotic cells, of which $20.5 \pm 10.5\%$ ($n = 5$) and $58.0 \pm 16.2\%$ ($n = 3$) were CLGN⁺ (spermatocytes) and SYCP3⁺ (spermatocytes to elongating spermatids) cells, respectively (Fig. 1I and J). However, ZBTB16⁺ (undifferentiated spermatogonia) and KIT⁺ (differentiating spermatogonia) cells in both *Cldn11* KO and control testes did not show increased apoptosis (*SI Appendix, Fig. S3B*). These results suggested that spermatocytes are the major cell type that undergoes apoptosis due to *Cldn11* deficiency.

SSC Activity of *Cldn11* KO Mice. In the first set of transplantation experiments, *Cldn11* KO mice were used as donors to examine whether *Cldn11* deficiency influences SSC activity. To introduce a donor cell marker, *Cldn11* KO mice were crossed with the transgenic mouse line C57BL6/Tg14 (act-EGFP-OsbY01) (green mouse). The testis cells were collected from both KO and littermate control WT mice. Total cell recovery from *Cldn11* KO testis cells was significantly decreased (Fig. 2A). The cells were then transplanted into the seminiferous tubules of busulfan-treated mouse testes after dissociation into single cells.

Two months after transplantation, the recipient mice were killed and their testes were examined under ultraviolet (UV) light to visualize donor cell colonization (Fig. 2B). The numbers of colonies generated by the *Cldn11* KO and control testis cells were 5.5 and 1.8 per 10^5 transplanted cells, respectively ($n = 22$ for *Cldn11* KO; $n = 20$ for WT) (Fig. 2C). The difference was statistically significant. When the total number of SSCs per testis was calculated (cell recovery \times colony counts), it was significantly smaller in *Cldn11* KO mouse testes than that in WT testes (831.2 vs. 2160.0 per testis) (Fig. 2D). Immunohistochemical staining confirmed normal spermatogenesis from the transplanted SSCs (Fig. 2E). These results suggested that *Cldn11* KO testes contain a smaller number of SSCs, which can differentiate normally into sperm once they are provided with a normal environment.

Impact of *Cldn11* Deficiency in SSC Homing. The results in the preceding section showed the enrichment of SSCs in *Cldn11* KO mice. However, it was possible that the SSCs were enriched due to the lack of haploid cells. Alternatively, a lack of *Cldn11* might have influenced SSC homing. To directly test the impact of *Cldn11* dosage on SSC homing, we used germ-line stem (GS) cells, cultured spermatogonia with enriched SSC activity (15). We first examined the impact of *Cldn11* overexpression (OE). In these experiments, GS cells that expressed enhanced green fluorescent protein (EGFP) were transfected with *Cldn11* cDNA by lentivirus, and GS cells that stably expressed *Cldn11* (*Cldn11* OE GS cells) were genetically selected by drug selection (Fig. 3A). Real-time PCR analysis showed that the transfected cells expressed *Cldn11* at ~ 160 -fold (Fig. 3B). The infected cells were subsequently transplanted into WBB6F1-W/W^v (W) mice. W mice have mutations in the *Kit* tyrosine kinase and contain only a small number of undifferentiated spermatogonia (4). However, they can support spermatogenesis after transplantation of WT SSCs. Quantification of colony numbers revealed a comparable number of germ cell colonies generated by *Cldn11* OE and control cells, i.e., 120.0 ± 34.0 and 120.0 ± 35.0 ($n = 10$) per

