Scl regulates the quiescence and the long-term competence of hematopoietic stem cells

*Julie Lacombe,[1] *Sabine Herblot,[1] Shanti Rojas-Sutterlin,[1] André Haman,[1] Stéphane Barakat,[1] Norman N. Iscove,[2] Guy Sauvageau,[1] and Trang Hoang[1,3]

[1]Institute of Research in Immunology and Cancer (IRIC), Montreal, QC; [2]Ontario Cancer Institute, Toronto, ON; and [3]Departments of Pharmacology and Biochemistry, University of Montreal, Montreal, QC

The majority of long-term reconstituting hematopoietic stem cells (LT-HSCs) in the adult is in G_0, whereas a large proportion of progenitors are more cycling. We show here that the SCL/TAL1 transcription factor is highly expressed in LT-HSCs compared with short-term reconstituting HSCs and progenitors and that SCL negatively regulates the G_0-G_1 transit of LT-HSCs. Furthermore, when SCL protein levels are decreased by gene targeting or by RNA interference, the reconstitution potential of HSCs is impaired in several transplantation assays. First, the mean stem cell activity of HSCs transplanted at approximately 1 competitive repopulating unit was 2-fold decreased when *Scl* gene dosage was decreased. Second, *Scl*$^{+/-}$ HSCs were at a marked competitive disadvantage with *Scl*$^{+/+}$ cells when transplanted at 4 competitive repopulating units equivalent. Third, reconstitution of the stem cell pool by adult HSCs expressing *Scl*-directed shRNAs was decreased compared with controls. At the molecular level, we found that SCL occupies the *Cdkn1a* and *Id1* loci in primary hematopoietic cells and that the expression levels of these 2 regulators of HSC cell cycle and long-term functions are sensitive to *Scl* gene dosage. Together, our observations suggest that SCL impedes G_0-G_1 transition in HSCs and regulates their long-term competence. (Blood. 2010;115:792-803)

Introduction

The lifelong production of blood cells depends on the regenerative capacity of a small population of hematopoietic stem cells (HSCs) that reside normally in the bone marrow, in the adult. This property forms the basis of HSC assays in which grafted donor cells are tested for their capacities for long-term reconstitution in all blood lineages of a transplanted host. The long-term persistence of clones initiated by HSCs depends critically on their ability to sustain self-renewal over time and over multiple HSCs divisions, thus their capacity for lifelong blood cell production. HSCs with reconstituting potential that do not sustain self-renewal activity generate clones that eventually lose stem cell activity and are detectable as transient reconstitutions in transplantation assays.[1] The mechanisms that sustain self-renewal remain to be further elucidated because only a few genes have been implicated in this process.[2]

In the adult, HSCs mostly reside in G_0[3] and are triggered into cycling by chemotoxic injuries,[4] by exposure to cytokines in vitro,[5] or by transplantation in vivo.[6] The state of quiescence in HSCs is reversible[3] and differs from quiescence associated with senescence, differentiation, or growth factor deprivation.[2,7] This suggests the existence of mechanisms that actively maintain HSCs in G_0. Such regulation is critical for long-term stem cell function,[2] as exemplified by several gene deletion models. HSCs from mice that are deficient for *Cdkn1a* or *Id1* exhibit impaired long-term activity, associated with increased cycling.[8,9] In addition, HSC quiescence is maintained by the opposing activities of *Gfi1*[10,11] and *Mef*,[12] although the transcriptional circuitry that actively maintains HSCs quiescence remains to be documented.[13]

Cell cycle and quiescence are actively controlled at critical transition points in the hematopoietic hierarchy. Unlike HSCs, a significant proportion of hematopoietic progenitors are found in S phase. For example, erythroid progenitors are highly proliferative,[14,15] whereas transition to terminal erythroid differentiation is coupled with growth arrest. We recently showed that the SCL transcription factor controls the transition between a proliferative state and commitment to differentiation and growth arrest in erythroid progenitors by up-regulating *Gfi1b* and *Cdkn1a* expression,[16] together with erythroid gene expression.[17,18] The critical role of *Scl* in generating HSCs during development is well documented,[19] but there is controversy with regards to its function in adult HSCs. For example, conditional knockout experiments suggest that *Scl* may be dispensable[20,21] and possibly redundant with other factors,[22] whereas decreasing SCL function by delivery of a dominant negative SCL or *Scl*-directed shRNA in human cells impaired their SCID-repopulating ability.[23,24] Nonetheless, gene profiling of HSCs ($Sca1^+Lin^-$ side population) identified a quiescence signature characterized by increased *Scl* and *Cdkn1a* expression.[25] In the present study, we address the role of SCL in HSC quiescence control and their long-term integrity.

Methods

Mice

Scl$^{+/-}$ mice[26] were a generous gift from Dr Glenn C. Begley (Amgen Inc). These mice were maintained on a C57BL/6 background for more than

Submitted January 23, 2009; accepted September 25, 2009. Prepublished online as *Blood* First Edition paper, October 22, 2009; DOI 10.1182/blood-2009-01-201384.

