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Cell Stem Cell

Rac Mediates Mouse Spermatogonial Stem Cell Homing to Germline Niches by Regulating Transmigration through the Blood-Testis Barrier

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SUMMARY

The homing ability of spermatogonial stem cells (SSCs) allows them to migrate into niches after being transplantated into infertile testes. Transplanted SSCs attach to Sertoli cells and transmigrate through the blood-testis barrier (BTB), formed by inter-Sertoli tight junctions, toward niches on the basement membrane. The most critical step is the passage through the BTB, which limits the homing efficiency to <10%. Here we demonstrated the involvement of Rac1 in SSC transmigration. *Rac1*-deficient SSCs did not colonize the adult testes, but they reinitiated spermatogenesis when transplanted into pup testes without a BTB. Moreover, a dominant-negative Rac1 construct not only reduced the expression of several claudin proteins, which comprise the BTB, but also increased SSC proliferation both in vitro and in vivo. Short hairpin RNA (shRNA) -mediated suppression of claudin3, which was downregulated by Rac inhibition, reduced the SSC homing efficiency. Thus, Rac1 is a critical regulator of SSC homing and proliferation.

INTRODUCTION

Spermatogenesis is a complex process that originates from the continuous division of SSCs. The mouse testis contains only 2–3 × 10^4 SSCs per testis, accounting for 0.02%–0.03% of the total testis germ cell suspension (de Rooij and Russell, 2000; Meistrich and van Beek, 1993). Despite their small number, these cells have a unique ability to undergo self-renewal division to reproduce themselves as well as produce committed daughter cells. Accumulating evidence indicates that SSCs are not randomly distributed in the testis, but that they reside within special microenvironments called "niches" where they can remain undifferentiated (Chiarini-Garcia et al., 2001). Self-renewal factors secreted from the niche are considered to maintain SSCs in the undifferentiated state, and this unique microenvironment is a prerequisite for self-renewal division; failure to remain in the niche induces differentiation or apoptosis. Although the precise location and cellular composition of these niches have long remained unknown, morphological analyses suggested that niches are located along the region of the basal lamina that faces the interstitium (Chiarini-Garcia et al., 2001). This area of the tubules has a relatively rich blood supply, and a recent study suggested that the germline niche is established in accordance with vasculature pattern formation (Yoshida, 2010).

Despite their close relationship, the interaction between SSCs and niches is dynamic. This is best illustrated by germ cell transplantation experiments, in which SSCs from a donor animal recolonized the seminiferous tubules when microinjected into those of infertile animals (Brinster and Zimmermann, 1994). Following introduction into the adluminal compartment of the seminiferous tubules, SSCs attach to the Sertoli cells, and within a few days migrate to the basal compartment following passage through the blood-testis barrier (BTB) between Sertoli cells. SSCs then proliferate to form chains or networks of spermatogonia on the basement membrane at around 2–3 weeks later and begin to differentiate adluminally at around 1 month, eventually producing mature spermatozoa by 2–3 months after transplantation (Nagano et al., 1999). These experiments demonstrated that SSCs have the ability to migrate toward the niche in a manner similar to hematopoietic stem cells (HSCs).

Although the homing ability of SSCs was discovered in 1994, very little is known about the molecular mechanism. However, β1-integrin expression in SSCs was recently shown to play a critical role in their homing ability (Kanatsu-Shinohara et al., 2008). In these experiments, the function of adhesion molecules in SSC homing was examined in *Itgb1* and *Cdh1* conditional knockout (KO) mice. These genes are strongly expressed in spermatogonia and were used as SSC markers (Oatley and Brinster, 2008). By combining with α6-integrin, β1-integrin comprises a laminin receptor and mediates the binding of SSCs to laminin in vitro. SSCs from *Itgb1* KO mice cannot form germ cell colonies and disappear after transplantation. Loss of β1-integrin expression probably prevents attachment of SSCs to the basal membrane because SSCs without β1-integrin neither attached to laminin in vitro nor migrated into the niche, even when transplanted into pup testes without a BTB. In contrast, SSCs without E-cadherin could home into the niche and produce normal germ

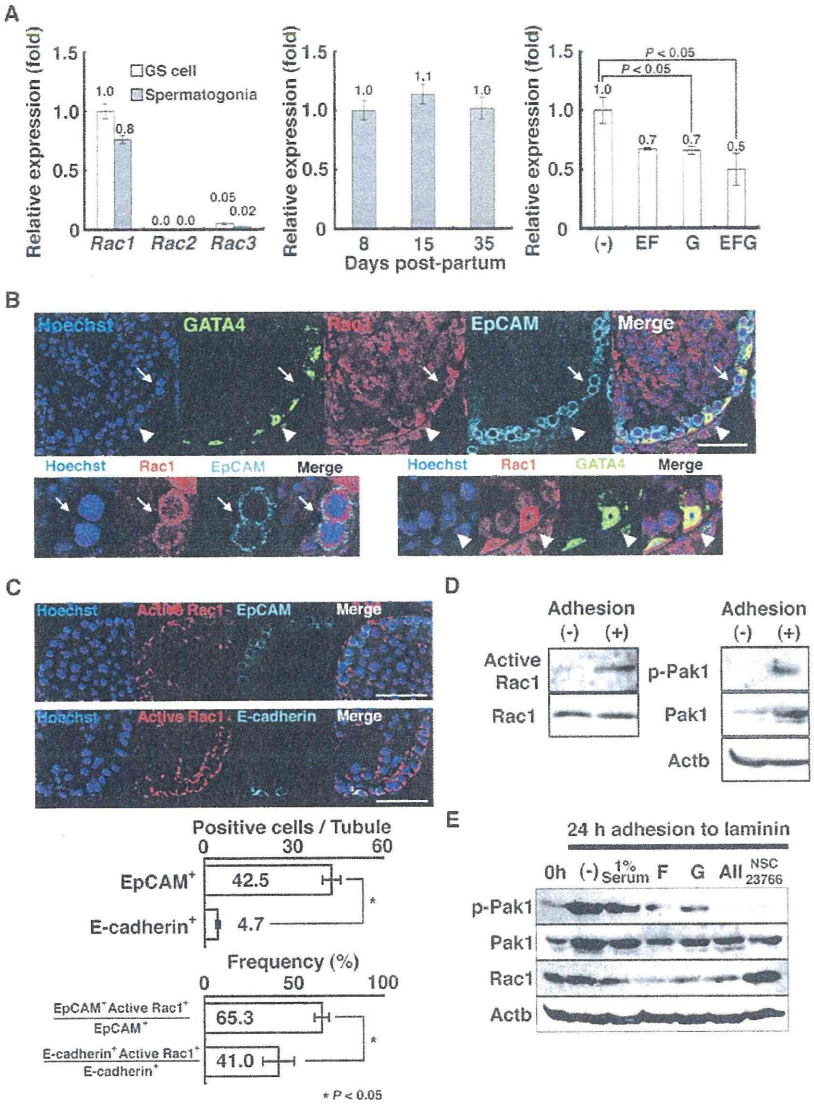

Figure 1. Expression and Activation of Rac

(A) (Left) Real-time PCR analyses of Rac expression. *Rac1* was predominantly expressed in both EpCAM$^-$ spermatogonia from 8-day-old pup testes and GSCs (n = 3). (Middle) Developmental changes in *Rac1* expression in EpCAM$^-$ spermatogonia. No significant changes were found (n = 3). (Right) Downregulation of *Rac1* expression in GSCs by cytokine treatment. GSCs were cultured on laminin for 3 days under the indicated culture conditions (n = 3).

(B) Rac1 expression in 8-week-old mouse testes. Rac1 is expressed not only in EpCAM$^-$ spermatogonia (arrow) but also in GATA4$^-$ Sertoli cells (arrowhead).

(C) Localization of activated Rac in spermatogonia. (Top) Immunostaining. (Middle) Calculation of EpCAM$^-$ or E-cadherin$^+$ cells per seminiferous tubule (n = 15). (Bottom) Quantification of cells with activated Rac1 expression (n = 637 for EpCAM; n = 70 for E-cadherin). Activated Rac1 was found more frequently in EpCAM$^-$ spermatogonia than in E-cadherin$^+$ spermatogonia. Double-positive cells on 15 tubules were counted for each cell type.

(D) Western blot analyses of Rac1 activation. GSCs were cultured on laminin without cytokines or serum, and the samples were recovered after 24 hr. (Left) Pull-down assay of activated Rac1. GST-fusion proteins containing Pak1 were used to affinity-precipitate active Rac1 from the cell lysate. (Right) The same cell lysate was used to detect phosphorylated Pak1.

(E) Effects of cytokines and Rac inhibitor on Pak1 phosphorylation. GSCs were cultured on laminin for 24 hr, and the indicated factors were added at the time of plating. E, EGF; F, bFGF; G, GDNF.

Scale bar = 50 μm (B and C). Error bars = SEM. See also Tables S1 and S2.

cell colonies and spermatozoa. These results were unexpected, because cadherin mediates the stem-cell-niche interaction in *Drosophila* gonads (Li and Xie, 2005). These experiments identified β1-integrin as a homing receptor for SSCs.

Here, we examined the roles of Rac1 small G protein in SSC homing. Because Rac is often activated downstream of the integrin receptor and mediates HSC homing (Cancelas et al., 2006), we hypothesized that Rac may also be involved in SSC adhesion to the basement membrane. However, our results showed that Rac was involved in a different step of homing, i.e., transmigration of SSCs through the BTB. We found that Rac regulates the expression of several claudins, critical components of the tight junction complex. Functional analyses by shRNAs showed that claudin3 downregulation is responsible for the loss of SSC activity.

RESULTS

Expression of Rac Genes in Primary Spermatogonia and Germline Stem Cells

Spermatogonia were collected from the testes of 8-day-old pups by magnetic-activated cell sorting (MACS) using the SSC marker EpCAM (Oatley and Brinster, 2008). We then examined the expression of Rac family molecules. Within the Rac subfamily, real-time polymerase chain reaction (PCR) analyses showed that spermatogonia predominantly express *Rac1* (Figure 1A, left and Table S1, available online). Although *Rac3* was expressed at significantly lower levels, *Rac2* expression was not detected. Despite the increased mitotic activity of pup spermatogonia (Nagano et al., 2001), *Rac1* expression was relatively constant at three different stages (8, 15, and 35 days old; Figure 1A, middle).

To understand the effect of the environment, we examined the expression of Rac genes in germline stem cells (GSCs), cultured spermatogonia with enriched SSC activity (Kanatsu-Shinohara et al., 2003). GSCs proliferate in the presence of self-renewal factors, including glial-cell-line-derived neurotrophic factor

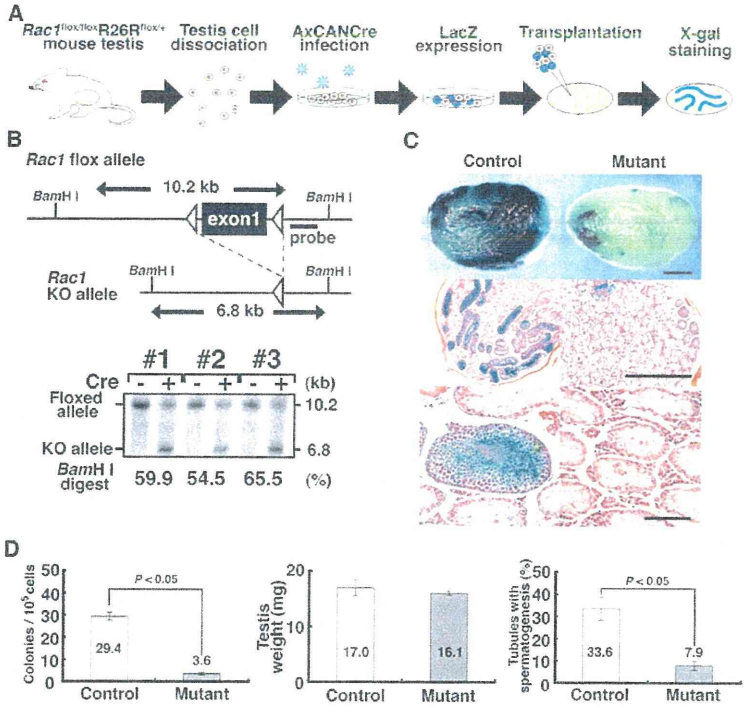

Figure 2. Reduced Colonizing Ability of Rac1 Mutant Cells

(A) Diagram of the experimental procedure. Testes from *Rac1* conditional mutant mice were dissociated and exposed to AxCANCre in vitro overnight. Cre-mediated deletion removed the target genes, and the cells were transplanted into recipient pup or adult testes to evaluate SSC activity. The colonization levels were determined by counting tubules with spermatogenesis by histology 3 months after transplantation. The colonization levels in adult recipients were also evaluated by counting the numbers of $LacZ^+$ colonies.