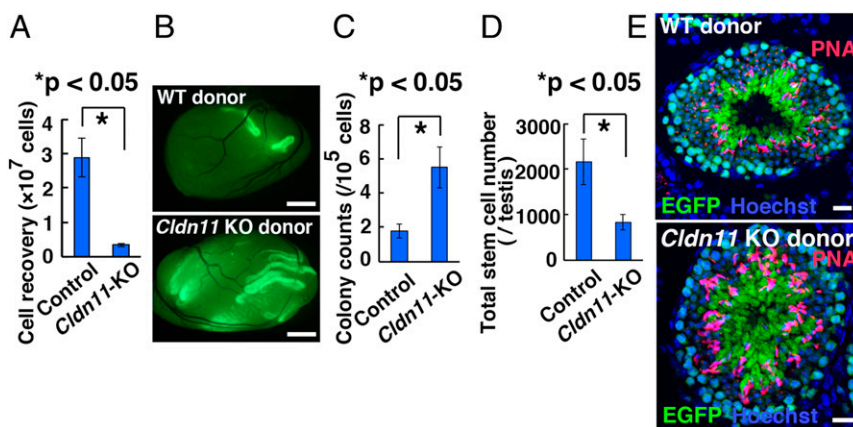


Fig. 2. Functional analysis of SSCs in *Cldn11* KO mice. (A) Cell recovery ($n = 4$). (B) Appearance of recipient testis transplanted with *Cldn11* KO mouse testis cells. (C) Colony counts ($n = 22$ for *Cldn11* KO; $n = 20$ for WT). (D) Total SSC number in *Cldn11* mouse testis ($n = 4$). (E) Lectin (PNA) and SYCP3 immunostaining of recipient testis. (Scale bars: B, 1 mm; E, 20 μ m.) Stain: Hoechst 33342 (E).

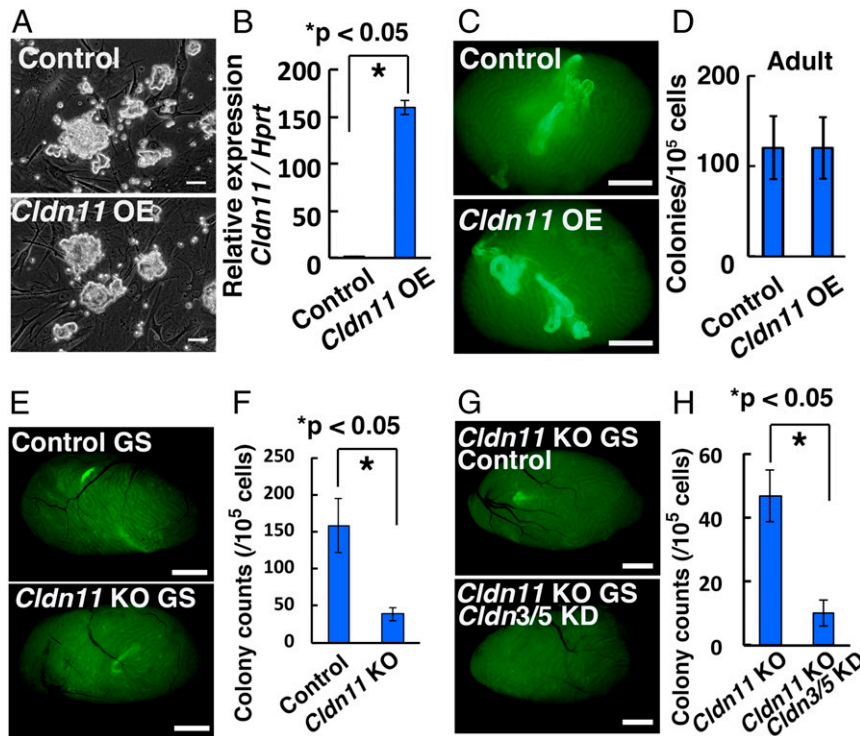


Fig. 3. Impact of *Cldn11* dosage in GS cells on SSC colonization. (A) Appearance of *Cldn11* OE GS cells. (B) Real-time PCR analysis of *Cldn11* expression in GS cells transfected with *Cldn11* ($n = 4$). (C) Appearance of recipient testes transplanted with *Cldn11* OE GS cells. (D) Colony count ($n = 10$). (E) Appearance or recipient testes transplanted with *Cldn11* KO GS cells. (F) Colony count ($n = 17$). (G) Appearance of recipient testis transplanted with *Cldn3/5* KD-*Cldn11* KO GS cells. (H) Colony count ($n = 18$ – 19). (Scale bars: A, 50 μm ; C, E, and G, 1 mm.)

10⁵ transplanted cells (Fig. 3 C and D), respectively, suggesting that *Cldn11* OE did not influence colony formation.

