同 意 撤 回 書

東京大学医学系研究科長・医学部長 殿

研究課題「家族性血小板異常症の遺伝子解析研究」

私は、上記研究への参加にあたり、説明文書の記載事項について説明を受け同意しましたが、同意の是非について再度検討した結果、同意を撤回いたします。

資料(試料)等の保存について(これまでの同意の状況):「はい」または「いいえ」にご自身で○を付けてください。
提供した資料(試料)等が、長期間保存され、将来、新たに計画・実施される研究に使用されることに同意しました。
はい　　いいえ
(本研究終了後も保存)　(本研究終了時に廃棄)

資料(試料)等の保存について(同意の撤回):
「はい」または「いいえ」にご自身で○を付けてください。
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はい　　いいえ
(本研究終了時に廃棄)　(本研究終了後も保存)

平成　年　月　日

氏名(研究参加者本人または代諾者)(自署)＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

(代諾者の場合は、本人との関係)＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

口腔粘膜からのゲノム DNA 用サンプル採取

恐れ入りますが、以下のように採取をお願い致したく存じます。
採取した検体は、封筒に入った状態でご送付下さい。
＊検体が唾液で湿った状態の場合、DNA が分解します。ビニール袋を検体送付に使わないようお願い致します。

・準備するもの
・綿棒(通常の小さいサイズ: φ4 mm x 1cm 程度)
・紙の封筒(綿棒を入れる:ビニール袋は不可)
＊封筒・綿棒は 1 人あたり 1 つずつ用意し、混同しないよう番号をつけて下さい。

手順
・普通のサイズ (φ4mm x 1cm 程度) の綿棒を用いる
・唾液をできるだけのみ込んでもらう
・採取する側の頬が上になるように首を傾けてもらう
・以上によって、なるべく頬粘膜が唾液で湿っていないようにする。可能なら、キムワイプかティッシュペーパーを軽く当てて唾液を吸い取る
・綿棒の 1 つの面で 5 回、その裏の面で 5 回、頬粘膜を強くこする
・綿棒を紙の封筒に入れる (採取した側が奥になるように入れる)

検体は常温でお送り下さい(末梢血検体とともにお送り頂く場合は冷蔵で結構です)。

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III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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著者氏名	論文タイトル	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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IV. 研究成果の刊行物・別刷

(主なもの)

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Article

Evi1 is essential for hematopoietic stem cell self-renewal, and its expression marks hematopoietic cells with long-term multilineage repopulating activity

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Ecotropic viral integration site 1 (Evi1), a transcription factor of the SET/PR domain protein family, is essential for the maintenance of hematopoietic stem cells (HSCs) in mice and is overexpressed in several myeloid malignancies. Here, we generate reporter mice in which an internal ribosome entry site (IRES)-GFP cassette is knocked-in to the *Evi1* locus. Using these mice, we find that Evi1 is predominantly expressed in long-term HSCs (LT-HSCs) in adult bone marrow, and in the hematopoietic stem/progenitor fraction in the aorta-gonad-mesonephros, placenta, and fetal liver of embryos. In both fetal and adult hematopoietic systems, Evi1 expression marks cells with long-term multilineage repopulating activity. When combined with conventional HSC surface markers, sorting according to Evi1 expression markedly enhances purification of cells with HSC activity. Evi1 heterozygosity leads to marked impairment of the self-renewal capacity of LT-HSCs, whereas overexpression of Evi1 suppresses differentiation and boosts self-renewal activity. Reintroduction of Evi1, but not Mds1-Evi1, rescues the HSC defects caused by Evi1 heterozygosity. Thus, in addition to documenting a specific relationship between Evi1 expression and HSC self-renewal activity, these findings highlight the utility of Evi1-IRES-GFP reporter mice for the identification and sorting of functional HSCs.

