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Mouse differentiating spermatogonia can generate germinal stem cells *in vivo*

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In adults, stem cells are responsible for the maintenance of many actively renewing tissues, such as haematopoietic, skin, gut and germinal tissues. These stem cells can self-renew or be committed to becoming progenitors. Stem-cell commitment is thought to be irreversible but in male and female *Drosophila melanogaster*, it was shown recently that differentiating germ cells can revert to functional stem cells that can restore germinal lineage[1,2]. Whether progenitors are also able to generate stem cells in mammals remains unknown. Here we show that purified mouse spermatogonial progenitors committed to differentiation can generate functional germinal stem cells that can repopulate germ-cell-depleted testes when transplanted into adult mice. We found that GDNF, a key regulator of the stem-cell niche, and FGF2 are able to reprogram *in vitro* spermatogonial progenitors for reverse differentiation. This study supports the emerging concept that the stem-cell identity is not restricted in adults to a definite pool of cells that self-renew, but that stemness could be acquired by differentiating progenitors after tissue injury and throughout life.

In adult mouse testes, germinal stem cells (GSCs), also called A_{single} (A_s) spermatogonia, are located on the basal membrane of seminiferous tubules. GSCs can self-renew or produce A_{paired} (A_p) spermatogonia. After successive divisions, A_p spermatogonia differentiate and form chains of 4, 8 or 16 aligned spermatogonia (A_{al}) and migrate along the basal membrane. On the basis of morphological criteria, GSCs and committed A_p and A_{al} spermatogonia are classically called undifferentiated spermatogonia, although this classification can be confusing because this population contains progenitors undergoing differentiation. A_{al} spermatogonia differentiate into more committed A_1 spermatogonia that will divide and differentiate into A_2, A_3, A_4, intermediate and B spermatogonia, which will undergo meiosis after a final mitosis[3]. 'Undifferentiated' (GSC-A_s, A_p and A_{al}) and 'differentiating' (A_1, A_2, A_3, A_4, intermediate and B) spermatogonia differ according to the expression of genes for the receptor tyrosine kinase c-kit, the HMG transcription factor Sox3, the bHLH transcription factor neurogenin-3 (Ngn3) and E-cadherin (E-cadh) (Fig. 1a)[4–8]. In particular, GSCs do not express c-kit, as shown by transplantation of c-kit-negative and c-kit-positive cells from adult mouse cryptorchid testes in which c-kit-positive differentiating spermatogonia are absent[9].

We assessed the potential of mouse spermatogonial progenitors to regenerate spermatogenesis after transplantation. c-kit-positive differentiating spermatogonia from enhanced green fluorescent protein (EGFP)-transgenic mice were purified and transplanted into seminiferous tubules of γ-irradiated germ-cell-depleted testes. Ten weeks later, the donor EGFP–c-kit-positive cells had colonized the seminiferous tubules of the recipient testes (Fig. 1b). In testicular single-cell suspensions, EGFP-positive cells could easily be distinguished from recipient cells by flow cytometry (Fig. 1c). They showed the Hoechst 33342 profile, which characterizes normal spermatogenesis[10,11], and included meiotic and haploid spermatid cells (Fig. 1d). Elongated spermatids were observed by histology in EGFP-positive tubules (Fig. 1e). Thus, c-kit positive cells were able to colonize, proliferate and differentiate in recipient testes. Spermatogonial transplantation provides a functional and quantitative assay to estimate GSC activity, by the formation of a clonal spermatogenetic colony[12]. The number of EGFP-positive spermatogenic colonies generated 10 weeks after transplantation of total testicular cells and c-kit-positive cells was compared (Fig. 1f). The colony-forming activity of c-kit-positive cells was 1.57-fold higher than the activity of total cells (Fig. 1g, $P < 0.02$). Consistent with this result, previous work has suggested that c-kit-positive germ cells, purified using immunomagnetic microbeads, retained colony-forming activity that was slightly higher (1.7-fold higher) than total cells, although this increase was not statistically significant[13]. We then checked whether the selected c-kit-positive donor cells simultaneously expressed the GSC markers, Thy-1 and α-6 integrin[9,14]. GSCs were present at a very low frequency in this fraction and, therefore, were not responsible for the observed regeneration (Supplementary Information, Fig. S1). These results show that adult c-kit-positive spermatogonial progenitors, although committed to differentiate, retain the potential to regenerate spermatogenesis after transplantation, but with a low efficiency.

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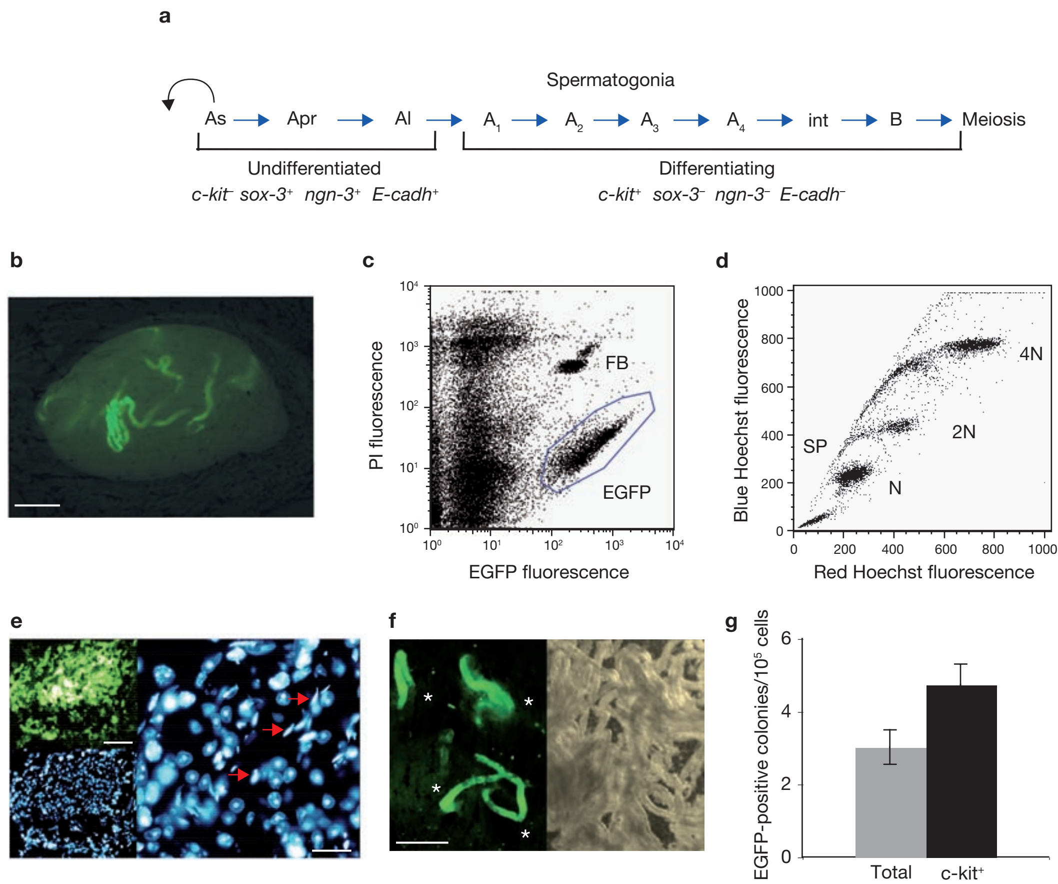

Figure 1 Adult c-kit-positive spermatogonial progenitors selected by MACS have the potential to regenerate spermatogenesis after transplantation in germ-cell depleted testis. (**a**) Diagram showing the first steps of the differentiation process of GSCs and spermatogonia, and the expression pattern of several markers of differentiation. (**b**) Detection by fluorescence microscopy of fluorescent seminiferous tubules in a recipient testis 10 weeks after transplantation with germinal EGFP–c-kit positive cells. Scale bar, 1 mm. (**c**) EGFP and propidium iodide (PI) fluorescence analysis of testicular cell suspension from a recipient testis. EGFP-positive cells (EGFP) and TruCount fluorescent beads (FB) are indicated. (**d**) Hoechst fluorescence (red/blue Hoechst fluorescence) analysis of EGFP-positive cells in a recipient testis gated from **c**. Side Population (SP), meiotic spermatocytes I (4N) and spermatocytes II (2N), and postmeiotic spermatids (N) are indicated. (**e**) Frozen section of a colony derived from EGFP–c-kit positive cells, analysed for EGFP (upper left) and DAPI staining (right and lower left). Arrows show elongated spermatids. Scale bar, 0.1 mm (left) and 25 μm (right). (**f**) Typical whole-mount EGFP fluorescence (left) and brightfield (right) observation of some seminiferous tubules from recipient testes 2 months after transplantation (asterisks indicate EGFP-positive colonies). Scale bar, 1 mm. (**g**) Colonization of a recipient testis by transplanted c-kit positive fraction, compared with total. The colonization efficiency is represented by the number of EGFP-positive spermatogenic colonies generated per 10^5 cells injected. The values are mean ± s.e.m. (n = 9 testes).

