fractions when sorted cells were collected from recipients that had received donor cells within 4 weeks (Figure 4F and G). The number of colonies generated was 3.45 ± 0.64 ($n=23$) and 1.07 ± 0.19 ($n=25$)/10^4 injected cells for Kit^- and Kit^+ cells, respectively. Although the difference was statistically significant, SSCs expressing Kit were found in 5 of 6 experiments. In contrast, $GFR\alpha1^+$ cells were significantly enriched for SSCs, and results from three experiments showed that the numbers of colonies were 0.3 ± 0.1 and $11.5\pm2.0/10^4$ injected cells ($n=15$) for $GFR\alpha1^-$ and $GFR\alpha1^+$ cells, respectively (Figure 4F and G).

When sorted cells were collected from primary recipients between 3 and 4 months after transplantation, results from two experiments showed that the difference in SSC activity became more pronounced and the average numbers of colonies were 0.37 ± 0.08 ($n=8$) and 0.03 ± 0.03 ($n=10$)/10^4 injected cells for Kit^- and Kit^+ cells, respectively. In contrast, SSCs were consistently positive for GFRα1, and 2.5 ± 1.0 colonies/10^4 injected cells ($n=10$) were generated only from $GFR\alpha1^+$ cells. Immunohistological staining of the recipient testes showed normal spermatogenesis from both Kit^- and Kit^+ cells. No significant differences in SYCP3 (meiotic cell marker) or PNA (acrosome marker) expression patterns were observed (Figure 4H). These results show that SSCs also change Kit expression levels during regeneration in vivo.

Discussion

Although both phenotypic and functional analyses suggested that most GS cells are progenitors without SSC activity, single-cell cloning experiments in our previous study showed that a significant proportion of GS cells maintain a potential to function as SSCs [16]. The current study was initiated to resolve the discrepancy between these findings, and we provide evidence that SSCs change their phenotype according to their microenvironment. Our conclusion was supported by our two transplantation experiments. First, in GS cell culture, Kit^+ cells proliferated as actively as Kit^- cells and frequency of SSCs was comparable between the two populations. Second, immediately after transplantation, we found weaker but distinct SSC activity in the Kit^+ donor cell population. These findings contrast with previous observations that SSCs do not express Kit. They also suggest that SSCs in vitro probably do not follow traditional scheme of SSC self-renewal [1,2].

One of the important factors that contributed to phenotypic changes was laminin. Several lines of evidence have suggested that laminin plays critical roles in SSC biology. First, SSCs express both α6- and β1-integrin strongly and preferentially attach to laminin compared with other extracellular matrix substrates in vitro [12]. Second, β1-integrin-deficient SSCs that failed to attach to laminin could not settle in the germline niche [6]. Third, SSCs from mice, rats and hamsters all proliferate on laminin for several months without losing germline potential, suggesting that the ability to bind to laminin is beneficial and conserved among species [15,24,25]. Therefore, we speculated that integrin-laminin interactions in vitro might partly mimic stem cell-niche interactions in vivo, and assumed that culturing on laminin would create a more hospitable environment for SSCs. Given these results, we did not expect that GS cells on laminin would strongly upregulate Kit, a marker of differentiating spermatogonia.

Another factor that influenced SSC phenotype was plating density. Cell density or shape has been shown to influence many biological processes, including the lineage-specific marker expression or differentiation of stem cells. For example, changes in mechanical tension mediated by RhoA-ROCK signaling pathway regulated the fate commitment of mesenchymal stem cells (MSC)[19]. Dominant-negative RhoA committed MSCs to become adipocytes, whereas constitutive-active RhoA caused osteogenesis. Low plating density also enhanced their proliferation. In contrast, GS cells proliferated more slowly at low density, but cytochalasin D or transfection with dominant-negative RhoA reduced Kit expression, suggesting the involvement of actin cytoskeleton in Kit expression. This finding suggests the importance of cell structure and mechanics in the modulation of SSC phenotype and heterogeneity.

Our retransplantation experiments showed that SSCs also change their phenotype in vivo. Retransplantation is a unique model to study SSC regeneration, because it allows SSCs to increase their number in vivo [26]. In normal testes, SSCs are kept under constant pressure to differentiate to produce sperm. SSCs undergo only two types of cell division, and they produce either two stem cells or two progenitor cells [1,2]. However, the concentration of GDNF in the Sl^d or W testis is upregulated by a deficiency of endogenous germ cells [27], and this probably promoted transplanted SSCs to preferentially undergo symmetric self-renewal divisions to fill empty niches. Indeed, undifferentiated spermatogonia in Sl^d mice take up BrdU more rapidly than those in WT mice [27]. However, as SSCs gradually repopulate to establish normal cycles of spermatogenesis with time, the probability of self-renewing division progressively decreases by downregulation of GDNF and they no longer exhibit an activated phenotype. On the other hand, in other models used to study SSC regeneration, such as experimental cryptorchidism or vitamin A deficiency [5,10], the number of SSCs remains constant, and this may explain why these treatments could not induce Kit in undifferentiated spermatogonia. Based on these observations, we suggest that, when SSCs are relieved from steady state kinetics, such as after germ cell transplantation or in vitro culture, they may be exempted from required differentiation and are induced to express Kit (Figure 5).

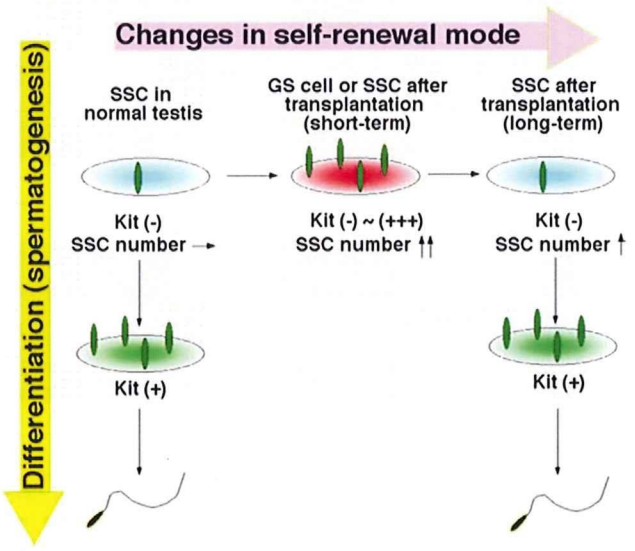

Figure 5. Expression of Kit on SSCs during active proliferation. SSCs in normal testes do not express Kit and maintain a constant number (non-activated state). However, when SSCs increase their number during culture or soon after transplantation, they upregulate Kit (activated state). Kit is downregulated in SSCs when germ cell colonies resume normal spermatogenesis.
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In some respects, our observation is reminiscent of the Clermont model of spermatogonial renewal, which proposes that A_1–A_4 spermatogonia, all of which express Kit, form a loop by recruiting a part of A_4 into A_1 [28,29]. The loop proposed by this model is limited within A_1–A_4 spermatogonia, and SSCs are thought to divide only when there is a problem in A_4 to A_1 transition. This model opposes the single stem cell (A_s) model, in which A_s spermatogonia differentiate unidirectionally. Although the experimental evidence favors the A_s model, studies in Drosophila initially showed that differentiated spermatogonia can dedifferentiate to form stem cells [30], and similar observations were also reported in mice. By taking advantage of lineage tracing, one study showed that undifferentiated spermatogonia that had already committed to differentiation reverted to SSCs [31]. Another study also showed that Kit^+ differentiating spermatogonia in the "side population", defined by the higher efflux of DNA-binding dye Hoeschst 33342, have SSC activity [32]. It will be interesting to study whether Kit^+ cells also developed from progenitor cells in GS cell culture.

Since the development of germ cell transplantation technique, SSC phenotype was thought to be fixed, and SSCs have been isolated in deterministic manner. However, our analyses now show that phenotype of SSCs can change according to their microenvironment. Thus, caution is necessary when analyzing SSCs without functional assay. Because effects of enzymatic digestion on surface antigens cannot be excluded, different experimental approaches are required to test our hypothesis that activated and non-activated SSCs show distinct phenotypes. Identifying SSC-specific markers and factors that influence the mechanism of fate commitment in vitro will have important implications in studies of stem cells in other self-renewing tissues.

Materials and Methods

Ethics Statement

We followed the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, and all of the protocols for animal handling and treatment were reviewed and approved by the Animal Care and Use Committee of Kyoto University.

Cell Culture

GS cells used in the present study were derived from a transgenic mouse line C57BL/6 Tg14(act-EGFP)OsbY01 that was backcrossed to DBA/2 background. The method for GS cell culture using StemPro-34 SFM (Invitrogen, Carlsbad, CA) was described previously [13]. For laminin culture, GS cells were transferred on dishes that had been coated with 20 μg/ml laminin (BD Biosciences, Franklin Lakes, NJ) for 2 h at room temperature [15]. For transfection, cDNAs encoding mouse Kit-G559 (a gift from Dr. T. Tsujimura, Hyogo College of Medicine), and dominant-negative RhoA-N19 (a gift from Dr. D. M. Pirone, University of Pennsylvania) was cloned into pCAG-IRES2-neo, whereas cDNA mouse Sl (a gift from Dr. Y. Matsui, Tohoku University) was cloned into a CSII-EF-IRES2-puro lentivirus vector. Virus particles were produced by transient transfection of 293T packaging cells, as previously described [25]. Transfected cells were selected by 40–120 μg/ml G418 (Invitrogen) or 110 ng/ml puromycin (Sigma, St. Louis, MO)[16,33]. ISCK03 was added at 1 or 5 μM (EMD Chemicals, San Diego, CA). F-36P cells (a gift from Dr. I. Matsumura, Osaka University) were maintained in RPMI supplemented with 10% fetal bovine serum (FBS). Increases in cell number were measured 5 days after initiation, whereas F-36P cells were cultured for 3 days.

Animals and Transplantation

W and Sl^d mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). For transplantation of cultured cells, cells were incubated with 0.25% trypsin/1 mM EDTA to obtain single-cell suspensions. For serial transplantation, testis cells from primary recipients were dissociated at indicated time points with a two-step digestion method using type IV collagenase and trypsin (both from Sigma), as described [4]. Donor cells were introduced into seminiferous tubules of W or Sl^d mice via efferent duct (4–6 weeks old). Approximately 4 μl of the donor cell suspension could be injected. To avoid rejection of donor cells, recipient animals were treated with anti-CD4 antibody (GK1.5, gift from Dr. T. Honjo, Kyoto University), as described previously [34].

Cell Staining and Selection

Dissociated cells were suspended (5×10^6 cells/ml) in 1 ml of phosphate buffered saline containing 1% FBS (PBS/FBS). Cells were then incubated with primary antibodies for 20 min on ice, washed twice with PBS/FCS, and used for cell separation. Primary antibodies used in this study were anti-rat GFRα1 (81401; R&D systems, Minneapolis, MN), R-phycoerythrin (PE) or allophycocyanin (APC)-conjugated rat anti-mouse Kit (2B8; BD Biosciences), APC-conjugated anti-rat α6-integrin (GoH3; BioLegend, San Diego, CA), anti-mouse E-cadherin (ECCD2; Takara Biomedicals, Shiga, Japan) and biotinylated anti-mouse β1-integrin (Ha2/5, BD Biosciences). For MACS, cells were further incubated for 20 min with Dynabeads M-450 sheep anti-rat IgG (Invitrogen) with agitation, and target cells were separated according to the manufacturer's protocol. For flow cytometric analysis and sorting, APC-conjugated streptavidin, and ant-mouse or -rat IgG (all from BD Biosciences) were used as secondary reagents. After the final wash, 1 μg/ml of propidium iodide was added to samples to eliminate dead cells. Stained cells were analyzed by FACSCalibur or sorted by FACSAria II (both from BD Biosciences).

Analyses of Recipient Testes

The number of colonies was counted under a stereomicroscope equipped with UV light. We defined a donor cell cluster as a colony when it occupied the entire basal surface of the tubule and was longer than 0.1 mm. For immunohistological staining, the recipient testes were fixed in 4% paraformaldehyde and then frozen in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) for cryosectioning. The slides were analyzed under confocal laser scanning microscopy. Meiosis was detected by immunofluorescence using anti-synaptonemal complex protein 3 (SYCP3) antibodies, which was prepared in our laboratory using a synthetic oligopepetide [35]. The anti-SYCP3 antibody was detected by Alexa 488-conjugated anti-rabbit immunoglobulin G antibodies (Molecular Probes, Eugene). Rhodamine-conjugated Peanut agglutinin (PNA) was used to detect acrosomes (Vector, Burlingame, CA). For preparation of paraffin slides, testis samples were fixed in 10% neutral-buffered formalin and processed for paraffin sectioning. Sections were stained with hematoxylin and eosin.

Western Blot Analysis

Samples were separated by SDS/PAGE, transferred to Hybond-P membranes (Amersham Biosciences, Buckinghamshire, UK), and incubated with anti-phospho-Akt (Ser 473) or anti-phospho-c-kit

(Tyr 719) antibody. After washing, peroxidase-conjugated anti-rabbit IgG was used as the secondary antibody (all from Cell Signaling, Danvers, MA).

Real-Time PCR

Total RNA was isolated using Trizol reagent (Invitrogen). First-strand cDNA was synthesized using Superscript^{TM} II (RNase H^{-} reverse transcriptase, Invitrogen). For quantification, StepOnePlus^{TM} Real-Time PCR system and *Power* SYBR Green PCR Master Mix were used according to the manufacturer's protocol (Applied Biosystems, Warrington, UK). Transcript levels were normalized to those of Hprt1. PCR conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. Experiments were performed on each subpopulation purified from three independent sorting experiments. Each PCR was run at least in triplicate using specific primers (Table S1).

Statistical Analysis

Results are presented as mean±SEM. Data were analyzed by Student's *t*-tests. Significant difference in the ISCK03 effect was determined by Tukey's HSD multiple comparisons test.

Supporting Information

Table S1 Real-time PCR primers used in the experiments. Found at: doi:10.1371/journal.pone.0007909.s001 (0.04 MB DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: TS. Performed the experiments: HM MKS SC TS. Analyzed the data: HM MKS NN TS. Contributed reagents/materials/analysis tools: ST MT TS. Wrote the paper: MKS TS.

