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Article

Reconstitution of Mouse Spermatogonial Stem Cell Niches in Culture

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SUMMARY

Spermatogonial stem cells (SSCs) reside in specific niches within seminiferous tubules. These niches are thought to secrete chemotactic factors for SSCs, because SSCs migrate to them upon transplantation. However, the identity of these chemotactic molecules remains unknown. Here, we established a testis feeder cell culture system and used it to identify SSC chemotactic factors. When seeded on testis cells from infertile mice, SSCs migrated beneath the Sertoli cells and formed colonies with a cobblestone appearance that were very similar to those produced by hematopoietic stem cells. Cultured cells maintained SSC activity and fertility for at least 5 months. Cobblestone colony formation depended on GDNF and CXCL12, and dominant-negative GDNF receptor transfection or CXCL12 receptor deficiency reduced SSC colonization. Moreover, GDNF upregulated CXCL12 receptor expression, and CXCL12 transfection in Sertoli cells increased homing efficiency. Overall, our findings identify GDNF and CXCL12 as SSC chemotactic factors in vitro and in vivo.

INTRODUCTION

Several self-renewal systems are present in the body, which are maintained by stem cell division. Stem cells in these tissues are thought to reside in a special microenvironment called a ‘‘niche’’ (Spradling et al., 2001). Stem cells in the niche have a high probability for self-renewal division, whereas committed daughter cells leave the niche to terminally differentiate. Therefore, the niche must provide factors to maintain stem cells and exclude those that trigger differentiation. Although the niche theory was originally proposed to explain the behavior of transplanted hematopoietic stem cells (HSCs) (Schofield, 1978), it has been extended to other self-renewing tissues, including *Drosophila* germline niches, which have simplistic structures that allow detailed molecular analyses (Spradling et al., 2001). However, self-renewing tissues in mammals are generally more complex, and much remains unknown about the mammalian niche and how it maintains stem cells.

Spermatogonial stem cells (SSCs) in testes provide the foundation for spermatogenesis throughout life (de Rooij and Russell, 2000; Meistrich and van Beek, 1993). The identification of SSCs depends on their ability to self-renew, not on their morphology. In 1994, a transplantation assay to study SSCs was developed (Brinster and Zimmermann, 1994). When SSCs are microinjected into the seminiferous tubules of infertile testes, SSCs reinitiate and maintain spermatogenesis for a long duration. Transplanted SSCs attach to Sertoli cells, the only somatic cell type that directly interacts with SSCs in seminiferous tubules, and transmigrate through the tight junction between Sertoli cells from the adluminal side toward the basal compartment of the tubules. SSCs then recolonize empty niches, which are located on the basement membrane (Nagano et al., 1999). The interaction between SSCs and the niche is dynamic, as transplantation of a large number of SSCs results in colonization even in a nonablated wild-type (WT) environment (Shinohara et al., 2002). The spermatogonial transplantation technique has not only provided a functional assay for SSCs but has also demonstrated the homing activity of SSCs.

In 2000, GDNF was identified as a self-renewal factor for SSCs (Meng et al., 2000). In transgenic mice that overexpress GDNF, clusters of undifferentiated spermatogonia accumulate in the seminiferous tubules, whereas spermatogonial depletion and male infertility occur in *Gdnf* heterozygous knockout (KO) mice. Moreover, adding GDNF to SSCs is necessary for in vitro proliferation (Kanatsu-Shinohara et al., 2003). These results established that the level of GDNF is critical for SSC self-renewal and survival. Despite these findings, the identity and location of the niche remains elusive. For example, in one study, SSCs were shown to reside in areas of the seminiferous tubules that closely associate with blood vessels (Yoshida et al., 2007), but another study demonstrated that the number of Sertoli cells, not the location of blood vessels, dictates the niche distribution (Oatley et al., 2011). The difficulty in studying SSC-niche interactions in vivo is caused in part by the inability to identify SSCs by their morphology. Although the transplantation assay is a reliable method for detecting SSCs, stable colonies are found only after

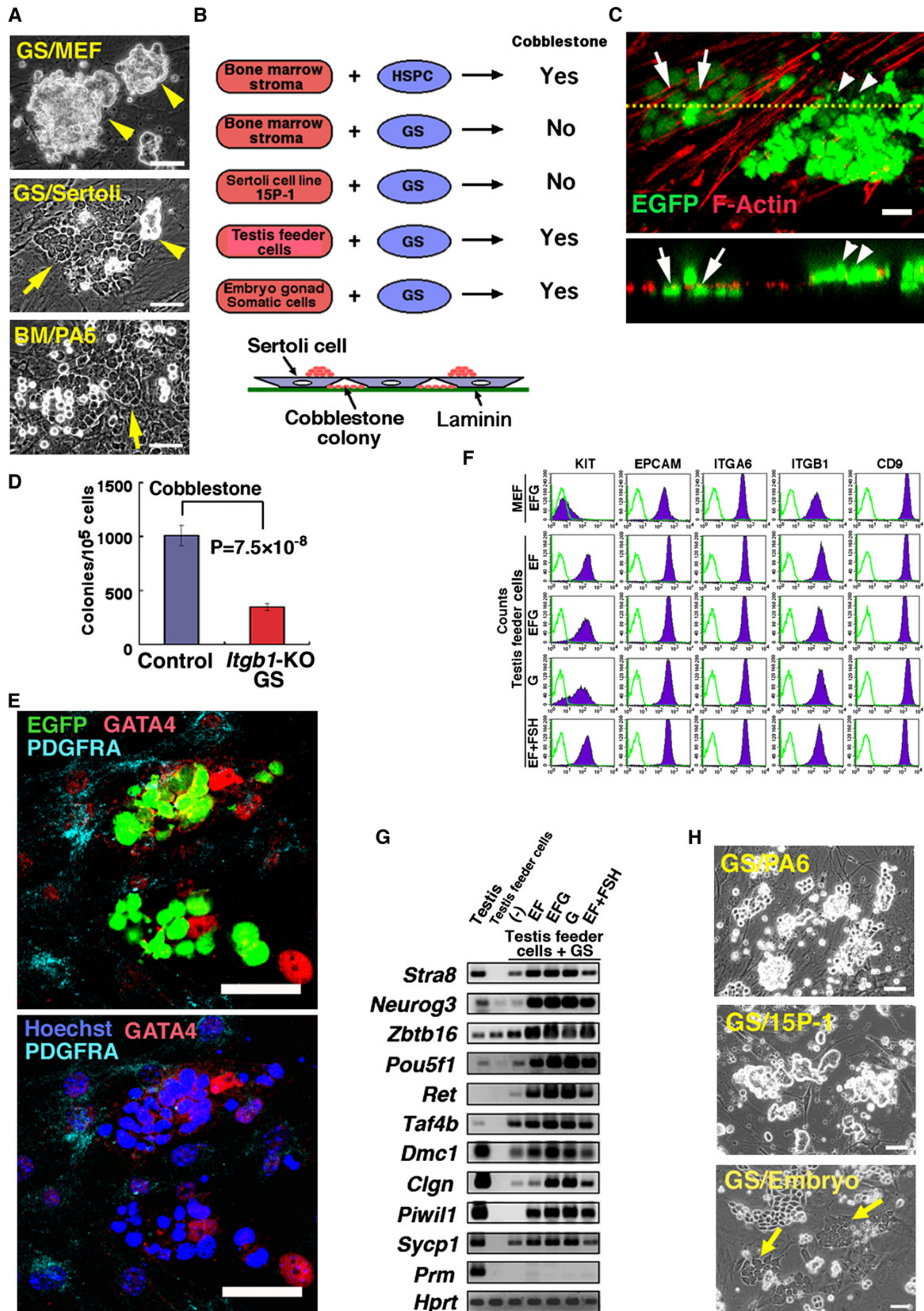

Figure 1. Cobblestone Formation of GS Cells

(A) Development of cobblestone colonies. GS cells on MEFs proliferate in morula-like clumps (top), migrate beneath the testis feeder cells, and develop cobblestone colonies (middle), which are indistinguishable from bone-marrow-derived cobblestones (bottom). Cobblestone and clump colonies are indicated by arrows and arrowheads, respectively.