*J.L. and S.H. contributed equally to this study.

An Inside *Blood* analysis of this article appears at the front of this issue.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

10 generations. B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ mice are from The Jackson Laboratory. All mice were kept under pathogen-free conditions according to institutional animal care and use guidelines. The protocols for the transgenic and knockout mice, as well as bone marrow transplantation in mice, were approved by the Committee of Ethics and Animal Deontology of the University of Montreal.

Flow cytometry and cell-cycle analysis

Bone marrow cells from 2-month-old mice were depleted of lineage-positive (Lin^+) cells through immunomagnetic bead cell separation as detailed in supplemental data (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). The remaining Lin^+ cells were further excluded by flow cytometry using fluorescein isothiocyanate (FITC)– or biotin-conjugated antibodies against lineage markers CD3, B220, Gr1, CD11b, and TER119.

$Kit^+Sca1^+Lin^-CD150^+CD48^-$ (long-term reconstituting hematopoietic stem cells [LT-HSCs]) labeling and cell sorting were performed according to Kiel et al[27] (supplemental data). $Kit^+Sca1^+Lin^-$ (KSL), common myeloid progenitor (CMP), granulocyte-monocyte progenitor (GMP), and megakaryocyte-erythroid progenitor (MEP) were purified as reported[28] with further exclusion of $Fc\alpha R^+$ and IL7-$R\alpha^+$ cells. Common lymphoid progenitors (CLPs) were purified as described[29] (supplemental data). Dead cells were excluded by propidium iodide staining. Analyses were performed on the LSRII (BD Biosciences) and cell sorting on the FACSAria.

For cell-cycle analysis, lineage-depleted bone marrow cells were stained with Hoechst and Pyronin Y with rotation. Cells were incubated at 37°C with Hoechst 33342 (10 μg/mL; Invitrogen) for 45 minutes in Dulbecco modified Eagle medium supplemented with 2% fetal calf serum, followed by Pyronin Y labeling (1 μg/mL; Sigma-Aldrich) for 30 minutes. Cells were then washed and labeled for cell-surface markers (Kit, Sca1, CD150, CD48, and Lin). Where indicated, lineage-depleted cells were stimulated in culture for 24 hours with Steel Factor (100 ng/mL), interleukin-11 (IL-11; 100 ng/mL), and interleukin-6 (IL-6; 10 ng/mL). Cell-cycle analyses were performed on the LSRII at low flow rate.

For bromodeoxyuridine (BrdU) labeling and staining, mice received an initial intraperitoneal injection of 3 mg of BrdU, followed by 3 days of BrdU in drinking water (1 mg/mL). BrdU incorporation in LT-HSCs was detected by flow cytometry with the FITC-BrdU flow kit (BD Biosciences).

Fluorescein-di-β-D-galactopyranoside staining

β-Galactosidase activity was analyzed as described using the *Scl-lacZ*-knockin mouse model[26] to track *Scl* gene expression in HSCs and progenitors.[30]

Gene expression analysis and Western blotting

For reverse-transcribed polymerase chain reaction (RT-PCR) analysis, total RNA was extracted from 10 000 FACS-purified cells (FACSAria) for reverse transcription, as described.[31] PCR primers and antibodies used for Western blotting are described in supplemental data.

Transplantation assays

Limiting dilution analysis was performed essentially as described.[32] Briefly, $Scl^{+/-}$ test cells ($CD45.2^+$) were transplanted into irradiated hosts ($CD45.1^+$) at various cell doses ranging from 5000 to 10^6 per recipient mouse, together with a life-sparing dose (10^5) of bone marrow cells ($CD45.1^+$; supplemental data).

The level of lympho-myeloid reconstitution was assessed by flow cytometric analysis of the peripheral blood with antibodies against Gr1 (granulocytes), CD11b (myeloid cells), B220 (B lineage), and CD3 (T cells) 6 months after transplantation. Mice were scored positive when multilineage reconstitution was more than 1%. Competitive repopulating unit (CRU) frequencies were calculated by applying Poisson statistics using the Limit Dilution Analysis software (StemCell Technologies).

Competitive repopulating assays were performed using total bone marrow cells as follows. $Scl^{+/-}$ test cells ($CD45.2^+$) were transplanted with $Scl^{+/+}$ competitor cells from age-matched donors ($CD45.1^+$) at 2 ratio of test (T) over competitor (C) cells (T/C of 1/1 or 1/5) into congenic lethally irradiated (850 cGy) hosts ($CD45.1^+$), for a total of 2×10^6 donor cells per recipient. Five recipients were injected for each condition. The contribution of $Scl^{+/-}$ cells to hematopoiesis was evaluated 4 and 8 months after transplantation by flow cytometry and the relative competitive advantage (RCA) of $Scl^{+/-}$ cells was calculated as follows:

$$RCA = [\text{output T/C}]/[\text{input T/C}].$$

The input mixture was assessed by flow cytometry before transplantation to obtain the exact input T/C ratio. For secondary transplantation, bone marrow cells from primary recipients were harvested 4 months after transplantation and 2×10^7 cells were injected into irradiated recipient mice. Reconstitution of secondary recipients was analyzed 4 to 5 months after transplantation.