References

1. Meistrich ML, van Beek MEAB (1993) Spermatogonial stem cells. In: Desjardins C, Ewing LL, eds. Cell and Molecular Biology of the Testis. New York: Oxford University Press. pp 266–295.
2. de Rooij DG, Russell LD (2000) All you wanted to know about spermatogonia but were afraid to ask. J Androl 21: 776–798.
3. Oatley JM, Brinster RL (2008) Regulation of spermatogonial stem cell self-renewal in mammals. Annu Rev Cell Dev Biol 24: 263–286.
4. Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci USA 91: 11298–11302.
5. Shinohara T, Orwig KE, Avarbock MR, Brinster RL (2000) Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc Natl Acad Sci USA 97: 8346–8351.
6. Kanatsu-Shinohara M, Takehashi M, Takashima S, Lee J, Morimoto H, et al. (2008) Homing of mouse spermatogonial stem cells to germline niche depends on β1-integrin. Cell Stem Cell 3: 533–542.
7. Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, et al. (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287: 1489–1493.
8. He Z, Jiang J, Hofmann MC, Dym M (2007) Gfra1 silencing in mouse spermatogonial stem cells results in their differentiation via the inactivation of ret tyrosine kinase. Biol Reprod 77: 723–733.
9. Yoshinaga K, Nishikawa S, Ogawa M, Hayashi S, Kunisada T, et al. (1991) Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. Development 113: 689–699.
10. Schrans-Stassen BHGJ, van de Kant HJG, de Rooij DG, van Pelt AMM (1999) Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. Endocrinology 140: 5894–5900.
11. Ohta H, Yomogida K, Dohmae K, Nishimune Y (2000) Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF. Development 127: 2125–2131.
12. Shinohara T, Brinster RL (2000) Functional analysis of spermatogonial stem cells in Steel and cryptorchid infertile mouse models. Dev Biol 220: 401–411.
13. Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, et al. (2003) Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod 69: 612–616.
14. Kanatsu-Shinohara M, Ogonuki N, Iwano T, Lee J, Kazuki Y, et al. (2005) Genetic and epigenetic properties of mouse male germline stem cells during long-term culture. Development 132: 4155–4163.
15. Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, et al. (2005) Long-term culture of mouse male germline stem cells under serum- or feeder-free conditions. Biol Reprod 72: 985–991.
16. Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2005) Genetic selection of mouse male germline stem cells in vitro: Offspring from single stem cells. Biol Reprod 72: 236–240.
17. Braydish-Stolle L, Kostereva N, Dym M, Hofmann MC (2007) Role of Src family kinases and N-Myc in spermatogonial stem cell proliferation. Dev Biol 304: 34–45.
18. Lee J, Kanatsu-Shinohara M, Inoue K, Ogonuki N, Miki H, et al. (2007) Akt mediates self-renewal division of mouse spermatogonial stem cells. Development 134: 1853–1859.
19. McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS (2004) Cell shape, cytoskeletal tension, and rhoA regulate stem cell lineage commitment. Dev Cell 6: 483–495.
20. Chiba S, Takaku F, Tange T, Shibuya K, Misawa C, et al. (1991) Establishment and erythroid differentiation of a cytokine-dependent human leukemic cell line F-36: a parental line requiring granulocyte-macrophage colony-stimulating factor or interleukin-3, and a subline requiring erythropoietin. Blood 78: 2261–2268.
21. Brannan CI, Lyman SD, Williams DE, Eisenman J, Anderson DM, et al. (1991) Steel-Dickie mutation encodes a c-kit ligand lacking transmembrane and cytoplasmic domain. Proc Natl Acad Sci USA 88: 4671–4674.
22. Kitayama H, Kanakura Y, Furitsu T, Tsujimura T, Oritani K, et al. (1995) Constitutively activating mutations of c-kit receptor tyrosine kinase confer factor-independent growth and tumorigenicity of factor-dependent hematopoietic cell lines. Blood 85: 790–798.
23. Nagano M, Avarbock MR, Brinster RL (1999) Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. Biol Reprod 60: 1429–1436.
24. Hamra FK, Chapman KM, Nguyen DM, Williams-Stephens AA, Hammer RE, et al. (2005) Self-renewal, expansion, and transfection of rat spermatogonial stem cells in culture. Proc Natl Acad Sci USA 102: 17430–17435.
25. Kanatsu-Shinohara M, Muneto T, Lee J, Takenaka M, Chuma S, et al. (2008) Long-term culture of male germline stem cells from hamster testes. Biol Reprod 78: 611–617.
26. Kanatsu-Shinohara M, Toyokuni S, Morimoto T, Matsui S, Honjo T, et al. (2003) Functional assessment of self-renewal activity of male germline stem cells following cytotoxic damage and serial transplantation. Biol Reprod 68: 1801–1807.
27. Tadokoro Y, Yomogida K, Ohta H, Tohda A, Nishimune Y (2002) Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. Mech Dev 113: 29–39.
28. Clermont Y, Bustos-Obregon E (1968) Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted "in toto". Am J Anat 122: 237–247.
29. Dym M, Clermont Y (1970) Role of spermatogonia in the repair of the seminiferous epithelium following x-irradiation of the rat testis. Am J Anat 128: 265–282.
30. Brawley C, Matunis E (2004) Regeneration of male germline stem cells by spermatogonial dedifferentiation in vivo. Science 304: 1331–1334.
31. Nakagawa T, Nabeshima Y, Yoshida S (2007) Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. Dev Cell 12: 195–206.
32. Barroca V, Lassalle B, Coureuil M, Louis JP, Le Page F, et al. (2009) Mouse differentiating spermatogonia can generate germinal stem cells in vivo. Nat Cell Biol 11: 190–196.
33. Kanatsu-Shinohara M, Ikawa M, Takehashi M, Ogonuki N, Miki H, et al. (2006) Production of knockout mice by random and targeted mutagenesis in spermatogonial stem cells. Proc Natl Acad Sci USA 103: 8018–8023.
34. Kanatsu-Shinohara M, Ogonuki N, Inoue K, Ogura A, Toyokuni S, et al. (2003) Allogeneic offspring produced by male germ line stem cell transplantation into infertile mouse testis. Biol Reprod 68: 167–173.
35. Chuma S, Nakatsuji N (2001) Autonomous transition into meiosis of mouse fetal germ cells in vitro and its inhibition by gp130-mediated signaling. Dev Biol 229: 468–479.

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Experimental Hematology

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Bone marrow engraftment but limited expansion of hematopoietic cells from multipotent germline stem cells derived from neonatal mouse testis

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Objective. **Multipotent germline stem (mGS) cells derived from neonatal mouse testis, similar to embryonic stem (ES) cells, differentiate into various types of somatic cells in vitro and produce teratomas after inoculation into mice. In the present work, we examined mGS cells for hematopoietic progenitor potential in vitro and in vivo.**

Materials and Methods. **mGS cells were differentiated on OP9 stromal cells and induced into Flk1$^+$ cells. Flk1$^+$ cells were sorted and replated on OP9 stromal cells with various cytokines and emerging hematopoietic cells were analyzed for lineage marker expression by fluorescein-activated cell sorting, progenitor activity by colony assay, and stem cell transplantation assay.**

Results. **mGS cells, like ES cells, produce hematopoietic progenitors, including both primitive and definitive erythromyeloid, megakaryocyte, and B- and T-cell lineages via Flk1$^+$ progenitors. When transplanted into the bone marrow (BM) of nonobese diabetic/severe combined immunodeficient (NOD/SCID) γc^{null} mice directly, mGS-derived green fluorescent protein (GFP)-positive cells were detected 4 months later in the BM and spleen. GFP$^+$ donor cells were also identified in the Hoechst33342 side population, a feature of hematopoietic stem cells. However, these mGS-derived hematopoietic cells did not proliferate in vivo, even after exposure to hematopoietic stressors, such as 5-fluorouracil (5FU) injection or serial transplantation.**

Conclusion. **mGS cells produced multipotent hematopoietic progenitor cells with myeloid and lymphoid lineage potential in vitro and localized in the BM after intra-BM injection but, like ES cells, failed to expand or show stem cell repopulating ability in vivo.** © **2009 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Hematopoietic stem cells (HSCs) are defined as blood cells displaying the potential for self-renewal and multilineage differentiation. HSC transplantation has been widely used for treating hematological malignancies and inherited disorders. Peripheral blood and cord blood stem cells, as well as bone marrow cells, have been intensively studied and shown to be effective for clinical use. Recently, embryonic stem (ES) cells have been proposed as an alternative candidate source of HSCs. Many approaches have been attempted to obtain HSCs from ES cells, but this is challenging unless using enforced expression of genes, such as *Hoxb4* [1] or *Cdx4* [2] in ES cells. Even if a robust method for HSC derivation from ES cells were discovered, one would still need to address donor–host differences in histocompatibility antigens to permit ES-derived HSC engraftment in patients.

Multipotent germline stem (mGS) cells have been established from neonatal mouse testis and have been proven to have similar potential to ES cells, including germline transmission [3]. If mGS cells could be isolated from human testis and were utilized to produce HSC, then the problem of major histocompatibility complex (MHC) incompatibility would be solved because it might be possible to establish the patient's own mGS cells. In that sense, mGS cells may have a big advantage over ES cells in human application for cell therapies.

The methods for inducing hematopoietic cells from ES cells have been well-developed [4]. Flk1 is a candidate marker for mesoderm [5] and hemangioblast [6,7] cells, and Flk1 progeny have been proven to differentiate into

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all hematopoietic cell lineages [8]. Using the OP9 stromal cell line as a feeder layer, hematopoietic cells are effectively induced from ES cells [9], and FLK1$^+$-derived definitive hematopoietic cells are obtained [10].

In a previous report [3], mGS cells have been shown to differentiate into CD45$^+$ hematopoietic cells, including Gr-1$^+$Mac1$^+$ myeloid cells and Ter119$^+$ erythroid cells, but the potential for mGS cells to differentiate into hematopoietic stem/progenitor has not been reported. We have observed multipotent hematopoietic progenitor cells with myeloid and lymphoid potential emerging from mGS FLK1$^+$ cells using the OP9 feeder cell system and describe the localization of mGS-derived hematopoietic cells in the bone marrow (BM) cavity when directly injected into the BM of immunodeficient mice. However, the mGS-derived hematopoietic cells present in the host, like differentiated wild-type ES-derived hematopoietic cells, do not proliferate or display multilineage repopulating ability in vivo.

Materials and methods

Cell culture

mGS cells were established from mouse neonatal testis of DBA/2 mice or a green fluorescent protein (GFP)–expressing transgenic mouse (C57BL6 x DBA/2 F1 background), as described previously [3]. The CCE ES cell line was kindly provided by Dr. S. Nishikawa (RIKEN, Kobe, Japan). The ES cell line D3 was transfected with GFP gene driven by the ubiquitous CAG promoter. These CCE and D3 cell lines are derived from the 129 mouse strain. GFP$^+$ mGS and D3 were used for the transplantation assay. The mGS or ES lines were maintained as described previously [3].

Differentiation to hematopoietic progenitor cells was induced as described [8,11]. Briefly, 10^4 of undifferentiated mGS and ES cells were seeded onto T-25 flask with confluent OP9 stromal cells (a gift from Dr. Kodama) in α–minimum essential medium supplemented with 10% fetal bovine serum and 5×10^{-5}M 2-mercaptoenthanol. After 4 days, cultured cells were harvested with cell dissociation buffer (Gibco, Grand Island, NY, USA) and Flk1$^+$ cells were collected using a FACSVantage flow cytometer (Becton Dickinson, Mountain View, CA, USA). The 5–10 $\times$ 10^3 Flk1$^+$ cells per well in six-well plate with confluent OP9 stromal cells were cocultured again with added cytokines, such as 100 ng/mL mouse stem cell factor (SCF), 10 ng/mL human thrombopoietin (TPO), 10 ng/mL mouse Flt-3 ligand (FL), 4 u/mL human erythropoietin (EPO), and 100 u/mL mouse interleukin (IL)-7. Mouse SCF, human TPO, and human EPO were kindly provided from Kirin Brewery (Tokyo, Japan). Mouse FL and IL-7 were purchased from R&D Systems (Minneapolis, MN, USA). For T-cell induction, Flk1$^+$ cells were cocultured with OP9-DL1 [12] stromal cells (kindly provided by Dr. Zuniga-Pflucker, University of Toronto) with 50 ng/mL IL-7.

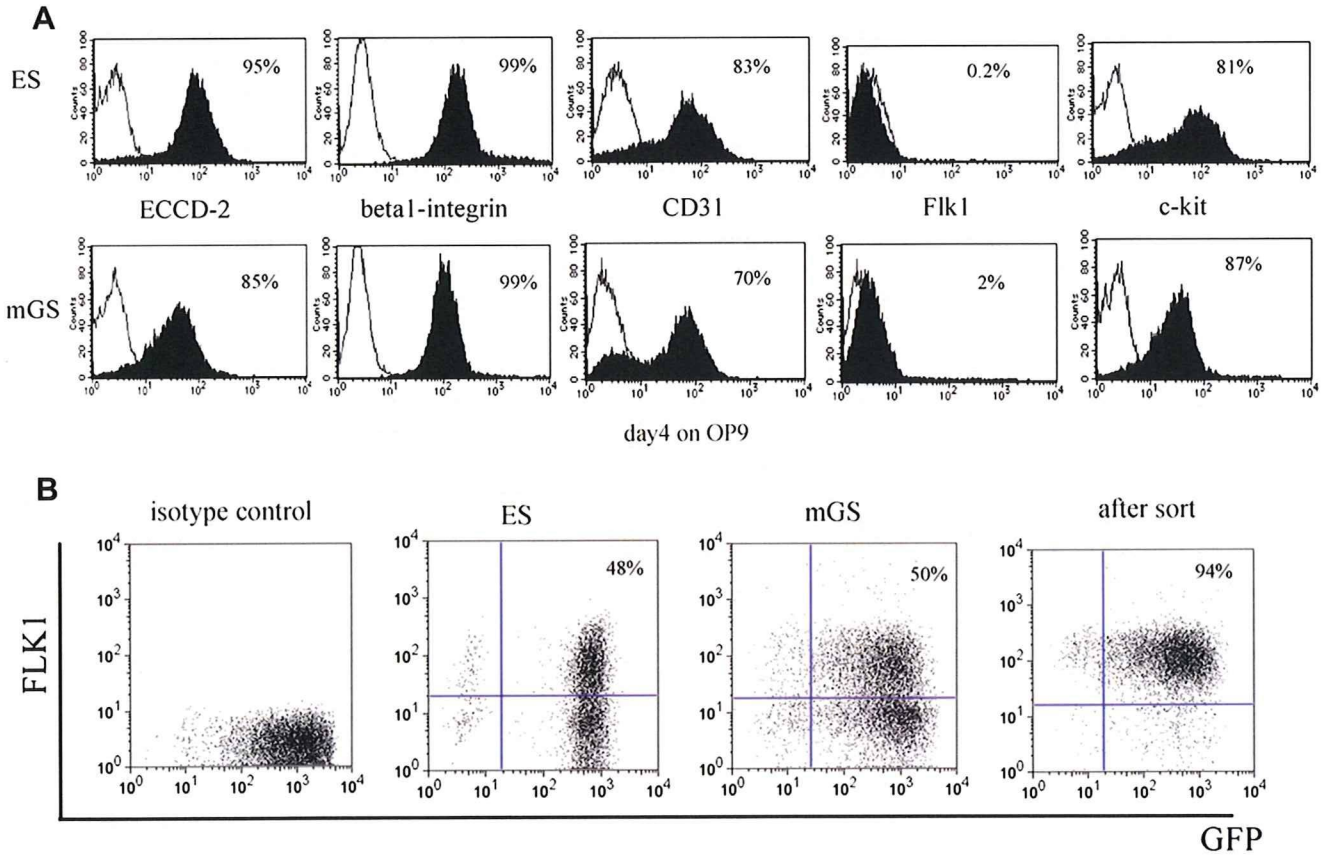

Figure 1. Surface markers of mGS and ES cells. Surface markers of undifferentiated mGS cells (**A**, lower panel) are similar to those of ES cells (**A**, upper panel). mGS cells were induced on OP9 stromal cells for 4 days and were confirmed to express Flk1 (**B**).