We have previously shown that GSCs and spermatogonial progenitors in adult mice express α-6 integrin and harbour the side population (SP) phenotype, based on the Hoechst 33342 efflux by the ABC transporter *Bcrp1*[10,11]. To further characterize the c-kit-positive cells responsible for regeneration, we analysed testicular populations according to Hoechst staining, α-6 integrin and c-kit expression (Fig. 2a–c). α-6$^+$c-kit$^+$cells and α-6$^+$c-kit$^{-/low}$ cells represented 55 ± 9% and 10 ± 2%, respectively, of the SP. SP α-6$^+$c-kit$^+$cells and SPα-6$^+$c-kit$^{-/low}$ cells were sorted and the expression of *sox3, E-cadh,* and *ngn3* markers[6–8] confirmed that the SPα-6$^+$c-kit$^{-/low}$ fraction consisted of undifferentiated spermatogonia (Fig. 2d). The absence of expression of these markers in the SPα-6$^+$c-kit$^+$ fraction further indicated that these cells were differentiating spermatogonia.

As expected, SPα-6$^+$c-kit$^{-/low}$ undifferentiated spermatogonia cells, which contain GSCs, showed a regenerative potential when transplanted into the seminiferous tubules of germ-cell-depleted testes. EGFP-donor SPα-6$^+$c-kit$^{-/low}$ cells colonized the seminiferous tubules of the recipient testes after ten weeks (Fig. 2e) and produced normal spermatogenesis (Fig. 2f). We observed a 50-fold increase in the colony-forming activity of SPα-6$^+$c-kit$^{-/low}$ cells, compared with total cells (Fig. 2g-2h; P = 0.003). Given that 151 clones were generated per 10^5 donor cells, and that colonization efficiency is around 5% in the GSC transplantation assay[15], it means that 1 in 33 SPα-6$^+$c-kit$^{-/low}$ cells (10^5/151 × 20) is GSC. The stem-cell activity of this population (151 colonies/10^5 donor cells) is very close to previously published data of transplantation with adult wild-type MHC-I$^-$ Thy-1$^+$α-6$^+$ donor cells (162 colonies/10^5 donor cells)[14]. These data show that SPα-6$^+$c-kit$^{-/low}$ population is highly enriched in GSCs and confirm our previous data and other data showing that SP contains the GSCs in normal adult and immature mice[11,16], although it has been reported that SP does not contain GSCs in the cryptorchid mouse model[17].

Transplanted SPα-6$^+$c-kit$^+$ cells, that is, differentiating spermatogonia, from EGFP-transgenic mice also showed a regenerative potential, colonized the seminiferous tubules of the recipient testes (Fig. 3a) and generated normal spermatogenesis (Fig. 3b). The colony-forming activity was about 5-fold higher in SPα-6$^+$c-kit$^+$ cells, compared with total

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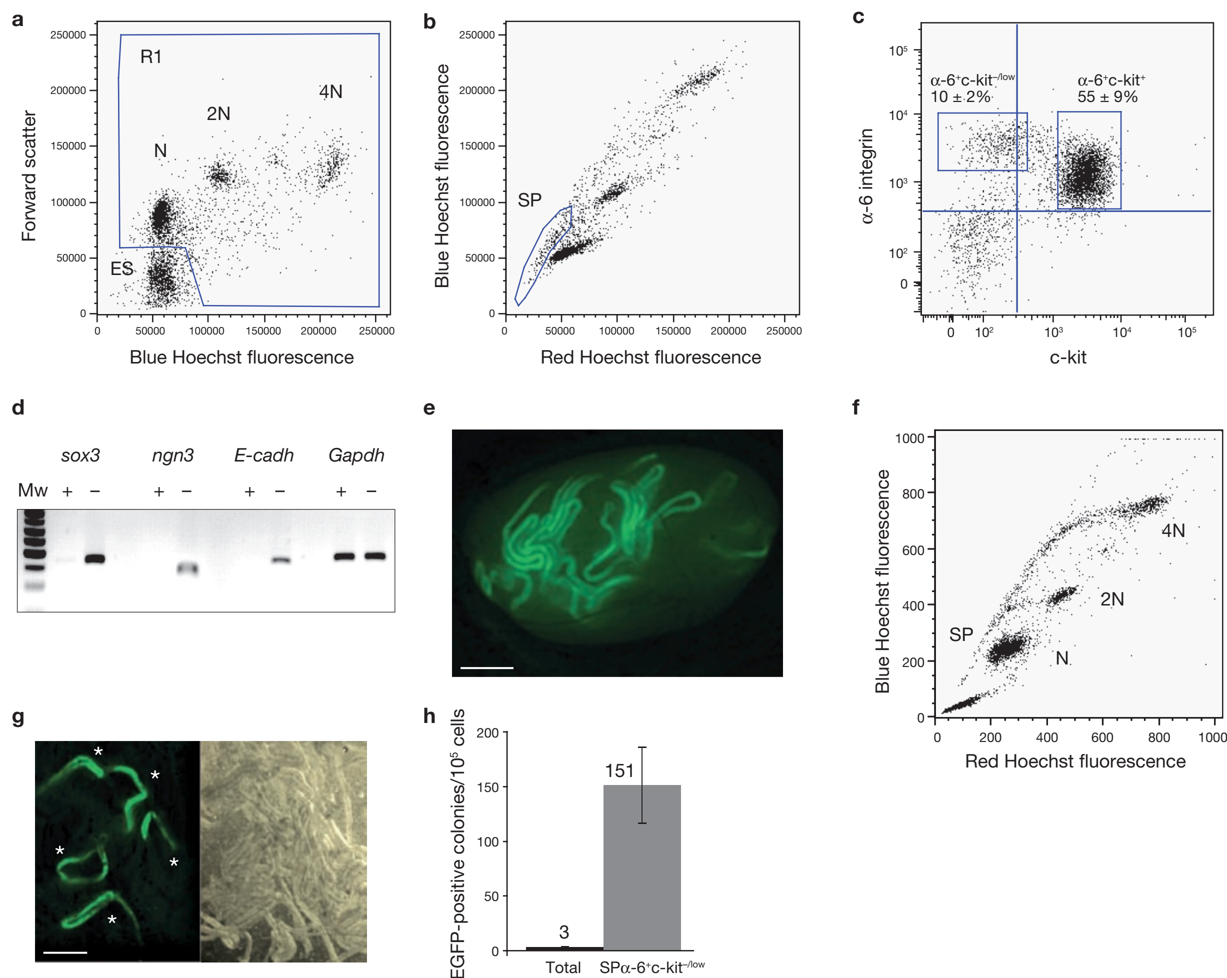

Figure 2 Flow cytometric analysis of c-kit and stem-cell activity in SPα-6^{+}c-kit$^{-/low}$ subpopulation of wild-type adult testes cells. (**a**) Blue Hoechst 33342 fluorescence/forward scatter analysis of viable cells. Diploid (2N), meiotic (4N) and postmeiotic (N) cells are indicated, as elongated spermatids (ES). A fraction of the population was gated (R1) to discard elongated cells from further analysis. (**b**) Red/blue Hoechst 33342 fluorescence of cells gated in R1. Side population (SP) is indicated. (**c**) α-6 integrin and c-kit expression of the SP cells. SPα-6^{+}c-kit^{+} and SPα-6^{+}c-kit$^{-/low}$ populations, and cell distribution are indicated (mean ± s.e.m., $n = 6$). (**d**) RT–PCR analysis of *sox3, E-cadh, ngn3* markers of SPα-6+c-kit+ (+) and SPα-6^{+}c-kit$^{-/low}$ (-) populations. (**e**) Detection of fluorescent seminiferous tubules in a recipient testis transplanted with EGFP SPα-6^{+}c-kit$^{-/low}$ cells 10 weeks after transplantation. Scale bar, 1 mm. (**f**) Red/blue Hoechst 33342 fluorescence analysis of EGFP-positive cells in a recipient testis. (**g**) Whole-mount EGFP fluorescence (left) and brightfield (right) observation of some seminiferous tubules from recipient testis 2 months after transplantation (asterisks indicate EGFP-positive colonies). Scale bar, 1 mm. (**h**) Enhanced colonization of recipient testis by transplanted SPα-6^{+}c-kit$^{-/low}$ ($n = 7$) fraction, compared with total ($n = 9$). The colonization efficiency is represented by the number of EGFP-positive spermatogenic colonies generated per 10^5 cells transplanted (values are mean ± s.e.m.).