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~6 weeks posttransplantation, and difficulty in distinguishing SSCs from progenitors in early phases of colonization makes it impossible to localize the niche in situ. In fact, only 5%–10% of A_{single} spermatogonia may have SSC potential by transplantation (Nagano et al., 1999; Nagano, 2003), and in situ SSC identification is difficult due to the lack of an SSC-specific marker.

In mammals, the interaction between stem cells and their niche has been studied most extensively in HSCs, for which partial in vitro reconstruction of the stem cell microenvironment is possible (Dexter et al., 1977). In long-term bone marrow cultures, HSCs closely associate with the pre-established stromal layer that facilitates HSC survival and differentiation. HSCs undergo apoptosis if they are prevented from attaching to stromal cells. Primitive cells, including HSCs, migrate through the stromal layer and produce colonies of primitive cells, called cobblestone areas, which consist of tightly packed nonrefractile phase-dark cells growing underneath the stroma. Under these conditions, HSCs are thought to maintain a steady state of cell production that continues for several months, and some differentiating progenitors are released into the culture medium. The technique has been used to quantify HSCs or primitive progenitors by counting cobblestone colonies (Ploemacher et al., 1989). Although the in vivo transplantation assay has been the ultimate demonstration of HSC activity, this cobblestone-area-forming cell assay has served as an alternative in vitro method for assessing HSC potential. This method has been particularly useful for evaluating HSC activities in humans, for which direct transplantation experiments are not feasible.

In this study, we report a testis cell culture method that can maintain SSCs for a long duration. In this culture system, SSCs produce cobblestone colonies that are morphologically indistinguishable from HSCs growing under bone marrow stroma. The cobblestone formation depends on GDNF and CXCL12, which are secreted from the testis stroma cells. Involvement of these cytokines in SSC homing to the niche in vivo was also demonstrated using spermatogonial transplantation.

RESULTS

Cobblestone Formation of Germline Stem Cells on Testis Feeder Cells

To establish testis cell culture conditions that mimic the in vivo seminiferous tubule environment, testis cells were prepared from 5- to 6-week-old congenitally infertile WBB6F1-W/W^v (designated W) mice, which are enriched with Sertoli cells due to the loss of differentiating germ cells by *Kit* tyrosine kinase mutations (Geissler et al., 1988). A testis cell suspension was then plated on laminin-coated dishes in serum-free medium, supplemented with EGF (E), FGF2 (F), and FGF9. The testis feeder cells were used for coculture after mitomycin C (MMC) treatment. When germline stem (GS) cells from C57BL/6 Tg14(act-EGFP) OsbY01 (Green) mice were plated on the feeder cells with EGF, FGF2, and GDNF (G) and 1% fetal bovine serum (FBS; EFG condition), some of the cells migrated beneath the feeders, whereas others produced clumps on top of the feeders within 7–14 days after coculture. The former colonies, which constituted 12.3% ± 0.6% of total colonies (n = 12) at 7 days after coculture, were composed of flat phase-dark cells with clear nuclei and cell borders, closely resembling hematopoietic cobblestone colonies (Figures 1A and 1B) (Dexter et al., 1977). The latter colonies resembled those on mouse embryonic fibroblasts (MEFs) (Figure 1A) and consisted of morula-like clumps with an unclear border between cells. The cobblestone colonies were not derived from residual spermatogonia of W mice, because they expressed enhanced green fluorescence protein (EGFP) under UV light (Figure 1C). *Itgb1*-KO GS cells, which proliferate to form clump colonies on MEFs (Kanatsu-Shinohara et al., 2008), showed reduced cobblestone formation, suggesting the involvement of ITGB1 on cobblestone generation (Figure 1D). The feeder layer predominantly consisted of $GATA4^+$ Sertoli cells, covering more than >80% of the total area (Figure 1E). $PDGFRA^+$ Leydig cells were also found in the culture (~10%), but they were not necessarily associated closely with the cobblestone colonies. Both Sertoli cells and Leydig cells were mitotically active before MMC treatment, as shown by Ki67 expression in both VIM^+ Sertoli cells (51.6%) and $PDGFRA^+$ Leydig cells (42.2%) (Figure S1A available online).

Flow cytometric analyses demonstrated that the cultured cells expressed SSC markers, including EPCAM, ITGA6, ITGB1, and CD9 (Figure 1F). Although the expression levels of these markers were comparable to those cultured on MEFs, coculturing with testis feeder cells strongly upregulated KIT, a marker for differentiating spermatogonia. We also assessed germ cell differentiation levels using reverse-transcription polymerase chain reaction (RT-PCR). The expression of spermatogonia markers, such as *Stra8*, *Neurog3*, *Zbtb16*, *Pou5f1*, *Ret*, and *Taf4b*, or meiotic germ cell markers, such as *Dmc1*, *Clgn*, *Piwil1*, and *Sycp1*, did not change significantly depending on the type of cytokines used to induce cobblestone colonies (Figure 1G and Table S1). Because we did not find expression of postmeiotic markers,

(B) Summary of coculture experiments. Cobblestone formation occurs only in cocultures with testis feeder cells or embryonic gonad-derived somatic cells.
(C) Confocal image of cobblestone colonies. $EGFP^+$ GS cells (green) were cultured on feeder cells for 10 days. Cobblestone-type colonies (arrow) are localized beneath actin filaments of feeder cells (phalloidin, red), whereas clump-type (arrowhead) colonies formed on top of feeder cells. The broken line indicates the plane of the section shown at the bottom.
(D) Reduced cobblestone formation of *Itgb1*-KO GS cells (n = 24).
(E) Immunohistochemistry of cobblestone culture. GS cells expressed EGFP (green). Sertoli and Leydig cells were detected by the expression of GATA4 (red) and PDGFRA (cyan), respectively. Counterstained with Hoechst 33342 (blue).
(F) Flow cytometric analysis of GS cells cultured with MEFs or testis feeder cells for 9 days. Only EGFP-expressing cells were gated for analysis. Note the upregulation of KIT expression in the coculture with testis feeder cells. Green lines indicate controls.
(G) RT-PCR analysis of GS cells cultured with testis feeder cells for 2 weeks.
(H) GS cells cultured under EFG condition with PA6 (top), 15P-1 (middle), and somatic cells derived from embryonic gonads of W mice 13.5 dpc (bottom), 7 days after culture initiation. Arrows indicate cobblestone colonies. HSPC, hematopoietic stem/progenitor cells.
The results are presented as means ± SEMs. Bar = 50 μm (A, E, and H) and 20 μm (C). E, EGF; F, FGF2; G, GDNF. See also Table S1 and Figure S1.