The mean activity of stem cells (MAS) is calculated according to the Harrison formula[33,34]: MAS = [RU]/[CRU] where RU represents the repopulating activity of 10^5 bone marrow cells and CRU was derived by limiting dilution analysis[32] as above. RU was calculated as previously described: $RU = [T \times \text{number of competitors} \times 10^{-5}]/C$.

Because the number of competitors was 10^5, the formula was applied as follows: RU = [T/C]. In mice transplanted with a dose of test cells corresponding to 1 CRU, then MAS = RU; therefore, MAS = [T/C].

Chromatin immunoprecipitation, production of lentivirus, shRNA gene transfer, and transplantation of puromycin-selected cells

This information is described in supplemental data. Primer sequences are shown in supplemental Table 1.

Statistical analyses

Where indicated, groups were compared using the Student *t* test.

Results

Elevated *Scl* expression in dormant adult LT-HSCs

To monitor *Scl* expression in LT-HSCs, we purified the $Kit^+Sca1^+Lin^-CD150^+CD48^-$ population[27] (Figure 1), which was independently confirmed by transplantation to be enriched in long-term HSCs in the proportion of one-half to one-fifth of cells[35] and will be referred to as cells with an LT-HSC phenotype. The KSL population is more heterogeneous and includes both short-term reconstituting HSCs (ST-HSCs) and LT-HSCs, whereas $Kit^+Sca1^-Lin^-$ progenitors are devoid of stem cell activity and could be further fractionated into common myeloid progenitor, granulocyte-monocyte progenitor, and MEP[28,29] (Figure 1A,C). We found that cells with an LT-HSC phenotype were mostly in G_0; ie, these cells exhibit low labeling with the DNA dye, Hoechst 33342, and the RNA dye, Pyronin Y,[36] whereas the KSL population was equally distributed in G_0 and G_1, and the Kit^+Sca1^- population was mostly in G_1 (Figure 1B).[37] *Scl* expression was first assessed by quantitative RT-PCR and was found to be present in all populations, including CLP. Nonetheless, we observed significant variations in *Scl* transcript levels, which were reproducibly highest in cells with an LT-HSC phenotype and lower in KSL cells (Figure 1C). In progenitors, *Scl* levels were higher in MEP compared with the others, consistent with previous reports.[38] Interestingly, these differential *Scl* levels were also observed when the activity of the *Scl* gene was monitored using the *Scl-LacZ* mouse model (hereafter $Scl^{+/-}$ mice) in which the *LacZ* gene was inserted into the *Scl* locus.[26] Indeed, β-galactosidase activities were 2.4- and 3.5-fold higher in cells with an LT-HSC phenotype than in KSL and