cells (Fig. 3c; $P < 0.002$). To evaluate the production of differentiated daughter cells by stem cells and the expansion of the colony, we calculated the number of spermatids generated per clone for each recipient testis. The clones generated by SPα-6^{+}c-kit^{+} cells showed a lower capacity to produce spermatids, compared with clones derived from total cells 10 weeks after transplantation ($P < 0.02$; Fig. 3d), suggesting that colonies generated from c-kit-positive cells should expand with less efficiency. To exclude the possibility that the sorted SPα-6^{+}c-kit^{+} fraction was contaminated with GSCs, we determined the percentage of SPα-6^{+}c-kit$^{-/low}$ (containing GSCs) in these sorted fractions before transplantation (Supplementary Information, Fig. S2). The frequency of SPα-6^{+}c-kit$^{-/low}$ cells was 0.35 ± 0.04% (mean ± s.e.m., $n = 7$), corresponding to 350 cells for 10^5 donor cells. This number of SPα-6^{+}c-kit$^{-/low}$-contaminating cells generated only 0.5 clones ($350 \times 151/10^5$ clones), a minor contribution when compared with the 14 clones generated for 10^5 SPα-6^{+}c-kit^{+} donor cells. Hence, the contamination by GSCs cannot be responsible for the regeneration that we observed for differentiating spermatogonia. These data confirm that the late spermatogonial progenitors, the differentiating spermatogonia SPα-6^{+}c-kit^{+}, which are already committed to differentiate, still retain regenerative potential.

The regeneration of spermatogenesis by differentiating c-kit-positive spermatogonia could result from at least two mechanisms. Differentiating spermatogonia could pursue their differentiation process after transplantation and produce meiotic and postmeiotic cells, a process that could be compared with short-term repopulating processes of progenitors in haematopoiesis. However, we analysed the testes repopulation 10 weeks after transplantation. This interval corresponds to two cycles of spermatogenesis, which means that the differentiated postmeiotic spermatids

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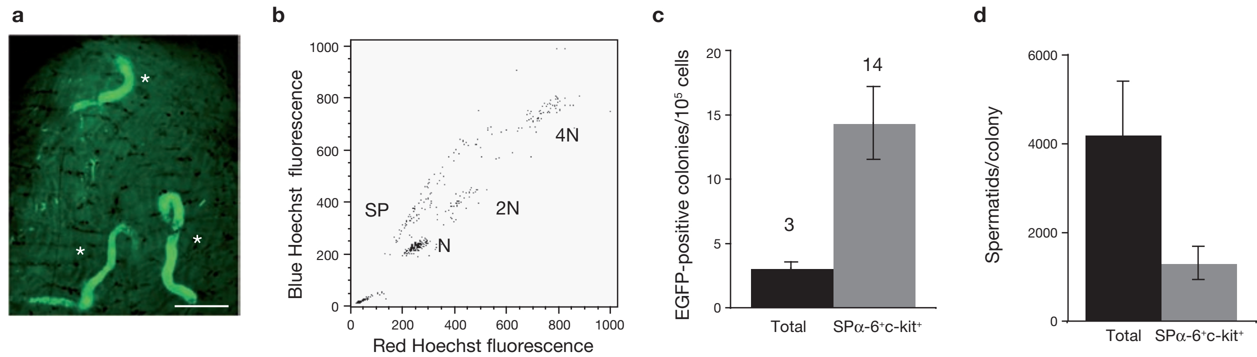

Figure 3 Adult SPα6$^+$c-kit$^+$ cells differentiating spermatogonia have the potential to regenerate spermatogenesis after transplantation. (**a**) Typical whole-mount EGFP fluorescence of some seminiferous tubules from a recipient testis 2 months after transplantation (asterisks indicate EGFP-positive colonies; scale bar, 1 mm). (**b**) Red/blue Hoechst 33342 fluorescence analysis of EGFP-positive cells in recipient testes. (**c**) Enhanced colonization of recipient testes by transplanted SPα6$^+$c-kit$^+$ fraction ($n = 7$), compared with total ($n = 9$). The colonization efficiency is represented by the number of EGFP-positive spermatogenic colonies generated per 10^5 cells injected (values are mean ± s.e.m.). (**d**) Levels of production of spermatids per colonies in recipient testes transplanted with SPα6$^+$c-kit$^+$ fraction ($n = 7$) and total population ($n = 9$). For each transplanted testis, the number of EGFP-positive spermatids per recipient testis was determined by flow cytometry, and was divided by the number of EGFP-positive colonies generated (number of spermatids produced/number of colonies generated; values are mean ± s.e.m.).

are derived from cells with self-renewal potential and that a steady-state spermatogenesis is established. Consistent with this, we have observed EGFP-donor-derived spermatogenesis one year after transplantation with donor EGFP–c-kit-positive fraction (Fig. 4a), and spermatogenetic colonies could be clearly identified (Fig. 4b). Spermatogenesis regeneration could also result from a de-differentiation process of differentiated germ cells to revert to stem cells, as previously observed in genetic models of male and female *Drosophila*[1,2]. To test this hypothesis, the EGFP–c-kit positive fraction was transplanted into depleted recipient testes and ten weeks later, EGFP-positive cells were analysed according to the SP phenotype, α-6 integrin and c-kit expression. We found that 30 ± 4% (mean ± s.e.m., $n = 3$) of EGFP-positive SP cells did not express the c-kit receptor (Fig. 4c), demonstrating that differentiating spermatogonia reverted to early undifferentiated spermatogonia during the regenerative process after transplantation. This observation was strengthened by the detection of a subpopulation (26 ± 4%) that expresses the GSCs markers SP, α-6 integrin and Thy-1 (Fig. 4d).

Serial transplantations were also performed to examine the self-renewal capacity of cells derived from transplanted EGFP c-kit-positive differentiating spermatogonia. Total EGFP-positive cells from two primary recipient testes were transplanted into new germ-cell-depleted recipient testes. Colonization of seminiferous tubules of the secondary recipient by EGFP-donor c-kit-positive derived cells from the primary recipient was observed ten weeks after transplantation (Fig. 4e); diploid, meiotic tetraploid and haploid spermatid cells were detected after DNA staining of EGFP cells (Fig. 4f). These serial transplantations demonstrated the long-term repopulating ability and the self-renewal of cells derived from the c-kit-positive spermatogonia transplanted in the first recipient testis. Together, our data demonstrate that c-kit-positive differentiating spermatogonia de-differentiate in functional stem cells after transplantation.

Stem cells reside in a local tissue microenvironment or niche, which regulates their cell fate. The somatic Sertoli cells are a major component of the GSC niche, and factors produced by these cells, such as the glial-cell-derived neurotrophic factor (GDNF) and the transcription factor ets-related molecule (ERM), are crucial for GSC maintenance[18,19]. The tyrosine kinase receptor c-kit and its ligand KitL, which is expressed by the Sertoli cells as a soluble or transmembrane isoform, have been previously shown to be involved in proliferation, survival and migration of gonocytes and spermatogonia[20]. Moreover, soluble KitL, released after cleavage by MMP-9, has a role in directing haematopoietic stem- and progenitor-cell recruitment, promoting haematopoietic reconstitution[21]. Hence, we investigated whether KitL and c-kit are involved in the recruitment of differentiating spermatogonia to the testicular stem-cell niche. First, we examined *in vitro* the influence of KitL on the migration of c-kit-positive spermatogonia using Transwell chambers. When the lower chamber was loaded with 10 or 40 ng ml^{-1} of KitL, we observed an increase in chemotactic migration of differentiating spermatogonia, which was impaired by the addition of the ACK45 antibody, which neutralizes c-kit (Fig. 5a). A weak chemokinetic response (enhanced cellular motility) was noted when the lower and upper chambers were loaded with KitL. Thus, KitL acts mainly as a chemoattractant to differentiating spermatogonia. We studied the effects of c-kit/KitL interaction *in vivo* by transplantation of SPα-6$^+$c-kit$^+$ pre-treated with the ACK45 neutralizing antibody to c-kit. We did not observe any effects of c-kit neutralization on the regenerative potential by this strategy (data not shown).