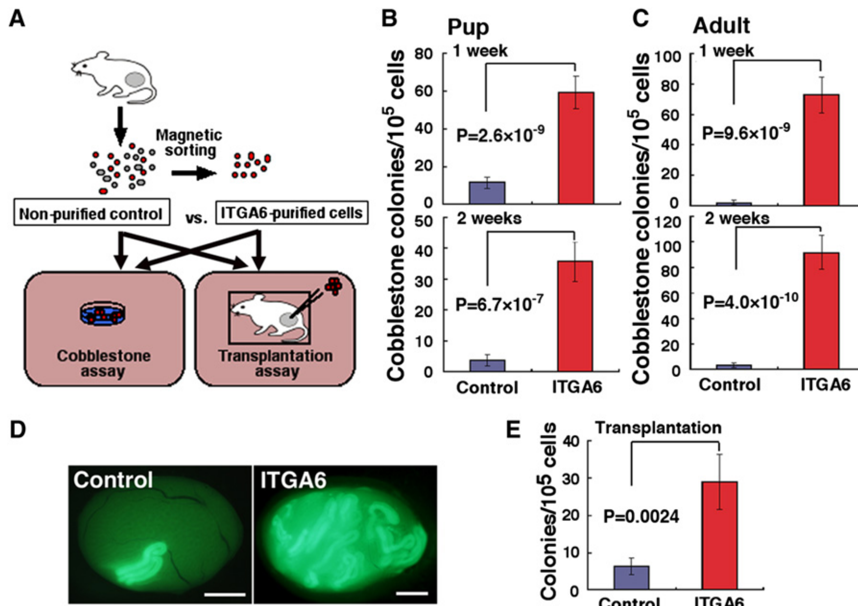

Figure 2. Enhanced Cobblestone Formation of ITGA6-Selected Primary Testis Cells

(A) Experimental procedure.

(B and C) Cobblestone formation of ITGA6-selected pup (B) or adult (C) testis cells (n = 112 for pups; n = 96 for adults).

(D) Macroscopic appearances of recipient testes transplanted with control (left) or ITGA6-selected (right) pup testis cells. Green fluorescence indicates donor cell origin.

(E) Colony counts in the recipient testes. Note the enhanced colonization of ITGA6-selected cells (n = 16). Results of three experiments are shown. The results are presented as means ± SEMs. Bar = 1 mm (D).

such as *Prm*, these results suggested that the cultures contained premeiotic cells that could not complete meiosis.

These cobblestone colonies were induced on freeze-thawed testis feeder cells prepared from W mice as well as on testis cells from WT mice, albeit at significantly lower levels (data not shown). The ability to support cobblestone formation is acquired by somatic cells in the gonad in the early stage of the embryonic period, as shown by the presence of cobblestone colonies on somatic feeder cells prepared from W mouse male gonads 13.5 days postcoitus (dpc) (Figure 1H). However, cobblestone colonies were not found on cell lines, such as PA6 bone marrow stromal cells or the 15P-1 Sertoli cell line (Kodama et al., 1982; Rassoulzadegan et al., 1993). When primary testis feeder cells were compared with 15P-1 cells by immunohistochemistry, we found that 15P-1 cells lacked expression of CLDN11 (Figure S1B). Although ITGB1 and VIM were similarly expressed in both 15P-1 and primary testis feeder cells, not all the Sertoli cells expressed SOX9 and WT1 in testis feeder cells. This was in contrast to 15P-1 cells, because all of the cells in culture expressed these two molecules. These results suggested that several features of primary Sertoli cells are lost or modified in 15P-1 cells, which may have contributed to the lack of cobblestone colonies in cultures with 15P-1 cells.

Increased Cobblestone Formation by SSC-Enriched Primary Testis Cells

Because GS cells do not necessarily exhibit the same characteristics as SSCs in the testes (Morimoto et al., 2009), we next examined whether SSCs freshly isolated from testes have cobblestone-forming activity. For this experiment, an SSC-enriched population was collected from 12- to 16-day-old or 8-week-old Green mice using an antibody against ITGA6, which is expressed on SSCs (Shinohara et al., 1999). The same population of cells was used simultaneously for spermatogonial transplantation in experiments using pups (Figure 2A). After magnetic sorting, 20.9% ± 7.3% and 11.0% ± 2.7% (n = 3) of the input cells were recovered from pup and adult testes, respectively. The selected cells were plated on testis feeder cells under the EFG condition to quantify the cobblestone areas.

In both pup and adult experiments, the numbers of cobblestone colonies, as defined by clusters of six or more cells, increased after ITGA6-mediated selection at both time points. For pup experiments, the numbers of cobblestone colonies from ITGA6-expressing cells were 59.8 ± 8.6 and 35.7 ± 6.3/10^5 cells (n = 112) at 1 and 2 weeks, respectively, and the numbers were significantly higher than those of control whole testes cells, which produced 11.6 ± 3.0 and 3.6 ± 1.8 colonies/10^5 cells (n = 112) for 1 and 2 weeks, respectively (Figure 2B). Similarly, ITGA6-selected adult testis cells generated 72.9 ± 11.7 and 91.7 ± 13.3 cobblestone colonies/10^5 cells (n = 96) at 1 and 2 weeks, respectively (Figure 2C). Consistent with the low SSC frequency in the adult testis (Shinohara et al., 2001), cobblestone colonies were rarely produced by the control whole adult testis cells, and the numbers of colonies were 2.1 ± 1.5 and 3.1 ± 1.8/10^5 cells (n = 96) for 1 and 2 weeks, respectively. When the SSC activities of pup testis cells were determined by spermatogonial transplantation, ITGA6-selected cells produced 28.9 ± 7.4 colonies/10^5 cells (n = 16), whereas nonselected control cells produced 6.4 ± 2.3 colonies/10^5 cells (n = 16), showing a 4.5-fold increase (Figures 2D and 2E). Taken together, these results show that cobblestone-forming activities are not limited to GS cells but also found in freshly prepared pup and adult testis cells.

Enhancement of Cobblestone Forming Activity by GDNF and CXCL12

To understand the mechanism of cobblestone formation, we compared the impact of cytokines on cobblestone forming activity of GS cells. Besides GDNF, EGF, and FGF2, we investigated the effect of follicle-stimulating hormone (FSH), which induces GDNF expression in Sertoli cells in vivo (Tadokoro et al., 2002). Cobblestone formation was induced by the combination of EGF and FGF2 (EF condition), but the frequency was significantly lower than that induced only by GDNF (G condition). However, the low frequency under the

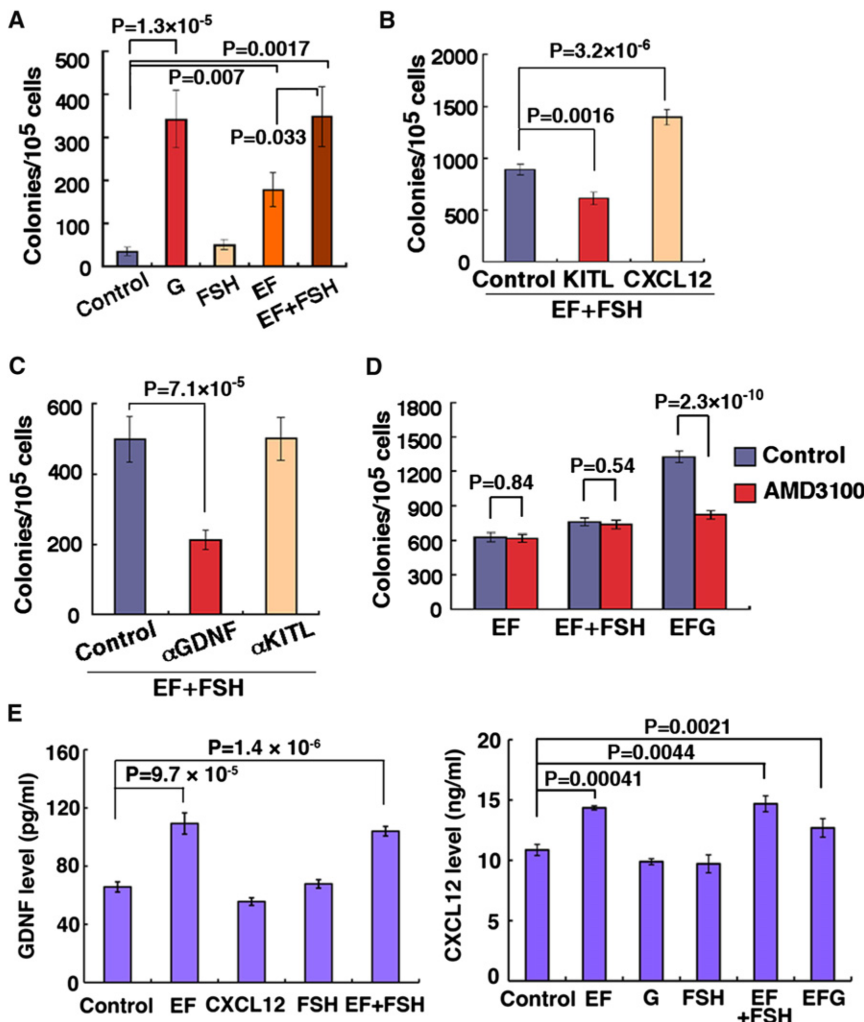

Figure 3. Enhanced Cobblestone Colony Formation by GDNF and CXCL12

(A) Effect of cytokines on cobblestone colonies (n = 46–48). The indicated reagents were added in the presence of 1% FBS.