Hematopoietic colony-forming cell assay

In order to analyze emergence of immature hematopoietic progenitor cells from mGS cells, on each day after induction of Flk1$^+$ cells on OP9, cells were harvested and plated in methylcellulose for colony-forming assay using a modification of the technique described previously [13,14]. All cultures were performed in triplicate and the number of colony-forming cells was scored at day 8 to 10. For megakaryocyte colonies, the cells were plated in Megacult (Stem Cell Technologies, Vancouver, Canada) according to manufacturer's instruction.

Antibodies and staining

The following primary antibodies were used: rat anti-mouse E-cadherin (ECCD2, from Calbiochem. San Diego, CA, USA), fluorescein isothiocyanate (FITC)–conjugated hamster anti-rat β1 integrin (Ha2/5), rat anti-mouse CD31 (MEC 13.3), allophycocyanin (APC)-conjugated rat anti-mouse *c-kit* (2B8), phycoerythrin (PE)-conjugated rat anti-Flk1 (AVAS12), PE-conjugated rat anti-mouse Ter119 (TER-119), APC-conjugated rat anti-mouse CD45 (30-F11), biotin-conjugated rat anti-mouse Gr-1 (RB6-8C5), biotin-conjugated rat anti-mouse Mac-1 (M1/70), purified rat CD41 antibody (MWReg30), and rabbit anti-embryonic hemoglobin antibody [15] (a gift from Dr. Takakura, Osaka University). PE-conjugated or alkaline phosphatase–conjugated anti-rat IgG, APC-conjugated streptavidin, or FITC–conjugated anti-rabbit immunoglobulin G were used as secondary antibodies. All antibodies except ECCD2 were purchased from Pharmingen (San Diego, CA, USA). For detection of side population (SP) cells, BM cells were stained with Hoechst 33324, as described previously [16–18]. Stained cells were analyzed using FACSCalibur or LSRII (Becton Dickinson).

Immunohistochemistry

Femurs of recipient mice were fixed with 4% paraformaldehyde, embedded in the optimal cutting temperature compound and frozen sections of 7-μm thickness were mounted on silan-coated glass slides and were stained with rabbit anti-GFP antibody (BD Bioscience Clontech, Palo Alto, CA, USA), as described previously [19]. Cytospin preparations and culture dishes were also stained with various antibodies, as described previously [20,21].

Mice and transplantation

Nonobese diabetic/severe combined immunodeficient (NOD/SCID) γc^{null} mice were kindly provided from the Central Institute of Experimental Animals (Kawasaki, Japan) and kept under specific pathogen-free conditions in accordance with the guidelines of the facility. Cultured mGS-derived hematopoietic cells and OP9 cells were collected and injected into the femoral BM of NOD/SCIDγc^{null} mice that were irradiated with 2.4 Gy before transplantation. The 2×10^5 BM mononuclear cells were injected as a positive control. After transplantation, mice were prophylactically provided sterile water with neomycin sulfate (Gibco BRL).

For serial transplantation, BM cells were collected from transplanted NOD/SCIDγc^{null} mice 4 months after primary transplantation and stained with anti-CD45.1$^+$ (recipient) and CD45.2$^+$ (donor) antibodies. CD45.2$^+$ donor cells were sorted on FACSVantage (Becton Dickinson) and injected into the BM of 2.4-Gy irradiated NOD/SCIDγc^{null} mice. Peripheral blood (PB) and BM were analyzed 3 to 4 months after secondary transplantation.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared using Trisol (Gibco BRL). Complementary DNA (cDNA) synthesis was performed using Superscript II

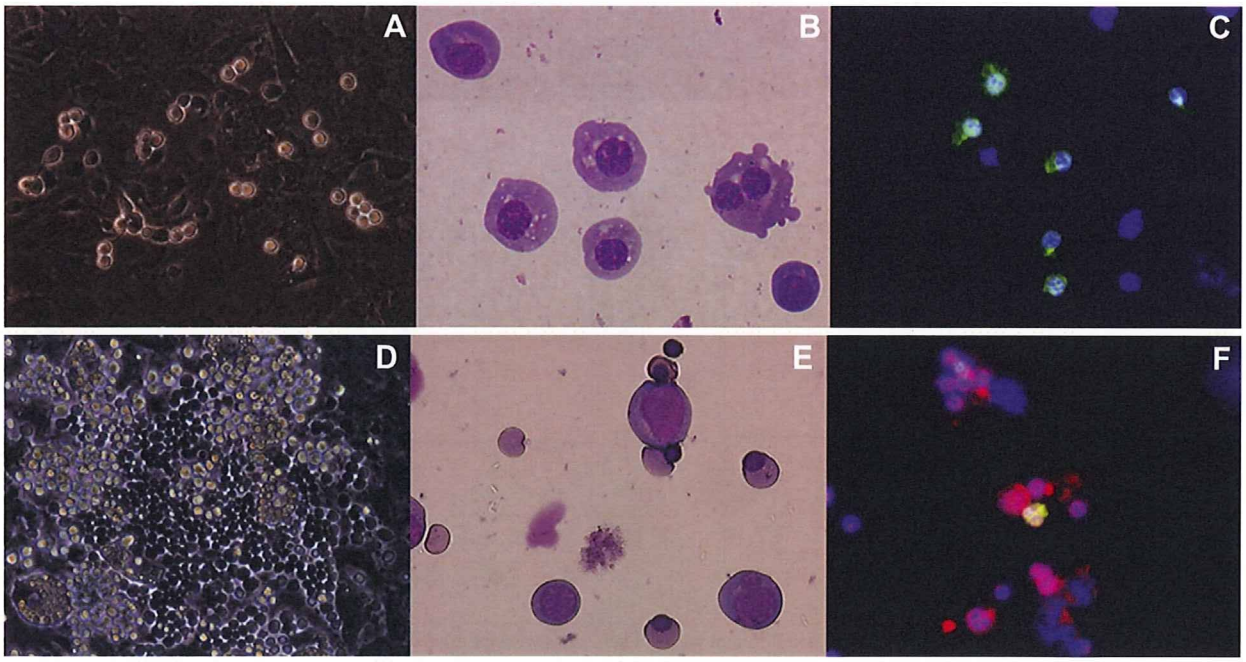

Figure 2. Primitive and definitive erythroid cells are differentiated from mGS–derived Flk1$^+$ cells. mGS-derived Flk1$^+$ cells were cultured on OP9 cells and small round cells and cobblestone-forming areas were found [(**A**) day 3, (**D**) day 9). May-Giemsa staining of a cytospin preparation [(**B**); day 3, (**E**); day 9)]. Immunostaining of E1 antigen and Ter119 (**C**,**F**). (**C**) E1 antigen; green. (**F**) E1 antigen; green Ter119; red. Nuclear staining with Hoechst 33324; blue. Magnification (**A**,**D**): ×100; (**B**,**C**,**E**,**F**): ×200.

and oligo $(dT)_{12\text{-}18}$ primers (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. The same cDNA sample was used for PCR amplification with different primer sets, using standard protocols using AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). The primer sequences were as follows: βH1 forward: AGTCCCCATGGAGTCAAAGA, reverse: CTC AAG GAG ACC TTT GCT CA, β major forward: CTG ACA GAT GCT CTC TTG GG, reverse: CAC AAA CCC CAG AAA CAG ACA, GAPDH forward: TCC CAC TCT TCC ACC TTC, reverse: CTG TAG CCG TAT TCA TTG TC. For PCR to detect GFP, the primer sequence were as follows: GFP forward: CTG GTC GAG CTG GAC GGC GAC G, reverse: CAC GAA CTC CAG CAG GAC CAT G. Cycling parameters included: denaturation at 94°C for 30 seconds; annealing at various temperatures for 30 seconds; elongation at 72°C for 40 seconds. The number of cycle varied between 25 and 35 cycles.

Results

mGS cells can differentiate into Flk1$^+$ cells on OP9 stromal cells

It has been reported that mGS cells are very similar to ES cells in their differentiation ability into multiple cell lineages in vitro and in vivo [3]. Undifferentiated mGS and ES cells express ECCD-2, β1-integrin, CD31 and c-kit, but are negative for Flk-1 expression (Fig. 1A). In order to examine the differentiation potential of these cells, we cocultured mGS or ES cells with OP9 stromal cells and assayed for emergence of Flk1$^+$ cells 4 days after induction because Flk1 is thought to be a representative marker for mesodermal cells [5]. The percentage of Flk1$^+$ cells derived from mGS cells (50%) was very similar to ES cells (48%) (Fig. 1B).

Primitive and definitive erythropoiesis can be derived in vitro in mGS culture

In order to examine red blood cell emergence from mGS-derived Flk1$^+$ cells, Flk1$^+$ cells were sorted and cocultured on OP9 stromal cells with added EPO. Three days later, small round hematopoietic cells were found (Fig. 2A). The cytospin preparation of these cells and May-Giemsa staining revealed the round, nucleated cell morphology of primitive erythrocytes (Fig. 2B). Immunostaining with an antiembryonic hemoglobin antibody confirmed that these cells were primitive erythrocytes (Fig. 2C). After 9 days of coculture, cobblestone-forming areas were observed in

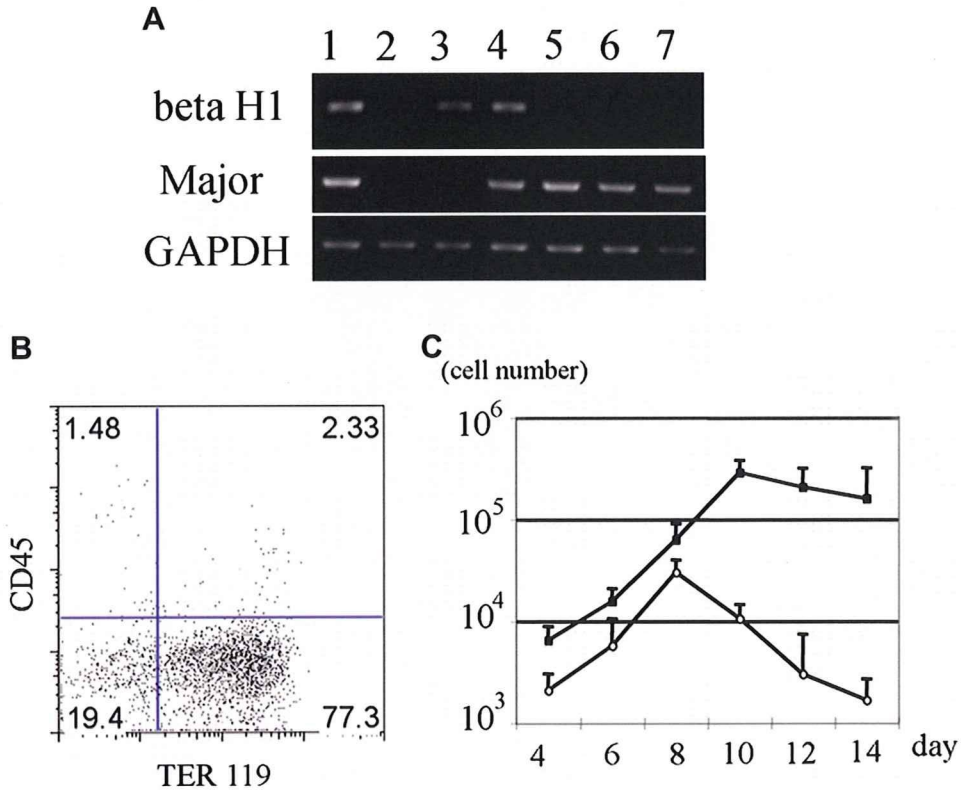

Figure 3. Erythropoiesis from mGS Flk1$^+$ cells shows two different waves. Flk1$^+$ cells were cultured on OP9 with erythropoietin (EPO) and with or without ACK45. The nonadherent cells in the culture were collected every other day, counted the number (**C**), examined the surface markers (**B**) and hemoglobin gene expression (**A**). Sequential RT-PCR analysis shows expression of β H1 hemoglobin mRNA, followed by β-major hemoglobin mRNA expression (**A**). E12.5 fetal liver (lane 1), E8.0 embryo (lane 2), mGS- Flk1$^+$ cells on OP9 for 4 days (lane 3), for 6 days (lane 4), for 8 days (lane 5), for 10 days (lane 6), for 12 days (lane 7). Flk1$^+$ cells were cultured on OP9 cells with EPO and with (white circle) or without ACK45 (black square) (**C**). Most cells in the culture were Ter119$^+$ erythrocyte (**B**). Definitive erythropoiesis was blocked by ACK45 (**C**). GAPDH = glyceraldehydes phosphate dehydrogenase.

the cultures (Fig. 2D). Morphologic analysis of nonadherent cells revealed enucleated red blood cells (Fig. 2E). Immunostaining confirmed the cells to be definitive erythrocytes that express Ter119, but not embryonic hemoglobin molecules (Fig. 2F). Next, mGS Flk1$^+$ cells were cocultured on OP9 cells in the presence of EPO or EPO and Ack45 (a blocking antibody of the c-kit signaling pathway). In this culture system, most of the emerging blood cells were Ter119$^+$ erythroid cells (Fig. 3B), but the growth factor requirements of the cultured cells in each condition showed different patterns: an initial c-kit–independent period (days 4–8; Fig. 3C) and a second c-kit–dependent period during which erythropoiesis was blocked by the presence of the Ack45 antibody (days 8–14, Fig. 3C). Erythrocytes in the initial wave expressed βH1 hemoglobin mRNA, while only β-major hemoglobin mRNA was expressed in the second wave of erythropoiesis (Fig. 3A). These results indicated that cells in the initial wave represented primitive erythrocytes, while definitive erythrocytes comprised the second wave [22]. Thus, mGS cells can give rise to primitive and definitive erythropoiesis in vitro in the same manner as differentiated ES cells.