GSCs can be maintained and propagated in long-term culture in serum-free medium in the presence of specific growth factors[14,22]. The *in vitro* germinal cluster formation assay provides a reliable technique to detect GSCs and to study the effects of specific components of the niche[23]. We developed a culture method on a MEF feeder that supports long-term growth of adult GSCs in presence of GDNF, FGF2 and GFRα1 (Supplementary Information, Fig. S3). We examined the potential of SPα6$^+$c-kit$^+$ differentiating spermatogonia to grow and form germ-cell clumps in long-term culture. EGFP–SPα6$^+$c-kit$^+$ were sorted and seeded on the MEF feeder in GSC medium. After 2 weeks, germ-cell clumps were clearly observed in cultures of EGFP–SPα6$^+$c-kit$^+$ cells (Fig. 5b). However, the capacity of SPα-6$^+$c-kit$^+$ cells (K$^+$) to form germ-cell clumps was low, compared with the GSC-enriched SPα-6$^+$c-kit$^{-/low}$ population (K$^-$), (2 clusters/500 cells versus 46 clusters/500 cells, respectively; Fig. 5c). Contamination with SPα-6$^+$c-kit$^{-/low}$ cells of the sorted SPα-6$^+$c-kit$^+$ fraction should only contribute to 0.16 cluster ([0.35 × 500/100] × [46/500]

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clusters) and, therefore, is not responsible for the observed germ-cell clumps (Supplementary Information, Table S1). Five hundred cells from SPα-6$^+$c-kit$^+$ and SPα-6$^+$c-kit$^{-/low}$ 2-week cultures were seeded on new feeders. Two weeks later, cells derived from SPα-6$^+$c-kit$^+$ (DK$^+$) continued to form germ-cell clumps, showing their long-term growth and maintenance. They also showed a capacity to generate cell clusters similar to SPα-6$^+$c-kit$^{-/low}$ cells (DK$^-$) (Fig. 5c). In a culture experiment, germ-cell clumps derived from SPα-6$^+$c-kit$^+$ after 4 weeks of culture were collected and cells were transplanted into seminiferous tubules of germ-cell-depleted testes. They colonized the seminiferous tubules of the recipient testes (Fig. 5d), and normal spermatogenesis was observed with the presence of spermatids (Fig. 5e). Hence, differentiating spermatogonia could be reprogrammed *in vitro* in the presence of GDNF and FGF2 to acquire the same capacity of GSCs to grow and form germ-cell clumps in the long-term, and regenerate spermatogenesis after transplantation. In addition, we observed that two other growth factors, KITL and LIF, did not improve *in vitro* the formation of germinal cluster, and therefore seem not to be involved in the reprogramming of SPα-6$^+$c-kit$^+$ cells (Fig. 5f).

Undifferentiated spermatogonia Ap-Al, which are considered to be early steps of differentiation, could still retain stem-cell properties when the intracellular cytoplasmic bridges are broken after irradiation[3,24]. A recent study suggested that actual and potential stem cells could be found in this compartment and that potential stem cells should be the immediate descendants of the stem cells, that is, a fraction of A_s and A_p spermatogonia[25]. However, mouse differentiating spermatogonia are considered so far to be irreversibly committed to differentiation, leading to meiosis and production of spermatozoa[3]. Here we show that c-kit-positive differentiating spermatogonia can reverse their differentiation program and act as functional stem cells, contributing to clonogenic activity and regeneration of spermatogenesis after transplantation in depleted recipient testes.

Recent landmark studies reported the possibility of reprogramming differentiated cells to pluripotency after genetic modification[26,27]. Our results underscore the remarkable plasticity of the late c-kit transit-amplifying progenitors in mouse and the conservation of the de-differentiation ability of spermatogonial progenitors between *Drosophila* and mouse[2]. The testicular niche, which controls the fate of GSCs, could also modulate reversibility of the commitment process of spermatogonial progenitors, notably in the case of tissue injury. After busulphan treatment, damaged testes devoid of germ cells showed a strong increase in GDNF expression[28], which appears to be a crucial factor in the *in vitro* reprogramming of differentiating spermatogonia that we observed. c-kit/KitL could also have a role in the chemotactic migration of differentiating spermatogonia to the niche. In addition, it will be interesting to test the regenerative potential of the A_{al} progenitors of the undifferentiated spermatogonia population, when specific markers are available.

Many unanswered questions remain about the contribution of progenitors to regeneration of the stem-cell pool in normal steady spermatogenesis, about the regenerative potential of specific differentiation stages among the pool of spermatogonial progenitors, and their potential use as an alternative source of cells for regenerative medicine. Moreover, mechanisms regulating this reprogramming are unknown. In this regard, the acquisition of long-term reconstitution capacity by multipotent progenitors in the haematopoietic lineage from triple *p16^{Ink4a}p19^{Arf}Trp53* mutant mice is promising[29]. Whether the ability of spermatogonial progenitors to de-differentiate could be shared by other mammalian self-renewing tissues, remains to be further investigated.

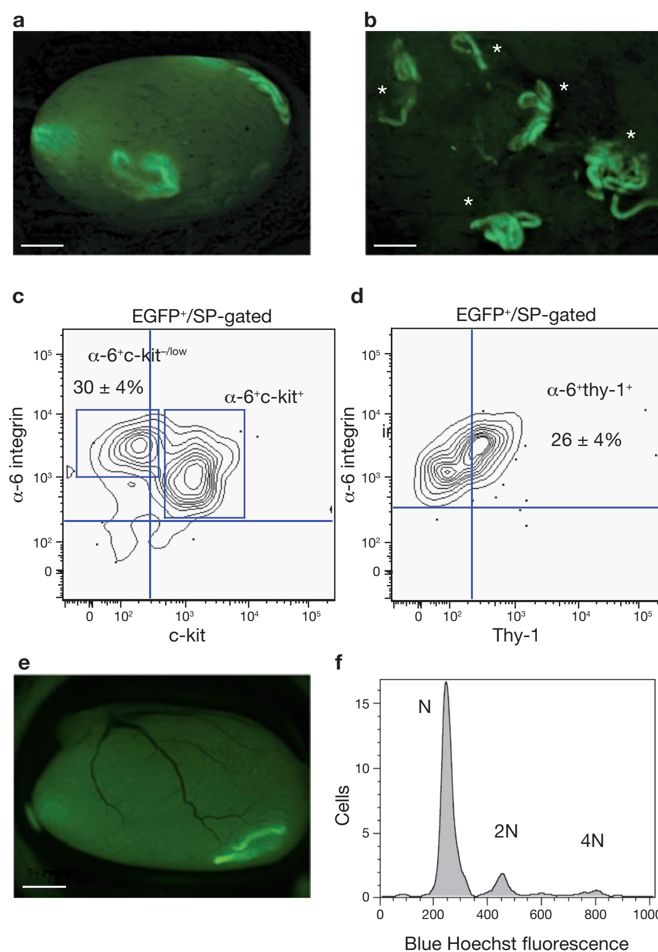

Figure 4 Differentiating c-kit-positive spermatogonia de-differentiate and act as functional GSCs after transplantation. (**a**, **b**) Macroscopic observation of fluorescence in recipient testes (**a**) and whole-mount observation of the seminiferous tubules (**b**) 1 year after transplantation with donor EGFP c-kit positive spermatogonia (asterisks indicate donor-derived EGFP-positive spermatogenic colonies; scale bar, 1 mm). (**c**, **d**) SP/EGFP-positive cells from recipient testes showing α-6 integrin and c-kit expression (**c**), and α-6 integrin and thy-1 (**d**) expression 10 weeks after transplantation with donor EGFP–c-kit positive spermatogonia. α6$^+$c-kit$^+$, α-6$^+$c-kit$^{-/low}$ and α-6$^+$thy-1$^+$ populations are indicated, as the cell distribution of α-6$^+$c-kit$^{-/low}$ and α-6$^+$thy-1$^+$ populations (mean ± s.e.m., $n = 7$). (**e**) Detection of fluorescent seminiferous tubules in a secondary recipient testis after serial transplantation of cells from a primary recipient transplanted with c-kit positive differentiating spermatogonia. Scale bar, 1 mm. (**f**) Hoechst fluorescence analysis of EGFP-positive cells from the secondary recipient testis after serial transplantation. Diploid (2N), meiotic (4N) and postmeiotic (N) cells are indicated.

Nevertheless, the germinal lineage seems to have a remarkable and singular plasticity, compared with other tissues in mice, as also shown by the recent derivation of pluripotent embyonic stem-like cells from adult spermatogonia[30,31], and the switch of developmental fate of spermatogonia into cells with mammary epithelial progenitor properties when transplanted into mammary fat pads[32].