(B) (Top) Conditional mutant mice used in the experiment. The *Rac1* mutant strain was crossed with a ROSA26 reporter mouse strain (R26R) to visualize donor cell colonization. (Bottom) Southern blot analysis of deletion efficiency. Genomic DNA was hybridized with the indicated probe.

(C) Macroscopic (top) and histological (middle and bottom) appearances of the recipient testes. Blue tubules indicate donor cell colonization. Note the reduced colonization levels in recipients with *Rac1* mutant cells.

(D) Evaluation of donor-derived spermatogenesis. (Left) Colony count (n = 10 for control; n = 12 for mutant). (Middle) Testis weight (n = 12). (Right) Tubules with spermatogenesis (n = 15 for control; n = 14 for mutant). The numbers of tubules counted were 1,321 (control) and 1,256 (mutant).

Scale bar = 1 mm (C, top and middle); 100 μm (C, bottom). Stain, hematoxylin and eosin (C). Error bars = SEM. See also Figure S1.

(GDNF) and epidermal growth factor (EGF)/basic fibroblast growth factor (bFGF), and produce germ cell colonies following transplantation into seminiferous tubules. Real-time PCR analyses showed that *Rac1* expression also predominated in GSCs (Figure 1A, left). Moreover, the addition of self-renewal factors inhibited *Rac1* expression. While the combination of EGF and bFGF showed a comparable effect to GDNF, the addition of all cytokines reduced the Rac1 levels to 50% (Figure 1A, right). Because these results suggest that Rac1 is downregulated around the niche, we performed immunostaining for Rac1 to examine its expression in testis. Rac1 expression was found not only in spermatogonia, but also in Sertoli cells (Figure 1B and Table S2). Also, activated Rac1 staining was found more frequently in $EpCAM^+$ cells than in E-$cadherin^+$ cells (Figure 1C). Because E-cadherin is expressed in a more primitive subset of spermatogonia than those with EpCAM (Yoshida, 2010), these results suggest that Rac activation occurs more frequently in differentiating cells.

Using GSCs, we then performed pull-down assays to determine how Rac1 is activated. A GST-fusion protein containing the Rac-binding domain of Pak1 was used to precipitate active Rac1. We examined the effects of laminin, because SSCs preferentially attach to laminin via β1-integrin (Kanatsu-Shinohara et al., 2008). Consistent with previous studies, we found that Rac1 was activated by adhesion to laminin-coated plates within 1 day after plating (Figure 1D, left). Due to the relatively low sensitivity of the assay, we also evaluated the Rac activation levels by examining Pak1 phosphorylation. Binding of Rac to Pak1 causes autophosphorylation of Ser residues in the amino-terminal inhibitory domain, which induces conformational changes and subsequent Pak1 activation by PDK (Knaus and Bokoch, 1998). As expected from the results of the pull-down assay, laminin binding also induced Pak1 phosphorylation (Figure 1D, right). Pak1 phosphorylation was no longer observed once the cells were detached from the laminin-coated dishes by trypsin digestion. Increases in total and phosphorylated Pak1 levels were also observed in response to laminin binding, which was probably caused by Rac translocation to the membrane upon binding (del Pozo et al., 2000). In contrast, addition of individual self-renewal factors reduced Pak1 phosphorylation, while serum had no significant effect (Figure 1E). A combination of all cytokines not only strongly suppressed Pak1 phosphorylation but also reduced Rac1 protein expression, consistent with the results of real-time PCR analyses (Figure 1A, right). This Pak1 phosphorylation was inhibited by the Rac inhibitor NSC23766, confirming that Rac activation is necessary for Pak1 phosphorylation. Together, these results suggest that adhesion to laminin can activate Rac1, which is downregulated by self-renewal factors.

Rac1 Gene Deletion Inhibits SSC Homing after Germ Cell Transplantation

To understand the role of Rac1 in SSC homing, we deleted the *Rac1* gene in SSCs in mice carrying a *Rac1* gene flanked by loxP sites (*Rac1* floxed mice) generated by homologous recombination (Glogauer et al., 2003) (Figure 2A). The Rac1 mutant mouse strain was crossed with the ROSA26 reporter mouse strain (R26R) to visualize the pattern of proliferation and differentiation of mutant SSCs (Soriano, 1999). Heterozygous R26R mice were used as controls.

Testis cells were collected from 8- to 12-day-old pups by enzymatic digestion. Testes at this stage are enriched in SSCs

due to the lack of differentiated cells (Shinohara et al., 2001). Single-cell suspensions were then exposed to adenovirus expressing Cre (AxCANCre) overnight in vitro. After incubation, ~60% of the infected cells could be recovered by trypsin digestion. Cre infection did not significantly enhance apoptosis of total testis cells (Figure S1 available online). Southern blot analyses showed that 60.0% ± 3.2% (n = 3) of the floxed alleles were deleted from the *Rac1* gene locus at the time of transplantation (Figure 2B). To quantify the SSC number, ~1.2×10^5 testis cells were microinjected into the seminiferous tubules of WBB6F1-W/W^v (W) mice. W mice lack endogenous spermatogenesis and serve as recipients for donor SSCs (Brinster and Zimmermann, 1994). Three sets of experiments were carried out, and the recipient testes were recovered and stained for β-galactosidase activity with X-gal 3 months after transplantation. This period corresponds to approximately three cycles of mouse spermatogenesis, thereby providing a sufficient time window for spermatogenesis regeneration (de Rooij and Russell, 2000).

Macroscopic analyses of the recipient testes showed significant reduction of blue germ cell colonies, while transplantation of control cells resulted in extensive colonization (Figures 2C and 2D). Moreover, the mutant recipient testes showed weaker blue staining, suggesting poorer spermatogenesis recovery (Figure 2C). The average weights of the recipient testes were not significantly different (Figure 2D). We further evaluated the colonization levels of the donor cells by histological analyses. As germ cells in W mice cannot differentiate beyond the undifferentiated spermatogonia stage (Ohta et al., 2004), all spermatogenesis was derived from transplanted donor cells. We assessed the numbers of tubules with spermatogenesis by counting tubules with multiple layers of germ cells. In total, the mutant cells showed spermatogenesis in 7.9% ± 2.0% (n = 14) tubules, while wild-type (WT) control cells showed spermatogenesis in 33.6% ± 5.2% (n = 15) tubules (Figure 2D); this difference was statistically significant. These results indicated that deletion of the *Rac1* gene inhibited SSC colonization.

Analysis of Rac1 Function Using GSCs

To understand the effects of Rac1 in SSC homing, GSCs expressing a dominant-active (RacV12; DA-Rac) or a dominant-negative Rac1 (RacN17; DN-Rac) construct were produced by transfecting GSCs with enhanced green fluorescent protein (EGFP; Figure 3A). DA-Rac cells showed increased cellular protrusion, while such protrusions were rarely found in DN-Rac cells (Figure 3B). Flow cytometric analyses indicated that both DA- and DN-Rac cells had normal levels of α6- and β1-integrins, which are considered to be involved in SSC homing (Figure 3C). We observed no significant changes in other SSC markers, such as EpCAM and CD9. The mutant cells expressed c-kit, suggesting the presence of differentiating cells. However, they did not express SSEA-1, a marker of ESCs. Reverse transcription PCR (RT-PCR) analyses also confirmed the normal spermatogonia phenotype of both DA- and DN-Rac cells (Figure 3D and Table S3). Several spermatogonia markers, including *Pou5f1*, *Zbtb16*, *Neurog3*, and *Taf4b*, were expressed in both cell types, but they did not express *Nanog*, another ESC marker. These results indicated that the changes in Rac activity did not induce apparent abnormalities in the spermatogonia phenotype.

We then evaluated the laminin binding abilities of DA- and DN-Rac cells (Figure 3E). In these experiments, GSCs were plated on laminin-coated plates, which were washed several times to remove floating cells after incubation. The cells that adhered to the plates were recovered by trypsinization. Despite the normal expression levels of α6- and β1-integrins, DN-Rac cells showed increased adhesion to laminin. While 58.4% ± 4.9% of the DN-Rac cells could attach to laminin-coated plates, 43.6% ± 4.1% and 47.6% ± 3.8% of control and DA-Rac cells, respectively, adhered after 30 min incubation (n = 12). However, we found no significant differences in colony morphology of DA- and DN-Rac cells on either mouse embryonic fibroblasts (MEFs) or laminin-coated dishes. The cells did not attach to the fibronectin- or collagen-type-I-coated plates. These results indicated that changes in Rac1 activity modulate laminin-binding activity.

In addition to the increased adhesion, DN-Rac cells showed more active proliferation than control and DA-Rac cells (Figure 3F). While both control and DA-Rac GSCs expanded 6.4 ± 0.3 and 6.6 ± 0.9 -fold, respectively, during 6 days, DN-Rac cells expanded 11.9 ± 0.9 -fold during the same period (n = 6). The effect of DN-Rac was not mimicked by NSC23766 (Figure S2), suggesting that an interaction with Rac-specific guanine nucleotide exchange factor Trio or Tiam1 is not involved in the enhanced proliferation (Gao et al., 2004). As cyclin overexpression can enhance GSC proliferation and has an impact on SSC colonization, we also examined the pattern of cyclin D expression (Lee et al., 2009). Real-time PCR analyses showed increased expression of *Ccnd1* in DA-Rac cells (Figure 3G), consistent with a previous study reporting that Rac activation increases *Ccnd1* transcription (Joyce et al., 1999). *Ccnd3* also decreased in both types of cells. However, no significant difference was seen between DN-Rac and control cells in *Ccnd2* expression, which enhances in vitro proliferation of GSCs (Lee et al., 2009). These results suggest that enhanced proliferation of DN-Rac cells was mediated by a cyclin-D2-independent mechanism.

Because Rac1 was downregulated in vitro by cytokine treatment, a serial transplantation technique was next used to examine the effects of Rac activity on SSC self-renewal under physiological conditions (Figure 4A). In these experiments, approximately 4×10^3 cells expressing the EGFP gene were microinjected into the seminiferous tubules of W mice (primary recipients). Two months after transplantation, the primary recipients were killed and the number of colonies in the testis was determined under UV illumination. Consistent with the decreased homing of Rac1 KO SSCs, DN-Rac cells produced significantly fewer colonies, but we found no significant difference between DA-Rac and control cells (Figures 4B and 4C). The number of cells recovered from the three types of recipients ranged from 0.5 to 1.6 × 10^6 cells (average of 1.1 × 10^6 cells). Differences between donors were not significant.