Flk1$^+$ cells derived from mGS cell can differentiate into multiple lineages including B and T cells

When mGS-derived Flk1$^+$ cells were cocultured on OP9 cells with added SCF, granulocyte colony-stimulating factor (G-CSF), IL-3, and EPO, Flk1$^+$ cells can also differentiate into Mac1$^+$Gr1$^+$ cells and Ter119$^+$ cells (7.3% and 20.9%, respectively) similar to ES cells (7.6% and 24.3%, respectively) (Fig. 4A). Furthermore, mGS-derived Flk1$^+$ cells also gave rise to CD19$^+$ B cells and CD4$^+$CD8$^+$ T cells when cultured on OP9 cells or OP9-DL1 cells with added IL7 (Fig. 4C and D).

Flk1$^+$ cells derived from mGS cell can differentiate into multipotent progenitors

To determine whether mGS cells can give rise to clonogenic hematopoietic progenitors, Flk1$^+$ cells derived from mGS cells were cocultured on OP9 cells and all the cells after 4 to 10 days culture were plated in methylcellulose with added growth factors. Burst-forming unit-erythroid (BFU-E), colony-forming unit granulocyte-macrophage (CFU-GM), and CFU-Mix were observed (Fig. 5Aa–d). Interestingly, the number of CFU-mix was decreased after 8 days' coculture (Fig. 5Ba), and CFU-GM was the main

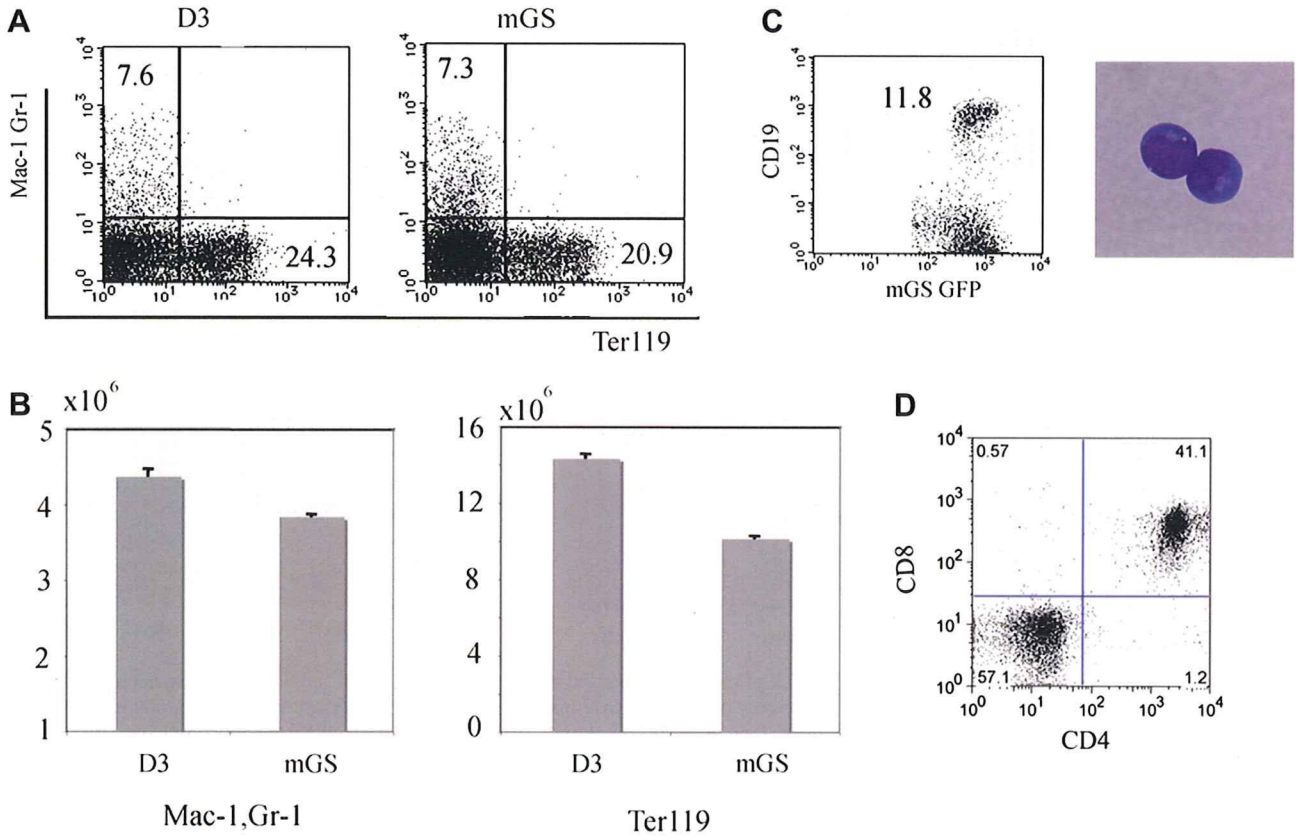

Figure 4. Myelolymphoid potential of mGS cells. Mac1$^+$Gr1$^+$, Ter119$^+$, CD19$^+$, and CD4$^+$CD8$^+$ cells were differentiated from mGS-derived Flk-1$^+$ cells within OP9 culture (**A**,**B**,**C**) or OP9-DL1 culture (**D**). For Mac1$^+$Gr1$^+$, Ter119$^+$ cells, cells were collected from 8–10 days culture (**A**,**B**). The numbers of myeloid and erythroid cells differentiated from mGS and ES cells were similar (**B**). For CD19$^+$ (**C**), and CD4$^+$CD8$^+$ cells (**D**), cells were collected from 14–21 days culture. These FACS data are representative among three independent experiments.

population detectable after 10 day's culture of mGS cells on OP9 cells. To examine whether production of these clonogenic progenitors from Flk1$^+$ cells was influenced by cytokines, Flk1$^+$ cells were cocultured on OP9 cells with only EPO or with SCF, TPO, and FL. The numbers of all kind of colonies were increased when cocultured with SCF, TPO, and FL (Fig. 5Bb). Thus, mGS-derived Flk1$^+$ cells generate multipotent hematopoietic progenitors, and this effect was promoted by addition of multiple cytokines, similar to differentiated ES cells.

Megakaryopoiesis through Flk1$^+$ cells derived from mGS cells

We also evaluated megakaryocyte production from mGS-derived Flk1$^+$ cells. Eight days after Flk1$^+$ cells were cocultured on OP9 cells in the presence of TPO and SCF, large round cells with proplatelets were observed (Fig. 5Ca). These cells expressed the cell surface protein CD41, a well-recognized marker of the platelet lineage (Fig. 5Cb). Cytospin preparations of culture fluid from these culture dishes showed large multinucleated cells that were CD41-positive (Fig. 5Cc and Cd). These cells contained both CFU-megakaryocyte and CFU-mega-mix confirmed by specific megakaryocyte progenitor cultures (Fig. 5Ce and Cf). CFU-megakaryocyte progenitors were maintained during culturing between day 8 and day 12 of mGS-derived Flk1$^+$ cells in the presence of TPO and SCF (Fig. 5D). Differentiated ES cells also showed similar trends of megakaryocyte colony production (data not shown). Thus, Flk1$^+$ cells derived from mGS cells were shown to produce megakaryocytes as well as megakaryocyte progenitor cells.

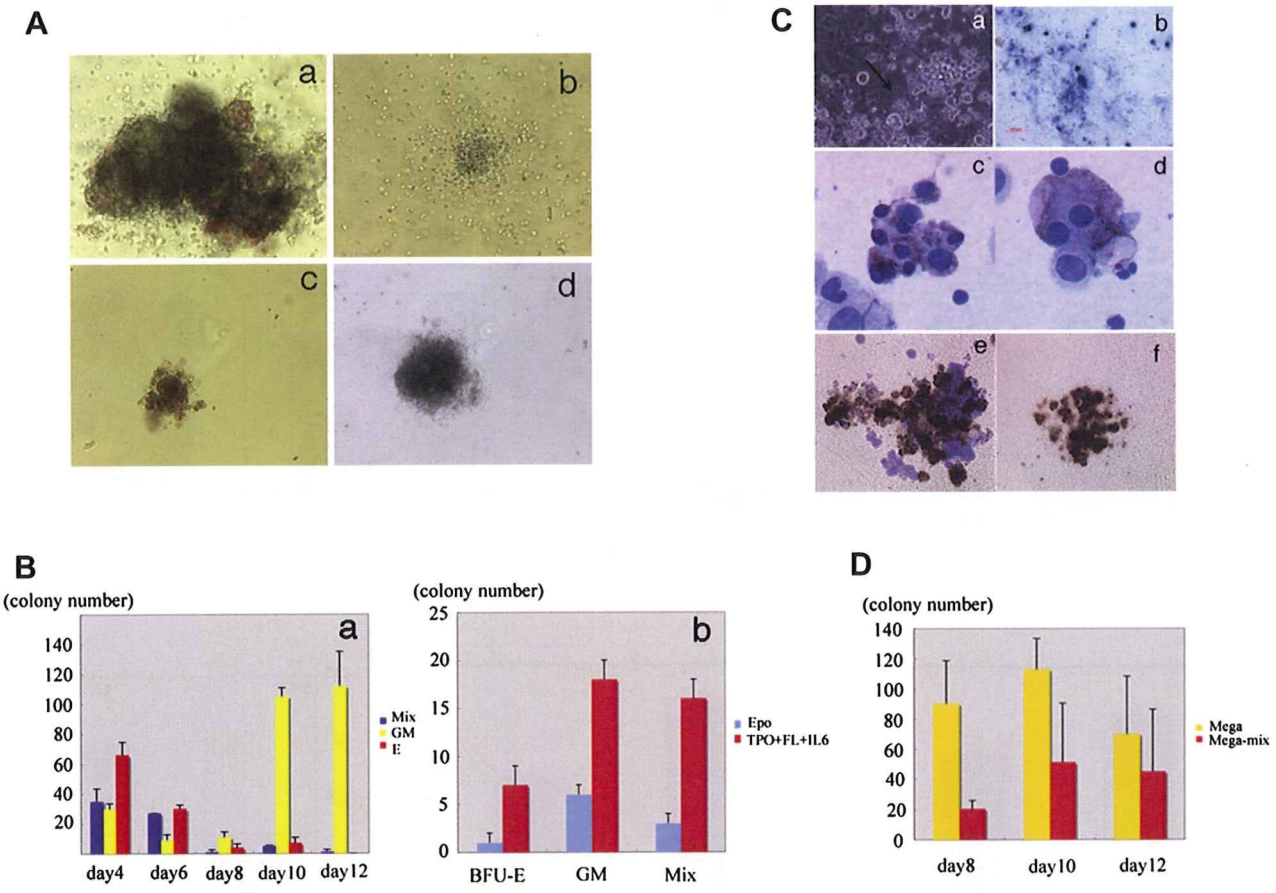

Figure 5. Colony-forming ability and megakaryocyte potential of mGS Flk1$^+$-derived cells. mGS-derived Flk-1$^+$ cells produce hematopoietic progenitor cells that can form various kinds of colonies [(**Aa**) mixed colony, (**Ab**) granulocyte-macrophage (GM) colony, (**Ac**) burst-forming unit erythroid (BFU-E), (**Ad**) mast cell colony]. Mixed colonies were predominant when mGS-derived Flk1$^+$ cells were cultured on OP9 cells for 4 to 6 days (**Ba**). Blue bar: mixed colony, yellow bar: GM colony, red bar: Erythroid colony. The number of all kinds of colonies was increased when mGS Flk1$^+$ cells were cultured on OP9 with thrombopoietin (TPO) and interleukin (IL)-6 (**Bb**). Blue bar: with erythropoietin (EPO), red bar: with TPO and IL6. Megakaryocyte or proplatelet mGS-derived Flk1$^+$ cells were observed within OP9 culture for 8–12 days. Proplatelet like cells (arrow **Ca**), Immunostaining of cultured cells with CD41 antibody secondary detected by alkaline phosphatase–conjugated antibody (**Cb** blue). Immunostaining of a cytospin preparation with CD41 antibody secondary detected by peroxidase conjugated antibody (**Cc**, **Cd** brown). After transferring cultured cells into Megacult conditions, megakaryocyte colonies (**Cf**) include mega-mix colonies (**Ce**) were found. These cells expressed acethylcholinestrase as evidenced by brown staining. The CFU-Mega was maintained during culturing day 8 to 12 in the presence of TPO and stem cell factor (**D**). Yellow bar: Megakaryo-colony, red bar: Mega-Mix colony. Magnification: (**Ca, Cb**) ×100; (**Cc, Cd**) ×200; (**Ce, Cf**) ×100.