METHODS

Mice. Adult male C57BL/6 (Charles River) and EGFP-transgenic mice[33] (C57BL/6-TgN(β-act–EGFP)01Osb, a gift from M. Okabe, Research Institute for Microbial Diseases, Osaka, Japan) were raised in our animal facility. All animal procedures reported in this paper were carried out in accordance with French

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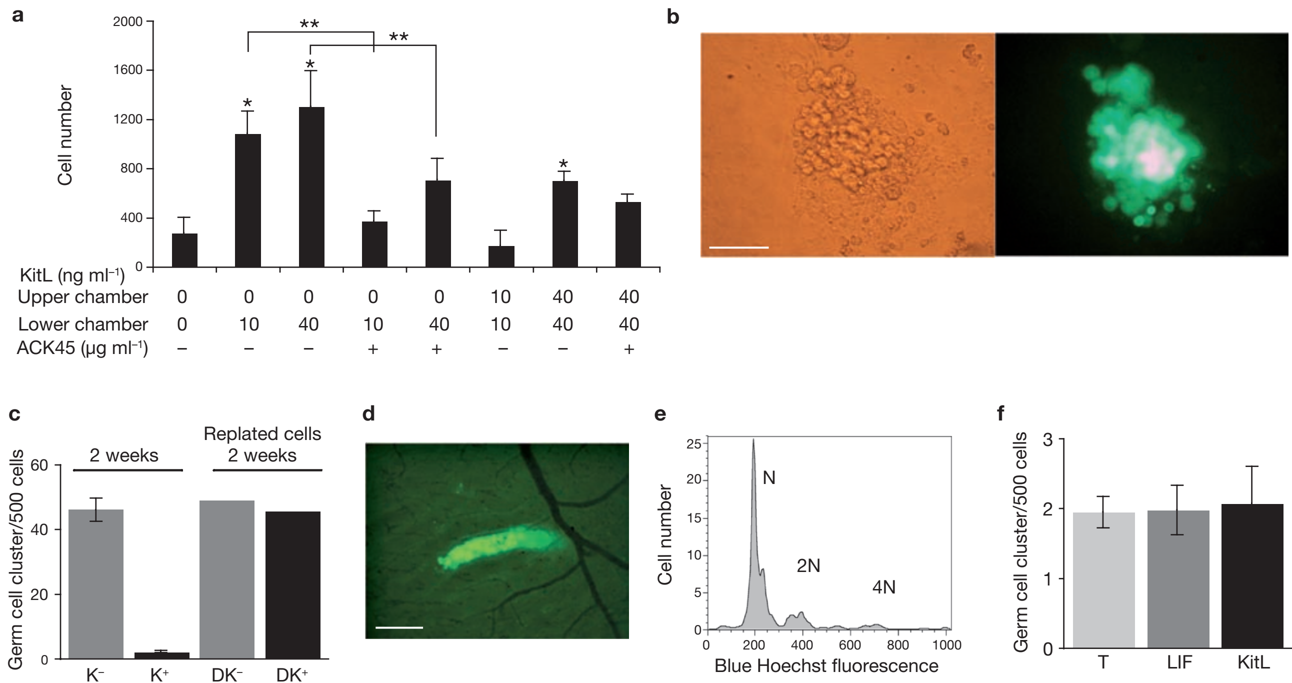

Figure 5 Chemoattraction of c-kit positive spermatogonia by KitL *in vitro*, and generation of germ-cell clusters with long-term clonogenic activity from c-kit positive spermatogonia in culture in presence of GDNF and FGF2. (**a**) *In vitro* migration assay. KitL concentrations in upper and lower chambers are indicated, as well as the addition of ACK45 neutralizing antibody to c-kit in the upper chamber (+). Mean ± s.e.m. of triplicate measurements are shown. Differences are significant (*$P \leq 0.01$) between 0/0 (control) and 0/10, 0/40, 40/40 conditions. Inhibition with the ACK45 neutralizing antibody was significant (**$P < 0.002$) in 0/10, 0/40 conditions (one-way ANOVA test with Fisher's LSD post-hoc). (**b**) *In vitro* cluster formation assay. Phase microscopic (left) and EGFP fluorescence (right) appearance of a germ-cell cluster after 2 weeks of culture of sorted EGFP-positive SPα6$^+$c-kit$^+$ cells in presence of GDNF and FGF2 on feeder cells. Scale bar, 50 μm. (**c**) Number of germ-cell clusters generated after 2 weeks from sorted SPα-6$^+$c-kit$^+$ (K$^+$) and SPα-6$^+$c-kit$^{-/low}$ (K$^-$) cells ($n = 10$), and from 2-week cultures derived SPα-6$^+$c-kit$^+$ cells (DK$^+$) and SPα-6$^+$c-kit$^{-/low}$ cells (DK$^-$) after two weeks, that is, 4 weeks after the initial seeding (mean of $n = 2$). (**d**) Macroscopic observation of fluorescence in recipient testes 10 weeks after transplantation with 4-week-culture cells derived from EGFP-positive SPα-6$^+$c-kit$^+$ spermatogonia. Scale bar, 100 μm. (**e**) Hoechst fluorescence analysis of EGFP-positive cells from the recipient testis after transplantation. Diploid (2N), meiotic (4N) and postmeiotic (N) cells are indicated. (**f**) Number of germ-cell clusters generated from sorted SPα–6$^+$c-kit$^+$ cultivated in presence of GDNF and FGF2 (T, $n = 8$), GDNF, FGF2 and LIF (LIF, $n = 8$), GDNF, FGF2 and KitL (KitL, $n = 4$). Mean ± s.e.m. are shown.

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Flow cytometry analysis, immunomagnetic and flow-cell sorting. Testicular single-cell suspensions from 2–3-month-old mice were obtained as described previously[11]. MACS (Miltenyi Biotech) c-kit-positive fraction of cells was obtained using anti-c-kit microbeads according to the manufacturer's protocol. Hoechst staining of cell suspensions was performed as described previously[11]. Cells were then labelled with monoclonal antibodies (1 μg per 10^6 cells) from BD Pharmingen: anti-c-Kit-biotin (2B8), anti-CD45-PC5 or FITC (30-F11), anti-Thy-1-APC (53-2.1) and anti–α-6 integrin-PE (GoH3). Analyses and cell-sorting were performed on ARIA, LSR and LSR II flow cytometers (Becton Dickinson).

Testicular transplantation and analysis of recipient mice. Flow- and MACS-sorted cellular fractions obtained from EGFP-transgenic males as donors were transplanted through the efferent ductules into testes of C57BL6 germ-cell-depleted recipient mice, except for conditioning of recipient mice, and analysed ten weeks after transplantation, as described previously[10,11]. γ-irradiation (^{137}Cs source, IBL637; CIS Biointernational) with a total dose of 10 Gy was used for recipient conditioning of 10-week-old mice, as a substitute to busulphan treatment. Total bone marrow (10^6 cells) transplantation was performed to support haematopoiesis 24 h after irradiation. The recipient testes were transplanted 4 weeks after irradiation. Efficiency of this conditioning was verified (Supplementary Information, Fig. S4). For serial transplantations, two recipient testes transplanted with EGFP–c-kit-positive cells were removed, and reconstitution was determined. Then, EGFP cells were flow-sorted and 25,000 EGFP cells were transplanted into the testis of a second irradiated recipient.

Reconstitution was monitored 10 weeks after transplantation and recipient testes were collected. Individual EGFP-positive stretches of spermatogenesis are spermatogenetic colonies derived from one single EGFP donor GSC, as previously reported[9,12]. Dissection of the testes and spread-out of the seminiferous tubules were performed under a fluorescent microscope and EGFP colonies were counted. Seminiferous tubules were then collected and cell suspensions were obtained for Hoechst 33342 flow cytometric analysis, as previously described using TruCount microbeads[11]. Statistical analysis was performed using Student's *t* test (unpaired, one-tailed).

RT–PCR. mRNAs were prepared from flow-sorted SPα-6$^+$c-kit$^+$ and SPα-6$^+$c-kit$^{-/low}$ cells using microMACS mRNA isolation kit (Miltenyi), and reverse-transcribed using Quantitect enzyme (Qiagen). The cDNA was then amplified by PCR using the following primers: *Sox3* (U ACAACTCCGAGATCAGCAAGC, L CTTGAGCAGCGTCTTGGTCTT), *NgN3* (U AACAGGCCCAAGAGCGAGTT, L GCCGAGTTGAGGTTGTGCAT), *E-Cad* (U TGAAGGGACGGTCAACAACTG, L GCTCTTTGACCACCGTTCTCC), *GAPDH* (U GAGCGAGACCCCACTAACAT, L TTCACACCCATCACAAACAT). RT–PCR assays were performed twice on each subpopulation purified from three independent sorting experiments.