Hematopoietic stem cells (HSCs) are distinguished by their inherent capacity to perpetuate themselves through self-renewal and to generate multiple blood cell lineages through differentiation. To maintain a steady-state pool of self-renewing HSCs and prevent HSC exhaustion, these defining properties of HSCs must be tightly regulated. Fine-tuning of stem cell properties requires stem cell–specific expression of their regulatory genes. To elucidate the stemness transcriptional profile, several gene expression microarray analyses have identified quite a few number of HSC-specific gene candidates (Ramalho-Santos et al., 2002; Akashi et al., 2003; Forsberg et al., 2010). However, most of the molecules established to be associated with the regulation of self-renewal capacity in HSCs are widely expressed in the hematopoietic system, and their mutations in genetic models are exclusively accompanied with other hematological abnormalities. Thus, a bona fide stem cell–specific regulator of their function has not been identified, and the functional identification of HSCs based on their ability to self-renew remains difficult.

Ecotropic viral integration site 1 (Evi1) is an oncogenic transcription factor that belongs to the SET/PR domain protein family (Goyama and Kurokawa, 2009). We and others have reported that Evi1 accomplishes an important regulatory function in hematopoietic stem/progenitor

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Abbreviations used: AGM, aorta-gonad-mesonephros; AML, acute myeloid leukemia; BFU-E, burst-forming unit-erythrocyte; CFU-S, CFU-spleen; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; CRA, competitive repopulation assay; EC, endothelial cell; ES, embryonic stem; Evi1, ecotropic viral integration site 1; FL, fetal liver; GEMM, granulocyte/erythrocyte/ macrophage/megakaryocyte; GM, granulocyte/macrophage; GMP, GM progenitor; HSC, hematopoietic stem cell; HSPC, hematopoietic stem/progenitor cell; IRES, internal ribosome entry site; Lin, lineage; LSK, Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$; LT-HSC, long-term HSC; ME, Mds1-Evi1; MEP, megakaryocyte/erythrocyte progenitor; MPP, multipotent progenitor; MSC, mesenchymal stem cell; MSL, $Mac\text{-}1^+$ $Sca\text{-}1^+$ Lin^-; OB, osteoblast; pA, polyadenylation; PB, peripheral blood; RQ-PCR, real-time quantitative PCR; SCF, stem cell factor; ST-HSC, short-term HSC; TPO, thrombopoietin.

K. Kataoka and T. Sato contributed equally to this paper.

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cells (HSPCs) during fetal and adult development. Evi1 expression is limited to HSPCs in the embryonic and adult hematopoietic systems. HSCs in *Evi1*$^{-/-}$ embryos are markedly decreased in numbers with defective repopulating capacity (Yuasa et al., 2005). Moreover, conditional deletion of Evi1 in adult mice revealed that Evi1 is essential for the maintenance of HSCs, but is dispensable for lineage commitment (Goyama et al., 2008). Besides the importance of Evi1 in normal hematopoiesis, dysregulation of Evi1 expression can have distinct oncogenic potential in various myeloid malignancies (Goyama and Kurokawa, 2009). Indeed, aberrant EVI1 expression defines a unique subset of acute myeloid leukemia (AML), and predicts adverse outcome in patients (Lugthart et al., 2008; Gröschel et al., 2010). Furthermore, Evi1 overexpression in hematopoietic cells leads to myelodysplasia in a murine BM transplant model (Buonamici et al., 2004).

In this study, using newly generated Evi1-GFP reporter mice, we demonstrate that Evi1 is preferentially expressed in LT-HSCs, and its expression can mark in vivo long-term multilineage repopulating HSCs and improve the conventional HSC isolation strategy in both adult BM and embryo, which suggests a distinctive relationship between Evi1 and HSC function. Consistent with this, heterozygosity of Evi1 causes a striking reduction in the number of LT-HSCs, with a specific defect of self-renewal capacity caused by accelerated differentiation. Our results point to a potential utility of an Evi1-GFP reporter mouse line for the functional identification of HSCs based on their self-renewal activity, and a central role of Evi1 in regulating the homeostasis of HSCs.