Next, we examined whether decreased *Cldn11* expression influenced the SSC activity of GS cells. We derived GS cells from *Cldn11* KO mice (*Cldn11* KO GS cells). The cells were labeled with a Venus-expressing lentivirus, and transplantation experiments were conducted. The recipient testes revealed reduced colonization of *Cldn11* KO GS cells (Fig. 3 E and F). The numbers of colonies generated by the *Cldn11* KO and control GS cells were 38.8 and 158.8 per 10⁵ transplanted cells, respectively ($n = 17$). The difference between the experimental and control samples was significant. We also depleted CLDN3 and CLDN5 in *Cldn11* KO GS cells, which further decreased the colonization levels (Fig. 3 G and H), suggesting that CLDN3 and CLDN5 were necessary for colonization of *Cldn11* KO SSCs. These results indicated that a synergy between the three claudins in SSCs was necessary for successful colonization, and that increased colonization of *Cldn11* KO testis cells was likely caused by a lack of differentiating germ cells, considering the lower seeding frequency of *Cldn11* KO SSCs.

Enhanced Colonization of SSCs in *Cldn11* KO Mouse Testes. Next, we used *Cldn11* KO mice as recipients to examine the impact of *Cldn11* on SSC colonization. We also investigated the effect of a GnRH analog (leuprolide acetate) treatment, which increases donor cell colony numbers and length by suppressing the hypothalamus-pituitary axis (16). Because CLDN11 is reportedly influenced by testosterone and testosterone regulates the permeability of the BTB (17, 18), we anticipated that leuprolide might interfere with the integrity of the BTB and enhance donor cell colonization.

Cldn11 KO mice were treated with busulfan when the animals were 7 wk old. Some of the KO and control mice were also treated with leuprolide at 4 and 9 wk after busulfan treatment for

maximum effect (19). Donor cells were prepared from green mouse testes, and the cells were transplanted into *Cldn11* KO mice at least 4 wk after the busulfan treatment. In the leuprolide experiments, the donor cells from green mice were transplanted the week after the last leuprolide treatment. Leuprolide treatment down-regulated testosterone levels, but did not disrupt the functional BTB, which was confirmed by biotin tracer experiments (SI Appendix, Fig. S2 A and B).

The number of colonies generated in leuprolide-treated *Cldn11* KO, untreated *Cldn11* KO, leuprolide-treated WT, and untreated WT mice were 9.6, 5.2, 2.6, and 1.6 per 10⁵ transplanted testis cells, respectively ($n = 14$ for *Cldn11* KO; $n = 16$ for WT) (Fig. 4 A and B). Because the same donor cells generated significantly more colonies in *Cldn11* KO mice than in the control mice, this confirmed that CLDN11 expression in Sertoli cells inhibits SSC colonization. Although the number of colonies was increased in leuprolide-treated WT mice, the difference between the leuprolide-treated and untreated mice was not statistically significant. However, the difference between leuprolide-treated and untreated *Cldn11* KO mice was statistically significant. These results indicated that leuprolide treatment increased donor cell colonization in *Cldn11* KO mice, suggesting that leuprolide-mediated colonization enhancement does not depend on *Cldn11*.

Enhanced Colonization of Donor SSCs by *Cldn11* Depletion in Recipient Testes. In the next set of experiments, we examined the feasibility of manipulating *Cldn11* in WT seminiferous tubules, which already have an established BTB. Here, we used W mice. Although conflicting observations were reported for the BTB in W mice (20–22), complete spermatogenesis from transplanted SSCs suggested that spermatogenesis can occur even with defective BTB (4). To test whether *Cldn11* knockdown (KD) could enhance SSC colonization in W mice, lentivirus particles expressing a short hairpin RNA (shRNA) against *Cldn11* were