(B) Suppression of cobblestone colonies by KITL and enhanced cobblestone colonies by CXCL12 (n = 32).

(C) Suppression of cobblestone colonies by GDNF neutralizing antibody (n = 41–44).

(D) Effect of a CXCR4 antagonist on cobblestone colonies. Although cobblestones developed under the EF or EF + FSH conditions were not significantly suppressed by AMD3100, those that developed under the EFG condition were suppressed by the same treatment (n = 24).

(E) Levels of GDNF (left) and CXCL12 (right) in culture. Culture supernatant was collected 5 days after adding the cytokines, and the protein levels were determined by ELISA (n = 8 for GDNF; n = 4 for CXCL12).

The results are presented as means ± SEMs.

EF condition was significantly improved by adding FSH (EF + FSH condition) (Figure 3A), although FSH itself did not induce cobblestones.

We also compared the effects of KITL and CXCL12, both of which increase HSC cobblestone formation (Szilvassy et al., 1996; Tzeng et al., 2011). Adding CXCL12 increased the cobblestone frequency when the cells were cultured under the EF + FSH condition, whereas KITL downregulated the cobblestone frequency to ~0.7-fold (Figure 3B). Cobblestone formation under the EF + FSH condition was inhibited by GDNF neutralizing antibody, whereas KITL neutralizing antibody did not show any effect (Figure 3C), suggesting the contribution of GDNF to cobblestone formation under the EF + FSH condition. In contrast, cobblestone formation under the EF + FSH condition was not suppressed by AMD3100, which inhibits the binding of CXCL12 to its receptor CXCR4 (Figure 3D) (Donzella et al., 1998). Notably, AMD3100 inhibited the cobblestone that developed under the EFG condition, suggesting that cobblestone formation under EFG condition depends partly on CXCL12 produced in the cultures.

The results of an enzyme-linked immunosorbent assay (ELISA) showed that CXCL12 was expressed at a significantly higher level than GDNF in the culture supernatants and that both GDNF and CXCL12 expression increased under the EF condition (Figure 3E). However, neither GDNF nor CXCL12 could upregulate each other's expression. Furthermore, FSH also did not influence the expression of these cytokines, suggesting the involvement of other molecules.

Long-Term Maintenance of SSC Activity under Cobblestone Cultures

To determine whether the cultured cells maintain SSC activity, we collected a portion of cells during culture and measured SSC activity by transplantation into W mice during a 5-month culture (Table S2). We compared three types of cultures maintained with different sets of cytokines. Although costimulation by GDNF and FGF2 is a prerequisite for SSC proliferation cultured on laminin-coated plates (Lee et al., 2007), the total germ cell number increased exponentially when the cells were cultured only with GDNF (Figure 4A), which could have been due to FGF2 expression from testis feeder cells (Figure S2A). In contrast, the number of germ cells was relatively constant under the EF and EF + FSH conditions, in which KIT expression was upregulated (Figure 1F). KIT upregulation was only slightly suppressed by GDNF, suggesting that the feeder cells secrete differentiation-inducing molecules.

Germ cell colonies were consistently found in recipients after spermatogonial transplantation with all types of cultures (Figure 4B and Table S2). The concentrations of SSCs, as estimated by the number of germ cell colonies, were comparable among the three types of culture. However, the fact that cell number increased under the G condition indicates that SSCs preferentially increase their number even on testis cells as long as exogenous GDNF is present in the medium (Table S2). Although KIT

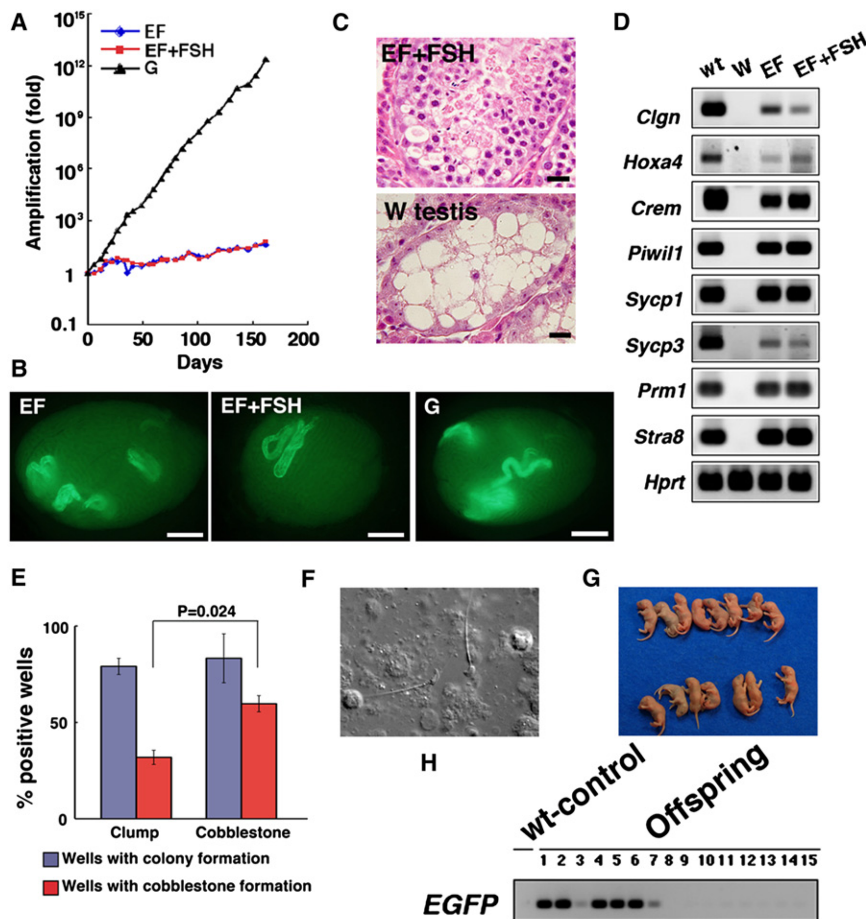

Figure 4. Maintenance of SSCs In Vitro

(A) Proliferation of GS cells maintained by coculture with testis feeder cells.

(B) Macroscopic appearances of recipient testes transplanted with the indicated cultured cells.

(C) Histological appearances of a recipient testis transplanted with GS cells cultured under the EF + FSH condition (top). No spermatogenesis was observed in control W testis (bottom).

(D) RT-PCR analysis of the recipient testes, showing spermatogenic cell differentiation.

(E) Single colony replating. The frequency of secondary cobblestone colony formation was higher from cobblestone colonies than from clump colonies (n = 48). Colonies were counted 7 days after replating.

(F) Spermatozoa retrieved from the recipient testis.

(G) Offspring produced by microinsemination.

(H) PCR analysis of the transgene. A total of 7 of the 15 offspring had the EGFP gene.