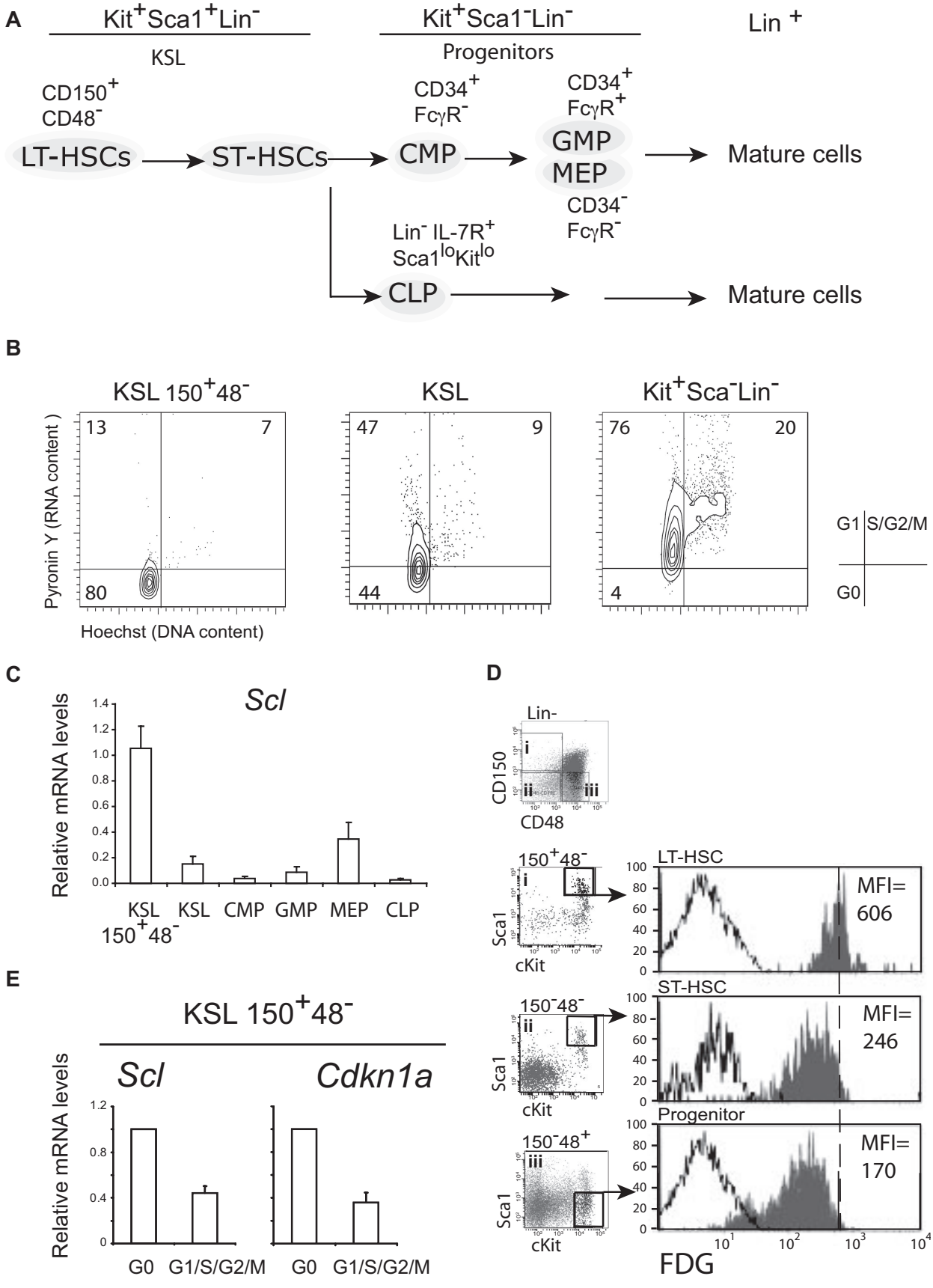

Figure 1.