Migration experiments. Transwell chambers (Costar) were used to assess cell migration through filters (pore size 8 μm). The c-kit-positive spermatogonial fraction was purified by MACS after a negative enrichment using the dead-cell removal kit (Miltenyi). For ligand-dependent cell migration, 10^5 cells were plated with Stemspan medium (Stemcell Technologies) supplemented with B27 (Gibco)

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onto polycarbonate filters pre-coated with laminin (4 μg/cm^2; Sigma). KitL was added to the bottom chamber or to both bottom and top chambers at either 10 or 40 ng ml^{-1}. ACK45 neutralizing antibody to c-kit (5 μg ml^{-1}) was added to the top chamber when necessary. After a 12-h incubation, cells in the bottom of the chamber were stained with Hoechst 33342 (5 μg ml^{-1}) and spermatogonial cell number was quantified by flow cytometry using the TruCount (BD Biosciences) methodology. Elongated spermatids were eliminated by FSC-gating. Each experiment was repeated three times in triplicate.

Long-term *in vitro* culture of adult GSCs and c-kit-positive spermatogonial fractions. We developed a culture method for adult GSC based on previous work[22], with slight modifications. For the *in vitro* cluster formation assay, sorted EGFP-positive SPα-6$^+$c-kit$^+$ and SPα-6$^+$c-kit$^{-/low}$ were cultured in 96-well tissue culture plates on a feeder layer of mouse embryonic fibroblasts, mitotically inactivated after mitomycin C (1 μg ml^{-1}) treatment. The GSC medium was composed of StemSpan (Stem cell) and B27 supplement (Invitrogen). Recombinant human GDNF (40 ng ml^{-1}, R&D Systems), recombinant rat GFRα1 (300 ng ml^{-1}, R&D Systems) and FGF2 (1 ng ml^{-1}, Invitrogen) were added, as described previously[22]. KitL (100 ng ml^{-1}, R&D Systems) and mouse LIF (1000 U ml^{-1}, Millipore) were added when indicated. SPα-6$^+$c-kit$^{-/low}$ and SPα-6$^+$c-kit$^+$ cells (500 and 2000, respectively) were flow-sorted and seeded per well. After 2 weeks of culture, EGFP cell clusters were counted. Cell clusters were then digested using 0.25% trypsin-EDTA and subcultured by direct flow-sorting of cells according to EGFP fluorescence in 96-well tissue culture plates (500 cells per well) and cultured for an additional 2 weeks.

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS
V.B. and B.L. contributed equally to the experimental work, with help from M.C., L.R.and P.F.; V.B., B.L., L.R. and P.F. conceived and designed the experiments; J.P.L. provided valuable material and initiated the breeding of the EGFP mice; F.L.P., J.T., I.A., B.L. and L.R. revised the manuscript. All authors participated in data analysis. P.F. coordinated the study; P.F. and V.B. wrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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1. Kai, T. & Spradling, A. Differentiating germ cells can revert into functional stem cells in *Drosophila melanogaster* ovaries. *Nature* **428,** 564–569 (2004).
2. Brawley, C. & Matunis, E. Regeneration of male germline stem cells by spermatogonial dedifferentiation *in vivo*. *Science* **304,** 1331–1334 (2004).
3. de Rooij, D. G. Proliferation and differentiation of spermatogonial stem cells. *Reproduction* **121,** 347–354 (2001).
4. Schrans-Stassen, B. H., van de Kant, H. J., de Rooij, D. G. & van Pelt, A. M. Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. *Endocrinology* **140,** 5894–5900 (1999).
5. Ohta, H., Yomogida, K., Dohmae, K. & Nishimune, Y. Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF. *Development* **127,** 2125–2131 (2000).
6. Yoshida, S. *et al.* Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. *Dev. Biol.* **269,** 447–458 (2004).
7. Raverot, G., Weiss, J., Park, S. Y., Hurley, L. & Jameson, J. L. Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. *Dev. Biol.* **283,** 215–225 (2005).
8. Tokuda, M., Kadokawa, Y., Kurahashi, H. & Marunouchi, T. CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. *Biol. Reprod.* **76,** 130–141 (2007).
9. Shinohara, T., Orwig, K. E., Avarbock, M. R. & Brinster, R. L. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. *Proc. Natl Acad. Sci. USA* **97,** 8346–8351 (2000).
10. Bastos, H. *et al.* Flow cytometric characterization of viable meiotic and post-meiotic cells by Hoechst 33342 in mouse spermatogenesis. *Cytometry A* **65,** 40–49 (2005).
11. Lassalle, B. *et al.* 'Side population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. *Development* **131,** 479–487 (2004).
12. Zhang, X., Ebata, K. T. & Nagano, M. C. Genetic analysis of the clonal origin of regenerating mouse spermatogenesis following transplantation. *Biol. Reprod.* **69,** 1872–1878 (2003).
13. Shinohara, T., Avarbock, M. R. & Brinster, R. L. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. *Proc. Natl Acad. Sci. USA* **96,** 5504–5509 (1999).
14. Kubota, H., Avarbock, M. R. & Brinster, R. L. Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. *Biol. Reprod.* **71,** 722–731 (2004).
15. Shinohara, T., Orwig, K. E., Avarbock, M. R. & Brinster, R. L. Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. *Proc. Natl Acad. Sci. USA* **98,** 6186–6191 (2001).
16. Falciatori, I. *et al.* Identification and enrichment of spermatogonial stem cells displaying side-population phenotype in immature mouse testis. *FASEB J.* **18,** 376–378 (2004).
17. Kubota, H., Avarbock, M. R. & Brinster, R. L. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. *Proc. Natl Acad. Sci. USA* **100,** 6487–6492 (2003).
18. Chen, C. *et al.* ERM is required for transcriptional control of the spermatogonial stem cell niche. *Nature* **436,** 1030–1034 (2005).
19. Meng, X. *et al.* Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* **287,** 1489–1493 (2000).
20. Bedell, M. A. & Mahakali Zama, A. Genetic analysis of Kit ligand functions during mouse spermatogenesis. *J. Androl.* **25,** 188–199 (2004).
21. Heissig, B. *et al.* Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* **109,** 625–637 (2002).
22. Kubota, H., Avarbock, M. R. & Brinster, R. L. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc. Natl Acad. Sci. USA* **101,** 16489–16494 (2004).
23. Yeh, J. R., Zhang, X. & Nagano, M. C. Establishment of a short-term *in vitro* assay for mouse spermatogonial stem cells. *Biol. Reprod.* **77,** 897–904 (2007).
24. de Rooij, D. G. & Grootegoed, J. A. Spermatogonial stem cells. *Curr. Opin. Cell Biol.* **10,** 694–701 (1998).
25. Nakagawa, T., Nabeshima, Y. & Yoshida, S. Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. *Dev. Cell* **12,** 195–206 (2007).
26. Hanna, J. *et al.* Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. *Cell* **133,** 250–264 (2008).
27. Aoi, T. *et al.* Generation of pluripotent stem cells from adult mouse liver and stomach cells. *Science* **26,** 101–106 (2008).
28. Ryu, B. Y., Orwig, K. E., Oatley, J. M., Avarbock, M. R. & Brinster, R. L. Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. *Stem Cells* **24,** 1505–1511 (2006).
29. Akala, O. O. *et al.* Long-term haematopoietic reconstitution by $Trp53^{-/-}$ $p16^{Ink4a-/-}p19^{Arf-/-}$ multipotent progenitors. *Nature* **453,** 228–232 (2008).
30. Guan, K. *et al.* Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* **440,** 1199–1203 (2006).
31. Seandel, M. *et al.* Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. *Nature* **449,** 346–350 (2007).
32. Boulanger, C. A., Mack, D. L., Booth, B. W. & Smith, G. H. Interaction with the mammary microenvironment redirects spermatogenic cell fate *in vivo*. *Proc. Natl Acad. Sci. USA* **104,** 3781–3786 (2007).
33. Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. & Nishimune, Y. 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* **407,** 313–319 (1997).