When the secondary recipients were analyzed, DN-Rac cells produced significantly fewer secondary colonies than control and DA-Rac cells (Figure 4D and Table S4). Although both control and DA-Rac cells showed increased colony numbers (total regenerated colony number – primary colony number used for transplantation) in 9/9 (100%) and 11/12 (91.7%) transplantations, only 8/12 (66.7%) transplantations showed

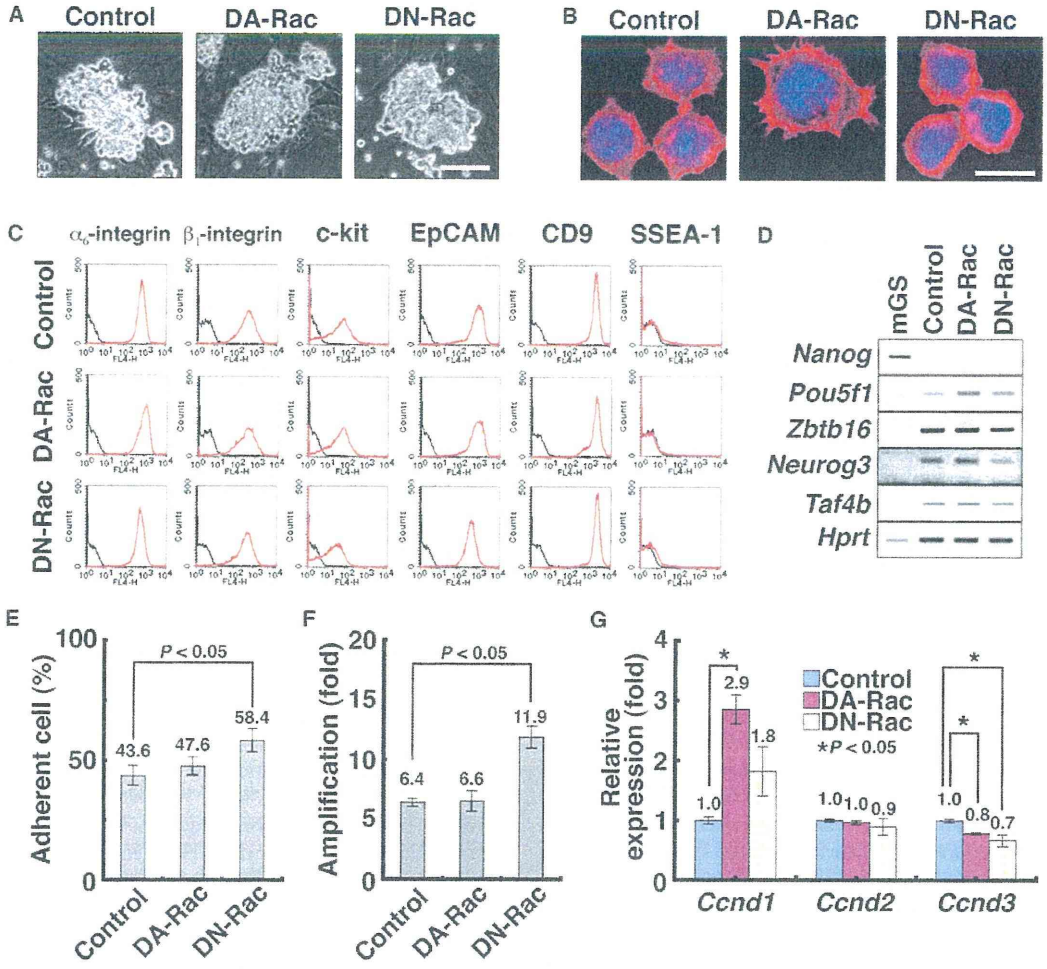

Figure 3. Characterization of DA-Rac and DN-Rac Cells

(A) Colony morphology on MEFs.

(B) Microscopic appearance of transfectants. Cells were plated on laminin for 6 hr and stained with rhodamine-labeled phalloidin. Counterstain is with Hoechst 33342.

(C) Flow cytometric analyses of surface molecules. Black lines indicate control staining.

(D) RT-PCR analyses showing normal spermatogonia marker expression. mGS, multipotent GSCs (Kanatsu-Shinohara et al., 2004). These cells are rare occurrences in GSC cultures and express *Nanog*, unlike GSCs that are unipotent.

(E) Enhanced laminin binding of DN-Rac cells. Cells were plated on laminin-coated plates for 30 min, and adherent cells were recovered by trypsinization.

(F) Enhanced proliferation of DN-Rac cells. Cells were cultured on MEFs for 6 days.

(G) Real-time PCR analyses of cyclin D expression levels (n = 6).

Scale bar = 100 μm (A); 10 μm (B). Error bars = SEM. See also Figure S2 and Tables S1, S2, and S3.

increases in DN-Rac cells. Assuming that each colony is produced by one SSC and that seeding efficiency is 10% (Nagano et al., 1999), the doubling times of the SSCs during the 2 month period were 12.7, 13.2, and 15.7 days for the control, DA-Rac, and DN-Rac SSCs, respectively. We examined the expression of p27 cyclin-dependent kinase inhibitor (CDKI), because *p27* KO SSCs also preferentially undergo differentiating divisions (Kanatsu-Shinohara et al., 2010). We found that *p27* is significantly suppressed in DN-Rac cells (Figure 4E). These results suggest that the inhibition of Rac induced more differentiating divisions of SSCs in vivo during regeneration.

Transplantation into Immature Testis Rescues Defective Colonization of Rac1 KO SSCs

To understand the mechanism of the homing defect, we followed the pattern of donor cell colonization at different time points after transplantation (Figure 5A). Although LacZ-expressing cells were similarly found until 10 days posttransplantation, we were unable to find patches or networks of spermatogonia on the basement membrane, which occurs 2–3 weeks after transplantation. Considering the relatively slow doubling time of the SSCs after transplantation (~7.9 days) (Nagano, 2003), this observation suggested that the loss of the typical colonization pattern was

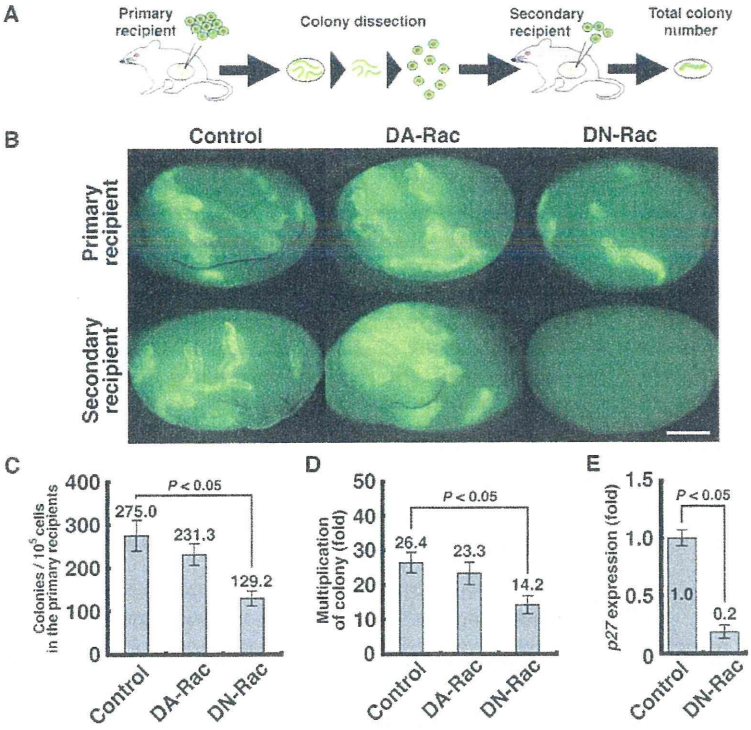

Figure 4. Serial Transplantation of GSCs

(A) Experimental procedure. GSCs were transplanted into W recipients (primary recipients). Two months after transplantation, EGFP-expressing germ cell colonies were dissected out using fine forceps, and enzymatically dissociated into single-cell suspensions. Portions of the cells were then transplanted into two testes of a W recipient (secondary recipients).

(B) Macroscopic appearance of recipient testes. Green fluorescence indicates donor cell colonization. Note the reduction of colonies in the recipients that received DN-Rac cells.

(C) Numbers of colonies in the primary recipients (n = 9 for the control; n = 12 for DA-Rac and DN-Rac cells).

(D) Multiplication of colony numbers (total regenerated colony number × 10 / primary colony number used for serial transplantation) (n = 9 for the control; n = 12 for DA-Rac and DN-Rac cells).

(E) Real-time PCR analyses showing *p27* downregulation in DN-Rac cells (n = 6).

Scale bar = 1 mm (B). Error bars = SEM. See also Tables S1 and S4.

not due to defects in proliferation and that the homing defect occurs at an early stage of colonization. SSC homing is thought to occur in several steps: attachment to Sertoli cells, passage through the tight junctions between Sertoli cells, and migration to the germline niche on the basement membrane (Nagano et al., 1999). To determine whether decreased SSC homing was caused by the defective migration of SSCs through the tight junctions between Sertoli cells, we next used immature 5- to 10-day-old recipient testes lacking tight junctions. The BTB develops around 12–14 days after birth, and immature pup testes before this period lack tight junctions between Sertoli cells and exhibit enhanced colonization of donor SSCs after transplantation (Shinohara et al., 2001). Although adult W mice were reported to have leaky BTBs in a previous study (Morrow et al., 2009), injection of dextran (~10 kD) or biotin (557 D) into the interstitium of pup, but not adult, W testes resulted in leakage into the adluminal compartment of the seminiferous tubules (Figure S3), showing the normal integrity of BTB in adult W mice.

Three experiments were performed, and approximately 1.2 × 10^5 *Rac1* KO or control cells were transplanted into pup testes after overnight incubation with AxCANCre in vitro. Cre treatment successfully deleted 68.6% ± 3.1% (n = 3) of the floxed alleles, and analyses at 3 months after transplantation revealed significant colonization by SSCs lacking the Rac1 gene (Figures 5B and 5C). Although testicular size was slightly smaller in recipients that received mutant cells, the difference was not significant (Figure 5D, top). Histological analyses showed comparable levels of colonization by mutant and control cells (Figure 5D, bottom). In total, 61.4% ± 7.9% and 47.1% ± 4.2% (n = 11) tubules showed spermatogenesis with control and mutant cells, respectively. Both histological analyses and RT-PCR analyses confirmed normal differentiation of *Rac1* mutant cells (Figures 5B and 5E).

Similar results were obtained using DN-Rac GSCs that showed reduced colonization (Figure 5F). We microinjected approximately 0.5 to 1.0 × 10^2 control and DN-Rac GSCs into pup seminiferous tubules, and the number of colonies was determined after 5 weeks. Compared with the adult recipients, the colonization efficiency of control and DN-Rac GSCs increased by 6.1- and 7.7-fold in the pup recipients, respectively. However, the difference between the two types of cells was not significant (Figure 5G). We then used the primary recipient testes for serial transplantation (Table S5). The number of cells recovered from the recipients ranged from 0.7 to 1.8 × 10^6 cells (average of 1.2 × 10^6 cells). In contrast to the adult recipient transplantation, the analyses of the secondary pup recipients revealed that DN-Rac GSCs produced significantly more colonies than did control cells (Figure 5H). Assuming that seeding efficiency is 100% in pup testes, the doubling times of the SSCs during the 5 week period were 7.8 and 6.1 days for the control and DN-Rac SSCs, respectively. Taken together, these results showed that loss of SSC activity in *Rac1* KO cells or DN-Rac cells was caused by the defective transmigration through the BTB.

Decreased Expression of Tight Junction-Associated Proteins in Rac Mutant Cells

The results described in the previous subsection suggested that *Rac1* KO SSCs have difficulty in migrating through the BTB. Because successful migration through the BTB would depend on the modulation of tight junction-associated protein expression on both SSCs and Sertoli cells, we hypothesized that homing defects were caused by abnormal expression of tight junction-associated adhesion molecules in SSCs. Therefore, we next examined the expression of occludin and claudins, components of tight junctions, in GSCs and W testis. RT-PCR

analyses showed that GSCs expressed all of these molecules except *Cldn5* (Figure 6A). In contrast, W testis lacked expression of *Cldn9*, *13*, *14*, *16*, *17*, *18*, *21*, and *24*. Using real-time PCR, we then quantitatively assessed the expression levels of these commonly expressed genes in control and DN-Rac cells (Figure 6B). Although we found no significant changes in *Ocln* or *Cldn1*, *2*, *10b*, *11*, *12*, *19*, *20*, or *22* expression, *Cldn3*, *4*, *6*, *7*, *8*, *10a*, *15*, and *23* were downregulated in DN-Rac cells. These cells also showed normal expression of *Etv5*, which regulates claudin5 expression and is involved in SSC self-renewal (Morrow et al., 2009). Of the differentially regulated claudins, western blotting showed reduced expression of Cldn3, 7, and 8 in DN-Rac cells. Ocln and Cldn10 expression, however, were not significantly altered (Figure 6C). While Cldn4, 6, and 15 were not expressed in either GSCs or W testis at the protein level, Cldn11 and 23 were found only in the W testis. Immunohistological staining confirmed the expression of Cldn3, 7, and 8 in spermatogonia in vivo (Figure 6D). While Cldn3 and 8 were expressed in cells on the basement membrane, Cldn7 was more widely expressed in the seminiferous tubules.