Flk1-derived hematopoietic cells can engraft in the BM of NOD/SCIDγc^{null} mice by intra-BM injection

Finally, day-6 cocultured mGS-derived Flk1$^+$ cells and OP9 cells were recovered and injected directly into the BM of NOD/SCIDγc^{null} mice (n = 4). As a positive control, 2 × 10^5 BM mononuclear cells were injected into the BM of NOD/SCIDγc^{null} mice. Every 4 weeks after transplantation, PB was analyzed for evidence of donor-cell engraftment. While the BM cells engrafted, mGS-derived cells were barely detected (<0.1%, Fig. 6A). After 7 weeks, fluorescein-activated cell sorting (FACS) analysis revealed donor mGS-derived GFP$^+$ cells in the BM, but at low chimerism of <0.1% (data not shown). PCR analysis confirmed the presence of GFP-DNA in the BM and the spleen (Fig. 6C). Four months after transplantation, a very small number of donor CD45$^+$GFP$^+$ cells were detected in the BM and the spleen by FACS analysis and confirmed by PCR (Fig. 6A, C, and D). Furthermore, when BM cells of transplanted mice were stained with Hoechst33324, GFP$^+$ cells were detected in the SP region (Fig. 6E and F). Immunostaining of the femur revealed that GFP$^+$ cells were attached to the endosteal region, where HSCs are considered to reside (Fig. 6B). Three of four transplanted mice were confirmed to display this type of "stem cell-like" engraftment (0.03% ± 0.03%). Thus, mGS-derived hematopoietic cells are found in the BM 4 months after transplantation. It is of note that no teratoma formation was observed in any of the transplanted animals.

mGS-derived hematopoietic cells did not show stem cell potential by serial transplantation

Because HSCs are enriched in the SP fraction and normally reside in the endosteal region of the BM [23–25], we speculated that hematopoietic progeny of mGS-derived Flk1$^+$ cells engrafted in the BM and remained in the stem cell fraction. In order to prove that these GFP$^+$ cells in the BM were HSCs, we hypothesized that hematopoietic stress may induce further expansion of the donor HSCs and thus, 5FU injection and serial transplantation were performed. 5FU was injected intraperitoneally at a standard dose of 100 ug/g into transplanted mice that received mGS-derived hematopoietic cells and the PB was analyzed with CD45.1 and CD45.2 antibodies every week after 5FU injection. When the blood cell count recovered, we expected donor-derived cells would increase in number in PB, however, no donor mGS-derived cells were observed (data not shown). For secondary transplantation analysis, we

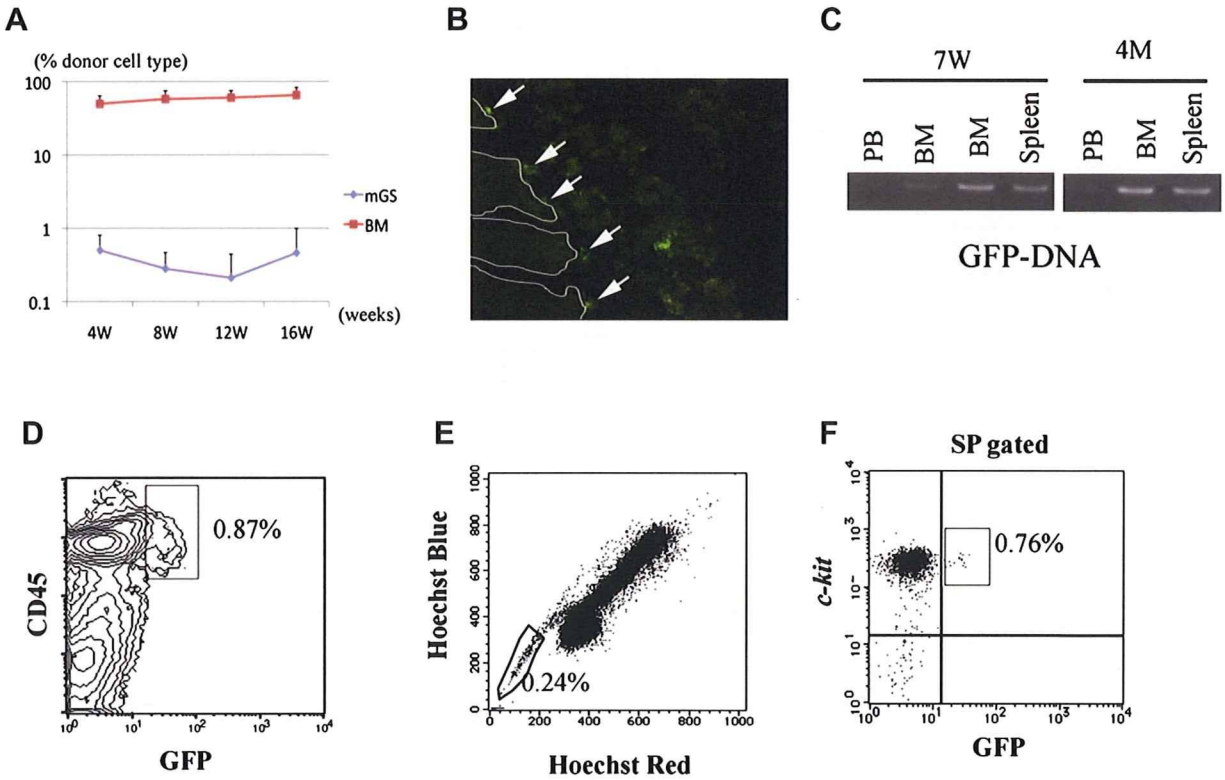

Figure 6. Transplanted hematopoietic cells from GFP$^+$ mGS cells can be detected in bone marrow (BM) 4 months after transplantation and displays stem cell phenotype. After transplantation, peripheral blood (PB) was analyzed every 4 weeks (**A**). BM cells from recipient mice 4 months after transplantation were analyzed by LSR II (**D**, **E**, **F**). GFP$^+$CD45$^+$ cells were detected (**D**). When the BM cells of the recipient mice were stained with Hoechst 33324, GFP$^+$ cells were detected in the SP region (**E**, **F**). In the section of recipient BM, GFP$^+$ cells were found attached to the endosteal region (**B**, arrow). RT-PCR shows donor-derived DNA in the BM and spleen at 7 weeks and 4 months after transplantation (**C**).

collected BM cells of primary recipient mice. Interestingly, more donor cells were detected in the lineage-negative population of the recipient BM cells (Fig. 7A) than in the whole BM (Fig. 7B). There were only 0.001% donor cells that displayed myelolymphoid lineage markers (Fig. 7B and C). Then we sorted CD45.2$^+$ mGS-derived cells from the BM of primary recipient mice (Fig. 7A) and injected 10^4 mGS-derived donor cells into the BM of irradiated secondary recipient NOD/SCIDγc^{null} mice. However, none of the host mice displayed donor-derived cells in the PB or BM after 4 months of the secondary transplantation. Thus, mGS-derived multipotent hematopoietic cells engrafted in the BM, but could not show stem cell potential by expanding and repopulating all the hematopoietic lineages posttransplantation for >16 weeks.

mGS-derived hematopoietic cells did not express CXCR4 or CDX4, but expressed HOXB4

Because mGS-derived hematopoietic cells did not engraft in the BM efficiently, we examined these cells for evidence of expression of a well-known and important homing receptor, CXCR4 (Fig. 8C). Indeed, very few mGS-derived CD45$^+$ cells expressed CXCR4. Also, c-kit expression was only 3% and the c-kit$^+$Sca-1$^+$ population represented only 0.09% of the culture elements. Because CDX4 and HOXB4 overexpression have enabled ES cells to engraft in vivo [2], the expression level of these genes was examined in mGS-derived cells using both RT-PCR and quantitative PCR. mGS-derived cells, as well as fetal liver and BM stem/progenitor cells, did not express mRNA for CDX4 (Fig. 8A). However, using the embryonic aorta-gonado-mesonephros (AGM) tissue as a reference (positive control tissue), the relative expression of HOXB4 in mGS-derived cells (0.76) was higher than embryonic day 16.5 fetal liver cells (0.02), BM lin$^-$ cells (0.09), BM Sca-1$^+$c-kit$^+$lin$^-$ cells (0.43), and CCE-derived cells (0.15) (Fig. 8B). From these results, it appears that the lack of CXCR4 expression of mGS-derived cells may be a primary reason that the transplanted cells could not expand in the recipient BM compartment.

Discussion

We report that multipotent hematopoietic progenitor cells are derived from mGS cells. mGS cells differentiate into Flk1$^+$ cells similar to differentiated ES cells. From mGS-derived Flk1$^+$ cells, erythroid, myeloid, lymphoid, and megakaryocyte hematopoietic progenitors were induced

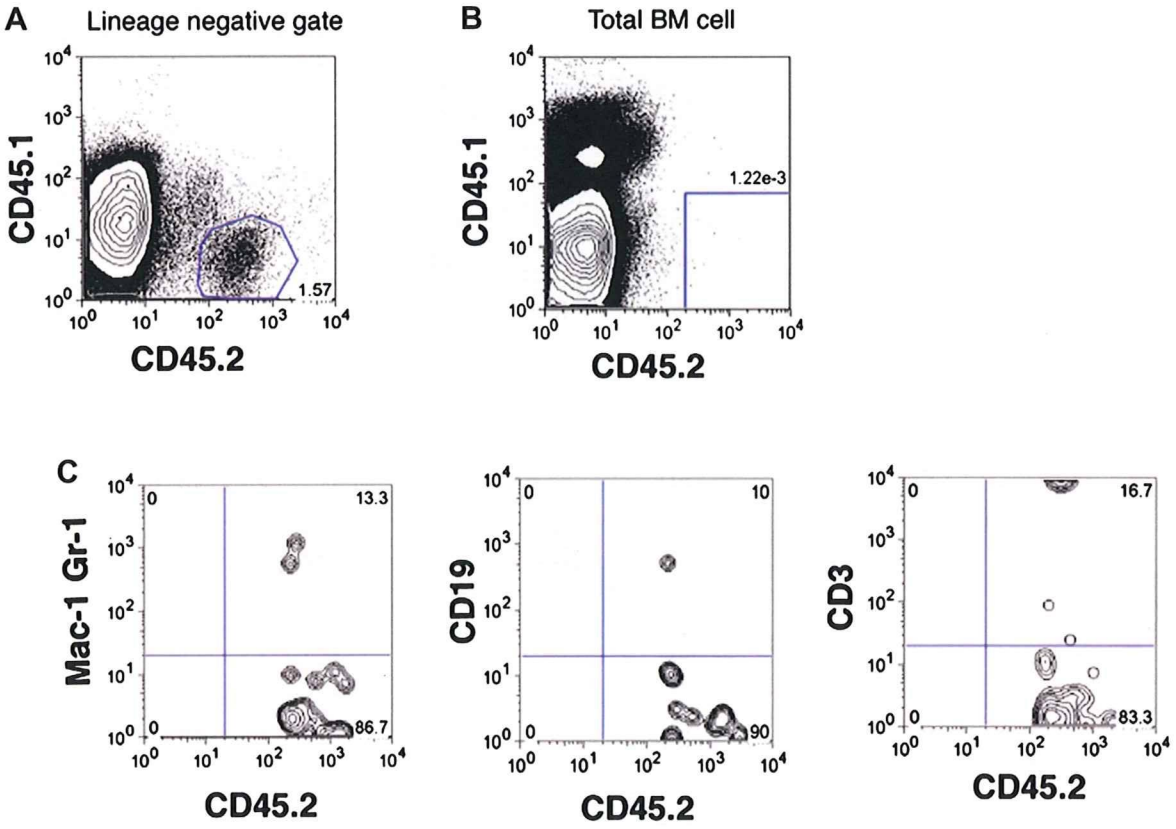

Figure 7. Analysis of primary recipient mice transplanted with mGS–derived hematopoietic cells. There are small percentages of mGS-derived cells in lineage negative fraction in the recipient bone marrow (BM) cells (**A**). When analyzing total recipient BM cells, mGS-derived cell can be detectable in very small percentage (**B**), but showed multi-lineage cell types (**C**).

efficiently in vitro in coculture with OP9 stromal cells. Furthermore, mGS-derived hematopoietic cells engrafted in the BM of NOD/SCIDγc^{null} mice, although their chimeric rate was very low. We have also transplanted hematopoietic cells differentiated from mGS-derived Flk1^{+} cells into lethally irradiated mice via tail vein, but found no significant number of donor cells in the PB or BM after transplantation (more than 30 transplanted mice, data not shown). mGS-derived cells were found in the recipient BM only when directly transplanted into the femoral BM of NOD/SCIDγc^{null} mice.

There may be several reasons why the mGS-derived Flk1^{+} progeny only engraft upon direct injection into the marrow. It is known that emergence of hematopoiesis from ES cells in vitro mirrors the emergence of hematopoiesis in the murine embryos [26]. Considering this observation, hematopoietic cells derived from mGS and ES cells should have similar characteristics to hematopoietic cells in embryos during early–mid gestation. Yolk sac cells and para-aortic splanchnopleura cells as early as E9.0 engraft in sublethally conditioned neonatal mice, but not in adult BM [27,28]. These facts lead us to speculate that mGS-derived hematopoietic cells may be too immature to express homing receptors and thus failed to find the HSC niche in the adult BM when circulating. Indeed, very few mGS-derived cells expressed CXCR4, c-kit, or Sca-1 (Fig. 8C). Furthermore, the mGS-derived Flk1^{+} progeny may fail to express high levels of human leukocyte antigen (HLA) and thus are trapped by host natural killer (NK) cells. However, NOD/SCIDγc^{null} mice have no NK cell activity [29,30] and are thus suitable for transplantation with embryonic HSCs that have just emerged from the aorta-gonado-mesonephros region at E10.5 [31]. Finally, we may have observed low chimerism due to the limited number of cells infused (1×10^5). Transplanting a larger number of cells might improve the chimerism. This limited proliferative ability of transplanted cells is similar to the behavior of human ES-derived cells transplanted in NOD/SCID mice [32].