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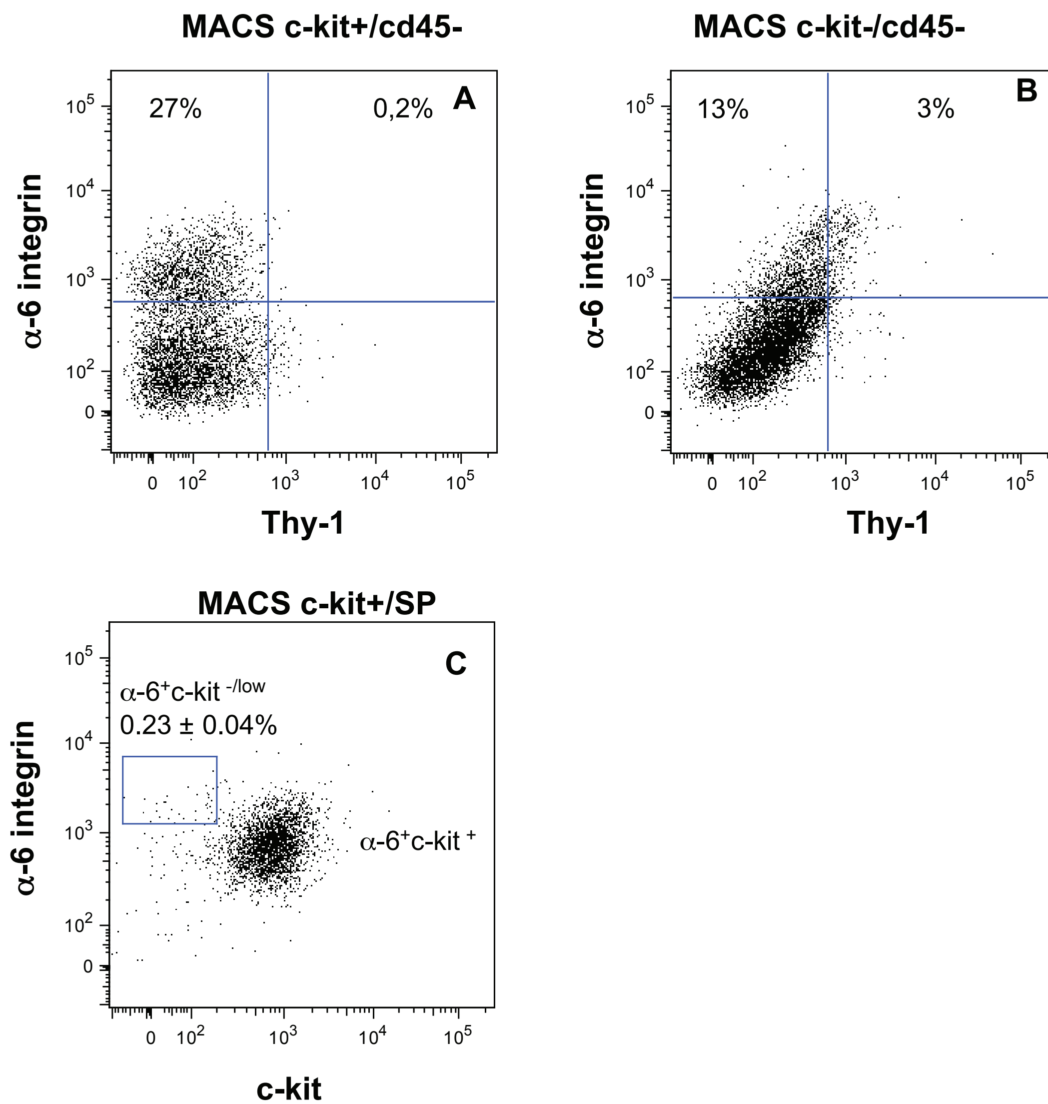

Figure S1 GSCs are found at a very low frequency in the MACS c-kit-positive cell population as shown by the absence of the joint expression of the GSC markers Thy-1 and α-6 integrin. Thy-1 and α-6 integrin expression in CD45 negative MACS c-kit-positive cell population **(a)** and CD45 negative MACS c-kit-negative cell population **(b)**. As expected, $Thy\text{-}1^+$ $\alpha\text{-}6^+$ positive cells were present at a low frequency (3%) in the c-kit-negative fraction. Quadrants were placed according to the IgG controls. Flow cytometry data are representative of three independent experiments. **(c)**. c-kit and α -6 integrin expression in MACS c-kit-positive cell population gated on the Side Population. The frequency of $SP\alpha\text{-}6^+c\text{-}kit^{-/low}$ cells was 0.23 ± 0.04 % (n=4), corresponding to 230 cells for 10^5 donor cells. Given that the stem cell activity of the $SP\alpha\text{-}6^+c\text{-}kit^{-/low}$ is 151 colonies/10^5 donor cells (see Figure 2), 230 $SP\alpha\text{-}6^+c\text{-}kit^{-/low}$ contaminating cells would generate only 0.35 clone (230 X 151 /10^5 clones), and this contribution is minor compared to the 4.7 clones generated from 10^5 $SP\alpha\text{-}6^+c\text{-}kit^+$ donor cells. Therefore, the contamination by GSCs cannot be responsible for the regeneration that we observed for differentiating spermatogonia.

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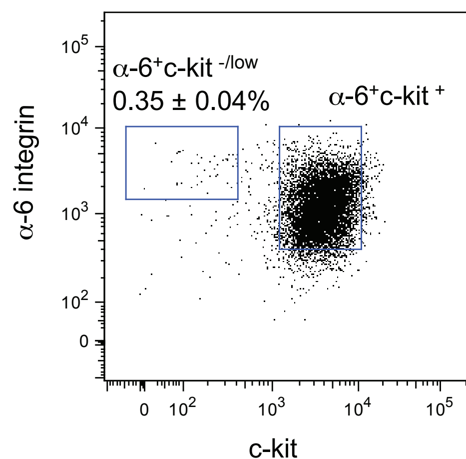

Figure S2 Analysis by flow cytometry of the sorted SPα-6^+c-kit$^+$ fraction according to α-6 integrin and c-kit expression, in order to determine the contamination by SPα-6^+c-kit$^{-/low}$ cells (containing GSC) before transplantation. Cells are gated on SP. α6+c-kit+ and α-6^+c-kit$^{-/low}$ populations and cell distribution are indicated (mean ± SEM , n=7).

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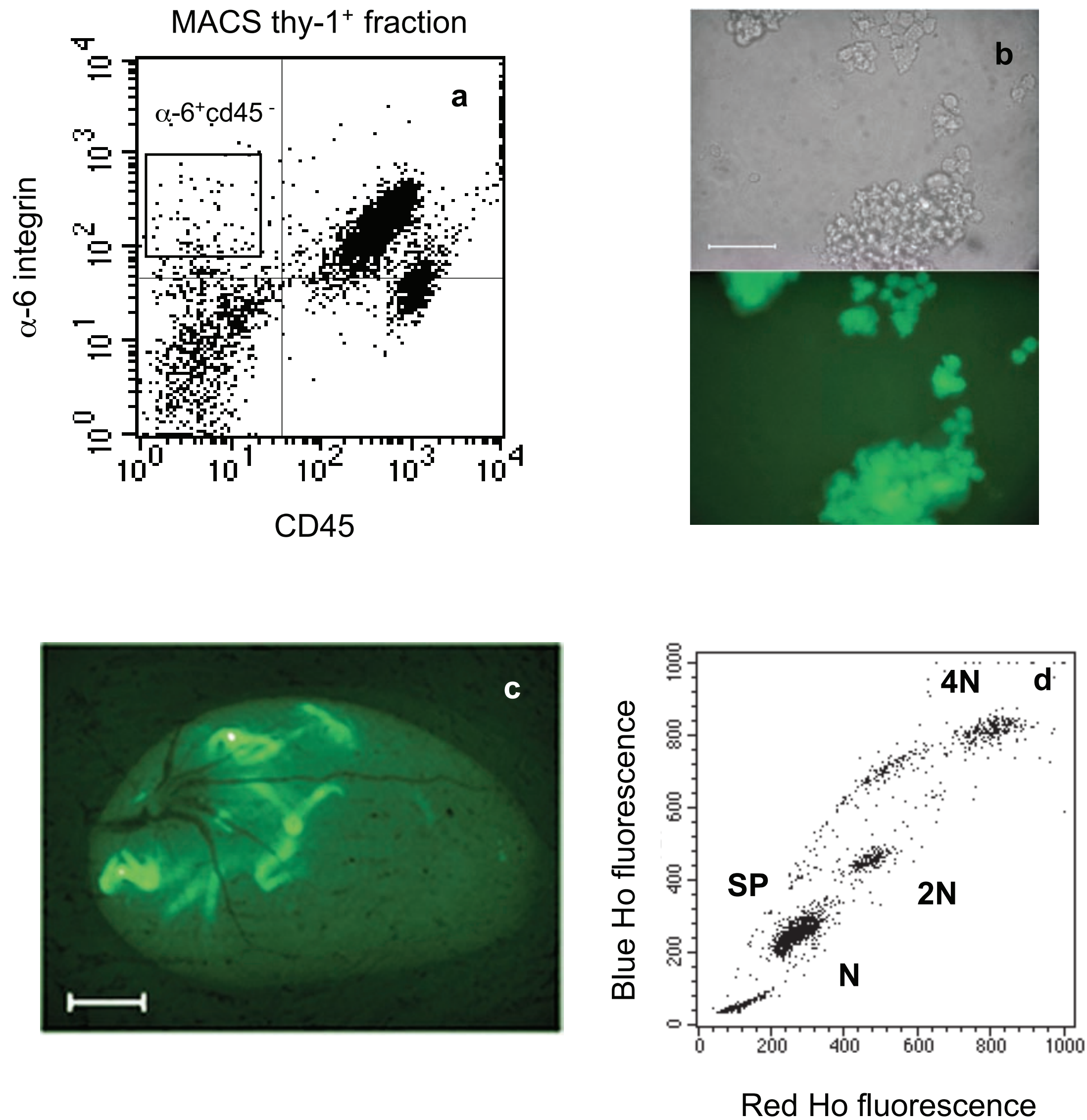