Functional Analyses of Tight Junction-Associated Proteins by Germ Cell Transplantation

In the final set of experiments, we investigated the impact of these candidate tight junction-associated molecules on SSC homing. We first evaluated the SSC activities of *Ocln* and *Cldn11* KO testis cells (Figure 6E). While *Ocln* KO mice gradually lose spermatogenesis and become infertile, *Cldn11* KO mice lack Sertoli cell tight junction strands and spermatozoa are never observed (Gow et al., 1999; Saitou et al., 2000). In two experiments, ~4×10^5 adult mutant testis cells were microinjected into the seminiferous tubules of adult W mice. Three months after transplantation, we found normal-appearing spermatogenesis from both mutant donors. In experiments with *Ocln* KO mice, 18.8% ± 1.7% tubules showed spermatogenesis with control cells, and 18.3% ± 2.7% (n = 6) tubules showed spermatogenesis with mutant cells. Likewise, in experiments with *Cldn11* KO mice, 4.7% ± 1.6% tubules showed spermatogenesis with control cells, and 6.2% ± 2.0% (n = 6) tubules showed spermatogenesis with mutant cells. Both *Ocln* and *Cldn11* KO SSCs reinitiated spermatogenesis with normal appearance (Figure S4A), and no significant differences were found in both experiments. These results indicate that these mutant SSCs can migrate through the BTB and reinitiate spermatogenesis as long as tight junction proteins are available in the host environment.

We further evaluated the functions of claudin3, 7, and 8 that were downregulated in DN-Rac cells. To examine the effects of claudin downregulation, we suppressed endogenous expression of claudins by delivering shRNAs via lentiviruses (Figure S4B and Table S6). ROSA GSCs were transduced with claudin knockdown (KD) vectors, and shRNA against EGFP was used as a control. Four days after infection, ~4×10^3 cells were microinjected into the seminiferous tubules of adult W mice. Claudin3 KD by shRNA significantly reduced ROSA26 GSC colonization in three experiments (Figure 6F). Although claudin7 and 8 KD also decreased colonization, the differences were not significant. Claudin shRNA treatment did not enhance apoptosis of GSCs (Figure S4C). Taken together, these results indicate that claudin3 is involved in the SSC transmigration through the BTB after transplantation.

DISCUSSION

Rac is involved in many biological processes, including cell migration and regulation of cell shape, by eliciting actin polymerization at the plasma membrane to produce lamellipodia and membrane ruffles (Burridge and Wennerberg, 2004). The involvement of Rac in stem cell homing was previously demonstrated for HSCs (Cancelas et al., 2006). While *Rac2*-deficient mice showed defects in long-term engraftment, *Rac1*-deficient HSCs were incapable of short-term engraftment. Interestingly, loss of both *Rac1* and *Rac2* led to massive mobilization of HSCs from the bone marrow. Our study shows that spermatogonia express *Rac1* predominantly, and this is the second molecule demonstrated to be involved in SSC homing. Unlike β1-integrin, which mediates attachment of SSCs to the basement membrane, our results suggest that Rac1 operates in a different step of SSC homing by regulating the transmigration of SSCs through the BTB.

The migration of SSCs through tight junctions at the BTB is a striking feature of the homing process. While preleptotene spermatocytes migrate adluminally from the basal side of the seminiferous tubules during normal spermatogenesis, transplanted SSCs migrate in the opposite direction toward the germline niche on the basement membrane. The ability of transmigration is not limited to spermatogonia in the postnatal testis; prospermatogonia in the fetus as early as 13.5 days postcoitum, which lack expression of spermatogonia markers, can also colonize adult seminiferous tubules (Ohta et al., 2004), indicating that homing ability is an important characteristic acquired in the early stages of development. To successfully cross the BTB, SSCs must bypass or block the tight junctions between Sertoli cells, which consist of claudins or occludin. Not surprisingly, this process is inefficient because <10% of the transplanted SSCs can reinitiate spermatogenesis after transplantation into the adult testes (Oatley and Brinster, 2008). On the other hand, the transplantation efficiency increased by about 10-fold when SSCs were transplanted into pup testes without a BTB, indicating that this is the most important step of SSC homing (Shinohara et al., 2001). However, the early phases of SSC colonization are difficult to study because no SSC-specific markers exist, and by definition, SSCs are detected only after producing germ cell colonies. In this study, the involvement of Rac1 in transmigration was examined using pup and adult recipients for transplantation. While *Itgb1*-deficient SSCs disappeared within 3 weeks post-transplantation in either pup or adult testes (Kanatsu-Shinohara et al., 2008), *Rac1*-deficient SSCs could colonize pup, but not adult, seminiferous tubules, indicating that the condition of the host testes is important in Rac-mediated SSC homing.

Spermatogonia are known to express several tight junction proteins as well as adherens junction proteins, despite the apparent lack of either type of junction (Kanatsu-Shinohara et al., 2006; Morrow et al., 2009). They may nonetheless play important roles in spermatogenesis, because loss of occludin causes spermatogenic failure and male infertility (Saitou et al., 2000). Moreover, *Cldn11* KO mice lose tight junction intramembranous strands in Sertoli cells, and are sterile (Gow et al., 1999).

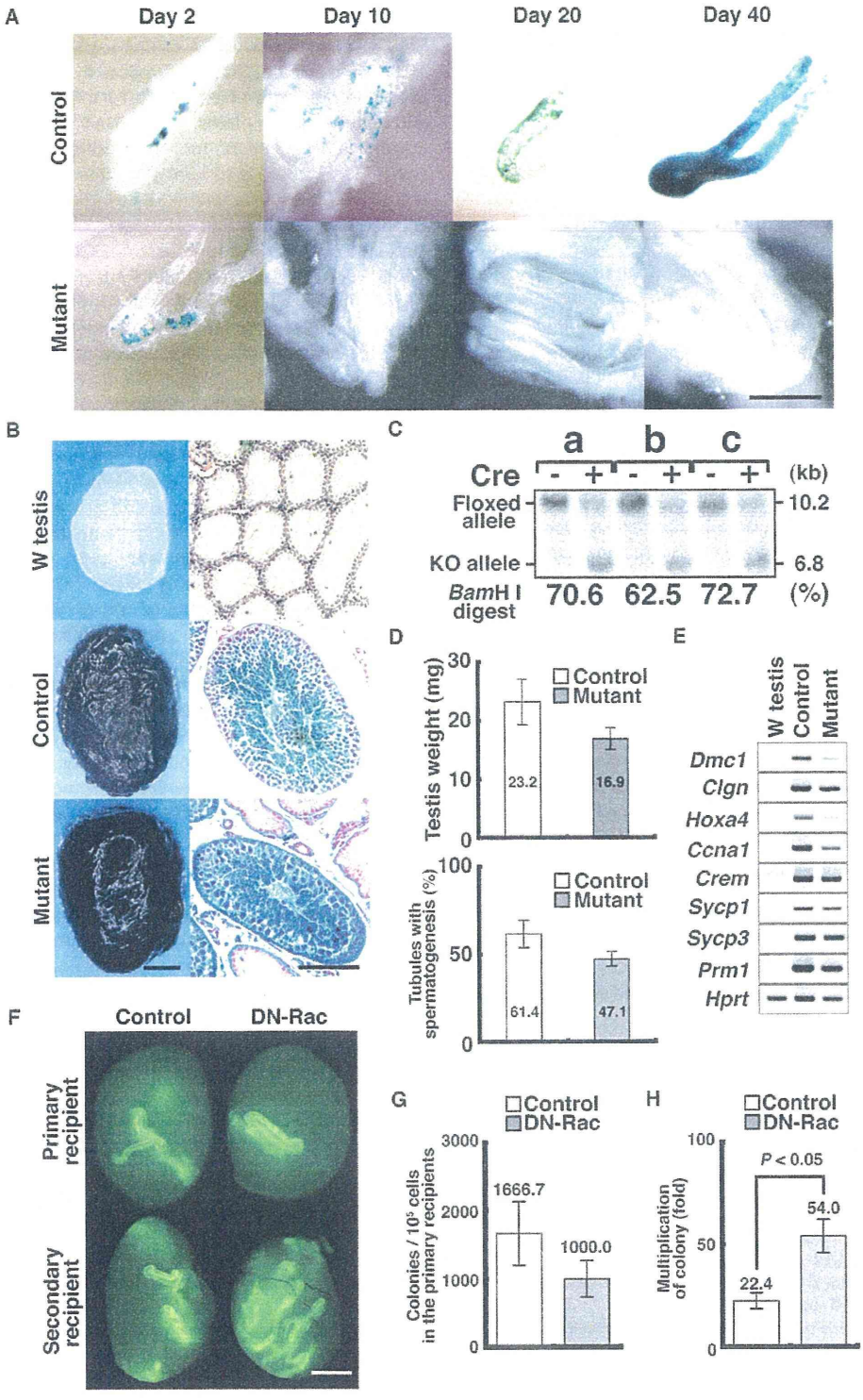

Figure 5. Colonization of Rac1 Mutant Cells in Immature Pup Testis

(A) Colonization of adult recipient mouse seminiferous tubules that received Cre-treated homozygous mutant and control testis cells. Although mutant cells were found at 2 days posttransplantation, they disappeared rapidly after 10 days. In contrast, control cells formed networks of spermatogonia at 20 days. Colonies with an intense blue color were found at 40 days, indicating multiple layers of germ cells.

Claudin expression in Sertoli cells is under complex regulation, because androgens change the permeability of the BTB by regulating claudin3 expression (Meng et al., 2005). Furthermore, claudin5 expression increases when spermatogenesis is established after transplantation in W mice (Morrow et al., 2009). Although whether infertility phenotypes in these mice are caused by abnormalities in germ cells or Sertoli cells is unclear, tight junction proteins on both germ cells and Sertoli cells are likely regulated dynamically and abnormalities in this interaction cause infertility or homing defects (Figure 7).

The results of the present study indicate that several claudins were downregulated in DN-Rac GSCs. To our knowledge, this is the first report showing the regulation of claudins by Rac. Although most of the claudins are expressed in GSCs, not all of them were translated into proteins, and functional analyses of these claudins revealed that claudin3 is involved in the transmigration of SSCs. Claudin3 is unique because it is transiently associated with newly formed tight junctions at the time when germ cells move from the basal to the adluminal compartment during stages VIII–IX of the seminiferous epithelial cycle (Meng et al., 2005). This is in contrast with claudin11 and occludin, which remain associated with the tight junction during the entire cycle of the seminiferous epithelium. In the transfer of spermatocytes to the adluminal compartment, Sertoli cells were previously postulated to play an active role and a transient intermediate compartment was thought to be created, thereby sealing these cells off from the rest of the seminiferous epithelium (Russell, 1977). This would accommodate the passage of germ cells without disrupting the integrity of the BTB. The validity of this model, however, has remained controversial (Mruk and Cheng, 2004). Whether SSCs create a similar compartment when they move to the opposite direction and how many different kinds of claudins on Sertoli cells comprise the BTB remain unknown. We speculate that SSCs and preleptotene spermatocytes may use different combinations of claudins for transmigration because *Rac1* KO SSCs underwent normal spermatogenesis in the pup recipients when the animals matured into adults with a BTB. Further analyses using the germ cell transplantation technique will provide insights into the mechanism of this unique biological process.