RESULTS

Evi1 is predominantly expressed in LT-HSCs in adult BM

To elucidate Evi1 expression within the hematopoietic system, we have generated gene-targeted mice in which an internal ribosome entry site (IRES)-GFP cassette is knocked-in to the *Evi1* locus by homologous recombination (Fig. 1 A). This knock-in allele functions in a bicistronic manner in that expression of both Evi1 and GFP is under the endogenous transcriptional regulatory elements of the *Evi1* gene, thus enabling us to track Evi1 expression on an individual cell basis. Appropriately targeted TT2 embryonic stem (ES) cell clones were identified by Southern blotting (Fig. 1 B). Mice heterozygous for the *Evi1-IRES-GFP* allele (*Evi1*$^{+/GFP}$) were distinguished from WT mice by genotyping PCR (Fig. 1 C). Western blot analysis showed the presence of GFP protein and comparable expression of Evi1 protein in embryonic fibroblast cells from *Evi1*$^{+/GFP}$ mice compared with WT mice (Fig. 1 D). *Evi1*$^{+/GFP}$ mice were phenotypically indistinguishable in survival, hematopoietic cellularity, and lineage composition from WT controls (unpublished data). Initial flow cytometric analysis of adult *Evi1*$^{+/GFP}$ mice revealed a small, but discrete, population of GFP$^+$ cells (0.15 ± 0.6%; Fig. 2 A), confirming the expression of the *Evi1-IRES-GFP* allele. To examine whether GFP expression levels correlated with those of endogenous *Evi1* mRNA expression, *Evi1* expression of sorted GFP$^-$ and GFP$^+$ cells from BM of *Evi1*$^{+/GFP}$ mice was analyzed by real-time quantitative PCR (RQ-PCR). *Evi1* mRNA was exclusively expressed in the GFP$^+$ cells, and almost no expression was found in the GFP$^-$ cells (Fig. 2 B), indicating that GFP expression in this mouse model faithfully marks cells with active Evi1 expression.