The results are presented as means ± SEMs. Bar = 1 mm (B) and 20 μm (C). Stain: hematoxylin and eosin (C). See also Tables S1–S4 and Figure S2.

upregulation suggested increased differentiation under the EF and EF + FSH conditions, SSCs were not only maintained successfully, but they also reinitiated normal spermatogenesis, which was shown by histological and RT-PCR analyses (Figures 4C and 4D and Table S1).

To examine whether cobblestone colonies possess SSC activity, we separated the two types of colonies by EDTA treatment and gentle pipetting. Cells enriched for each colony type were transplanted into testes. The numbers of colonies generated from cobblestone and clump colonies were comparable regardless of culture conditions, and we did not find statistical differences (Table S3). Cobblestone colonies could be reproduced after replating of cobblestone cells, whose SSC potential could be demonstrated at least up to 10 weeks (Table S4). Furthermore, when we picked single colonies by micromanipulation and replated the dissociated cells, cobblestone colonies showed a higher frequency of cobblestone formation compared to clump colonies (Figure 4E). These results suggested that not only clump colonies, but also cobblestone colonies, have SSC activity.

To confirm whether the cultured cells were fertile, we killed one of the W mice that received a transplant of cells cultured for 3 months under the EF condition. After cryopreservation of the testes for 7 days, germ cells from EGFP-expressing seminiferous tubules were used for microinsemination (Figure 4F). In total, 95 embryos were created, and 70 two-cell embryos were transferred into uteri of pseudopregnant mothers. Fifteen offspring were produced, seven males and eight females (Figures 4G and 4H). A combined bisulfite restriction analysis (COBRA) of their tail DNA showed that the offspring had normal DNA methylation patterns in imprinted genes (Figure S2B). These results showed that SSCs in cobblestone cultures are functionally normal.

Decreased Homing of *Cxcr4*-KO SSCs

On the basis of our in vitro observations, we hypothesized that CXCL12 and GDNF mediate SSC homing in vivo. In the first set of experiments, we examined the involvement of CXCR4 in SSC homing. While CXCL12 was expressed in Sertoli cells (Figure S3A), A_{single} or A_{paired} ZBTB16^{+} spermatogonia showed CXCR4 expression (Figures S3B and S3C). CXCR4 was found more frequently in A_{single} or A_{paired} than in $A_{aligned}$ cells (Table S5). Notably, the expression of CXCR4 in GS cells increased to a greater extent under the G condition than under the EF condition (Figure 5A) (Castellone et al., 2004; Lu et al., 2009). However, adding CXCL12 or AMD3100 did not have a significant effect on GS cell proliferation (Figures 5B and 5C), which suggests that CXCL12 does not influence SSC proliferation. To examine the involvement of CXCR4 in SSC homing, we used *Cxcr4* conditional KO mice, in which the *Cxcr4* gene is flanked by loxP sites (*Cxcr4* floxed mice) (Figures 5D and 5E). This mouse was crossed with the ROSA26 reporter mouse strain (R26R) to visualize the pattern of colonization after transplantation (Soriano, 1999; Tokoyoda et al., 2004). Testis cells were collected from 10- to 19-day-old homozygous *Cxcr4* floxed mice heterozygous for the R26R allele, and then single-cell suspensions were exposed to adenovirus expressing Cre (AxCANCre)

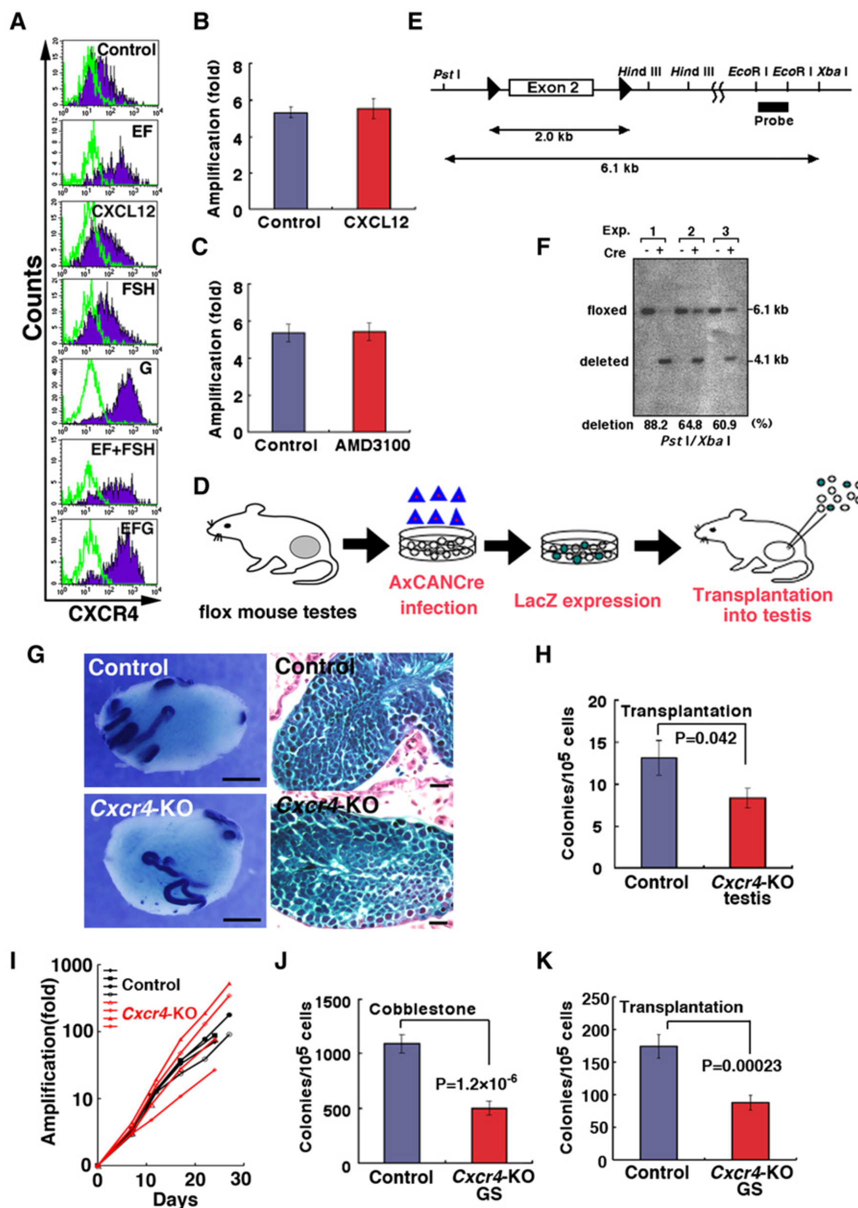

Figure 5. Reduced SSC Homing by *Cxcr4* Deficiency

(A) Flow cytometric analysis of CXCR4 expression in GS cells. The cells were cultured on testis feeder cells for 6 days with the indicated cytokines. Green lines indicate controls.

(B and C) Effect of CXCL12 (B) or AMD3100 (C) on GS cell proliferation on testis feeder cells under the EFG condition. The numbers of cells were counted after 6 days of culture (n = 6). Results of two experiments are shown.

(D) Experimental procedure. Testicular cells from *Cxcr4* floxed mice were dissociated and incubated with AxCANCre overnight and then injected into the seminiferous tubules of W mice.

(E) Conditional mutant mice used in the experiment. Exon 2 of the *Cxcr4* gene was deleted by Cre-mediated recombination. The indicated probe was used for Southern blot analysis.

(F) Southern blot analysis of deletion efficiency. Genomic DNA was digested with PstI/XbaI and hybridized with the indicated probe.

(G) Macroscopic (left) and histological (right) appearances of the recipient testes.

(H) Colony counts in the recipient testes, showing reduced colonization of *Cxcr4*-KO testis cells (n = 12 for controls; n = 15 for mutants). Results of three experiments are shown.