On the other hand, Rac1 is dispensable in tethering SSCs to the niche on the basement membrane. Because Rac is often activated downstream of integrin receptors and Rac deficiency causes mobilization of HSCs from bone marrow (Burridge and Wennerberg, 2004; Cancelas et al., 2006), we expected that Rac would reinforce integrin-mediated signaling and that *Rac1*-deficient cells would exhibit a phenotype similar to that of *Itgb1*-deficient SSCs. Unexpectedly, *Rac1* KO SSCs and DN-Rac cells were able to bind to the basement membrane and laminin-coated plates, respectively, indicating that Rac1 is dispensable for integrin-mediated adhesion. Enhanced adhesion of DN-Rac cells to laminin further suggests that Rac1 is one of the molecules that modulate integrin-mediated adhesion in a negative manner. Rac activation by integrin is thought to be mediated by the FAK-p130 Cas-Crk-DOCK180 pathway and the paxillin-GIT-PIX pathway (Legate et al., 2009). Cell adhesion influences the ability of Rac to activate Pak1 and induces phosphorylation. Also, Rac feeds back to the integrins by interacting with Rho (Parsons et al., 2010), and autophosphorylation of Pak1 triggers degradation of Pak1 (Weisz Hubsman et al., 2007), thereby creating another feedback loop to prevent further Pak1 activation. Interference with these feedback loops by a dominant-negative Rac construct may have enhanced cell adhesion. Because activated Rac was found less frequently in undifferentiated spermatogonia, SSCs in the niche may be less motile and adhere more strongly to the basement membrane (Figure 7).

The results of the present study also showed that Rac is involved in the proliferation of GSCs. Transfection of a dominant-negative Rac construct not only enhanced GSC proliferation, but also induced abnormal expression of cyclin D and p27 CDKI. In particular, decreased *p27* expression suggested that they undergo differentiating divisions, because *p27* KO SSCs preferentially undergo differentiating divisions (Kanatsu-Shinohara et al., 2010). Consistent with this, DN-Rac cells showed reduced colonization in the adult recipients. Although increased adhesion to the niche and promotion of differentiating divisions appear contradictory, adhesion to the niche is necessary but not sufficient for defining SSCs. SSCs must also be able to transmigrate through the BTB; those cells that cannot transmigrate are differentiating cells by definition even if they can attach strongly to the basement membrane.

Because a homing defect alone can impair SSC activity, it was difficult to determine whether reduced colonization of DN-Rac cells was due to impaired self-renewal activity or a migratory defect. This problem was resolved by serial transplantation using pup recipients, which showed that the concentration of SSCs in WT and DN-Rac GSCs in vitro is comparable if the transmigration process is omitted. Moreover, the serial transplantation also showed that the DN-Rac cells proliferate actively also in an in vivo environment and produce significantly more secondary colonies

(B) Macroscopic (left) and histological (right) appearances of the recipient testes. Both types of donor cell produced germ cell colonies. Note the increased size of the recipient testes after transplantation.
(C) Southern blot analysis of deletion efficiency.
(D) Evaluation of spermatogenesis. (Top) Testis weight (n = 9 for the control; n = 10 for mutant). (Bottom) Tubules with spermatogenesis (n = 11). The numbers of tubules counted were 783 (control) and 1,083 (mutant).
(E) RT-PCR analyses of the pup recipient testes, showing stage-specific spermatogenic gene expression. *Hprt* was used as an internal control. No apparent defects in differentiation were observed.
(F) Macroscopic appearance of recipient testes used for serial transplantation. GSCs were transplanted into W pup recipients (primary recipients). Five weeks after transplantation, EGFP-expressing germ cell colonies were dissected out using fine forceps and enzymatically dissociated into single-cell suspensions. Portions of the cells were then transplanted into two testes of a W pup recipient (secondary recipients). Green fluorescence indicates donor cell colonization. Note the increased secondary colonies in the recipients that received DN-Rac cells.
(G) Numbers of colonies in the primary recipients (n = 27 for the control; n = 21 for DN-Rac cells). (H) Multiplication of colony numbers (total regenerated colony number / primary colony number used for serial transplantation) (n = 11).
Scale bar = 500 μm (A); 1 mm (B, left and F); 100 μm (B, right). Stain, hematoxylin and eosin (B). Error bars = SEM. See also Figure S3 and Tables S3 and S5.

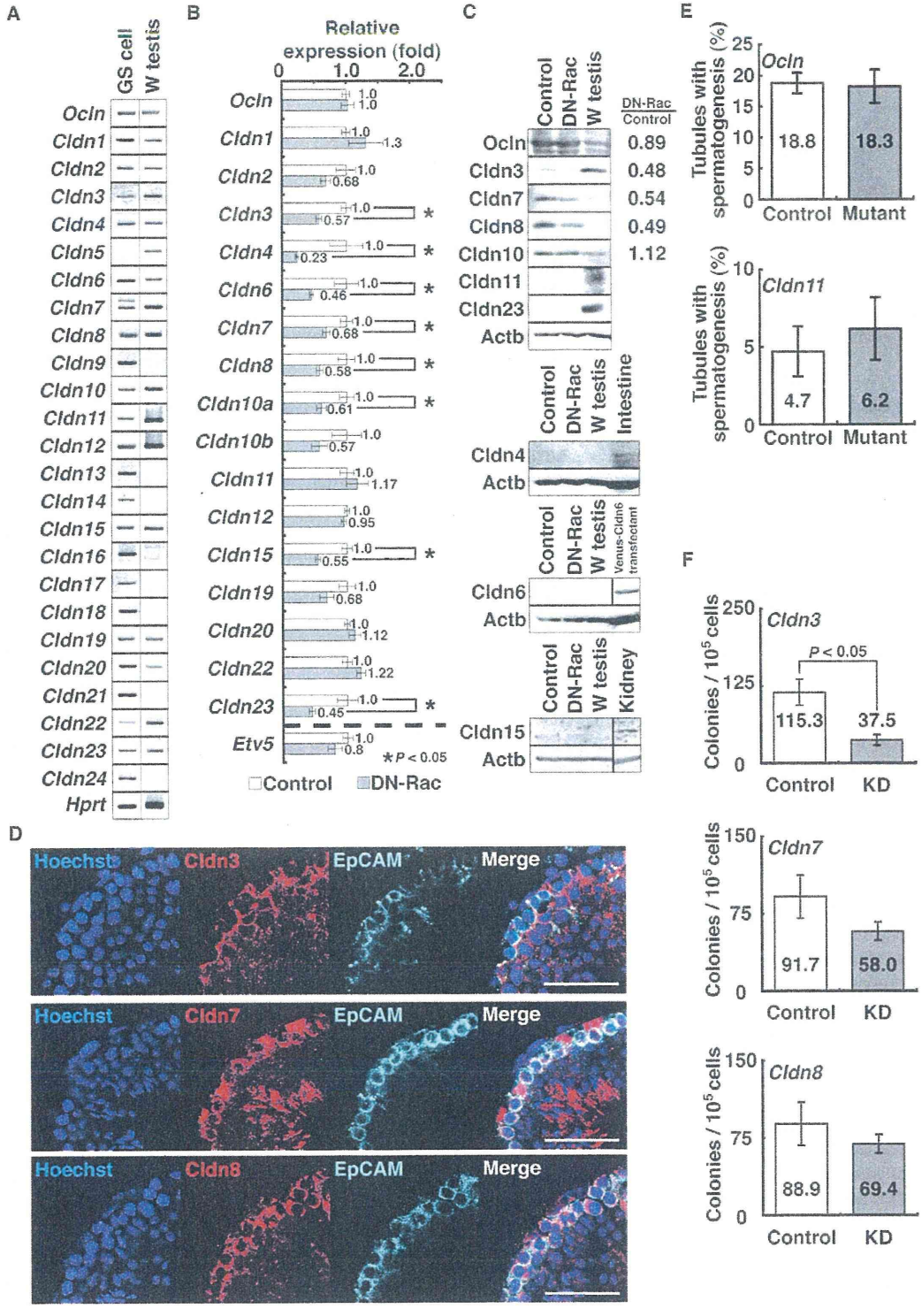

Figure 6. Functional Analyses of Tight Junction-Associated Proteins in SSC Homing

(A) RT-PCR analyses of *Ocln* and *Cldn* genes in GSCs and W testis.

(B) Real-time PCR analyses of commonly expressed genes and *Etv5* in control and Rac-DN cells (n = 6).

(C) Western blot analyses. The numbers along the gel represent the ratios of band intensities. The positive control was made by transfecting *Cldn6* cDNA into 293T cells.

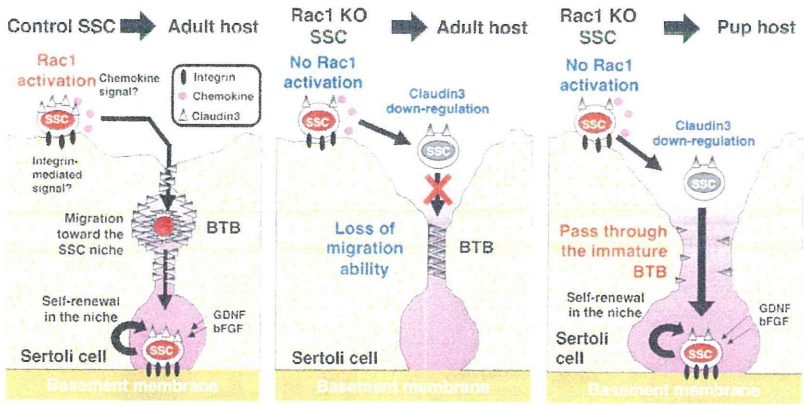

Figure 7. Model for SSC Homing

Rac in SSCs is activated either by chemokines or adhesion to Sertoli cells. SSCs then transmigrate through the BTB by modulating the expression of tight junction-associated proteins before they settle on the basement membrane via β1-integrin. The downregulation of tight junction-associated proteins, including claudin3, by Rac1 inhibition interfered with SSC transmigration. In contrast, SSCs can directly settle on the basement membrane of the seminiferous tubules in the pup testis without a BTB.

than WT cells do. Therefore, Rac inhibition enhances cell proliferation both in vivo and in vitro but does not necessarily compromise self-renewal activity. However, the potentially increased SSC activity, as suggested by pup transplantation, is offset by defective transmigration in the adult seminiferous tubules, which was interpreted as increased differentiating division using adult recipients. Although we currently do not know how Rac inhibition enhanced GSC proliferation, these results suggest that Rac is a critical molecule that controls both SSC transmigration and proliferation.

The next challenge is to identify upstream molecules of Rac signaling, which will be important for understanding the initial phases of SSC homing. At least two possibilities exist: chemokines and attachment to Sertoli cells. Although firm confirmation is lacking, SSCs are considered to be guided by chemokines to the appropriate niche to initiate migration (Nagano et al., 1999). Although GDNF is a chemoattractant factor for some cell types (Paratcha et al., 2006; Tang et al., 1998), no evidence yet exists to indicate that it also attracts SSCs toward the niche. Chemokine signals activate Rac in hematopoietic cells (Cancelas et al., 2006), but whether a similar scenario applies to SSCs is unknown. With regard to the second possibility (i. e., attachment to Sertoli cells), transplanted SSCs attach to the Sertoli cell surface before migrating toward the BTB (Russell et al., 1996). In leukocyte transmigration, integrins on leukocytes are activated by attachment to the endothelium and subsequently participate in firm arrest on the endothelium before transmigration (Worthylake and Burridge, 2001). Although we found that adhesion to laminin can activate Rac, different types of integrins on SSCs may be involved in Sertoli cell attachment and activate Rac, which then modulates the expression of tight junction proteins for transmigration. Gaining an understanding of the mechanism of Rac activation and its targets in endogenous spermatogonia is another important challenge. This may explain why pup spermatogonia proliferate more actively despite the same level of Rac expression. It also remains to be examined why SSCs with reduced Rac activity can undergo self-renewal division during normal spermatogenesis whereas inhibition of Rac activation promotes differentiation division by serial transplantation. Perhaps Rac expression as well as activation levels may change when SSCs are triggered to increase their number. Future studies are required to address these questions.