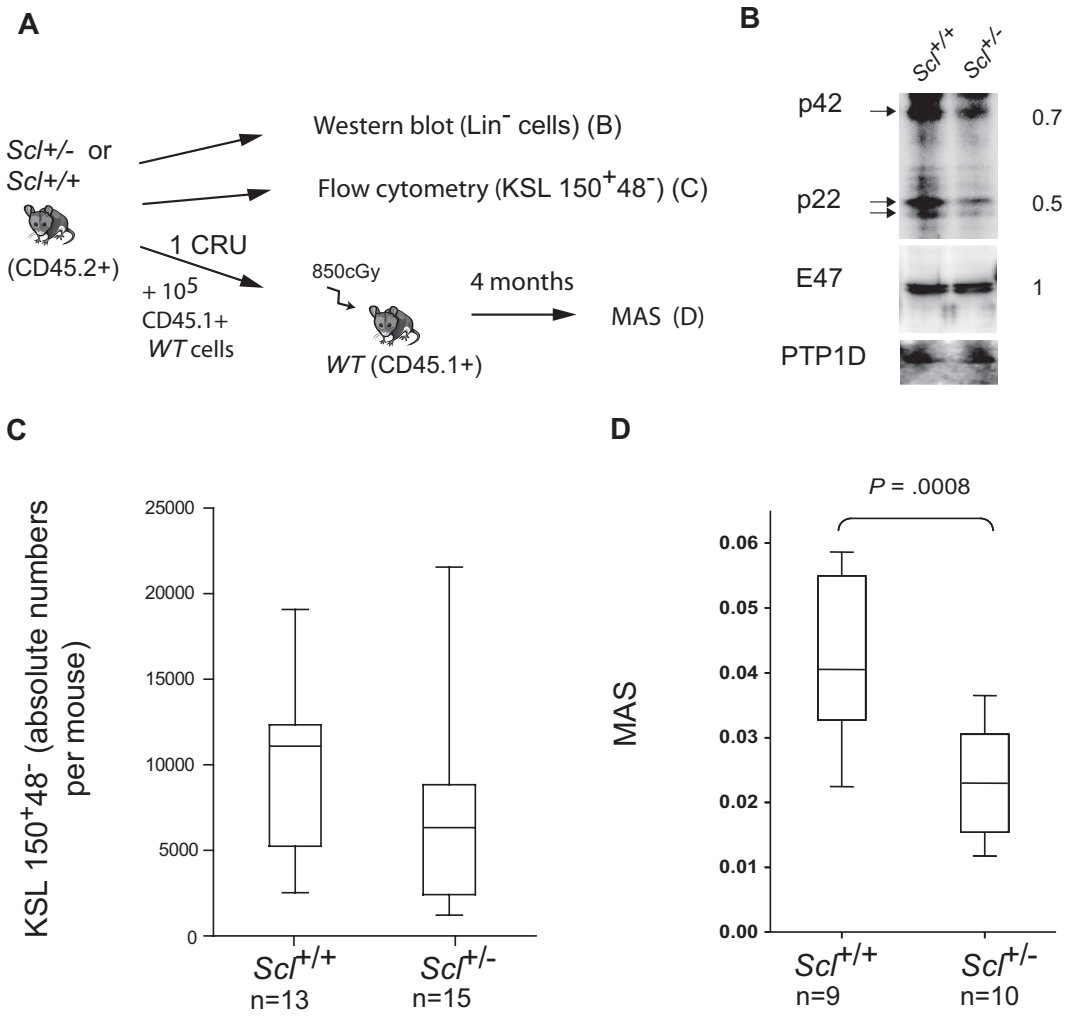

Figure 2. *Scl* haplodeficiency decreases mean stem cell activity. (A) Experimental scheme. Bone marrow cells from adult $Scl^{+/-}$ mice and $Scl^{+/+}$ littermates or age-matched controls were processed in parallel. (B) Western blot of total protein extracts were used to assess SCL and E2A protein levels in $Scl^{+/+}$ and $Scl^{+/-}$ lineage-depleted bone marrow cells. Anti-PTP1D was used as a loading control. Ratio of chemiluminescent intensities from $Scl^{+/-}$ over $Scl^{+/+}$ samples are shown on the right. (C) The frequency of LT-HSCs in $Scl^{+/+}$ and $Scl^{+/-}$ mice was assessed by flow cytometric analysis of $Kit^+Sca1^+Lin^-CD150^+CD48^-$ cells. Shown are box plots with the median and extreme values of each distribution (n mice per group). The 2 distributions are not significantly different ($P = .1$). (D) The mean stem cell activity (MAS) was calculated in mice receiving approximately 1 CRU, as described in "Transplantation assays." Box plots as above from n mice; *P* values as shown.

progenitors, respectively (Figure 1D). Furthermore, this experiment indicated that the *Scl* locus is transcriptionally active in all HSCs/progenitors at the single-cell level.

Figure 1. *Scl* expression in HSCs correlates with quiescence. (A) Schematic diagram of hematopoietic populations and their cell-surface markers. HSCs are KSL, which include LT-HSCs ($CD150^+CD48^-$) and ST-HSCs. Progenitors that are devoid of stem cell activity are $Kit^+Sca1^-Lin^-$, except for CLPs, which are $Kit^{lo}Sca1^{lo}Lin^-$ and express IL7R. Progenitors can be further differentiated on the basis of CD34 and FcγR expression. (B) The cell-cycle status of HSCs and progenitors assessed by Hoechst 33342 (DNA) and Pyronin Y (RNA) staining. G_0, G_1, and S-G_2-M are defined as shown (dot plots are representative of 5 independent experiments). (C) *Scl* mRNA levels in purified populations from adult mice were assessed by quantitative RT-PCR and normalized using *Hprt*. Expression levels in LT-HSCs were set as 1 (mean ± SD of 3 experiments). (D) *Scl* gene expression was monitored by β-galactosidase staining (FDG) and flow cytometric analysis in LT-HSCs, ST-HSCs, and progenitor populations from $Scl^{+/-}$ mice in which the *LacZ* gene was inserted into the *Scl* locus (shaded histograms) and wild-type (white histograms) mice. The mean fluorescence intensities (MFIs) are indicated for $Scl^{+/-}$ populations. Data shown are representative of 2 independent experiments with groups of 3 or 5 mice each. (E) LT-HSC–enriched populations were further purified into G_0 or G_1/S/G_2/M fractions by flow cytometry with Hoechst and Pyronin Y, as in panel B. *Scl* and *Cdkn1a* expression levels were assessed by quantitative RT-PCR. mRNA levels were normalized using *Hprt* and compared with expression levels in the G_0 population (mean ± SD of 4 replicates; 2 experiments).

To address the possibility that *Scl* expression correlates with quiescence (Figure 1A-D), we subdivided the LT-HSC population into G_0 and non-G_0 (G_1/S/G_2/M) fractions and found that *Scl* expression is higher in the G_0 fraction and lower in the other fractions (Figure 1E). Interestingly, this expression pattern was also observed with *Cdkn1a*, an SCL target gene in erythroid progenitors,[16] encoding a negative regulator of the cell cycle. Together, our observations indicate that *Scl* expression is highest in resting LT-HSCs, whereas its expression is lowered with cell-cycle entry.