It is interesting that donor mGS–derived hematopoietic cells that engrafted remained at the endosteal region, reported to be a niche for HSC, and that they were resident in the SP fraction 4 months after transplantation. However, these mGS-derived hematopoietic cells could not proliferate even after 5FU injection; a hematopoietic stress that normally recruits quiescent HSC into cycle and replenishment of the 5FU–depleted progenitor cell pool. In the secondary transplantation studies, as many as 30,000 to 40,000 cells were sorted from the BM of the primary recipients and injected into secondary recipient BM, and no donor cells were observed. While the most quiescent

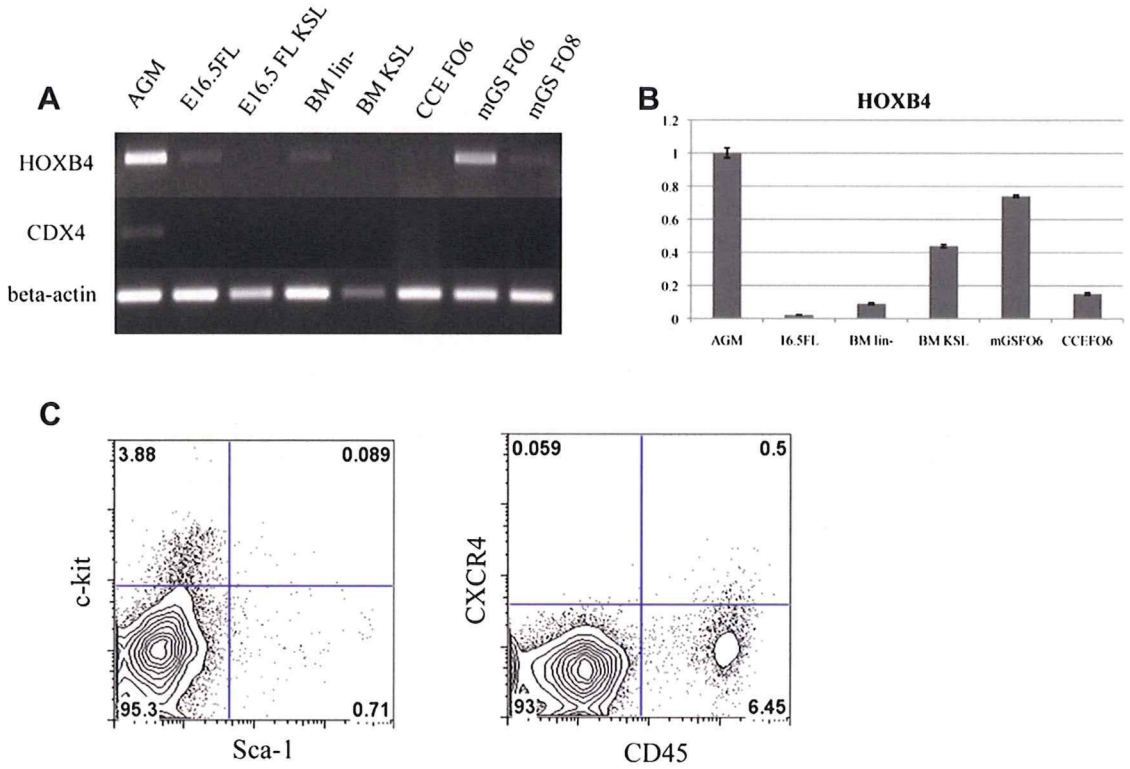

Figure 8. mGS–derived cells express HOXB4, but not CDX4 or CXCR4. RT-PCR for CDX4 and HOXB4 among various hematopoitic cells (**A**). KSL = c-kit^{+}Sca-1^{+}lineage^{-} cells, BM lin^{-} = bone marrow lineage-negative cells, FO6 = Flk1 culture on OP9 day 6, FO8 = Flk1 culture on OP9 day 8. Relative HOXB4 expression in the various hematopoitic cells (**B**). With AGM as a reference, the relative expressions of HOXB4 in each sample were follows; 16.5FL 0.02, BM lin^{-} 0.09, BM KSL 0.43, mGS FO6 0.74, CCE FO6 0.15. c-kit, Sca-1, and CXCR4 expression on mGS-derived cells [(**C**) 6 days culture].

dormant HSCs may divide every 145 days, even dormant HSC can proliferate after 5FU injection [33]. Therefore, it is unlikely that our mGS-derived multipotent cells represent dormant HSCs.

We have also shown that mGS-derived Flk-1$^+$ cells could produce B- and T-lymphoid cells, as well as erythromyeloid cells, using OP9 and OP9-DL1 stromal cell line cocultures. These results were very similar to ES cell differentiation studies reported previously [12,22]. Thus, mGS cells have at least the same hematopoietic potential as ES cells in vitro and in vivo.

Wild-type ES cells fail to reconstitute mouse BM without prior genetic modification [1,2]. Considering that only CDX4- and HOX B4-expressing ES cells can engraft and proliferate in vivo, it might be possible that ES or mGS-derived hematopoietic cells originally lack the proliferation potential required for in vivo expansion following engraftment. Actually, our mGS-derived cells, as well as fetal liver and BM stem/progenitor cells, did not express CDX4 transcripts. On the other hand, mGS-derived cells did express HOXB4. Because mGS-derived cells could not expand in vivo in spite of higher HOXB4 expression than fetal liver and BM progenitors, lack of CXCR4 expression may be a primary reason for the low level of donor cell chimerism rather than lack of CDX4 expression, although the question of whether upregulating CDX4 expression may enhance repopulating ability of the transplanted mGS cells needs further study. Nevertheless, this is the first report that transplanted hematopoietic cells derived from ES-like cells (mGS or ES cells) may reside in a long-lived multipotent hematopoietic cell fraction in vivo.

Recently, multipotent germline stem cells have been established from adult mouse testis [34]. Multipotent adult germline stem cells display similar characteristics to ES cells, including contributions to various organs (even germline transmission) when injected into an early blastocyst. The multipotent adult GS cells possess an advantage over ES or embryonic germ cells because they can be stably established from the postnatal mouse. Establishing multipotent adult GS cells from human subjects should be feasible both technically and ethically. Indeed, pluripotent stem cells from adult human testis have been generated [35,36]. Additionally, induced pluripotent stem cells have been established from mouse and human somatic cells and upon differentiation induced pluripotent stem cell progeny appear to have the same potential as ES-derived cells [37–39]. Which cell source will ultimately prove most efficacious for human therapy remains fertile ground for future translational research.

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Conflict of Interest Disclosure

No financial interests/relationships with financial interest relating to the topic of this article have been declared.

References

1. Kyba M, Perlingeiro RC, Daley GQ. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. Cell. 2002;109:29–37.
2. Wang Y, Yates F, Naveiras O, Ernst P, Daley GQ. Embryonic stem cell-derived hematopoietic stem cells. Proc Natl Acad Sci U S A. 2005;102:19081–19086.
3. Kanatsu-Shinohara M, Inoue K, Lee J, et al. Generation of pluripotent stem cells from neonatal mouse testis. Cell. 2004;119:1001–1012.
4. Kennedy M, Firpo M, Choi K, et al. A common precursor for primitive erythropoiesis and definitive haematopoiesis. Nature. 1997;386:488–493.
5. Kataoka H, Takakura N, Nishikawa S, et al. Expressions of PDGF receptor alpha, c-Kit and Flk1 genes clustering in mouse chromosome 5 define distinct subsets of nascent mesodermal cells. Dev Growth Differ. 1997;39:729–740.
6. Shalaby F, Rossant J, Yamaguchi TP, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 1995; 376:62–66.
7. Shalaby F, Ho J, Stanford WL, et al. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. Cell. 1997;89: 981–990.
8. Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. Development. 1998;125:1747–1757.
9. Nakano T, Kodama H, Honjo T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. Science. 1994;265:1098–1101.
10. Nishikawa SI, Nishikawa S, Kawamoto H, et al. In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. Immunity. 1998;8:761–769.
11. Iida M, Heike T, Yoshimoto M, Baba S, Doi H, Nakahata T. Identification of cardiac stem cells with FLK1, CD31, and VE-cadherin expression during embryonic stem cell differentiation. FASEB J. 2005;19:371–378.
12. Schmitt TM, de Pooter RF, Gronski MA, Cho SK, Ohashi PS, Zuniga-Pflucker JC. Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. Nat Immunol. 2004;5:410–417.
13. Nakahata T, Ogawa M. Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential hemopoietic colonies. Proc Natl Acad Sci U S A. 1982;79:3843–3847.
14. Nakahata T, Ogawa M. Hemopoietic colony-forming cells in umbilical cord blood with extensive capability to generate mono- and multipotential hemopoietic progenitors. J Clin Invest. 1982;70:1324–1328.
15. Miwa Y, Atsumi T, Imai N, Ikawa Y. Primitive erythropoiesis of mouse teratocarcinoma stem cells PCC3/A/1 in serum-free medium. Development. 1991;111:543–549.
16. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med. 1996;183:1797–1806.
17. Tsuchiya A, Heike T, Fujino H, et al. Long-term extensive expansion of mouse hepatic stem/progenitor cells in a novel serum-free culture system. Gastroenterology. 2005;128:2089–2104.

18. Nagato M, Heike T, Kato T, et al. Prospective characterization of neural stem cells by flow cytometry analysis using a combination of surface markers. J Neurosci Res. 2005;80:456–466.
19. Yoshimoto M, Chang H, Shiota M, et al. Two different roles of purified CD45+c-Kit+Sca-1+Lin- cells after transplantation in muscles. Stem Cells. 2005;23:610–618.
20. Umeda K, Heike T, Yoshimoto M, et al. Development of primitive and definitive hematopoiesis from nonhuman primate embryonic stem cells in vitro. Development. 2004;131:1869–1879.
21. Umeda K, Heike T, Yoshimoto M, et al. Identification and characterization of hemoangiogenic progenitors during cynomolgus monkey embryonic stem cell differentiation. Stem Cells. 2006;24:1348–1358.
22. Fujimoto T, Ogawa M, Minegishi N, et al. Step-wise divergence of primitive and definitive haematopoietic and endothelial cell lineages during embryonic stem cell differentiation. Genes Cells. 2001;6: 1113–1127.
23. Yoshimoto M, Shinohara T, Heike T, Shiota M, Kanatsu-Shinohara M, Nakahata T. Direct visualization of transplanted hematopoietic cell reconstitution in intact mouse organs indicates the presence of a niche. Exp Hematol. 2003;31:733–740.
24. Arai F, Hirao A, Ohmura M, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell. 2004;118:149–161.
25. Nilsson SK, Johnston HM, Coverdale JA. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. Blood. 2001;97:2293–2299.
26. Choi K. The hemangioblast: a common progenitor of hematopoietic and endothelial cells. J Hematother Stem Cell Res. 2002;11:91–101.
27. Yoder MC, Hiatt K, Dutt P, Mukherjee P, Bodine DM, Orlic D. Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. Immunity. 1997;7:335–344.
28. Yoder MC, Hiatt K, Mukherjee P. In vivo repopulating hematopoietic stem cells are present in the murine yolk sac at day 9.0 postcoitus. Proc Natl Acad Sci U S A. 1997;94:6776–6780.
29. Ito M, Hiramatsu H, Kobayashi K, et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood. 2002;100:3175–3182.
30. Hiramatsu H, Nishikomori R, Heike T, et al. Complete reconstitution of human lymphocytes from cord blood CD34+ cells using the NOD/SCID/gammacnull mice model. Blood. 2003;102: 873–880.
31. Cumano A, Ferraz JC, Klaine M, Di Santo JP, Godin I. Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. Immunity. 2001;15:477–485.
32. Wang L, Menendez P, Shojaei F, et al. Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression. J Exp Med. 2005;201:1603–1614.
33. Wilson A, Laurenti E, Oser G, et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell. 2008;135:1118–1129.
34. Guan K, Nayernia K, Maier LS, et al. Pluripotency of spermatogonial stem cells from adult mouse testis. Nature. 2006;440: 1199–1203.
35. Conrad S, Renninger M, Hennenlotter J, et al. Generation of pluripotent stem cells from adult human testis. Nature. 2008;456:344–349.
36. Kossack N, Meneses J, Shefi S, et al. Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. Stem Cells. 2009;27:138–149.
37. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131: 861–872.
38. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126:663–676.
39. Wernig M, Meissner A, Foreman R, et al. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature. 2007;448: 318–324.

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—Full Paper—

Genetic Influences in Mouse Spermatogonial Stem Cell Self-Renewal

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Abstract. Spermatogonial stem cells (SSCs) are slowly dividing cells that undergo self-renewal division to support spermatogenesis. Although the effects of genetic background in stem cell self-renewal have been well studied in hematopoietic stem cells, little is known about its effect on stem cells in other self-renewing tissues, including SSCs. To examine whether genetic factors are involved in regulation of SSC self-renewal, we first studied spermatogenesis in different inbred mouse strains (C57BL/6, DBA/2, AKR, BALB/C and C3H) after chemical damage caused by busulfan. Spermatogenesis in the DBA/2 and AKR strains was relatively resistant to busulfan treatment, whereas spermatogenesis was diminished in C57BL/6 mice and nearly ablated in C3H and BALB/C mice. Serial germ cell transplantation experiments provided functional evidence that SSCs with the DBA/2 background expanded more rapidly than those with the B6 background. Finally, we also employed the Germline Stem (GS) cell culture technique to examine the self-renewal activity *in vitro*. Although genetic manipulation of GS cells has been limited to those from the DBA/2 background, we produced transgenic offspring of the C3H background by electroporation of GS cells with a plasmid vector. Our results underscore the importance of genetic factors in SSC self-renewal. Furthermore, application of genetic modification techniques to GS cells with non-DBA/2 backgrounds extends the potential of a SSC-based approach in male germline modification.

Key words: Germ cell transplantation, Germline stem cell, Spermatogonial stem cell

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Stem cells are a unique population of cells with self-renewal activity. A single stem cell can proliferate to make two stem cells (self-renewal division), one stem cell and one differentiating cell (asymmetric division) or two differentiating cells (differentiating division) [1]. The mode of stem cell replication is altered by environmental factors. For example, stem cells have the potential to regenerate an entire organ following damage by chemicals or radiation treatment. In such cases, it is thought that the number of stem cells is initially increased by self-renewing division, followed by gradual production of differentiated cells by asymmetric division. Spermatogonial stem cells (SSCs) are the only type of stem cells that have germline potential [1, 2]. Like stem cells in other self-renewing tissues, SSCs can regenerate spermatogenesis after chemical treatment [3–5] or after germ cell transplantation [6]. SSCs migrate into empty niches to reinitiate spermatogenesis following their microinjection into seminiferous tubules. During the first several weeks, SSCs expand on the basal membrane to form patches or networks of spermatogonia, and these cells eventually produce differentiated sperm several months after transplantation [7]. However, the mechanisms by which SSCs regulate self-renewal and differentiation remain uncertain.

In 2003, we reported a culture method to expand SSCs *in vitro* [8]. The addition of glial cell line-derived neurotrophic factor (GDNF), a self-renewal factor for SSCs [9], induced proliferation of SSCs *in vitro*. The cultured cells, which we designated Germline Stem (GS) cells, continued to expand for more than 2 years without losing fertility [10]. They have an ability to convert into pluripotent stem cells [11] and are amenable for gene targeting [12]. In the course of these studies, we found that GS cells are easily derived from the DBA/2 (designated as DBA), C57BL/6 × DBA/2 F1 (B6D2F1) or ICR genetic backgrounds, but not from the B6 or 129 backgrounds. Although germ cells from the latter strains could form colonies, somatic cells overwhelmed the culture, and these colonies disappeared within several weeks [8]. Although a culture technique for SSCs in B6 mice was later developed, it was reported that they still grew poorly compared with those from DBA mice [13]. These *in vitro* studies suggest the involvement of genetic differences in SSC proliferation.