(I) Proliferation of *Cxcr4*-KO GS cells on testis feeder cells cultured under the EFG condition.

(J) Reduced cobblestone colonies by *Cxcr4*-KO GS cells under the EFG condition (n = 24). The cells were transplanted on the next day after Cre treatment.

(K) Colony counts in the recipient testes, showing reduced colonization of *Cxcr4*-KO GS cells (n = 23–24). Results of three experiments are shown. GS cells were labeled with Venus-expressing lentivirus and Venus-expressing cells were purified by sorting and expanded in vitro before adenovirus infection and transplantation.

The results are presented as means ± SEMs. Bar = 1 mm (G, left) and 50 μm (G, right). Stain: hematoxylin and eosin (G). See also Tables S1 and S5 and Figure S3.

overnight in vitro. Cre-treated heterozygous R26R mice were used as controls. After incubation, 63.8% ± 3.3% and 77.4% ± 11.4% (n = 6) of the infected cells were recovered from control and mutant testis cells, respectively. Southern blot analyses showed that 69.6% ± 5.4% (n = 6) of the floxed allele was deleted from the *Cxcr4* gene locus at the time of transplantation (Figure 5F).

Analyses of the recipient testes showed a significant reduction in germ cell colonies by *Cxcr4*-KO testis cells (Figures 5G and 5H). We also examined the effect of *Cxcr4* deficiency on GS cell proliferation and homing by establishing GS cells from *Cxcr4* floxed mice (*Cxcr4*-KO GS cells) (Figure S3D). Consistent with the results using AMD3100, *Cxcr4* deficiency did not significantly influence proliferation, as demonstrated by normal proliferation of *Cxcr4*-KO GS cells in vitro after the Cre treatment (Figure 5I). In addition, no increase occurred in the frequency of Annexin V^+ cells in *Cxcr4*-KO GS cells in vitro after cytokine removal or in vivo after transplantation (Figures S3E and S3F). Although the frequency of clump colonies did not change by *Cxcr4* deficiency (Figure S3G), these *Cxcr4*-KO GS cells showed impaired in vitro cobblestone formation and in vivo colony formation (Figures 5J and 5K). Taken together, these results indicate that *Cxcr4* deficiency inhibits SSC homing, but not their proliferation or survival.

Impaired Cobblestone Formation and SSC Homing by a *Ret* Mutation

We next examined the involvement of GDNF in SSC homing. However, because GDNF is involved in SSC self-renewal (Meng et al., 2000), confirming whether reduced homing is due to reduced cell migration or proliferation is difficult. To overcome this problem, we took advantage of a dominant-negative form

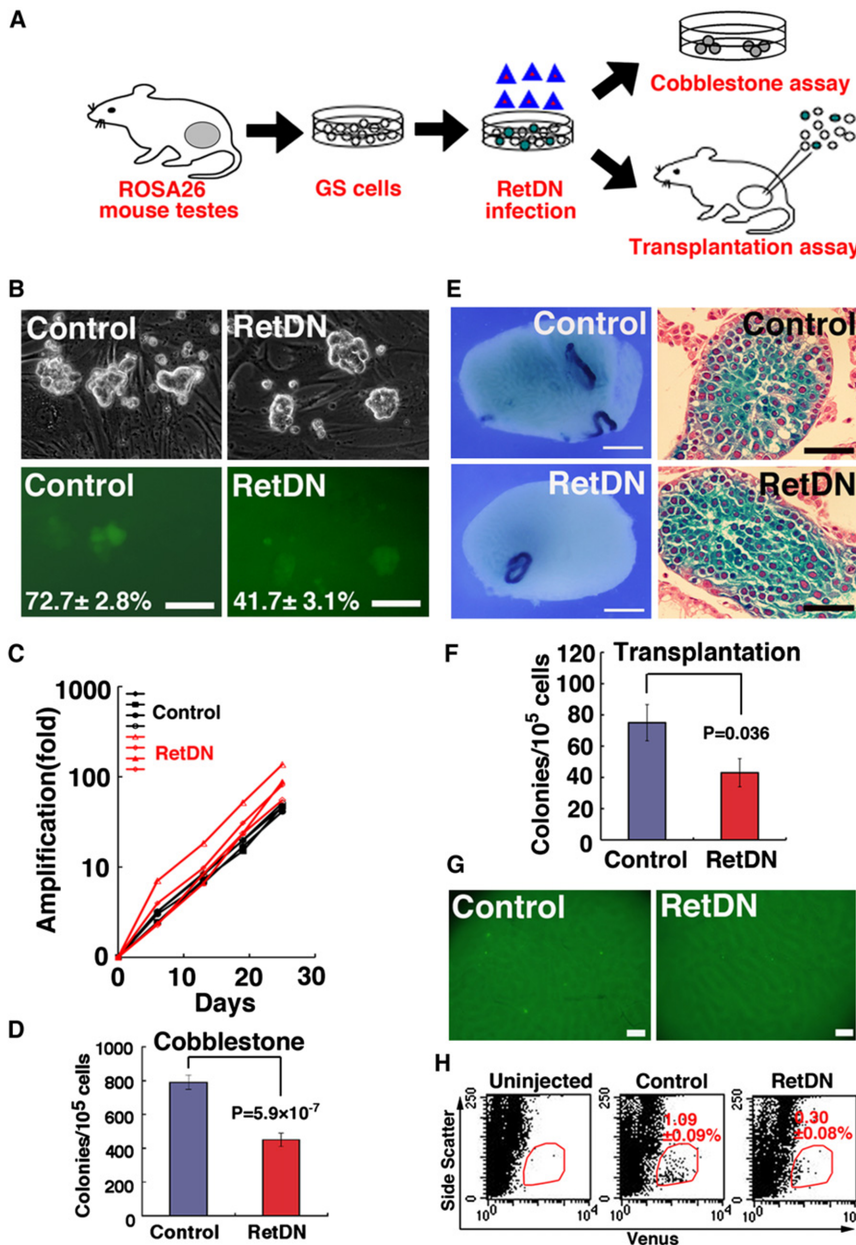

Figure 6. Reduced SSC Homing by RetDN Transfection

(A) Experimental procedure. ROSA GS cells were transduced with RetDN-expressing virus and then injected into the seminiferous tubules of W mice or used for cobblestone cultures.

(B) Appearance of GS cells transfected with RetDN 6 days after infection. The values indicate the percentages of Venus$^+$ cells (n = 8).

(C) Proliferation of Venus$^+$ RetDN-GS cells on testis feeder cells under the EFG condition.

(D) Reduced cobblestone colonies by RetDN. Green GS cells were transduced and plated onto testis feeder cells under the EF + FSH condition (n = 24). EGFP$^+$ cobblestone colonies were counted.

(E) Macroscopic (left) and histological (right) appearances of recipient testes.

(F) Colony counts in the recipient testes (n = 18), showing reduced colonization of RetDN-GS cells. Results of three experiments are shown.

(G and H) Macroscopic appearance (G) and flow cytometric analysis (H) of recipient testes at 6 days after the transplantation of control or RetDN-GS cells. Venus expression was quantified by gating the spermatogonia population (n = 13).