Since the development of the germ cell transplantation technique, the mechanism of SSC homing has remained unclear. In addition to the hematopoietic system, the spermatogenic system is the only other self-renewing tissue in which a functional transplantation assay exists for stem cell homing. In addition to β1-integrin, we have now determined that a role exists for Rac1 in regulating transmigration between Sertoli cells, which is the most critical aspect of SSC homing. These molecules operate in distinct steps of homing and also play important roles in HSC homing to the bone marrow niche. In contrast to the complex structure of the bone marrow, the relatively simple structure of the testis is advantageous for understanding the homing process, and Rac may regulate homing of other types of stem cells in a similar manner by regulating the expression of tight junction-associated molecules. Comparison between the two self-renewing systems will reveal the common molecular machinery of stem cell homing and will provide insight into the mechanism and regulation of stem-cell-niche interactions.

EXPERIMENTAL PROCEDURES

KO Animals and Transplantation

The generation of *Cldn11*, *Ocln* KO, and *Rac1* conditional mutant mice was described previously (Glogauer et al., 2003; Kitajiri et al., 2004; Saitou et al., 2000). For *Rac1* mutant experiments, male *Rac1* mutant mice were crossed with R26R female mice (Soriano, 1999) to introduce the LacZ reporter construct for Cre-mediated deletion (both from the Jackson Laboratory, Bar Harbor, ME).

Statistical Analyses

Results are presented as means ± SEM. Data were analyzed using Student's t test.

(D) Cldn3, 7, and 8 expression in 8-week-old mouse testes.
(E) Tubules with spermatogenesis after transplantation of *Ocln* and *Cldn11* KO testis cells (n = 6). The numbers of tubules counted were: 639 (control) and 659 (mutant) for occludin; 735 (control) and 663 (mutant) for claudin11.
(F) Colony count after claudin3, 7, and 8 KD in GSCs (n = 18).
Scale bar = 50 μm (D). Error bars = SEM. See also Figure S4 and Tables S1, S2, S3, and S6.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes four figures, six tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.stem.2011.08.011.

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—Original Article—

Unstable Side Population Phenotype of Mouse Spermatogonial Stem Cells *In Vitro*

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Abstract. Stem cells of the side population (SP) phenotype are found in many self-renewing tissues and can be identified by their unique ability to effectively exclude the dye Hoechst 33342. We previously established a method for expanding spermatogonial stem cells (SSCs) *in vitro*, but the frequency of SSCs is only about 1 to 2%, limiting detailed SSC analyses. In this study, we sought to isolate SSCs from *in vitro* cultures by exploiting their ability to exclude Hoechst 33342. In contrast to the findings of previous *in vivo* studies, we found that SP cells developed in a stochastic manner *in vitro*. Moreover, SP cells in culture were not enriched in SSCs, but they were interconvertible with non-SP cells. Although SP cells were consistently found in testes after transplantation of cultured cells, they were not enriched in SSCs. These results show that SSCs have an unstable SP phenotype and provide evidence that SSCs change their phenotype characteristics in response to their microenvironment.

Key words: Developmental biology, Gametogenesis, Sertoli cell, Spermatogenesis, Testis

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Spermatogonial stem cells (SSCs) are spermatogonia that continuously undergo self-renewal [1, 2]. Hence, although they comprise a very small percentage of the cells in the testis, SSCs provide an unlimited source of spermatogenesis throughout the male lifetime. Despite their biological importance, however, SSCs are difficult to study because distinguishing them from committed progenitor cells is technically problematic. No SSC-specific markers are available, and SSCs cannot be identified by their morphology, only by their ability to self-renew themselves. However, the technique of germ cell transplantation is widely used to identify SSCs, based on their biological function [3]. In this technique, dissociated testis cells are transplanted into the seminiferous tubules of infertile recipient testes. Those transplanted cells that reinitiate spermatogenesis after transplantation are SSCs by definition.

Development of the germ cell transplantation technique has been accompanied by development of methods for enriching SSCs [4–6] and identification of several SSC surface antigens. Although stem cells have traditionally been purified on the basis of their surface antigens, they can also be purified using fluorescent dyes. In particular, stem cells in many self-renewing tissues, including bone marrow and various types of epithelium, can be found within a side population (SP) of cells defined by the ability to exclude Hoechst 33342 [7, 8], which is a function associated with members of the ATP-binding cassette (ABC) transporter family, such as MDR1 and ABCG2/Brcp1. Although several groups have examined whether Hoechst 33342 exclusion can be used for enrichment of SSCs, results have been inconsistent. In fact, SSCs were initially thought to be non-SP cells [6], but they are now considered to be SP cells [9–12].

In 2003, a system for long-term culture of SSCs was established [13]. Development of this system was based on the observation of glial cell line-derived neurotrophic factor (GDNF) transgenic/knockout (KO) mice [14]: overexpression of GDNF in transgenic mice induced the accumulation of undifferentiated spermatogonia and produced germ cell tumors, whereas the decreased expression of GDNF in heterozygous KO mice depleted the spermatogonia and caused male infertility. SSCs from testes at any postnatal stage were then found to proliferate in the presence of self-renewal factors, including GDNF and fibroblast growth factor 2 (FGF2). These cells, designated as germline stem (GS) cells, can expand by a factor of 10^{85}-fold after 2 years in culture [15]; they can also be genetically modified to produce transgenic and KO mice and are potentially pluripotent [16, 17]. This GS cell culture technique allows researchers to greatly expand SSCs for molecular or biochemical analysis.

Although all GS cells are spermatogonia, the frequency of SSCs in GS cell cultures is only approximately 1–2% [18], and obtaining a purified SSC population has not been possible. In the present study, we sought to identify SSCs in a GS cell population by exploiting the ability of SP cells to exclude Hoechst 33342 dye. We determined the SSC activity of the SP cells using germ cell transplantation. We also examined the SP cell phenotype and SSC activity of GS cells after transplantation into seminiferous tubules.

Materials and Methods

Cell culture

The GS cells used in this study were derived from the transgenic mouse line C57BL/6 Tg14(act-EGFP)OsbY01, which was backcrossed to a DBA/2 background [13]. These cells were maintained on dishes coated with laminin (20 μg/ml; BD Biosciences, Franklin Lakes, NJ, USA). For culturing after cell sorting, sorted cells were

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Table 1. Summary of transplantation experiments

Experiment	Donor cells	No. of experiments	No. of cells injected[a]	No. of testes injected	No. of testes colonized (%)	No. of colonies/ testis/10^5 cells[b]	P value by t-test
In vitro	SP	4	150 – 1300	20	8 (40)	560.3 ± 432.0	0.12
	Total EGFP	4	1000 – 2400	20	16 (80)	142.5 ± 22.6	
In vivo (WT)	SP	3	650 – 2000	18	0 (0)	0	0.40
	Total EGFP	3	32000 – 70000	16	3 (19)	0.8 ± 0.4	
In vivo (Ras)	SP	3	640 – 5900	17	2 (12)	10.1 ± 9.1	0.30
	Total EGFP	3	5900 – 30000	17	2 (12)	0.5 ± 0.3	

Values are means ± SEM. [a] Approximate number of cells was calculated by assuming 4 μl of cells was transplanted. [b] Number of individual colonies in each testis. Results were normalized to 10^5 cells injected/testis.

maintained on mitomycin C-treated mouse embryonic fibroblasts (MEFs). For combined staining experiments, we used GS cells from the transgenic mouse line B6-TgR(ROSA26)26Sor (The Jackson Laboratory, Bar Harbor, ME, USA), which was back-crossed to a DBA/2 background. The growth factors used were mouse epidermal growth factor (EGF; 10 ng/ml), human FGF2 (10 ng/ml), and rat GDNF (15 ng/ml; all from PeproTech, Rocky Hill, NJ, USA). GS cells expressing constitutively active *Akt* or *H-RasV12* under CAG promoter were previously described [19].

Magnetic cell sorting (MACS) and SP cell analysis by flow cytometry

Cultured cells were dissociated by treatment with 0.25% trypsin, and testis cells were dissociated by two-step enzymatic digestion with collagenase type IV and trypsin, as previously described [20]. For MACS, primary spermatogonia were collected from 7-day-old DBA/2 pup testes using a rat anti-EPCAM antibody (G8.8; BioLegend, San Diego, CA, USA) and Dynabeads sheep anti-rat IgG (Invitrogen, Carlsbad, CA, USA), as previously described [4]. For SP cell analysis, single-cell suspensions were suspended at 10^6 cells/ml in Dulbecco's Modified Eagle's Medium containing 2% fetal bovine serum (FBS) and 10 mM Hepes. SP cells were detected as those remaining unstained after a 90-min incubation with 4 μg/ml Hoechst 33342 (Sigma, St. Louis, MO, USA) at 37 C. For combined staining, 0.1 μg/ml rhodamine 123 (Rh 123; Invitrogen) was added during the last 20 min of the Hoechst 33342 incubation. For immunostaining of SP cells, Hoechst 33342-stained cells were incubated with allophycocyanin- or Alexa 647-conjugated antibodies against KIT (2B8), EPCAM (G8.8), ITGB1 (HMβ1-1), ITGA6 (GoH3), CD9 (MZ3) and FUT4 (MC480; BioLegend) after washing the cells with ice-cold phosphate-buffered saline containing 1% FBS. Propidium iodide (1 μg/ml; Sigma) was added to exclude dead cells before analyses. Cells were analyzed on a FACSAria 2 flow cytometer equipped with a 375-nm UV laser (7 mV; BD Biosciences). The Hoechst dye was excited at 375 nm, and its dual emission wavelengths were detected using 450/20 (Hoechst 33342 blue) and 660/20 (Hoechst 33342 red) filters.

Germ cell transplantation

Single-cell suspensions of germ cells were microinjected into the seminiferous tubules of WBB6F1-W/W^v (W) mice (Japan SLC, Shizuoka, Japan) through the efferent duct [20]. Approximately 75–85% of the tubules were filled in each recipient testis. The recipient mice were treated with anti-CD4 antibody (GK1.5; a gift from Dr T Honjo, Kyoto University) to induce tolerance to the allogeneic donor cells [21]. Immediately after transplantation, some of the recipients were made cryptorchid by suturing the testicular fat pad to the lateral abdominal wall, as previously described [22]. All animal experimentation protocols were approved by the Institutional Animal Care and Use Committee of Kyoto University.

Analysis of recipient testes

Donor cell-derived colonies in the recipient testes were detected by enhanced green fluorescence protein (EGFP) fluorescence under UV light. Germ cell clusters were defined as colonies when they were longer than 0.1 mm and occupied the entire circumference of the seminiferous tubule. For histological analyses, samples were fixed in formalin, embedded in paraffin and sectioned. All sections were counterstained with hematoxylin and eosin.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol (Invitrogen), and first-strand cDNA was produced using Superscript II (Invitrogen). The specific RT-PCR primers are listed in Table S1. PCR was performed using a single incubation at 95 C for 5 min, followed by 30 cycles of 94 C for 30 sec, 60 C for 30 sec and 72 C 1 min.

Statistical analysis

Results are presented as means ± SEM. Data were analyzed using the Student's *t*-test.

Results

Identification of SP cells in GS cell cultures

SP cells in the testis population have been shown to express *Abcg2*, which is thought to be responsible for the SP phenotype [10]. To determine what types of ABC transporters are expressed in GS cells, we carried out RT-PCR analysis. The analysis showed that several ABC transporters are expressed in the testis (Fig. 1A). GS cells were found to express *Mrp1, Mrp4, Mrp5* and *Abcg2*, but not *Mrp2, Mrp3 or Mrp6*. This pattern of gene expression is different from that observed in EPCAM-expressing spermatogonia (Fig. 1A), which exhibit the SP cell phenotype [11].