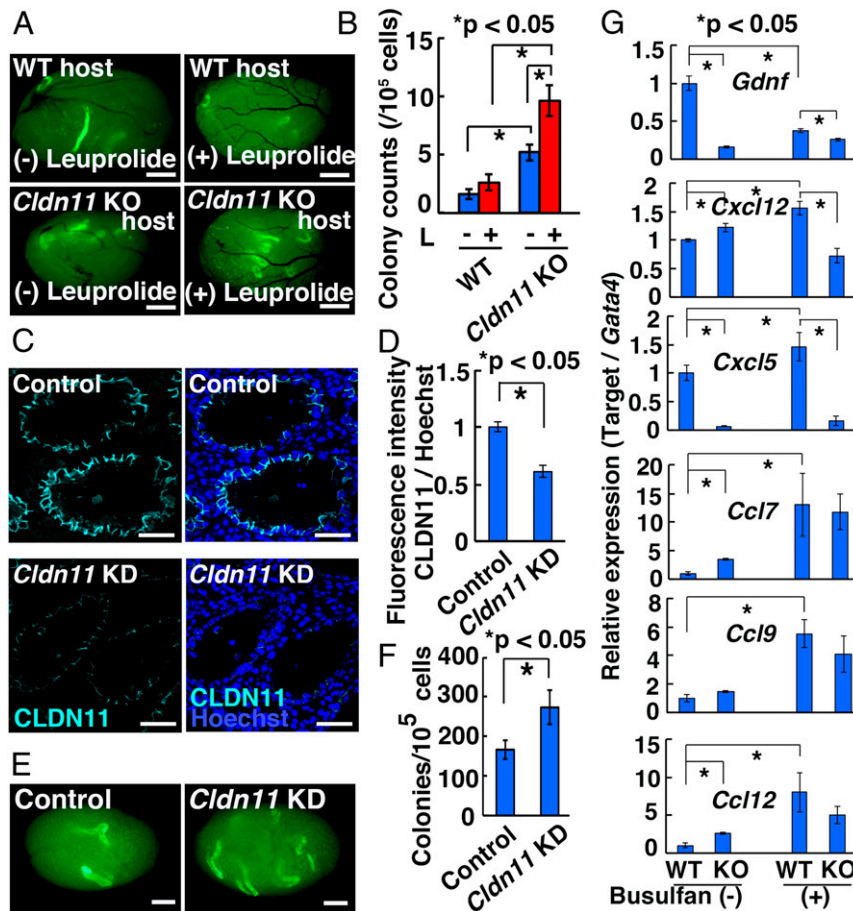


Fig. 4. Enhanced colonization of SSCs in *Cldn11* KO mouse testes. (A) Appearance of *Cldn11* KO recipient testes. (B) Colony count ($n = 14$ – 16). L, leuprolide. (C) Immunostaining of CLDN11 in a W mouse testis that received shRNA against *Cldn11* 5 d after microinjection. (D) Quantification of fluorescence intensity after *Cldn11* KD. At least 17 cells were counted. (E) Appearance of busulfan-treated WT recipient testes that were depleted of *Cldn11* expression. (F) Colony count ($n = 17$ – 18). (G) Real-time PCR analysis of chemokine expression in *Cldn11* KO mouse testes ($n = 4$). Both untreated and busulfan-treated mice were used. (Scale bars: A and E, 1 mm; C, 50 μ m.) Stain: Hoechst 33342 (C).

microinjected into the seminiferous tubules. Immunohistochemical analysis showed that the expression of *Cldn11* decreased to 61.1% in the recipient testes 5 d after microinjection (Fig. 4 C and D). GS cells were then transplanted into the seminiferous tubules 7 d after lentivirus injection.

We found significantly increased colony numbers when we injected shRNA against *Cldn11* prior to transplantation (Fig. 4 E and F). The numbers of colonies generated in *Cldn11* KD and control recipient testes were 273.5 and 166.7 per 10^5 transplanted cells, respectively ($n = 17$ for *Cldn11* KD; $n = 18$ for control). These results suggested that *Cldn11* down-regulation can increase colonization efficiency even after BTB formation.

Deregulated Expression of Chemokines in *Cldn11* KO Mouse Testes. Although these experiments suggested that the lack of *Cldn11* enhances SSC colonization, it was still possible that *Cldn11* KO mouse testes were secreting large volumes of chemokines involved in SSC homing. Increased chemokine expression might have attracted more SSCs than in the WT environment. To understand the molecular mechanism underlying the increased SSC colonization in *Cldn11* KO mice, we analyzed the expression of several chemokines, including *Gdnf*, *Cxcl12*, *Cxcl5*, *Ccl7*, *Ccl9*, and *Ccl12*, in both untreated and busulfan-treated mice. These genes are reportedly implicated in SSC migration into niches (23–26).