Reducing SCL level impairs HSC functions

To assess the role of *Scl* in HSC activity, we took advantage of the $Scl^{+/-}$ mouse (Figure 2A) in which we observed a 2-fold decrease of both p42 and p22 SCL protein isoforms in lineage-depleted bone marrow cells compared with $Scl^{+/+}$ cells, whereas E47 and PTP1D levels remained constant (Figure 2B). Decreased SCL protein levels did not affect the number of myeloid progenitors in $Scl^{+/-}$ mice at steady state or their proliferative potential compared with their $Scl^{+/+}$ counterparts (colony assays shown in supplemental Figure 1A-B). To estimate the pool of LT-HSCs, we first assessed

the numbers of $Kit^+Sca1^+Lin^-$ $CD150^+CD48^-$ cells in the femurs of $Scl^{+/-}$ and $Scl^{+/+}$ mice (Figure 2C) and found this number to be marginally lower in the $Scl^{+/-}$ group. These observations are consistent with a near-normal frequency of CRU estimated in transplantation assays (supplemental Figure 2A), in agreement with published results.[21] We next examined stem cell potentials under limiting conditions and assessed their mean repopulating abilities (MAS) in mice grafted with approximately 1 CRU. We observed that the MAS of $Scl^{+/-}$ HSCs was significantly decreased compared with $Scl^{+/+}$ HSCs (Figure 2D). Thus, despite a near-normal stem cell pool size, $Scl^{+/-}$ HSCs exhibit impaired repopulating potential under conditions of high proliferative demand.

We therefore assessed their competitive advantage against HSCs from $Scl^{+/+}$ littermates in competitive repopulation assays as well as serial transplantations. $Scl^{+/-}$ bone marrow cells ($CD45.2^+$) were mixed with an equal number of wild-type competitor bone marrow cells ($CD45.1^+$) and transplanted in congenic irradiated hosts ($CD45.1^+$; Figure 3A). Because HSCs transplanted under limiting conditions undergo extensive proliferation, which in turn can lead to impaired stem cell function,[2] we performed competitive repopulation assays at 2 doses of $Scl^{+/-}$ bone marrow cells: a limiting dose of 10^5 cells and a higher dose of 10^6 cells. Because HSC frequency derived by limiting dilution analysis was 1 of 22 000 for $Scl^{+/-}$ cells (supplemental Figure 2A) and the transplanted mixture was 45.7% $CD45.2^+$, we estimated that mice were transplanted with $Scl^{+/-}$ cell equivalents of 4 CRU and 40 CRU, respectively. Reconstitution in the peripheral blood, bone marrow, and thymus was assessed 4 and 8 months after transplantation (Figure 3B-D), and the RCA of $Scl^{+/-}$ over $Scl^{+/+}$ cells was calculated as described in "Transplantation assays." At the limiting dose of 4 CRU, $Scl^{+/-}$ HSCs were severely impaired in their capacity to repopulate blood myeloid and lymphoid cells except B cells at 4 months (Figure 3B left panel), consistent with decreased MAS at approximately 1 CRU (Figure 2D). At the saturating dose of 40 CRU equivalent, this competitive disadvantage was manifest in mature blood (Figure 3B right panel) and bone marrow cells (not shown) at 8 months and was normal (RCA $\sim$ 1) at 4 months, as reported for a conditional $Scl^{fl/-}$ model.[20] Thus, stem cell deficiency was readily detectable when transplanted at limiting dose, possibly because of extensive proliferation when the stem cell pool is limited.

Nonetheless, analysis of the KSL population in mice transplanted at high dose revealed that the contribution of $Scl^{+/-}$ cells was 2-fold decreased at 4 months (Figure 3C-D) and 10-fold decreased at 8 months (Figure 3D). These results indicate that $Scl^{+/-}$ cells are less competitive than $Scl^{+/+}$ cells in reconstituting the stem cell pool. This competitive disadvantage was observed in $IL7R\alpha^-$ myeloid progenitors at both time points.

In contrast to the myeloid lineages, $IL7R\alpha^+$ CLPs are slightly increased at 4 months and near-normal at 8 months (Figure 3D), consistent with a transient 2-fold increase in B cells in the peripheral blood followed by a normal contribution at 8 months when all other lineages are impaired (Figure 3B right panel). Because *Scl* is expressed at low levels in CLP (shown here)[39] and in pro-B cells[40] where SCL has been shown to inhibit E2A, $Scl^{+/-}$ cells might have a competitive advantage over $Scl^{+/+}$ cells in the B lineage. Finally, thymic repopulation as illustrated here for the $CD4^-CD8^-$ (double-negative [DN]) and $CD4^+CD8^+$ (double-positive [DP]) populations was transiently increased at 4 months and 5-fold impaired at 8 months (Figure 3F), consistent with decreased peripheral $CD3^+$ T lymphocytes at this time point (Figure 3B).

In secondary transplantations assays, $Scl^{+/-}$ HSCs were severely impaired in their abilities to reconstitute secondary recipients in stem cell and progenitor populations (Figure 3E left panel) and in almost all mature lineages, except mature B cells (Figure 3E right panel).

Finally, we ruled out a possible non–cell-autonomous contribution to the phenotype of $Scl^{+/-}$ HSCs. Indeed, the capacity of HSCs to reconstitute hematopoiesis was similar when wild-type bone marrow cells were transplanted into $Scl^{+/+}$ or $Scl^{+/-}$ irradiated mice (supplemental Figure 2B).