To determine whether GS cells exhibit the SP phenotype, GS cells expressing the gene for EGFP were cultured on laminin for 1–6 days (Fig. 1B), recovered by trypsin digestion, and stained with Hoechst 33342. Unlike the whole testis cell population, which yielded at least six distinct regions on a flow cytometry dot plot [9], GS cell cultures exhibited a simpler dot plot pattern, indicating a more uniform composition of the stained cells (Fig. 1C). However, SP cells were found in only 57.1% (24 out of 42) of the GS cell cultures, indicating that the SP population developed stochastically. At least three independent GS cell lines showed stochastic expression. Development of the SP population was not influenced by the length of time after passage, but was inhibited by the addition of verapamil (Fig. 1D), which inhibits dye efflux by ABC transporter activity [7]. In cultures with SP cells, the frequency of the SP population ranged from 0.3 to 22.3% (2.1 ± 0.9%; n=24), whereas non-SP cells were consistently found in all of these cultures.

Because *Akt* regulates the SP cell phenotype in hematopoietic and neuronal cells [23, 24], we examined the effect of *Akt* overexpression on SP cell development. However, GS cells stably expressing active *Akt* failed to develop SP cells in three independent experiments (Fig. 1E), suggesting that the mechanism regulating SP cell development in GS cell culture differs from that in hematopoietic or neuronal cells.

To characterize the SP cells, we stained them with several spermatogonia markers. Despite the significant difference in the Hoechst 33342 staining patterns of the SP vs. non-SP cells, both types of cells expressed the same surface markers, ITGA6, ITGB6, EPCAM and CD9 (Fig. 2A). They also exhibited strong expression of KIT, which is normally used as a marker for differentiating spermatogonia and spermatocytes. Neither SP nor non-SP cells expressed FUT4, a marker for primordial germ cells and embryonic stem cells. Efflux of Rh 123, which is often used to enrich stem cells in several tissues [25], was more pronounced in SP cells than in non-SP cells (Fig. 2B). Rh 123^{low} cells were not detected in the non-SP cell population.

Reversibility of the SP phenotype in GS cell culture

Because previous studies suggested that the SP population has higher stem cell activity, we expected that SP cells in GS cell cultures would be more undifferentiated than non-SP cells and that they would differentiate into non-SP cells. To test this hypothesis, we fractionated EGFP-expressing GS cells on laminin-coated dishes according to their Hoechst 33342 staining patterns (Fig. 3A). In these experiments, 2.5 to 5.0 × 10^3 SP cells and 1.8 to 2.9 × 10^5 non-SP cells were sorted per experiment. The separated SP and non-SP cells were then cultured on MEFs and examined for their phenotypes. The sorted cells were cultured on MEFs rather than on laminin because they survived better on MEFs, possibly because they were damaged during sorting [5].

The colonies that developed from the cultured SP and non-SP cells were morphologically indistinguishable and did not vary significantly in growth rate. To observe the SP phenotype of the developed colonies, the cells were stained again with Hoechst 33342 on days 29 and 34 after sorting. In two separate experiments, the cultured SP cells differentiated into non-SP cells, whereas the cultured non-SP cells produced both SP and non-SP cells. As they did on laminin, GS cells stochastically produced SP cells on MEFs and yielded a pattern of Hoechst 33342 similar to that of the original unsorted GS cell cultures. These results indicate that SP and non-SP cells are interconvertible *in vitro*.

SSC activity of SP cells in GS cell culture

To determine whether the SP cell population is enriched in SSCs, we used germ cell transplantation to compare the SSC activity of SP cells with that of total GS cells (Fig. 3B). These two populations of cells were microinjected into the seminiferous tubules of infertile W mice lacking endogenous spermatogenesis. In four separate experiments, a total of 20 recipient testes received transplantation of each cell type.

The recipients were sacrificed 2 months after transplantation. The isolated recipient testes were then analyzed for donor cell colonization levels by counting the number of EGFP-expressing germ cell colonies under UV light. Transplantation of the SP and total GS cell populations produced 560.3 ± 432.0 and 142.5 ± 22.6 (n=20) SSC-derived colonies per 10^5 donor cells, respectively. Although the frequency of SSC colony production was higher for the SP cells, the difference between the two cell types was not statistically significant (P=0.12; Table 1), indicating that SP cells are not enriched in SSCs.

Histological analyses of the recipient testes showed normal differentiation of the transplanted SP cells (Fig. 3C). No spermatogenesis was observed in the nontransplanted control W testes.

SP cell phenotype after transplantation in vivo

To examine the effect of the microenvironment on SP cell development, we transplanted EGFP-expressing GS cells into seminiferous tubules (Fig. 4A). In these GS cells, the EGFP gene is driven by the CAG promoter and is expressed at all stages from the spermatogonium to the round spermatid [21]. By gating EGFP-expressing cells, we excluded the possibility of somatic cell contamination of the germ cells.

Transplanted SSCs are known to form chains or networks of spermatogonia on the basement membrane 2–3 weeks after transplantation, when SSCs are thought to be preferentially undergoing self-renewal division [26]. However, at around 1 month, some of the cells in the colony start to differentiate vertically into the lumina. Normal spermatogenesis with spermatogenic cycles is restored as soon as 3 months after transplantation, which corresponds to approximately three cycles of spermatogenesis.

We transplanted approximately 4 × 10^6 EGFP-expressing GS cells grown on laminin into the seminiferous tubules of W mice. Some of the testes were surgically fixed at a higher position in the abdomen to induce cryptorchidism, which destroys differentiating germ cells [22]. Recipients were sacrificed at the early (7–14 days) or late (3–4 months) phase after transplantation, and the testes were dissociated into single cells using a collagenase type IV/trypsin, a two-step digestion technique that is commonly used to prepare single-cell suspensions for germ cell transplantation [20].

At the early phase after transplantation, approximately 1.8–2.0 × 10^6 cells were recovered per testis, and the proportion of EGFP-expressing cells was small (Fig. 4B). The overall Hoechst 33342

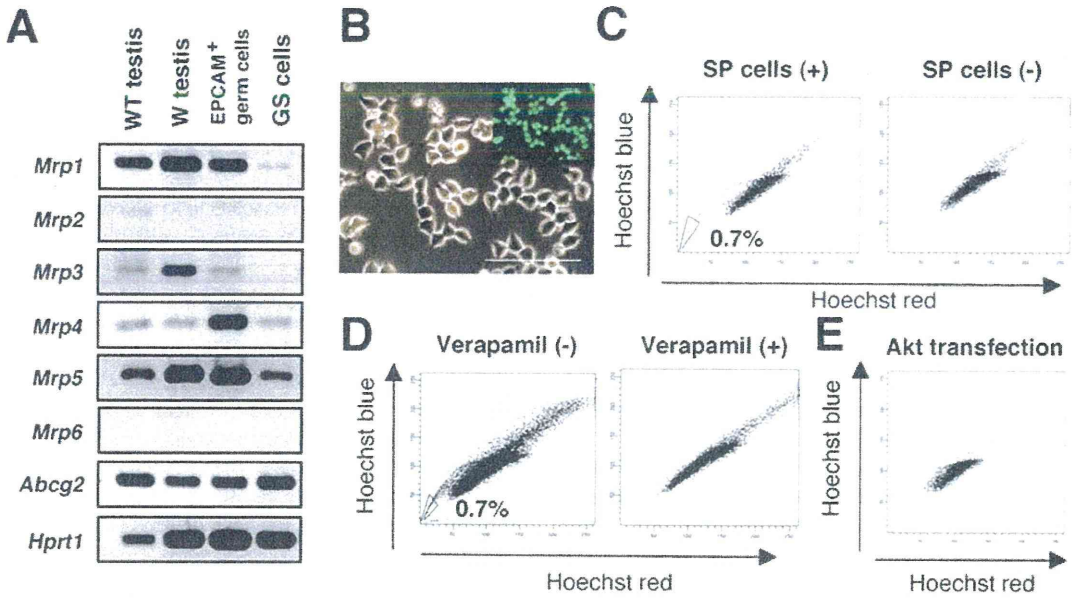

Fig. 1. SP cell phenotype of GS cells. A: RT-PCR analysis of transporter gene expression demonstrating that GS cells express *Mrp1*, *Mrp4*, *Mrp5* and *Abcg2*. B: EGFP-expressing GS cells on a laminin-coated dish exhibit strong green fluorescence under UV light (inset). C: Flow cytometric analysis of GS cells stained with Hoechst 33342. GS cells were imaged by using filters for Hoechst red and Hoechst blue emission. Whereas some of the GS cell cultures contained Hoechst 33342-excluding cells (SP cells: left), other did not (right). D: Inhibition of SP cell development by verapamil. After GS cells were dissociated, the recovered cells were stained with Hoechst 33342 in the absence (left) or presence (right) of verapamil. (E) Absence of SP cells in a population of GS cells stably expressing active *Akt*. Bar=50 μm (B).

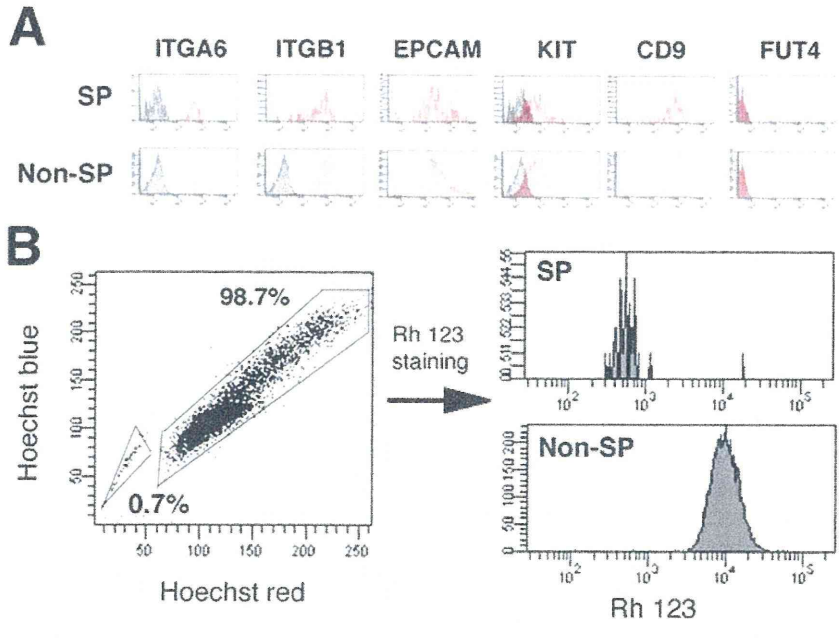

Fig. 2. Phenotypes of SP cells in GS cell culture. A: Expression of surface markers on SP and non-SP cells. The black-shaded area indicates control staining. No significant differences between SP and non-SP cells are evident. B: Rh 123 efflux characteristics of SP and non-SP cells. GS cells from ROSA26 mice were stained with Hoechst 33342 and Rh 123.