Real-time PCR analyses showed that busulfan treatment significantly altered the expression patterns of these chemokines (Fig. 4G). In untreated testes, only *Ccl9* levels were comparable between the *Cldn11* KO and WT testes. Although *Cxcl12*, *Ccl7*, and *Ccl12* levels were significantly increased in the *Cldn11* KO mouse testes, *Gdnf* and *Cxcl5* levels were significantly decreased. However, analysis after busulfan treatment showed a significant decrease in the levels of *Gdnf*, *Cxcl12*, and *Cxcl5* in *Cldn11* KO testes. *Gdnf* expression in busulfan-treated testes was decreased to 0.4-fold relative to that in untreated mice. *Cxcl12*, which is involved in primordial germ cell or SSC migration, also decreased significantly in *Cldn11* KO testes. *Cxcl5* was most significantly decreased in *Cldn11* KO mouse testes, but there were no significant changes in the rest of the genes tested. These results suggested that *Cldn11* deficiency reduced SSC chemokine gene expression and that neither *Cxcl12* nor *Gdnf*, both of which were shown to directly influence SSC homing in vivo (24), was responsible for the enhanced colonization of SSCs in *Cldn11* KO mice.

Restoration of Spermatogenesis in *Cldn11* KO Mouse Testes. Through analysis of *Cldn11* KO recipient testes, we noted that the donor WT cells formed colonies with multiple layers of germ cells (Fig. 5A), which suggested that SSCs differentiated into haploid cells despite the defective environment. To confirm the degree of differentiation, immunostaining of *Cldn11* KO recipient testes