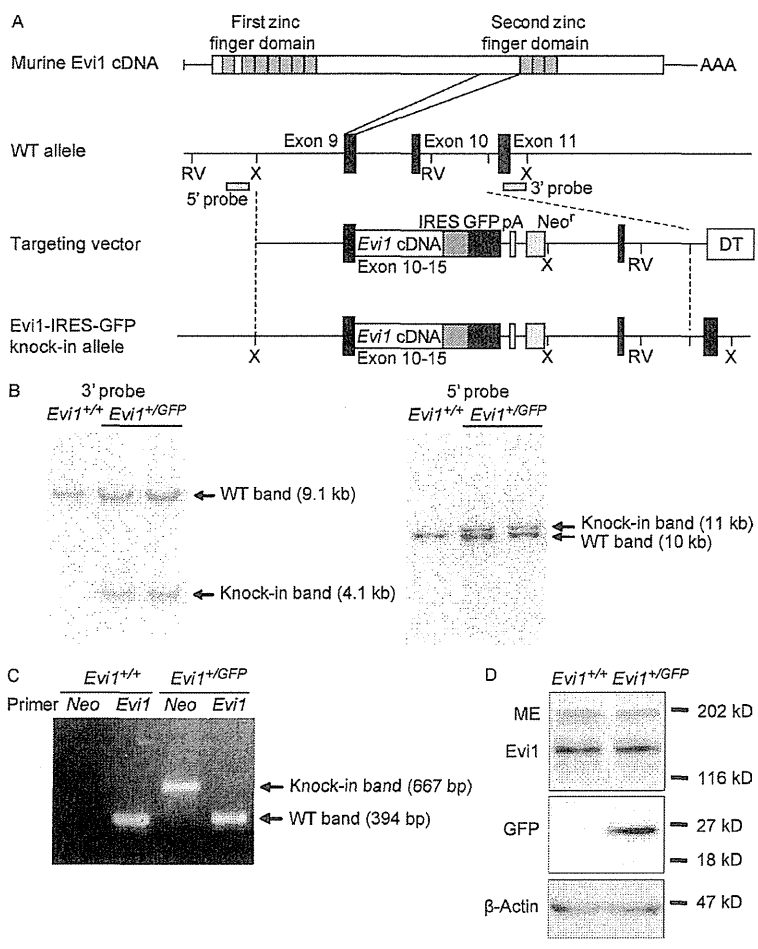

Figure 1. Generation of Evi1-IRES-GFP knock-in mice. (A) The structure of murine *Evi1* and the targeted *Evi1-IRES-GFP* locus is shown. RV, EcoRV; X, XbaI. (B) Southern blot analysis of genomic DNA isolated from WT ES cells (*Evi1*$^{+/+}$) and two independent clones of targeted ES cells (*Evi1*$^{+/GFP}$). DNA was digested with XbaI (left) or EcoRV (right), and hybridized with the indicated probes. (C) Genotyping of *Evi1*$^{+/GFP}$ mice by PCR. (D) Western blot analysis for GFP and Evi1 in embryonic fibroblast cells from *Evi1*$^{+/+}$ and *Evi1*$^{+/GFP}$ mice. β-Actin was used as a loading control. ME, Mds1-Evi1.

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Evi1 mRNA has been shown to be expressed at significantly higher levels in HSPCs (Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ [LSK]) and common lymphoid progenitors (CLPs) than in other hematopoietic cells (Yuasa et al., 2005; Chen et al., 2008). To gain insight into the biological function of Evi1 through its cell type–specific expression pattern, the distribution of GFP^+ cells was examined in adult BM from $Evi1^{+/GFP}$ mice. Beyond expectation, GFP expression was highly restricted to the LSK fraction (Fig. 2 A). To confirm stem/progenitor-specific expression of Evi1, we analyzed the GFP fluorescence of various hematopoietic cell populations from BM and spleen of $Evi1^{+/GFP}$ mice. We found a heterogeneous expression of GFP in the LSK fraction, in which about half of the cells were GFP^+ (Fig. 2, C and D). Conversely, only 2.5% of common myeloid progenitors (CMPs) expressed GFP, and almost no expression was found in granulocyte/monocyte progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs; Fig. 2 C). In contrast to the previous study (Chen et al., 2008), GFP was not expressed in CLPs (Fig. 2 C). In addition, no GFP expression was observed in mature hematopoietic lineages or nonhematopoietic cells in BM (Fig. 2 C). Together, these results suggest that Evi1 is uniquely expressed in HSPCs, but its expression is sharply down-regulated along with differentiation.

Because LSK cells, a population which contains multipotent progenitors (MPPs), short-term HSCs (ST-HSCs), and long-term HSCs (LT-HSCs), include both a GFP^+ fraction and a GFP^- fraction, we next resolved GFP expression within the LSK compartment for other markers characteristic of LT-HSCs. When LSK cells were subdivided according to CD34 and Flk-2 expression (Orford and Scadden, 2008), the $Flk\text{-}2^-$ $CD34^-$ LSK fraction, which is considered to contain most LT-HSC activity, had the highest expression of GFP, and its expression decreased with differentiation to hematopoietic progenitors (Fig. 2 E). In addition, further enrichment for LT-HSCs within the LSK fraction using SLAM family receptors (CD48 and CD150; Kiel et al., 2005) revealed that GFP^+ cells were found in greatest abundance within $CD48^-$ $CD150^+$ LSK cells, in which LT-HSCs are highly enriched. In contrast, GFP expression was substantially down-regulated in $CD48^+$ LSK cells, irrespective of CD150 expression (Fig. 2 F). When we examined how GFP^+ cells were distributed within the LSK fraction, GFP expression was highly enriched in the $Flk\text{-}2^-$ $CD34^-$ LSK or $CD48^-$ $CD150^+$ LSK fractions (Fig. 2, G and H). Therefore, these results indicate that Evi1 is dynamically regulated within HSPCs; its