The results are presented as means ± SEMs. Bar = 50 μm (B and E, right), 100 μm (G), and 1 mm (E, left). Stain: hematoxylin and eosin (E). See also Figure S4.

of *Ret* (RetDN). GDNF binds to GFRA1, which brings two molecules of RET together and triggers phosphorylation of specific tyrosine residues (Arighi et al., 2005). Although complete absence of GDNF-RET signaling kills spermatogonia soon after birth (Naughton et al., 2006), spermatogonia in the testes of RetDN transgenic mice survive and proliferate due to residual AKT phosphorylation despite defective spermatogenesis (Jain et al., 2004). A lentivirus expressing RetDN and Venus protein was used to transduce GS cells established from B6-TgR(ROSA26)26Sor (ROSA26) mice (ROSA GS cells), which ubiquitously express *β-galactosidase*. An empty lentivirus vector was used as a control. After overnight infection, the virus supernatant was removed, and GS cell culture medium containing EGF, FGF2, and GDNF was added to the transfected cells (Figure 6A). RetDN expression did not confer a selective advantage or disadvantage to GS cell proliferation, because Venus$^+$ cells in each culture proliferated at comparable speed for at least 3 weeks (Figures 6B and 6C). We also did not observe significant changes in the number of clump colonies (Figure S4A). However, RetDN-infected GS cells (RetDN-GS cells) showed significantly reduced cobblestone formation (Figure 6D).

Because the negative effect of RetDN on cobblestone formation suggested that GDNF is involved in SSC homing in the seminiferous tubules, we tested this hypothesis by spermatogonial transplantation. Flow cytometry showed that 43.8% ± 2.6% and 76.4% ± 2.8% (n = 8) of the RetDN-GS and control ROSA GS cells, respectively, expressed Venus at the time of transplantation. LacZ staining of the recipient testes showed that the RetDN-GS cells produced fewer colonies in vivo (Figures 6E and 6F). The reduced colonization was possibly due to decreased migration to the niche, because the number of RetDN-GS cells in recipient testes decreased significantly at 6 days posttransplantation and few Venus$^+$ cells were found in the recipient testes (Figures 6G and 6H). In contrast, significantly more control Venus-expressing cells were found on the basal membrane at the same time point. Considering the relatively slow doubling time of the SSCs after transplantation (~7.9 days) (Nagano, 2003), and because no

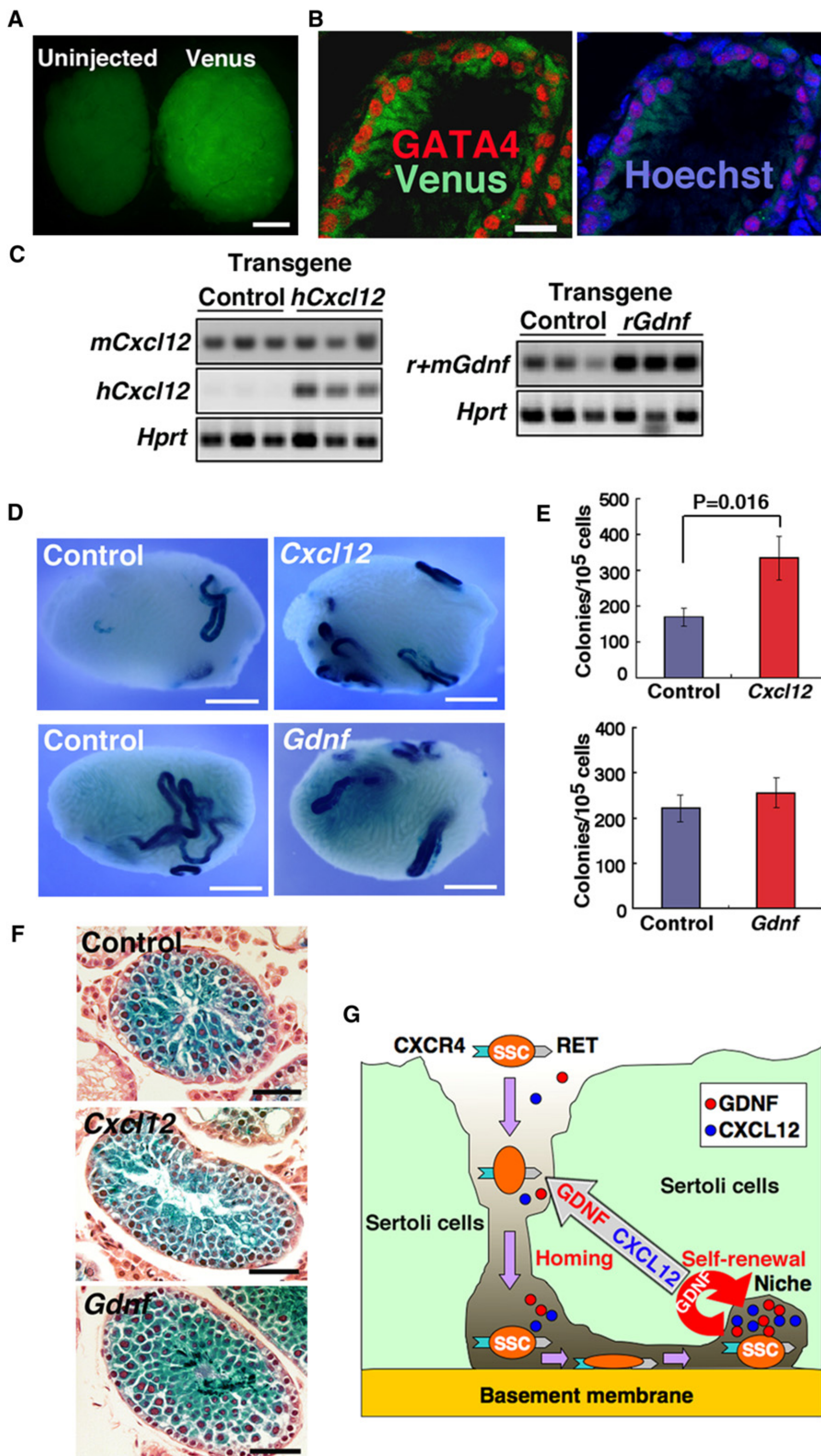

Figure 7. Lentivirus-Mediated Transduction of *Cxcl12* and *Gdnf* in Sertoli Cells

(A) Macroscopic appearance of uninjected testis (left) or testis injected with a Venus-expressing virus without cDNA insert (right).

(B) Histological appearance of the Venus-injected testis. Green fluorescence in GATA4 (red)-expressing Sertoli cells indicates successful transduction of Sertoli cells.

(C) RT-PCR analysis of recipient testes transduced with human *Cxcl12* (left) or rat *Gdnf* (right) expression vectors.

(D) Macroscopic appearance of *Cxcl12* (top)- or *Gdnf* (bottom)-transduced recipient testes transplanted with ROSA GS cells.

(E) Colony counts in the *Cxcl12*-transduced recipient testes (top; n = 23 for control; n = 22 for CXCL12; results of two experiments), showing increased colonization after CXCL12 treatment. No significant difference was noted for *Gdnf*-transduced testes (bottom; n = 33 for control; n = 27 for GDNF; results of four experiments).

(F) Histological appearance of recipient testes.

(G) A diagram showing the roles of GDNF and CXCL12 in SSC homing.

The results are presented as means ± SEMs. Bar = 1 mm (A and D), 20 μm (B), and 50 μm (F). m, mouse; r, rat; h, human. See also Table S1.

increase occurred in the frequency of Annexin V$^+$ cells in RetDN-GS cells in vitro after cytokine removal or in vivo after transplantation (Figures S4B and S4C), these observations suggested that RetDN expression significantly delays or compromises SSC migration to the niche.

Enhanced SSC Colonization by *Cxcl12* Transduction of Recipient Testes

In the final set of experiments, we examined whether GDNF or CXCL12 expression enhances SSC homing in vivo. Sertoli cells can be transduced efficiently by microinjection of lentivirus into the seminiferous tubules (Figure 7A) (Ikawa et al., 2002). Histological sectioning confirmed transgene expression in Sertoli cells (Figure 7B). Lentivirus vectors that express either rat GDNF or human CXCL12 were introduced into the seminiferous tubules of W mice. Direct injection of lentivirus resulted in increased mouse/rat *Gdnf* and human *Cxcl12* mRNA expression at 7 days postinjection (Figure 7C).