Figure S3 Long term growth of adult GSC. **a**, α-6 integrin and CD45 expression analysis of MACS Thy-1 positive cell fraction from adult testis. Thy-1⁺α-6⁺cd45⁻ cells were flow sorted and cultivated on MEF feeder in the GSC medium supplemented with GDNF, GFRalpha1 and FGF2. **b**, Observation of three dimensional germ cell cluster after 1 month of culture. Upper, phase microscopy. Lower, EGFP fluorescence. (Scale bar, 50 μm). **c**, Adult GSCs after two months of culture have the potential to regenerate spermatogenesis. Detection of fluorescent seminiferous tubules in a recipient testis transplanted with EGFP GSC cultivated two months as determined by fluorescence microscopic analysis of whole testis 10 weeks after transplantation. (Scale bar, 1 mm). **d**, Hoechst fluorescence (red/blue Hoechst fluorescence) analysis of EGFP positive cells in recipient testis from Fig. 1c shows that regenerated spermatogenesis is normal. Side Population (SP), meiotic spermatocytes I (4N) and spermatocytes II (2N) and postmeiotic spermatids (N) are indicated.

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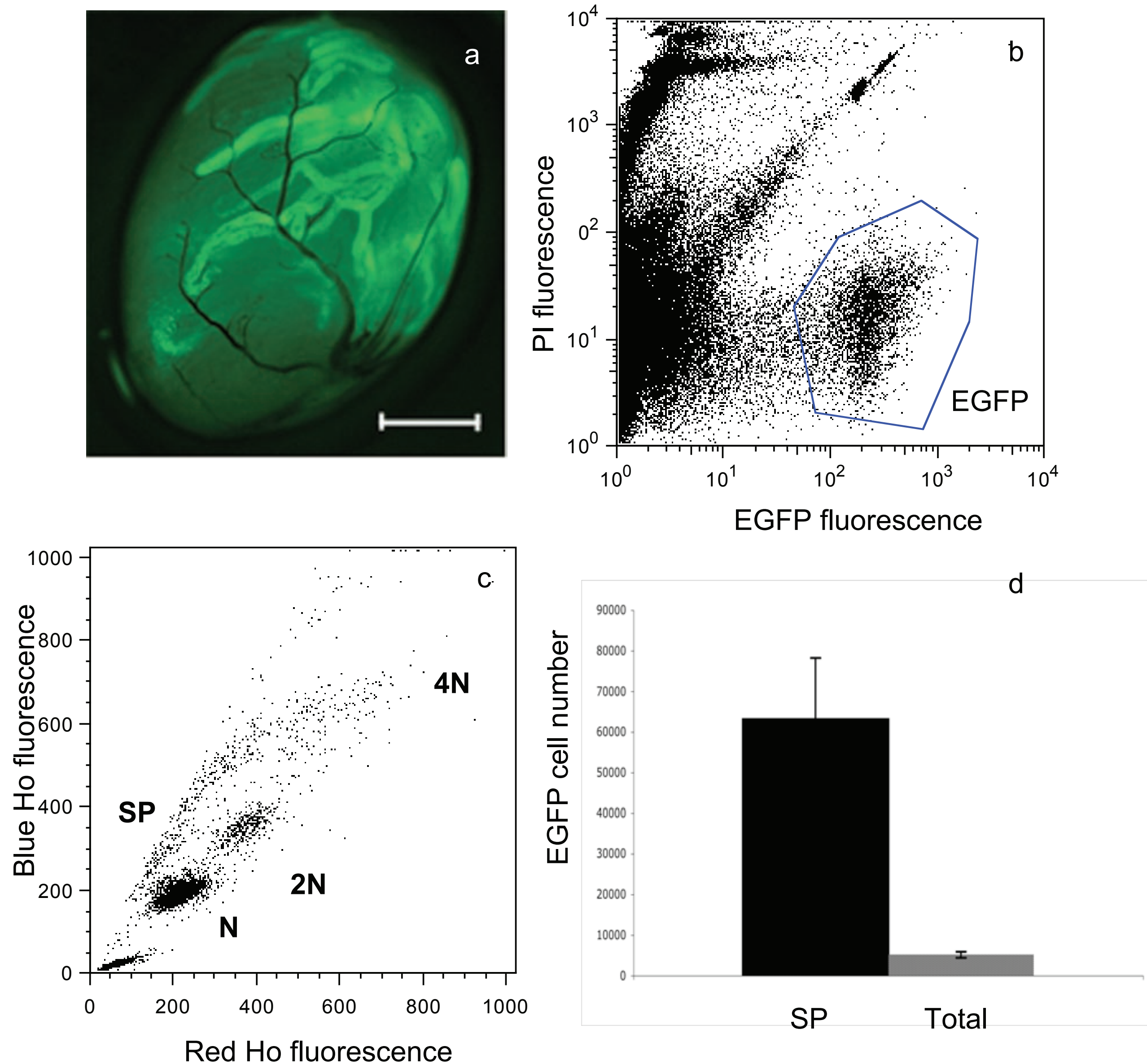

Figure S4 Efficiency of the γ-irradiation conditioning of recipient testis for the transplantation and regeneration of spermatogenesis. **a**, Detection of fluorescent seminiferous tubules in a recipient testis transplanted with EGFP total testicular cells 10 weeks after transplantation. (Scale bar, 1mm). **b**, EGFP and propidium iodide (PI) fluorescence analysis of testicular cell suspension from recipient testis in order to determine EGFP positive cell number per recipient testis by flow cytometry. EGFP positive cells (EGFP) are boxed **c**, Hoechst fluorescence (red/blue Hoecsht fluorescence) analysis of EGFP positive cells in recipient testis gated from Fig. S4b. Side Population (SP), meiotic spermatocytes I (4N) and spermatocytes II (2N) and postmeiotic spermatids (N) are indicated. **d,** Enhanced colonization of recipient testis by transplanted SP cells compared to total population. EGFP positive cell number per recipient testis was normalized to 10^5 cells injected. The values are mean ± SEM (n=3). The difference is significant ($P < 0.03$).

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Number of EGFP SPα-6$^+$c-kit$^{-/low}$ cells	Number of SPα-6$^+$c-kit$^+$ cells	Contamination level (%)	Number of EGFP clusters/500 cells (mean ± SEM)
8	2000	0.4	0.12 ± 0.04

Table S1 Number of clusters generated after 8 days of culture by SPα-6$^+$c-kit$^{-/low}$ cells in a mixture of C57Bl6J SPα-6$^+$c-kit$^+$ and EGFP SPα-6$^+$c-kit$^{-/low}$ cells. This mixture reproduces the level of contamination (0.4 %) by SPα-6$^+$c-kit$^{-/low}$ cells in flow sorted SPα-6$^+$c-kit$^+$ experiments. The cells were cultivated on MEF feeder which supports long term growth of adult GSCs in presence of GDNF, FGF2, and GFRalpha1, as *in vitro* germinal cluster formation assay provides a reliable technique to detect GSCs within one week [23]. After 8 days of culture, the number of EGFP germ cell clumps was determined. The frequency of EGFP clones was 0.12 ± 0.04 (n=15). Thus, SPα-6$^+$c-kit$^{-/low}$ contamination can not be responsible for the formation of the germ cell clumps observed with SPα-6$^+$c-kit$^+$ cells (2 ± 0.3 clusters/500 cells).

This result (0.12 clusters/500 input cells) is very close to the expected contribution of the contamination with SPα-6$^+$c-kit$^{-/low}$ cells that we calculated: 0.16 cluster/500 input cells ([0.35 X 500/100] X [46/500] clusters). Hence, it validates our methodology to calculate the expected contribution of the SPα-6$^+$c-kit$^{-/low}$ cells contamination to the cluster formation assay and to the regenerative capacity of the SPα-6$^+$c-kit$^+$ cells.

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