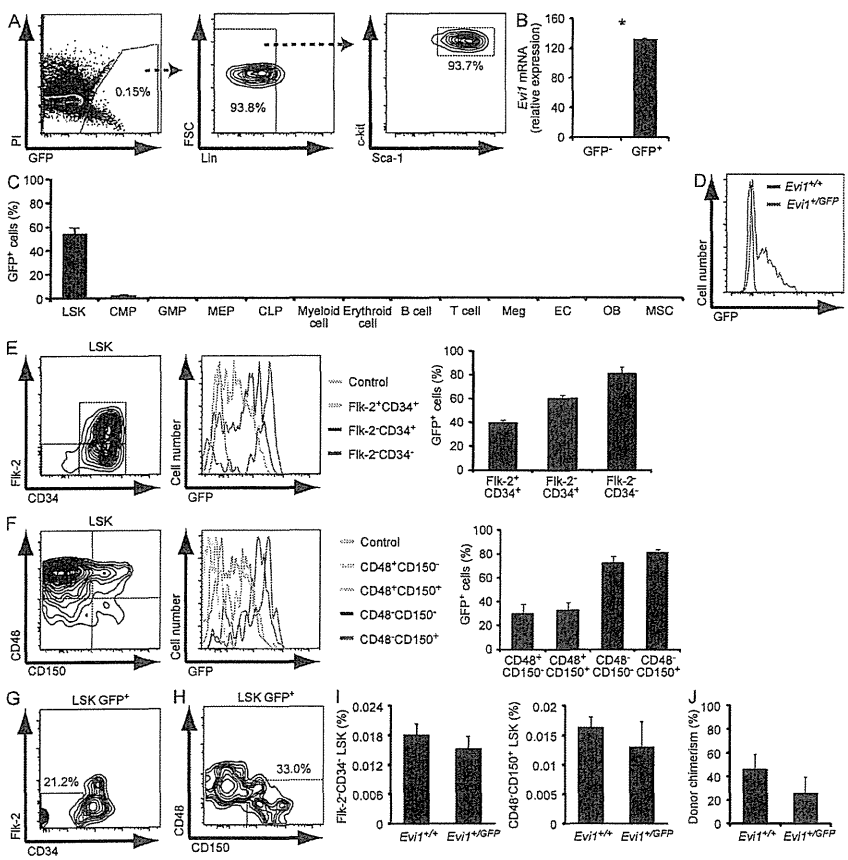

Figure 2. Evi1 is predominantly expressed in LT-HSCs in adult BM. (A) FACS analysis of expression of lineage markers (Lin), c-kit, and Sca-1 on GFP^+ cells in adult BM from $Evi1^{+/GFP}$ mice. Data are representative of three independent experiments. PI, propidium iodide; FSC, forward scatter. (B) RQ-PCR analysis of the expression of *Evi1* mRNA in sorted GFP^- or GFP^+ cells from BM of $Evi1^{+/GFP}$ mice, presented relative to GAPDH expression (*, $P < 0.0001$; $n = 2$). (C) Frequency of GFP^+ cells in indicated BM subpopulation and in splenic T cells from $Evi1^{+/GFP}$ mice ($n = 3$–5). Meg, megakaryocyte; EC, endothelial cell. (D) FACS analysis of expression of GFP in LSK cells from $Evi1^{+/+}$ and $Evi1^{+/GFP}$ mice. Data are representative of at least twenty independent experiments. (E and F) Frequency of GFP^+ cells in subpopulations of LSK cells divided using Flk-2 and CD34 (E) or CD48 and CD150 (F) in $Evi1^{+/GFP}$ mice. (left) Representative plot is shown. (right) Bar graph represents mean ± SD ($n = 3$–4). (G-H) FACS analysis of expression of Flk-2 and CD34 (G) or CD48 and CD150 (H) on LSK GFP^+ cells in BM from $Evi1^{+/GFP}$ mice. Data are representative of two independent experiments. (I) Frequency of $Flk\text{-}2^-$ $CD34^-$ LSK or $CD48^-$ $CD150^+$ LSK cells in BM from $Evi1^{+/+}$ and $Evi1^{+/GFP}$ mice ($n = 3$–5). (J) PB donor chimerism in CRAs, in which 2×10^5 BM cells from $Evi1^{+/+}$ and $Evi1^{+/GFP}$ mice (Ly5.1) were transplanted into lethally irradiated recipients (Ly5.2) together with 2×10^5 competitor BM cells (Ly5.2). Percentages of donor-derived cells (Ly5.1) in PB 16 wk after transplantation are shown ($P = 0.12$; $n = 3$). Data represent mean ± SD.