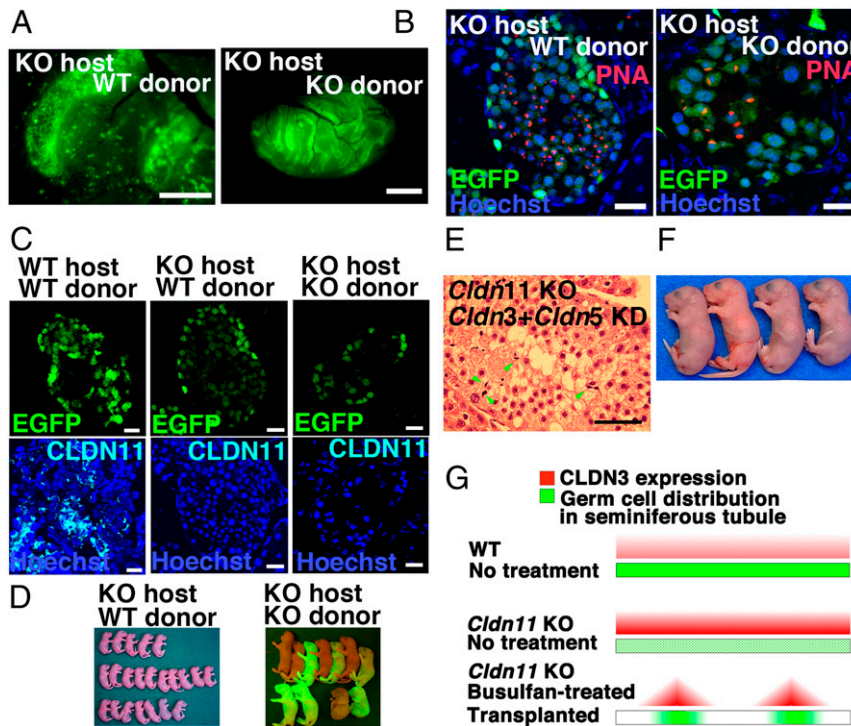


Fig. 5. Regeneration of spermatogenesis in *Cldn11* KO mouse testes after spermatogonial transplantation. (A) Appearance of EGFP-expressing WT or *Cldn11* KO testis cell colonies in busulfan-treated *Cldn11* KO testes showing tubules with adluminal donor cell differentiation. (B) Lectin (PNA) immunostaining of recipient testes. (C) CLDN11 expression in recipient testes after EGFP-expressing donor cell colonization. (D) Offspring born from *Cldn11* KO recipients after microinsemination using germ cells from Venus-labeled WT GS cells (Left) or *Cldn11* KO testis cells (Right). (E) Histological appearance of *Cldn11* KO testis after double depletion of *Cldn3* and *Cldn5*. Arrows indicate elongated spermatids. (F) Offspring born after microinsemination using round spermatids developed in *Cldn11* KO mouse after double depletion of *Cldn3* and *Cldn5*. (G) A hypothetical model of suppressed spermatogenesis in *Cldn11* KO mice. Although CLDN3 expression is enhanced in untreated *Cldn11* KO mice, it disappears after busulfan treatment. However, germ cell transplantation initiates CLDN3 expression. This probably starts in the center of the colonies in which most differentiated cells are found. A germ cell-induced increase in CLDN3 expression would create regions with normal levels of CLDN3, which may allow regeneration of spermatogenesis. (Scale bars: A, 1 mm; B and C, 20 μ m; E, 50 μ m.) Stain: Hoechst 33342 (B and C) and hematoxylin & eosin (E).

was carried out. While the germ cells in intact *Cldn11* KO testes did not progress beyond meiosis, we noted many tubules in *Cldn11* KO recipient testes with PNA⁺ haploid cells (Fig. 5B). We also confirmed the lack of CLDN11 in *Cldn11* KO recipient testes (Fig. 5C). Although no infiltrating CD4⁺ or CD8⁺ lymphocytes were found in these testes (SI Appendix, Fig. S4A), real-time PCR analysis revealed a significant increase in *Ccl2* and *Tnf* expression levels (SI Appendix, Fig. S4B). These results suggested that spermatogenesis occurred in the absence of the BTB despite inflammation.

Based on these observations, autologous transplantation of *Cldn11* KO testis cells was performed. Because *Cldn11* KO testis cells could undergo spermatogenesis in busulfan-treated WT recipients, we deduced that homologous interaction of CLDN11 between germ cells and Sertoli cells is dispensable for spermatogenesis progression at least after transplantation. In our initial experiments, we collected the right testis cells and stored for 4 wk to allow for busulfan treatment of the left testis prior to transplantation. Histological sections showed development of PNA⁺ haploid cells in all three recipients (SI Appendix, Fig. S5). Because regeneration of endogenous spermatogenesis cannot be excluded without a marker, we used green *Cldn11* KO testis cells into busulfan-treated *Cldn11* KO mouse testes in the next experiments, which confirmed donor cell-derived germ cell colonies (Fig. 5A). Immunohistochemistry showed differentiation of germ cells into PNA⁺ haploid cells (Fig. 5B).

Although PNA⁺ cells were found in *Cldn11* KO testes regardless of the donor genotype, it was possible that they lacked fertilization activity because they developed in the absence of the BTB. Microinsemination experiments were conducted to test

whether these haploid germ cells were functionally normal. In these experiments, we used *Cldn11* KO recipient testes transplanted with WT GS cells with a *Venus* transgene (lentivirus) or *Cldn11* KO testis cells with an *Egfp* transgene (green mouse). Seminiferous tubules with spermatogenic colonies were dissociated into a single cell suspension. Elongated spermatids or spermatozoa were microinjected into WT oocytes for fertilization (SI Appendix, Table S2). Both donor cell types produced normal offspring. While 26 offspring were born when WT donor GS cells were used, 11 offspring were born from *Cldn11* KO donor testis cells (Fig. 5D). The donor cell origin was confirmed by PCR using WT GS cells because *Venus* expression was not clear under a UV light (SI Appendix, Fig. S6A). These results suggested that SSCs can mature into functional gametes in *Cldn11* KO mouse testes regardless of their genotype.

Regeneration of Endogenous Spermatogenesis in *Cldn11* KO Mice. To understand the mechanism of spermatogenesis regeneration after autologous transplantation, immunostaining of other claudins was carried out. Although CLDN3 expression in Sertoli cells was lost after busulfan treatment in *Cldn11* KO mice (Fig. 1E), it was restored after spermatogonial transplantation (SI Appendix, Fig. S7A), suggesting that germ cells induce CLDN3 expression in *Cldn11* KO mice. However, CLDN5 expression was enhanced in *Cldn11* KO mice after busulfan treatment (Fig. 1F). However, CLDN5 expression decreased after spermatogonial transplantation into *Cldn11* KO mice (SI Appendix, Fig. S7B). When untreated *Cldn11* KO mice were examined, intensity of CLDN3 and CLDN5 staining was stronger (SI Appendix, Fig. S7 C–F).