The above observations suggest that *Scl* levels regulate long-term HSC competence. Alternatively, it is possible that these stem cell defects arose during development. To address this question, we took an independent approach to decrease *Scl* levels in adult HSCs, based on the shRNA technology[16] (Figure 4A). Three different *Scl*-directed shRNAs delivered by lentivirus efficiently knocked down protein levels in lineage-negative bone marrow cells as assessed by Western blotting, compared with control cells expressing a nontargeting control shRNA (Figure 4B). These cells were transplanted at 100 KSL 150^+48^- per mice. Long-term reconstitution in the bone marrow within the population of cells with an HSCs phenotype (KSL $CD34^-$, Figure 4C; or KSL 150^+48^-, not shown) was on average 95% donor-derived in mice transplanted with control cells. This proportion was, however, 2-fold decreased by *Scl* shRNAs. We therefore conclude that *Scl* levels regulate the long-term maintenance of adult HSCs after transplantation.

SCL restrains the cell cycle in LT-HSCs

The long-term competence of stem cells has been linked to quiescence control.[2,7] Furthermore, our observations indicate a correlation between *Scl* levels and quiescence (Figure 1). We therefore addressed the question of whether the cell-cycle status of LT-HSCs was affected by *Scl* RNA interference or reduced *Scl* gene dosage.

Analysis of cell-cycle parameters in the population enriched in LT-HSCs using Hoechst and Pyronin staining indicated that *Scl* shRNA infection resulted in a 3-fold increase in the G_1 fraction (Figure 4D). The same effects were observed with 3 different shRNAs compared with a nontargeting control, suggesting that these were specifically the result of decreased SCL protein levels. Furthermore, populations that are enriched in hematopoietic progenitors ($Kit^+Sca^-Lin^-$; Figure 4E) and mature cells (Lin^+; supplemental Figure 3B) were not affected by shRNA exposure. Together, our observations indicate that SCL specifically hinders G_0-G_1 progression in LT-HSCs.

We next examined the cell-cycle status of LT-HSCs isolated from $Scl^{+/-}$ mice and found that reducing *Scl* gene dosage reproducibly increased by 2-fold the proportion of cells in G_1 but not in $S/G_2/M$ (Figure 5A). To further evaluate the proportion of LT-HSCs that enter the cell cycle at steady state, we treated $Scl^{+/+}$ and $Scl^{+/-}$ mice with BrdU for 3 days, a thymidine analog that incorporates into newly synthesized DNA. Whereas the estimated doubling time of individual HSCs is between 18 and 145 days, a 3-day treatment with BrdU appears to be sufficient to label LT-HSCs because BrdU is toxic for mature hematopoietic cells, causing an injury response that triggers dormant HSCs into proliferation.[3] We found that the proportion of $BrdU^+$ cells within the LT-HSC fraction was 19% in $Scl^{+/+}$ mice, and this proportion was increased to 34% in $Scl^{+/-}$ mice (Figure 5B). Thus, $Scl^{+/-}$ LT-HSCs reproducibly exhibit a significant increase in the fraction of cells that undergo G_0-G_1 transition in vivo, either during steady state or in response to BrdU.