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expression is predominantly enriched in LT-HSCs and rapidly extinguished during early stages of lineage commitment.

To reinforce Evi1-IRES-GFP knock-in mice as a faithful tool for investigating HSCs, we assessed the number and function of LT-HSCs in BM from *Evi1*$^{+/GFP}$ mice. Flow cytometric analysis revealed that the frequencies of Flk-2$^-$ CD34$^-$ LSK or CD48$^-$ CD150$^+$ LSK cells were comparable between *Evi1*$^{+/+}$ and *Evi1*$^{+/GFP}$ mice (Fig. 2 I). In addition, a competitive repopulation assay (CRA) showed that *Evi1*$^{+/GFP}$ BM cells exhibited slightly less, but not significantly different, long-term reconstitution capacity (Fig. 2 J), indicating that the number and function of HSCs in *Evi1*$^{+/GFP}$ mice are similar to WT controls.

Evi1 expression represents a functionally distinct population that remains in an undifferentiated and quiescent state within HSPCs

As only a subset of LSK cells expressed GFP in *Evi1*$^{+/GFP}$ mice, we hypothesized that Evi1 expression functionally divides the LSK population and marks a more undifferentiated and quiescent state with multipotent differentiation properties in this population. To test this idea, we separated the LSK population into LSK GFP$^-$ and LSK GFP$^+$ cells and compared their biological functions. Initially, we confirmed that LSK GFP$^+$ cells had a much higher level of *Evi1* transcripts than LSK GFP$^-$ cells by RQ-PCR analysis (Fig. 3 A). Interestingly, despite the negative GFP expression, LSK GFP$^-$ cells expressed *Evi1* mRNA at a higher level compared with CMPs and GMPs (Fig. 3 A), which also suggests that Evi1 expression is inversely proportional to the differentiation status. To achieve an estimate of the differentiation stage of these two populations, LSK GFP$^-$ and LSK GFP$^+$ cells were cultured in serum-free medium containing stem cell factor (SCF) and thrombopoietin (TPO). After 3 d of culture, the proportion that remained in the LSK fraction was significantly higher in LSK GFP$^+$ cells than in LSK GFP$^-$ cells (Fig. 3 B), suggesting that LSK GFP$^+$ cells are more primitive HSCs. Next, to evaluate the differentiation potential of LSK GFP$^-$ and LSK GFP$^+$ cells, we performed colony-forming assays in vitro. Although both populations generated an equivalent number of myeloid colonies CFU-granulocyte/macrophage [CFU-GM]), LSK GFP$^+$ cells gave rise to greater numbers of erythroid (burst-forming unit-erythrocyte [BFU-E]) and multipotential (CFU-granulocyte/erythrocyte/macrophage/megakaryocyte [CFU-GEMM]) colonies than LSK GFP$^-$ cells (Fig. 3 C). These data suggest that Evi1 expression correlates with multipotent differentiation capacity. In addition, to assess the colony-forming capacity at the clonal level, single LSK GFP$^-$ and LSK GFP$^+$ cells were cultured in serum-free medium. LSK GFP$^-$ cells formed detectable colonies at a frequency comparable to LSK GFP$^+$ cells, but generated smaller numbers of highly proliferative colonies (>300 cells; Fig. 3 D), indicating that the LSK GFP$^+$ fraction comprises a higher proportion of HSPCs with enhanced proliferative capacity.