Therefore, we reasoned that strong CLDN3 and CLDN5 expression in Sertoli cells might inhibit spermatogenesis in untreated *Cldn11* KO mice.

To test this hypothesis, we microinjected lentivirus expressing shRNA against *Cldn3* and/or *Cldn5* into untreated *Cldn11* KO mouse testes. Two months after microinjection, we noted PNA⁺ haploid cells in *Cldn11* KO testes (Fig. 5E and SI Appendix, Fig. S8A). Although no significant differences were found among the three groups (*Cldn3*, *Cldn5*, and *Cldn3+Cldn5* KD), spermatogenesis occurred most extensively after double KD of *Cldn3* and *Cldn5* (SI Appendix, Fig. S8B). The same treatment did not show apparent impact in WT testes (SI Appendix, Fig. S8A). To confirm the fertility of the germ cells, the seminiferous tubules of *Cldn11* KO testes were dissociated ~2 mo after double KD of *Cldn3* and *Cldn5* and used for microinsemination. Although elongated spermatids were not recovered after cell dissociation, a total of 49 eggs were constructed using round spermatids and four offspring were born (Fig. 5F and SI Appendix, Table S2). As expected, all of the offspring were heterozygous for the *Cldn11* KO allele because oocytes from WT females were used (SI Appendix, Fig. S6B). PCR analysis confirmed the lack of transgenes in the offspring (SI Appendix, Fig. S6B).

Discussion

To understand the impact of the BTB on spermatogenesis and SSC homing, we used *Cldn11* KO mice. Our initial experiments showed that premeiotic *Cldn11* KO germ cells that are outside of the BTB are not completely normal. Moreover, transplantation studies showed impaired SSC colonization when *Cldn11* KO GS cells were transplanted into WT testes, suggesting that CLDN11 positively promotes SSC colonization through the BTB. Therefore, a lack of CLDN11 in the germ cells may prevent passage through the BTB during normal spermatogenesis. However, CLDN11 in the Sertoli cells inhibits SSC colonization because WT SSCs colonized more efficiently in *Cldn11* KO testes. These results showed that the function of CLDN11 differs between germ cells and Sertoli cells in SSC homing.

In the course of our analyses of recipient testes, we found haploid cells in *Cldn11* KO mice. Surprisingly, these haploid cells were capable of producing offspring. These results were unexpected because a lack of a BTB would change the composition of the seminiferous tubule fluid and disrupt the normal cytokine expression pattern. Moreover, although postmeiotic cell development is supported by mitotically quiescent, polarized Sertoli cells under physiological conditions, our results suggested that neither the cell cycle status nor the polarity of Sertoli cells is critical for completing functional spermatogenesis. Because inflammatory cytokines were up-regulated in recipient testes, production of haploid cells caused immune response. However, unlike after allogeneic transplantation (25), we did not find infiltration of lymphocytes and haploid cells were not rejected. Because conflicting observations on the BTB function are reported for busulfan-treated mice and W mice (20–22), more extensive studies need to be carried out to confirm the role of the BTB in normal spermatogenesis. Nevertheless, our results based on nonphysiological conditions revealed a remarkable flexibility of spermatogenesis. Perhaps, the BTB may simply amplify spermatogenesis efficiency.

There were significant changes in claudin expression patterns after transplantation in both the germ cells and Sertoli cells. In particular, CLDN3 expression depended on the germ cells because its expression disappeared after busulfan treatment. Unlike untreated *Cldn11* KO mice that contain germ cells in all areas of the seminiferous tubules, spermatogenesis from transplanted SSCs occurs in restricted areas because only a limited number of SSCs can colonize the recipient testes (26). Given the lack of CLDN3 in busulfan-treated *Cldn11* KO Sertoli cells, only CLDN5 was expressed in *Cldn11* KO Sertoli cells. If CLDN3 is induced by germ cells, CLDN3 expression would only occur when Sertoli cells interact with appropriate numbers of germ cells. In germ cell

transplantation, colonies tend to differentiate in the center (26). This would create a gradient of CLDN3 expression in developing colonies (Fig. 5G). However, such heterogeneity of CLDN3 expression may not occur during spermatogenesis in intact *Cldn11* KO testes because all areas of the seminiferous tubules are filled with germ cells to the same degree. We reasoned that this potential heterogeneity of claudin expression in germ cell colonies of busulfan-treated testes might have allowed some germ cells to pass through the BTB.