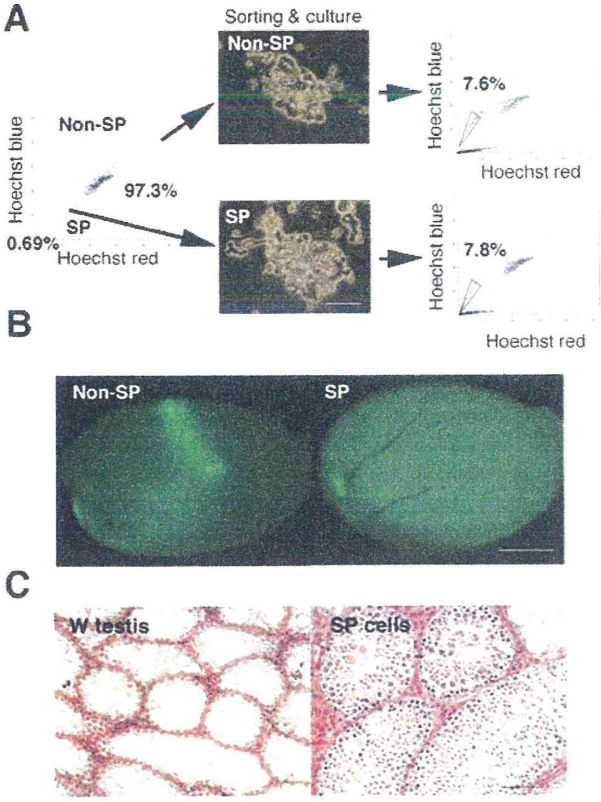

Fig. 3. Functional analyses of SP cells in GS cell culture. A: Reversibility of the SP cell phenotype. GS cells cultured on laminin-coated dishes were sorted into SP and non-SP cells according to their Hoechst 33342 staining patterns. The SP and non-SP cells were cultured for 29 and 34 days *in vitro* and stained again with Hoechst 33342. B: Macroscopic appearance of recipient W testes transplanted with SP (right) and total EGFP-expressing cells (left). Approximately 0.2–1.3 × 10^3 SP or 1.0–2.4 × 10^3 non-SP cells were transplanted into each testis. Green fluorescence indicates colonization by donor cells. C: Histological appearance of the recipient testes. W testes transplanted with SP cells produce colonies undergoing normal spermatogenesis (right). In contrast, non-transplanted W testes demonstrate no evidence of ongoing spermatogenesis (left). Bars=50 μm (A), 1 mm (B) and 100 μm (C).

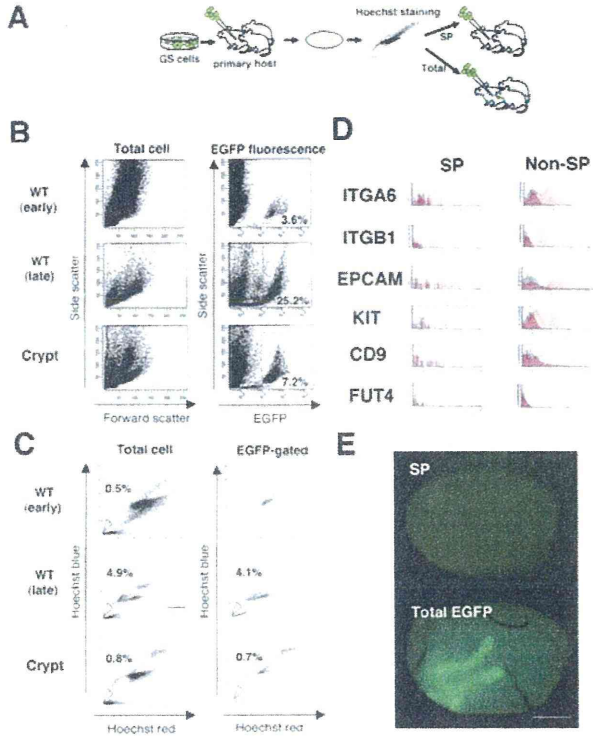

Fig. 4. SP cell analysis after transplantation of WT GS cells. A: A diagram showing the experimental strategy. EGFP-expressing GS cells were transplanted into the primary W recipient mice. Some of the recipients were made cryptorchid to eliminate differentiated germ cells. Recipient testes were dissociated at early (7–14 days) or late (3–4 months) phases after transplantation and stained with Hoechst 33342. After cells expressing EGFP were gated, SP and total EGFP-expressing cells in the EGFP-expressing cell population were sorted and transplanted into secondary W recipients. B: Comparison of light-scattering properties (left) and EGFP fluorescence (right) of dispersed testis cells from WT and cryptorchid recipients. Donor-derived cells were gated based on EGFP fluorescence and side scatter for SP cell identification. C: Comparison of Hoechst 33342 staining patterns of dispersed testis cells from WT and cryptorchid recipients. D: Expression of surface markers on SP and non-SP cells in WT W recipients. The black-shaded area indicates control staining. E: Macroscopic appearance of recipient testes that were transplanted with SP (top) and total EGFP-expressing cells (bottom). Approximately 0.7–2.0 × 10^3 SP or 3.2–7.0 × 10^4 non-SP cells were transplanted into each testis. Green fluorescence indicates colonization by donor cells. Bar=1 mm (E).

staining pattern of the recovered cells indicated no SP cells (Fig. 4B and C). At the later phase after transplantation, the recipient testes were significantly larger, and the two-step digestion technique described above yielded 2.8–5.9 × 10^6 cells per testis. The cryptorchid testes yielded only 1.0–1.5 × 10^6 cells per testis because germ cell differentiation was limited by the high temperature of the body cavity. In contrast to our findings *in vitro*, all nine experiments performed *in vivo* yielded SP cells. The pattern of Hoechst 33342 staining in normal W recipients appeared more complex in the later stage of transplantation than in the early stage (Fig. 4B and C), reflecting the colonization and differentiation of the transplanted GS cells over time. Although cryptorchid testes also yielded SP cells, the staining pattern of the dissociated testicular cells of the cryptorchid testes differed somewhat from that of the normal testes.

When only EGFP-expressing cells were gated for analyses, both wild-type (WT) and cryptorchid testes cells yielded staining patterns that were distinctly different from those of the total cells (Fig. 4B). Despite the elimination of some subpopulations by EGFP gating, the EGFP-expressing cell populations from both WT and

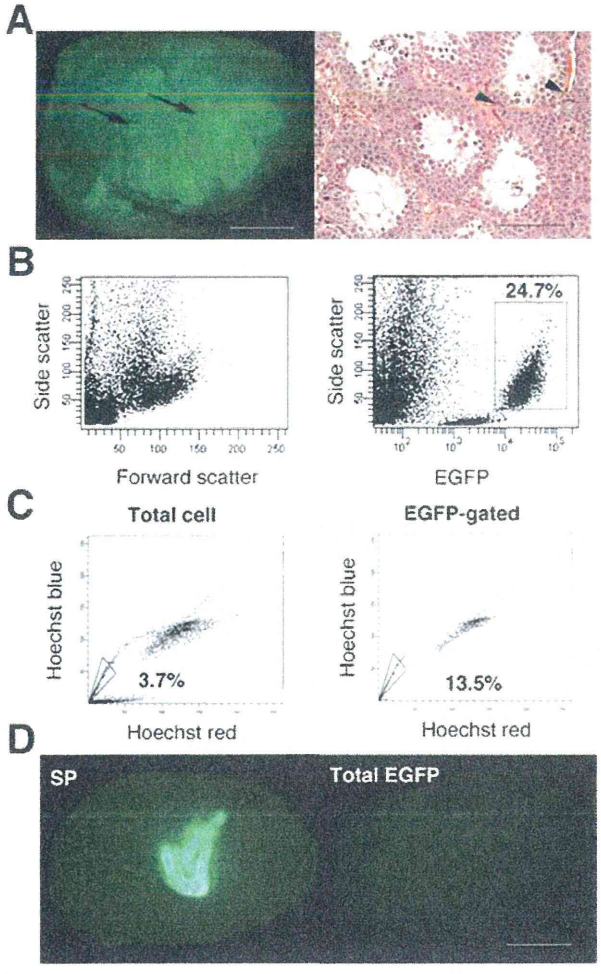

Fig. 5. SP cell analysis after transplantation of *H-RasV12*-transfected GS cells into W testes. A: Macroscopic (left) and histological (right) appearance of a recipient testis transplanted with *H-RasV12*-transfected GS cells. Arrows indicate abnormal germ cell clumps in the seminiferous tubules; arrowheads indicate invasion into interstitial tissue. Note the abnormal spermatogenesis in the seminiferous tubules. B: Light-scattering properties (left) and EGFP fluorescence (right) of dispersed testis cells from recipients that were transplanted with *H-RasV12*-transfected cells. Donor-derived cells were gated based on EGFP fluorescence and side scatter for SP cell identification. C: Hoechst 33342 staining patterns of dispersed testis cells from recipient testes that were transplanted with *H-RasV12*-transfected cells. Although SP cells were found in total testis cells (left) and in EGFP-expressing testis cells (right), the overall pattern of Hoechst 33342 staining was significantly different from that observed after transplantation of WT GS cells (see Fig. 4C). D: Macroscopic appearance of recipient testes transplanted with SP (left) and total EGFP-expressing cells (right). Approximately 0.6–5.9 $\times$ 10^3 SP or 0.6–3.0 $\times$ 10^4 non-SP cells were transplanted into each testis. Green fluorescence indicates colonization by donor cells. Bars=100 μm (A, right) and 1 mm (A, left; D).

cryptorchid testes consistently yielded SP cells (Fig. 4C). In WT recipients, the SP cells comprised 0.06 to 4.7% (1.9 ± 0.6%; n=7) of the EGFP-expressing cells, and they expressed typical SSC markers including ITGB1, ITGA6, and EPCAM. Compared with GS cells, however, CD9 is only weakly expressed in both SP and non-SP cells, suggesting that these populations have a reduced SSC concentration. Although they weakly expressed KIT, no FUT4 expression was detected (Fig. 4D).

To examine the SSC activity of SP cells found in the testis after GS cell transplantation, the SP cells were separated from the WT primary recipient testes 3 to 4 months after GS cell transplantation and implanted into secondary recipient testes (Fig. 4E). We gated EGFP-expressing cells and sorted 0.5–1.5 $\times$ 10^4 SP cells (0.8 ± 0.3 $\times$ 10^4 cells; n=3) and 2.4–5.3 $\times$ 10^5 total EGFP-expressing cells (3.4 ± 0.9 $\times$ 10^5 cells; n=3). All of the sorted cells were clearly positive for EGFP expression, and the total EGFP-expressing cell population was implanted into secondary recipient testes as a control. In three separate experiments, a total of 18 and 16 recipient testes were transplanted with SP cells or total EGFP-expressing cells, respectively. The secondary recipient testes yielded no colony after SP cell transplantation, whereas total EGFP cells yielded 0.8 ± 0.4 colonies per 10^5 donor cells (n=16; Table 1). The difference was not statistically significant (P=0.40).

SP cell phenotype in seminomatous tumors formed after transplantation

In our final set of experiments, we used GS cells overexpressing activated *H-Ras* (*H-RasV12*) to examine whether the SP phenotype could be used to enrich the population of cancer stem cells. *H-RasV12*-transfected GS cells proliferate even in the absence of exogenous cytokines, and produce seminomatous tumors after transplantation into W mice [19]. Three to four months after 4 $\times$ 10^6 of these cells were transplanted into W mice, the testes were recovered (Fig. 5A and B), and the EGFP-expressing cells were gated (Fig. 5B) and analyzed for their Hoechst 33342 staining pattern. *H-RasV12*-transfected GS cells exhibited SP cell development in each of four independent experiments. The Hoechst 33342 staining pattern appeared simpler than that observed after transplantation of WT GS cells (Fig. 5C), possibly reflecting abnormal spermatogenesis from the *H-RasV12*-transfected cells. SP cells comprised 0.7–13.5% (4.4 ± 3.0%; n=4) of the total EGFP-expressing cells.

The numbers of SP cells recovered by cell sorting was 0.4–4.4 $\times$ 10^4 cells (2.1 ± 1.2 $\times$ 10^4; n=3) per testis. On the other hand, 0.2–1.5 $\times$ 10^5 total EGFP-expressing cells (7.2 ± 3.9 $\times$ 10^4; n=3) per testis were sorted as a control for transplantation. The SP and total EGFP-expressing cells were collected and microinjected into W mice in three sets of experiments involving a total of 17 recipient testes for each cell type. Analyses of the recipient testes showed that transplantation of SP cells and total EGFP-expressing cells produced 10.1 ± 9.1 and 0.5 ± 0.3 colonies per 10^5 donor cells (n=17). However, the difference between the two cell types was not statistically significant (P=0.30)(Fig. 5D, Table 1).