The importance of genetic factors in stem cell self-renewal was originally suggested based on data from hematopoietic stem cells (HSCs). A series of classic studies established that HSCs from different genetic backgrounds show remarkable differences in cell cycle or repopulation kinetics [14–16]. The effect of the genetic background has most intensively been investigated in the DBA and B6 backgrounds. For example, HSCs from the DBA background proliferate faster than those from the 129 or B6 backgrounds. Moreover, AKR and DBA mice have significantly more HSCs than other strains [15]. Considering the many similarities between spermatogenesis and hematopoiesis [17], it is possible that similar genetic differences play important roles in the kinetics of SSC self-renewal.

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In this study, we addressed this hypothesis by examining the effects of the genetic background on SSC regeneration *in vivo*. The speed of spermatogenesis regeneration was analyzed by administering busulfan, a chemical that eradicates SSCs in several animal species [3–5]; significant strain differences in the regenerating potential of spermatogenesis were observed. Using serial germ cell transplantation techniques, we also found that SSCs with the DBA background proliferate faster than those with the B6 background. These results underscore the importance of genetic factors in understanding the regulation of SSC self-renewal and further indicate similarities between hematopoiesis and spermatogenesis.

Materials and Methods

Animals

AKR, BALB, C3H, DBA and B6 wild-type mice and WBB6F1-W/W^v (W) mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Busulfan solution was prepared as previously described [18]. In brief, busulfan (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide and mixed with an equal volume of sterile distilled water before intraperitoneal injection into the animals. All busulfan treatments were conducted when the animals were 8 weeks old. For transplantation experiments, we used the transgenic mouse line C57BL/6 Tg14(act-EGFP)OsbY01 (designated as Green) provided by Dr. M Okabe (Osaka University, Japan) [19]. To introduce the transgene into the DBA background, the transgenic mice were backcrossed for seven generations to the DBA background. The spermatogonia and spermatocytes of these mice express the enhanced green fluorescent protein (EGFP) [19]. The Institutional Animal Care and Use Committee of Kyoto University approved all of the animal experimentation protocols.

Culture conditions

For the derivation of GS cells, testis cells from the AKR and C3H mouse strains were initiated as described previously using StemPro-34 SFM (Invitrogen, Carlsbad, CA, USA) [8]. In brief, testis cells were plated on a gelatin-coated plate, and after vigorous pipetting, floating cells were transferred to another plate to initiate culture. The growth factors used were 20 ng/ml mouse epidermal growth factor (EGF) (BD Biosciences, Franklin Lakes, NJ, USA), 10 ng/ml human FGF2 (BD Biosciences) and 15 ng/ml recombinant rat GDNF (R&D Systems, Minneapolis, MN, USA). After three or four passages on laminin-coated dishes (BD Biosciences), the cells were transferred to mitomycin C-treated mouse embryonic fibroblast cells. For transfection, GS cells from C3H mice were electroporated with *pCAG-EGFP2-neo*, which carried a *neo*-resistance gene; stably transfected cells were established via G418 treatment by mixing with non-transfected cells, as described previously [20].

Germ cell transplantation

For the quantification of germ cell colonies, approximately 4 μl of cell suspension (4 $\times$ 10^5 cells per testis) were injected into W mice (4–6 weeks old). For serial transplantation, testis cells from primary recipients were dissociated by two-step enzymatic digestion using collagenase and trypsin [7]. Comparison between the single cell suspensions prepared from intact testes of W and wild-type mice showed that 93.5 $\pm$ 0.4% (mean $\pm$ SEM, n=6) of the cells were germ cells. All recovered cells were microinjected into two to four secondary recipient testes (1.9–9.3 $\times$ 10^5 cells per testis). For production of transgenic animals using EGFP-transfected GS cells from the C3H background, approximately 2 μl of cell suspension (6 $\times$ 10^4 cells per testis) were microinjected into immature W pups (5–10 days old); pup recipients allow enhanced donor cell colonization [21]. The cells were introduced into the seminiferous tubules of a W testis through the efferent duct. The recipient animals were administered with an anti-CD4 antibody (GK1.5) intraperitoneally on days 0, 2 and 4 after transplantation to induce tolerance to the allogeneic donor cells [22].

Analysis of the recipient testes

For histological analysis of the testes, testis samples were collected at 35 and 70 days after busulfan treatment. The samples were fixed in 10% neutral-buffered formalin and processed for paraffin sectioning. Two sections were made from each testis at 12-μm intervals. Each slide was viewed at 400 $\times$ magnification to confirm the level of spermatogenesis in the testis, and images of the sections under an inverted microscope equipped with a CCD camera (DP70, Olympus, Tokyo, Japan) were collected using Photoshop software (Adobe, San Jose, CA, USA). The total numbers of tubules and tubules demonstrating spermatogenesis (defined as tubules with multiple layers of germ cells in the entire circumference) were counted for one section from each testis. All sections were stained with hematoxylin and eosin.

For quantification of germ cell colonies, recipient testes were recovered 2 months after transplantation. The testes were then exposed to UV light, and the fluorescence of the donor cells was observed under a stereomicroscope. The donor cell clusters were defined as colonies when they occupied the entire basal surface of the tubule and were at least 0.1 mm in length.

Flow cytometry

Dissociated testis cells were suspended (1 $\times$ 10^6 cells/100 μl) in phosphate-buffered saline containing 1% fetal calf serum (PBS/FCS). Cells were then incubated with rat anti-mouse EpCAM (clone G8.8; BD Biosciences) for 20 min on ice, washed twice with PBS/FCS and incubated with allophycocyanin-conjugated anti-rat IgG (BD Biosciences). Propidium iodide (1 μg/ml) was added to each sample just before analysis to distinguish live and dead cells. The cells were analyzed by FACSCalibur (BD Biosciences), as described previously [17].

Polymerase Chain Reaction (PCR)

Genomic DNA (25 ng) was isolated from the tail by phenol/chloroform extraction followed by ethanol precipitation. The presence of the transgene was determined by PCR of the genomic DNA using *EGFP*-specific primers (5'-ACGGCAAGCTGACCCTGAAG-3' and 5'-GGGTGCTCAGGTAGTGGTTG-3').

Microinsemination

Spermatozoa were collected mechanically from tubule segments that showed EGFP fluorescence. Microinsemination was per-

formed as described previously using spermatozoa [23]. These cells were microinjected into B6D2F1 oocytes. Embryos that reached the four-cell stage after 24 h in culture were transferred to the uteri of ICR recipient females.

Statistical analysis

The results were analyzed using the Student's *t*-test for independent samples with equal variance. Analysis of spermatogenic regeneration or flow cytometry after busulfan treatment was performed with ANOVA followed by Tukey's HSD. The colonization efficiency of the primary recipient testis in the secondary recipients was analyzed using the Chi-squared test.

Results

Regeneration of spermatogenesis after busulfan treatment

In our previous study, we showed regeneration of spermatogenesis and an increase in SSC number in B6 mice after busulfan treatment. Regeneration of spermatogenesis depended on the dose of busulfan; little regeneration occurred after the administration of 45 mg/kg busulfan, whereas recovery was more pronounced after treatment with 15 mg/kg busulfan [18]. In the first set of experiments, we administered 15 mg/kg busulfan to B6, DBA, AKR, BALB/C (designated as BALB) and C3H inbred mouse strains at 8 weeks of age and compared their spermatogenic regeneration. These mice contain comparable numbers of EpCAM-positive cells according to flow cytometry (Table 1). EpCAM is a marker for spermatogonia, including SSCs [24]. Administration of busulfan did not significantly change the proportion of spermatogonia in any of the strains at 2 days after treatment (Table 1), indicating that the immediate busulfan toxicity is not significantly different among these strains. It also suggested that there were comparable numbers of SSCs in all the strains before the start of regeneration.

To analyze the levels of regeneration, the testes of the animals were recovered by 35 and 70 days after busulfan treatment. A period of 70 days corresponds to two cycles of mouse spermatogenesis and provides sufficient time for regeneration from the SSCs that survived the treatment [1, 2]. Each testis was weighed, and regeneration was measured by counting the numbers of seminiferous tubules with spermatogenesis in histological sections. In agreement with previous studies, busulfan administration caused a significant decrease in testis weight in all tested strains (Fig. 1A). The most pronounced decrease was observed in C3H mice, where the average weight of the testis at 35 days was 34% of the untreated control weight. In contrast, the decrease was relatively modest in mice with the B6 background, and the testis weight was 59% of the control weight. The number of tubules with spermatogenesis was also reduced in all strains (Fig. 1B). In particular, severe effects were observed in BALB and C3H mice: only 4.6 and 8.0% of the total tubules from these mice, respectively, showed spermatogenesis. The difference between these two strains was not statistically significant (Table 2). In contrast, the same treatment had a mild effect in DBA and AKR mice, in which > 60% of the tubules demonstrated spermatogenesis. Although the average weight of the testes was comparable between the B6 and AKR mice, spermatogenesis was lower in the B6 strain, with only 32% of the tubules showing evidence of spermatogenesis. The most differentiated cell type at this stage was predominantly round spermatids, but some elongated spermatids could be observed.

At 70 days after busulfan administration, the testis weight and spermatogenesis increased in all tested strains, suggesting regeneration of spermatogenesis from SSCs. The testis weight at 70 days did not necessarily correlate with the testis weight at 35 days. In the B6 mice, for example, the mean weight of the testes at 70 days was 2.6-fold than at 35 days. In contrast, the testis weights were < 2-fold heavier at 70 days than at 35 days in the other strains. Interestingly, testes from DBA mice were significantly larger than those from B6 mice at 35 days, but were smaller than those from B6 mice at 70 days. However, regeneration with respect to spermatogenic recovery was most dramatic in the C3H and BALB mice, as assessed by histological analyses (Fig. 2). Although both of these strains showed < 10% spermatogenic tubules at 35 days, the levels of spermatogenesis had increased by factors of 12.6 and 7.1, respectively, by 70 days. While the increases in testis weight in the DBA and AKR mice were modest compared with that in the B6 mice, higher levels of spermatogenesis were observed by histology in the DBA and AKR mice, and many tubules showed spermatozoa

Table 1. Percentage of EpCAM-expressing cells in different strains

Strain[a]	Wild-type mice[b]	Busulfan-treated mice[b] (2 days after busulfan)
B6	15.9 ± 1.6	14.2 ± 4.6
DBA	15.2 ± 2.7	17.6 ± 1.8
AKR	12.6 ± 2.2	13.4 ± 2.0
BALB	17.3 ± 2.3	18.0 ± 2.3
C3H	15.0 ± 2.6	15.4 ± 2.1

Values are means ± SEM. Results from four separate experiments. Testis cells were dissociated by collagenase and trypsin and stained with anti-EpCAM antibody (See Materials and methods for details). [a] No significant difference was found among the different strains by ANOVA followed by Tukey's HSD. [b] No significant difference was found between the wild-type and busulfan-treated mice by *t*-test.

Table 2. Comparison of spermatogenesis levels after busulfan treatment

Comparison between strains	Significance at 35 days after busulfan treatment	Significance at 70 days after busulfan treatment
B6 *vs.* DBA	P<0.01	Nonsignificant
B6 *vs.* AKR	P<0.01	Nonsignificant
B6 *vs.* BALB	P<0.01	P<0.05
B6 *vs.* C3H	P<0.01	P<0.01
DBA *vs.* AKR	P<0.01	Nonsignificant
DBA *vs.* BALB	P<0.01	P<0.01
DBA *vs.* C3H	P<0.01	P<0.01
AKR *vs.* BALB	P<0.01	P<0.01
AKR *vs.* C3H	P<0.01	P<0.01
BALB *vs.* C3H	Nonsignificant	Nonsignificant

The difference among the strains was determined by ANOVA followed by Tukey's HSD. The numbers of testes analyzed were 12, 12, 12, 14 and 12 for B6, DBA, AKR, BALB and C3H, respectively.

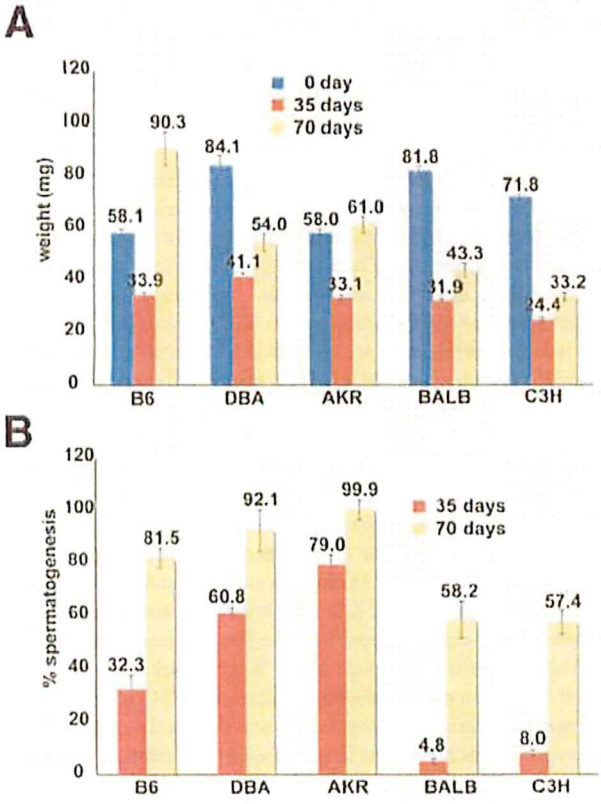


Fig. 1.

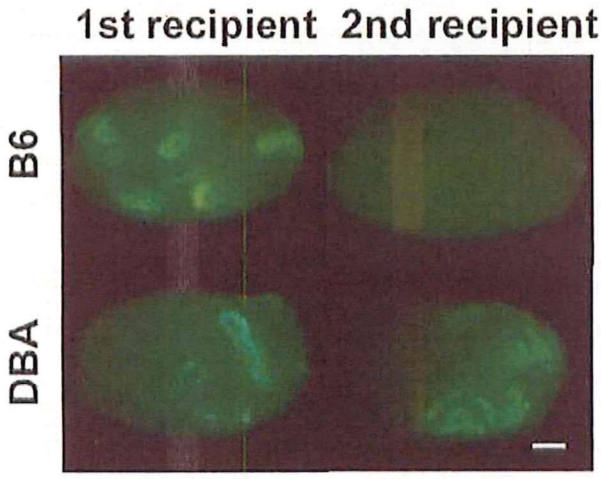
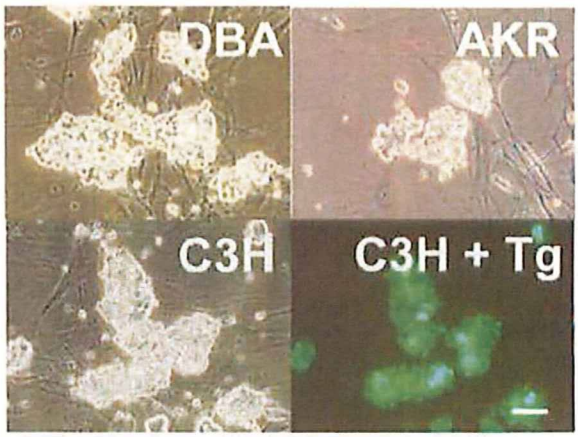


Fig. 3.