To test this hypothesis, we carried out in vivo KD experiments. Because both *Cldn3* and *Cldn5* KD induced spermatogenesis in intact *Cldn11* KO testis, both claudins are involved in suppression of endogenous spermatogenesis. Therefore, a balance in claudin expression levels appears to be critical for the completion of spermatogenesis. However, we still do not know how these claudins blocked spermatogenesis. Because transgenes were not found in the offspring and lentivirus cannot infect endogenous germ cells (27), claudin expression in Sertoli cells are probably responsible for regeneration after in vivo KD. One possibility is that increased expression of CLDN3 and CLDN5 in Sertoli cells enhanced the adhesiveness between Sertoli cells, which may physically block germ cell migration. Although *Cldn3* KO mice does not have apparent phenotype (28), increased *Cldn3* expression might physically block spermatogenesis. However, this is unlikely because Sertoli cells are mitotically active in *Cldn11* KO mice (14). It is difficult to conceive that proliferating Sertoli cells adhere more strongly to each other and block spermatogenesis. We rather think that increased claudin expression in Sertoli cells might have directly interacted with claudins on germ cells. Because *Cldn11* KO germ cells lacked CLDN5 expression, this interaction may occur via CLDN3. Perhaps CLDN3 on Sertoli cells may trap CLDN3-expressing spermatocytes and inhibit their differentiation into haploid cells. Given the success with *Cldn5* KD, however, CLDN5 may indirectly influence CLDN3 expression. Further investigations into the type of germ cells that induce CLDN3 expression and the nature of claudin interaction between germ cells and Sertoli cells are warranted.

SSC transplantation has been widely used for functional analysis of SSCs and dissection of germ cell–Sertoli cell interaction. However, the possibility of autologous transplantation has been overlooked. The current study demonstrated the involvement of *Cldn11* in SSC homing but also showed that the BTB is dispensable for spermatogenesis. In addition, lack of immunological rejection for haploid cells suggests that it is not the BTB per se, but rather the immunosuppressive properties of Sertoli cells are responsible for the immune privilege of regenerated germ cells. Our study also provides a possibility in stem cell transplantation therapy and suggests that other forms of congenital male infertility can be rescued by SSC transplantation or slight modification of testicular environment.

Materials and Methods

Animals and Microinjection Procedure. *Cldn11* KO mice were provided by S. Tsukita (Osaka University, Suita, Japan) (13). We crossed *Cldn11* KO mice with green mice (gift from M. Okabe, Osaka University, Suita, Japan) to introduce a donor cell marker. For the transplantation, 4-wk-old C57BL/6 (B6) or B6 × DBA/2 F1 (BDF1) mice were injected intraperitoneally with busulfan (44 mg/kg). For the transplantation experiments, busulfan was administered when the animals were 7 wk old. At least 1 mo following the busulfan treatment, the animals were used for transplantation. In some experiments, we also used 4- to 6-wk-old W mice for microinjection of *Cldn11* KD lentivirus particles (1.2 × 10⁸/mL with polybrene [333 μg/mL] 1 wk before transplantation (Japan Shizuoka Laboratory Animal Center). For the microinjection, dissociated single-cell suspensions or virus particles were inserted into seminiferous tubules via the efferent duct (29). Each injection filled 75–85% of the seminiferous tubules. Where indicated, we administered the GnRH agonist leuporelin acetate (0.19 mg per mouse; Takeda Pharmaceutical Co.) by subcutaneous injection 9 and 4 wk before transplantation, as described previously (19). The Institutional Animal Care and Use Committee of Kyoto University approved all of the animal experimentation protocols.

Statistical Analyses. Results are presented as the means \pm SEM. Data were analyzed using Student's *t* tests. Multiple comparison analyses were performed using ANOVA followed by Tukey's honestly significant difference (HSD) test.

SI Appendix. Additional data discussed in the paper and full details of methods are provided in *SI Appendix*.

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