To test whether the genes could increase homing efficiency, we transplanted 2 × 10^3 ROSA GS cells into the lentivirus-transduced recipient seminiferous tubules 7 days after lentivirus transduction. Analysis of the recipient testes showed significantly more colonies after *Cxcl12* transduction (Figures 7D and 7E). The number of colonies increased by 2-fold in *Cxcl12*-transduced recipient testes. However, transduction of *Gdnf* failed to increase SSC colonization (Figure 7E). Histological analyses showed normal appearing spermatogenesis in both types of recipients (Figure 7F).

DISCUSSION

In this study, we established a testis cell culture method that can sustain SSCs with the cobblestone formation for the long term. Although GDNF plays a pivotal role in SSC maintenance in vivo (Meng et al., 2000), our culture method allowed the long-term maintenance of SSCs without GDNF, suggesting that GDNF is secreted from testis feeder cells. Our results showed that cobblestone formation was enhanced not only by GDNF, but also by EGF + FGF2, and that FSH further enhanced cobblestone formation when added to the EF condition. While we were able to confirm enhancement of GDNF secretion under the EF condition, its level did not increase with the addition of FSH. Hence, the beneficial effect of FSH on cobblestone formation is likely mediated by additional self-renewal factors. This notion is also supported by the observation that SSCs from C57BL/6 (B6) testes, which do not proliferate on MEFs (Kanatsu-Shinohara et al., 2003), formed cobblestones when they were cultured with testis feeder cells. One candidate molecule was CXCL12, because adding AMD3100 inhibited cobblestones. However, adding CXCL12 or AMD3100 did not influence GS cell proliferation, and neither GDNF nor CXCL12 levels increased after FSH treatment. We also failed to observe B6 germ cell proliferation by CXCL12 on MEFs (data not shown), suggesting the involvement of other factors. It also should be noted that GDNF dramatically increased the SSC population without exogenous FGF2 in the current culture system. Although the combination of GDNF and FGF2 is a prerequisite for in vitro SSC expansion on laminin (Lee et al., 2007), Sertoli cells secrete FGF2 and additional FGF family molecules, which likely preclude exogenous FGF2 supplementation. These results strongly suggest that unknown cytokines maintain SSCs in this culture system. Therefore, the current culture method will be useful for studying SSC regulation according to environmental cues.

The cobblestone culture proved useful for identifying cytokines involved in SSC homing (Figure 7G). Studies on SSC homing have been hindered, because SSCs are defined by the generation of colonies; thus, determining whether reduced cobblestone formation is due to impaired proliferation or homing is difficult. This was particularly true for GDNF, which has diverse functions in other cell types, including a role in cell migration and proliferation (Arighi et al., 2005; Tang et al., 1998). In this study, we overcame this problem by taking advantage of a RET mutant with attenuated AKT activity. Although complete loss of GDNF signaling is detrimental to SSC survival (Naughton et al., 2006), transgenic mice expressing RetDN had proliferating spermatogonia, whereas those in *Ret* KO mice were quickly lost soon after birth (Jain et al., 2004). Consistent with this result, GS cells transfected with RetDN survived and proliferated in vitro, although they had impaired cobblestone-forming ability. Analyses of the recipient testes showed that RetDN transduction reduced the number of cells that persisted in the seminiferous tubules 6 days after transplantation. Because the doubling time of SSCs in vivo is ~7.9 days (Nagano, 2003), the difference between the control and RetDN-GS cells was not likely due to GS cell proliferation. Although *Gdnf* transduction of Sertoli cells was not effective for improving colonization, it is likely that GDNF may not have been sufficiently expressed in the seminiferous tubules, because overexpression of GDNF in Sertoli cells by electroporation not only induced abnormal accumulation of undifferentiated spermatogonia, but also impaired their differentiation (Yomogida et al., 2003). Because such colonies were not observed in our study, experiments using a stronger promoter may resolve this problem.

We also showed the involvement of CXCL12/CXCR4 in SSC homing. CXCL12 was first identified as a chemokine for HSCs and was later shown to be involved in primordial germ cell (PGC) migration into the embryonic gonad (Aiuti et al., 1997; Ara et al., 2003; Doitsidou et al., 2002). Involvement of this factor in spermatogonia chemotaxis or maintenance has been suggested (Chen et al., 2005; Payne et al., 2010), but no direct evidence has been obtained. In this study, the cobblestone assay showed the enhanced cobblestone formation by CXCL12. Although the CXCR4 antagonist AMD3100 was unable to suppress the cobblestones completely under the EF + FSH condition, those produced under the EFG condition were inhibited significantly. This was likely due to upregulation of CXCR4 by GDNF (Castellone et al., 2004: Lu et al., 2009). Although CXCL12 has been implicated in HSC proliferation (Tzeng et al., 2011), our analyses did not show any effect of CXCL12 on cell proliferation. The results of in vitro experiments were confirmed by an in vivo transplantation assay using *Cxcr4*-KO mice, which showed reduced colonization of *Cxcr4*-KO cells. Moreover, CXCL12 overexpression in Sertoli cells increased SSC colonization in vivo. Although these results implicate the CXCL12-CXCR4 pathway in SSC homing, CXCL12 also binds to CXCR7 and contributes to PGC migration in zebrafish (Boldajipour et al., 2008). Determining whether a similar pathway also contributes to SSC homing will be important.

Many regulatory molecules are involved in the SSC-niche interaction, but a lack of methods to prospectively identify SSCs or the niche has prevented the understanding of molecular action. We developed a testis cell culture system that mimics the in vivo SSC microenvironment and provide evidence that GDNF and CXCL12 are involved in SSC chemotaxis into the germline niche. Although the identification of SSCs must be based on a functional transplantation assay, the cobblestone assay provides a fast quantitative method for SSCs or for discovering molecules involved in the stem cell-niche interaction. This culture system may also be useful for culturing SSCs from other animal species, for which appropriate culture methods are not yet available. Thus our culture system not only provides a strategy for understanding niche function, but also adds a new dimension to the study and application of SSCs.

EXPERIMENTAL PROCEDURES

Preparation of Feeder Cells

Testes of 5- to 7-week-old W mice (Japan SLC, Shizuoka, Japan) were dissociated using a two-step enzymatic procedure with collagenase and trypsin to prepare the testis feeder cells, as described previously (Ogawa et al., 1997). The basal medium consisted of StemPro-34 SFM (Invitrogen, Carlsbad, CA), 25 μg/ml insulin (Nacalai Tesque Inc., Kyoto, Japan), 100 μg/ml transferrin, 60 μM putrescine, 30 nM sodium selenite, 30 μg/ml pyruvic acid, 1 μl/ml DL-lactic acid, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10^{-4} M ascorbic acid, 10 μg/ml d-biotin, 3 μg/ml heparin (all from Sigma, St. Louis, MO), MEM vitamin solution, and MEM nonessential amino acid solution (both from Invitrogen). The cells were plated on laminin-coated (20 μg/ml; BD Biosciences, Franklin Lakes, NJ) dishes at 2.0×10^5 cells/9.4 cm^2 in basal medium supplemented with 20 ng/ml EGF, 10 ng/ml FGF2,

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10 ng/ml FGF9 (all from Peprotech, Rocky Hill, NJ), 2 μg/ml heparin, 3 mg/ml Albumax II (Invitrogen), 1 mg/ml fetuin, 1:100 lipid mixture 1 (both from Sigma), and 1:1,000 lipoprotein-cholesterol concentrate (MP Biomedicals, Solon, OH). PA6 and 15P-1 cell lines were maintained with αMEM/10% FBS and Dulbecco's modified Eagle medium/10% FBS, respectively.

Statistical Analysis

Data were analyzed using the Student's t test. For analysis of double immunostaining using CXCR4 and ZBTB16 antibodies, the Chi-square test was used.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes four figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2012.06.011.

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