Our observations suggested that Evi1 reporter activity is down-regulated as HSCs differentiate. To examine this issue, we forced LSK GFP$^+$ cells to differentiate in vitro in response to SCF, TPO, IL-3, and IL-6. These LSK GFP$^+$ cells predominantly generated GFP$^-$ cells (Fig. 3 E). After culture, the majority of cells that had become GFP$^-$ lost the LSK phenotype, whereas most cells that remained in GFP$^+$ continued to express

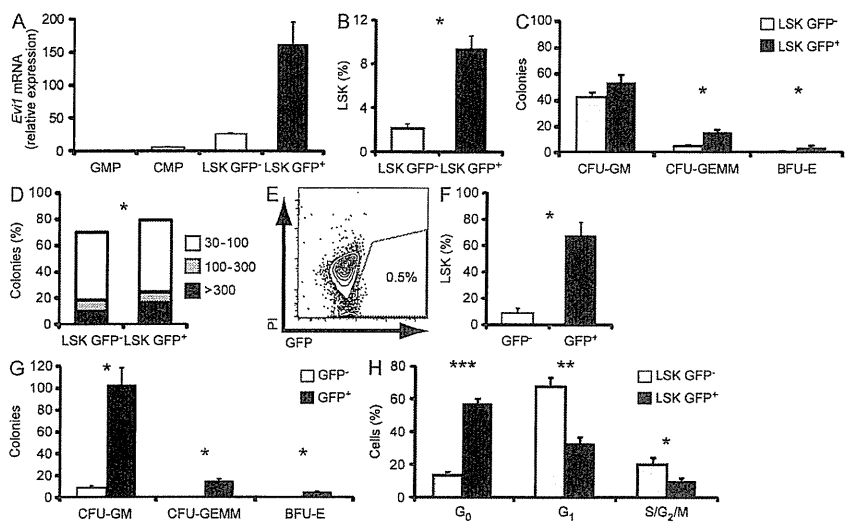

Figure 3. Evi1 expression represents a functionally distinct population that remains in an undifferentiated and quiescent state within HSPCs. (A) RQ-PCR analysis of the expression of *Evi1* mRNA in sorted GMPs, CMPs, LSK GFP$^-$ cells, and LSK GFP$^+$ cells from *Evi1*$^{+/GFP}$ mice, presented relative to *GAPDH* expression ($n = 2$). (B) LSK GFP$^-$ and LSK GFP$^+$ cells were cultured in serum-free medium with 20 ng/ml SCF and 20 ng/ml TPO for 3 d, and the percentage of the remaining LSK fraction was analyzed (*, $P < 0.001$; $n = 3$). (C) Numbers of CFU-GM, CFU-GEMM, and BFU-E colonies derived from 100 sorted LSK GFP$^-$ and LSK GFP$^+$ cells (*, $P < 0.05$; $n = 3$). (D) Single LSK GFP$^-$ and LSK GFP$^+$ cells from *Evi1*$^{+/GFP}$ mice were clone-sorted and cultured in serum-free medium. After 14 d of culture, cell numbers in each colony were analyzed. Their relative distribution is shown (*, $P < 0.05$; $n = 192$ clones from 2 independent experiments). (E) LSK GFP$^+$ cells were cultured in medium containing 10% serum with 50 ng/ml SCF, 50 ng/ml TPO, 10 ng/ml IL-3, and 10 ng/ml IL-6 for 5 d, and the percentage of the remaining GFP$^+$ fraction were analyzed. Data are representative of four independent experiments. (F) The percentages of the remaining LSK fraction in GFP$^-$ and GFP$^+$ cells after culture were analyzed (*, $P < 0.0001$; $n = 4$). (G) Numbers of CFU-GM, CFU-GEMM, and BFU-E colonies derived from 200 GFP$^-$ and GFP$^+$ cells were analyzed (*, $P < 0.0001$; $n = 4$). (H) Cell cycle status of LSK GFP$^-$ and LSK GFP$^+$ cells from *Evi1*$^{+/GFP}$ mice, analyzed by Hoechst 33342 and pyronin Y staining (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$, $n = 3$). Data represent mean ± SD.