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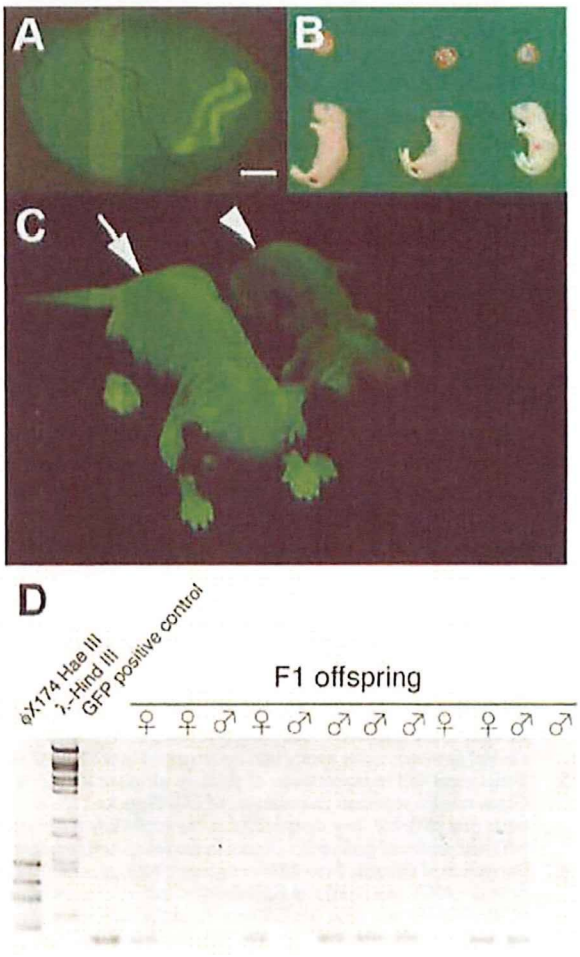

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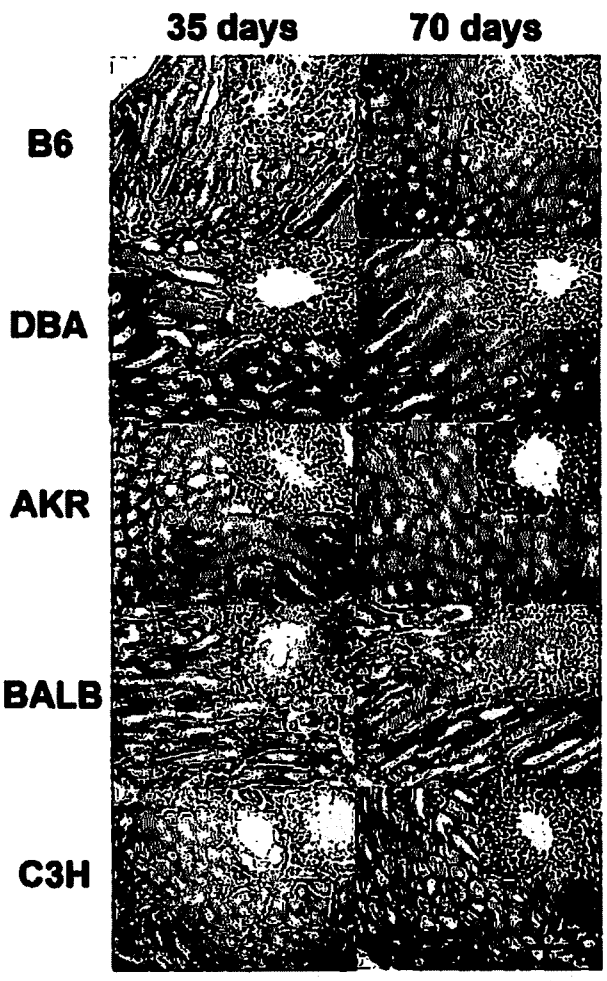

Fig. 2.

at this stage (Fig. 2, inset). The difference between DBA and AKR and between B6 and BALB was not statistically significant (Table 2). Thus, regeneration of spermatogenesis occurred in all tested inbred strains but was accompanied by wide variations in testis weight and tubule count.

Increase in SSC number during serial transplantation

The results in the preceding section suggested variability in SSC self-renewal activity; however, the actual changes in the number of SSCs could not be quantified because SSCs are defined only functionally by their ability to self-renew. Therefore, germ cell transplantation was used to measure the SSC number [6]. In the second set of experiments, we compared SSC activity between DBA and B6 mice because SSCs in DBA mice proliferated actively *in vitro*, whereas those in B6 mice did not. We wanted to examine whether they have different levels of self-renewal activity *in vivo*. We introduced the EGFP transgene into the DBA background by backcrossing the Green mouse and used the progeny as donor mice [19]. The Green mice ubiquitously express the EGFP transgene, which allowed us to evaluate the number of SSCs during serial transplantation experiments. Testis cells were collected from adult Green mice with the B6 and DBA backgrounds, and single-cell suspensions were microinjected into the seminiferous tubules of congenitally infertile W recipient mice. W mice lack all stages of differentiating germ cells, and administration of anti-CD4 antibodies allowed colonization of allogeneic donor SSCs at the same level as immunodeficient nude mice in germ cell transplantation studies [8, 25].

At 2 months after transplantation, the recipient testes were recovered, and the number of germ cell colonies was counted. It is thought that a single colony represents colonization originating from a single SSC [7, 26, 27]. The number of colonies per 10^5 transplanted cells ranged from 1.2 to 5.5, with an average of 2.3 ± 0.7 for DBA donor cells. On the other hand, the number of colonies per 10^5 transplanted cells ranged from 0.8 to 1.8, with an average of 1.1 ± 0.2 (mean ± SEM, n=28) for B6 donor cells (mean ± SEM, n=21). The DBA mice appeared to have more SSCs, but

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Fig. 1. Regeneration of spermatogenesis after busulfan treatment. (A, B) Changes in the testis weight (A) and levels of spermatogenesis (B) during regeneration of spermatogenesis. Note the extensive regeneration of spermatogenesis in the BALB and C3H testes. In contrast, the changes in the DBA and AKR mice were relatively modest.

Fig. 2. Histological changes in testes that received busulfan injection. The number of tubules with spermatogenesis was observed 35 and 70 days after treatment. Although many tubules were filled with mature germ cells in the AKR and DBA mice, the germ cells in the B6 mice were relatively immature, and there were many empty tubules in the BALB and C3H mice after 35 days. At 70 days, tubules with spermatozoa were found in all strains; however, many empty tubules remained in the BALB and C3H mice. Higher magnifications (inset). Stain: H & E. Bar=500 μm.

Fig. 3. Serial germ cell transplantation of primary recipient testis cells. Macroscopic appearance of recipient testes at 2 months after transplantation. Green tubules represent colonization of EGFP-marked donor cells. Germ cell colonies from B6 donor cells were found in the primary recipient testis (top left), but they disappeared in the secondary recipient testis (top right). In contrast, DBA donor cells in the primary recipient (bottom left) still produced germ cell colonies in the secondary recipient testis (bottom right). Bar=1 mm.

Fig. 4. Derivation of GS cells from different genetic backgrounds. Note the grape-like morphology of the GS cell colonies from all strains. The GS cells from the AKR (top right) and C3H (bottom left) mice were remarkably similar to those from the DBA mice (top left). After transfection with the *pCAG-EGFP2-neo* gene, the GS cells from the C3H mice showed green fluorescence under UV light (bottom right). Note that all of the cells expressed the EGFP transgene. Tg, transgene. Bar=100 μm.

Fig. 5. Production of transgenic offspring from EGFP-transfected GS cells of C3H mice. (A) Appearance of the recipient testis that was used for microinsemination. Note the green fluorescence from the transgene. (B) Newborn offspring after microinsemination. (C) Expression of the EGFP transgene in the offspring. While the offspring with the EGFP transgene showed strong fluorescence (arrow), the control littermate without the transgene did not fluoresce under UV light (arrowhead). (D) PCR analysis of the transgene. Seven of the twelve offspring contained the EGFP gene. Bar=1 mm (A).

Table 3. Serial transplantation of testis cells from primary recipients into secondary recipients

Type of original donor cells	Number of primary recipient testes used for serial transplantation	Number of cells recovered from the primary recipient testes ($\times 10^5$) [a]	Number of successful transplants[b] (%)	Number of colonies / 10^5 donor cells[a]	Number of secondary colonies /primary colony[a]
DBA	24	6.8 ± 0.6 [2.6–11.8]	18 (75.0)	0.56 ± 0.17 [0–3.4]	0.86 ± 0.20 [0–2.6]
B6	19	4.4 ± 0.6 [1.0–10.7]	7 (36.8)	0.06 ± 0.02 [0–1.7]	0.35 ± 0.13 [0–2.0]

Values are means ± SEM; the range is shown in brackets. [a] The difference between the two groups was statistically significant ($P<0.05$, analysis by *t*-test). [b] The difference between the two groups was statistically significant ($P<0.05$, analysis by Chi-squared test).

the difference was not statistically significant (P=0.33). Assuming that 1) all regenerating SSCs in the DBA and B6 mice colonize at the same efficiency in W mice (e.g., 10%) [7], 2) there was 100% harvesting efficiency during enzymatic digestion of the donor cells, 3) the colony number is linearly related to the number of injected cells and 4) W mice were immunologically tolerant to donor cells by anti-CD4 treatment, the total number of SSCs per testis (cell recovery × colony number) was 451 for the DBA cells and 384 for the B6 cells. Although the values were higher for the DBA background, the difference was not statistically significant. These results indicate that the SSC frequency and total number of SSCs are not different between these strains.

To evaluate the degree of self-renewal, we transplanted the primary testis cells into secondary recipients. In this experiment, all cells from the primary testis were recovered by enzymatic digestion using collagenase and trypsin. Significantly more cells were recovered from the testes that had received DBA cells than from those that had received B6 cells. The average recoveries of the cells from the primary recipient testes were $6.8 \pm 0.6 \times 10^5$ and $4.4 \pm 0.6 \times 10^5$ cells (means ± SEM, n=25 for DBA, n=19 for B6), respectively, suggesting that DBA cells proliferated more in the testis. The single-cell suspension from each testis was then microinjected into the seminiferous tubules of two to four secondary recipient testes. The secondary recipient testes were analyzed 2 months after transplantation, and the number of colonies was determined under UV light.

Serial transplantation of the recipient testes revealed that 18 of 24 primary testes (75%) that had received DBA donor cells produced germ cell colonies in the secondary recipients (Fig. 3). In contrast, only seven of 19 primary testes (37%) that had received B6 donor cells produced secondary colonies, suggesting that fewer SSCs were present in the B6 testis cell suspension. The average numbers of secondary colonies per primary colony were 0.86 ± 0.20 (mean ± SEM, n=24) and 0.35 ± 0.13 (mean ± SEM, n=19) for the DBA and B6 donor cells, respectively; the difference between the two groups was statistically significant. Assuming that 10% of the transplanted SSCs can colonize the recipient testis [7], this result indicates that one DBA or B6 SSC seeded in a W mouse underwent self-renewal divisions and produced 8.6 or 3.5 SSCs, respectively, in a germ cell colony generated in the primary recipient testes over a 2-month period (Table 3). These results confirm previous findings indicating that SSCs can increase in number during serial transplantation [18, 28]. Furthermore, these results indicate that SSCs from DBA mice proliferate to a greater extent than those from B6 mice *in vivo*.

Derivation and genetic manipulation of GS cells derived from C3H strains

We and others have previously reported that GS cells could be readily established from DBA or ICR mice, but that GS cells obtained from the B6 and 129 backgrounds grow poorly [8, 13, 29]. In the final set of experiments, we attempted to evaluate the genetic effect by comparing SSC proliferation *in vitro* using SSCs from AKR and C3H mice because these mouse strains showed significantly different responses to busulfan treatment. Testis cells from 0- to 2-day-old newborn mice were collected, and somatic cells were removed by overnight incubation on gelatin-coated plates. Germ cells from both of these strains could proliferate to form typical GS cell colonies, and the appearances of the GS cell colonies from each strain were indistinguishable from those of the DBA mice (Fig. 4).

To analyze whether the cultured cells contained SSCs, it was necessary to introduce a donor cell marker for germ cell transplantation. GS cells from C3H mice were regularly passaged at a 1:2 or 1:3 ratio every 5–6 days; they grow more slowly than GS cells from DBA mice (doubling time, 4.3 *vs.* 2.7 days) [30]. The cells were electroporated with the *pCAG-EGFP2-neo* transgene at 108 days after initiation of culture, and the transfected cells were selected by culture in the presence of G418. The sensitivity of the cells to G418 was not significantly different from that of DBA GS cells, and the cells could not grow clonally; hence, non-transfected cells had to be mixed with the cells during G418 drug selection. Stably transfected cells were established 67 days after transfection. The transfected cells showed EGFP fluorescence under UV light and produced typical germ cell colonies after transplantation into W mice (Fig. 5A).

To confirm whether the germ cells were fertile, we used microinsemination, a technique commonly used to treat infertility in animals and humans [23, 31]. Seminiferous tubules with green fluorescence were dissected using fine microforceps, and spermatogenic cells were recovered. Spermatozoa were microinjected into B6D2F1 oocytes. Of the 126 embryos constructed, 63 (50%) developed to the two-cell stage within 24 h in culture. All of these embryos were transferred into the oviducts of pseudopregnant females. Of these, 27 (42.9%) implanted in the uterus, and a total of 13 (20.6%) pups were born (Fig. 5B). The presence of the transgene in the offspring was confirmed by fluorescence under UV light (Fig. 5C). PCR analyses of 12 weaned animals also showed that seven of the offspring (4 males and 3 females) contained the EGFP transgene in tail DNA (Fig. 5D). These results show that GS