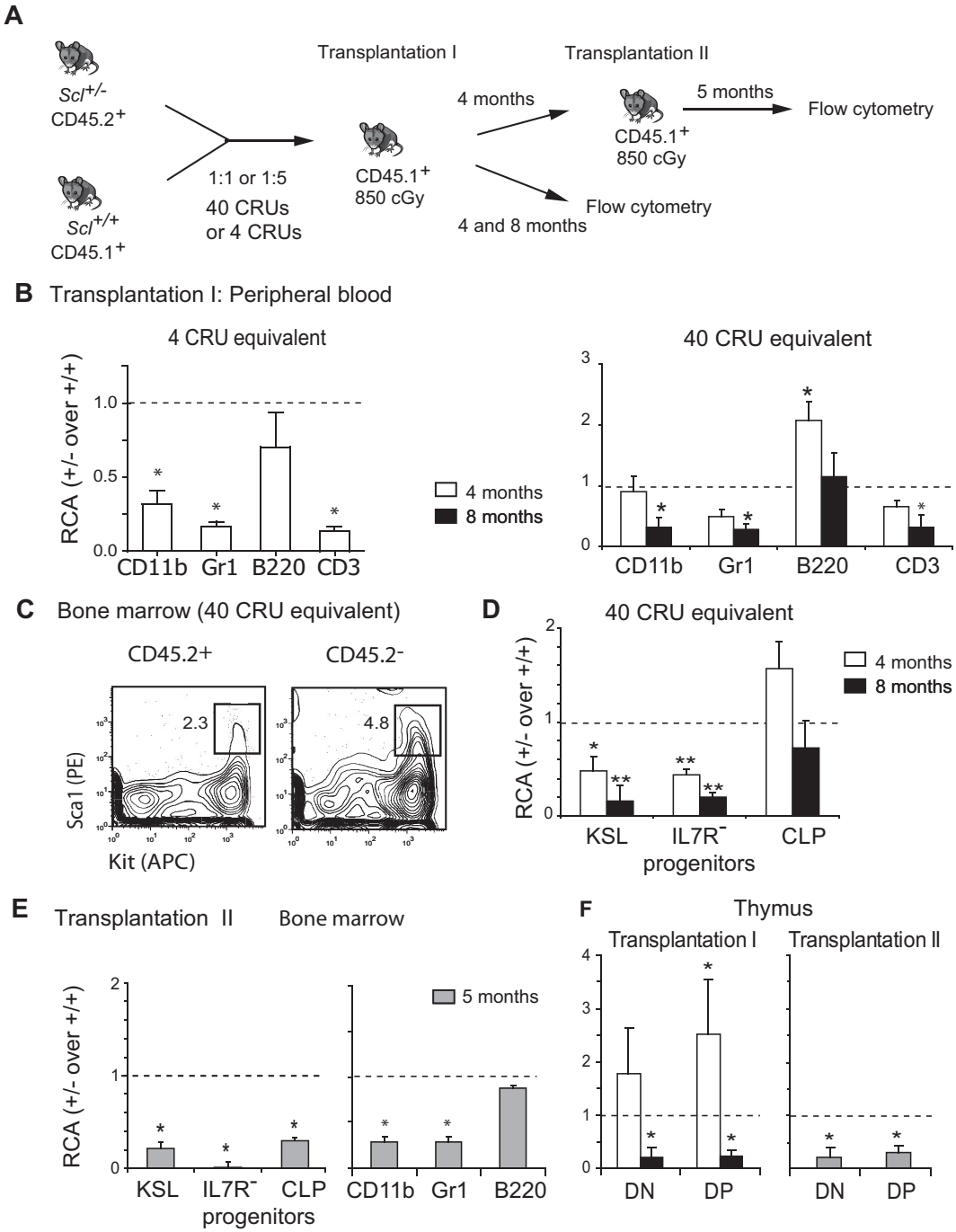

Figure 3. *Scl* is required for HSC long-term competence. (A) Diagram of the serial competitive transplantation strategy. $Scl^{+/-}$ bone marrow cells ($CD45.2^+$) were mixed with $Scl^{+/+}$ competitor bone marrow cells ($CD45.1^+$) at 1:1 or 1:5 ratio, and cells were transplanted in congenic irradiated hosts ($CD45.1^+$) at 2 $Scl^{+/-}$ cell equivalents of 4 CRU or 40 CRU. (B-F) Data illustrate results for the 1:1 ratio at 4 CRU cell equivalents (B left panel) and 40 CRU cell equivalents (B-F). The RCA of $Scl^{+/-}$ cells was calculated and shown as the median ± SEM (*$P \leq .05$). (B) Reconstitution by $Scl^{+/-}$ ($CD45.2^+$) and $Scl^{+/+}$ ($CD45.2^-$) cells after the first transplantation at 4 or 40 CRU equivalents. Reconstitution at 4 and 8 months after transplantation was assessed by flow cytometric analysis of myeloid (CD11b, Gr1) and lymphoid (B220, CD3) cells in the peripheral blood. Data are representative of 2 independent experiments with groups of 5 or 7 mice each. Input $Scl^{+/-}$ cells before transplantation were 45.7% and 54.5%, respectively. (C-D) Analysis of populations enriched in stem cells and progenitors in the bone marrow. A representative analysis at 4 months of the KSL population is shown (C). The RCA of $Scl^{+/-}$ cells within the HSCs ($Kit^+Sca1^+Lin^-$) population and myeloid ($Kit^+Sca1^-Lin^-IL7R^-$) as well as lymphoid (CLP) progenitor populations was assessed 4 and 8 months after transplantation (D) (**$P \leq .001$). (E) Reconstitution by $Scl^{+/-}$ cells in the HSC population (KSL) and myeloid and as well as lymphoid progenitor populations (E left panel) or the mature myeloid and lymphoid populations (E right panel) 5 months after secondary transplantation. (F) Thymic reconstitution in primary and secondary transplantation within the $CD4^-CD8^-$ (DN) and $CD4^+CD8^+$ (DP) populations is shown. Comparable results were observed with the $CD4^+$ or $CD8^+$ populations. All populations shown are $Thy1^+$.

During development, HSCs are mostly nonquiescent and then at approximately week 4 after birth switch to a quiescent state, which persists throughout adult life.[37] We therefore assessed the loss of one *Scl* allele on cell cycle in perinatal HSCs. Unlike adult HSCs, we found that half of perinatal HSCs are in $G_1/S/G_2/M$, and these are not significantly affected by *Scl* haploidy (Figure 5C). On week 4, however, these cells switch to the adult phenotype and their cycling state was affected by *Scl* gene dosage (not shown). Therefore, SCL does not control HSC cell cycle at the early postnatal stage. Consistent with this, *Scl* was shown to be more

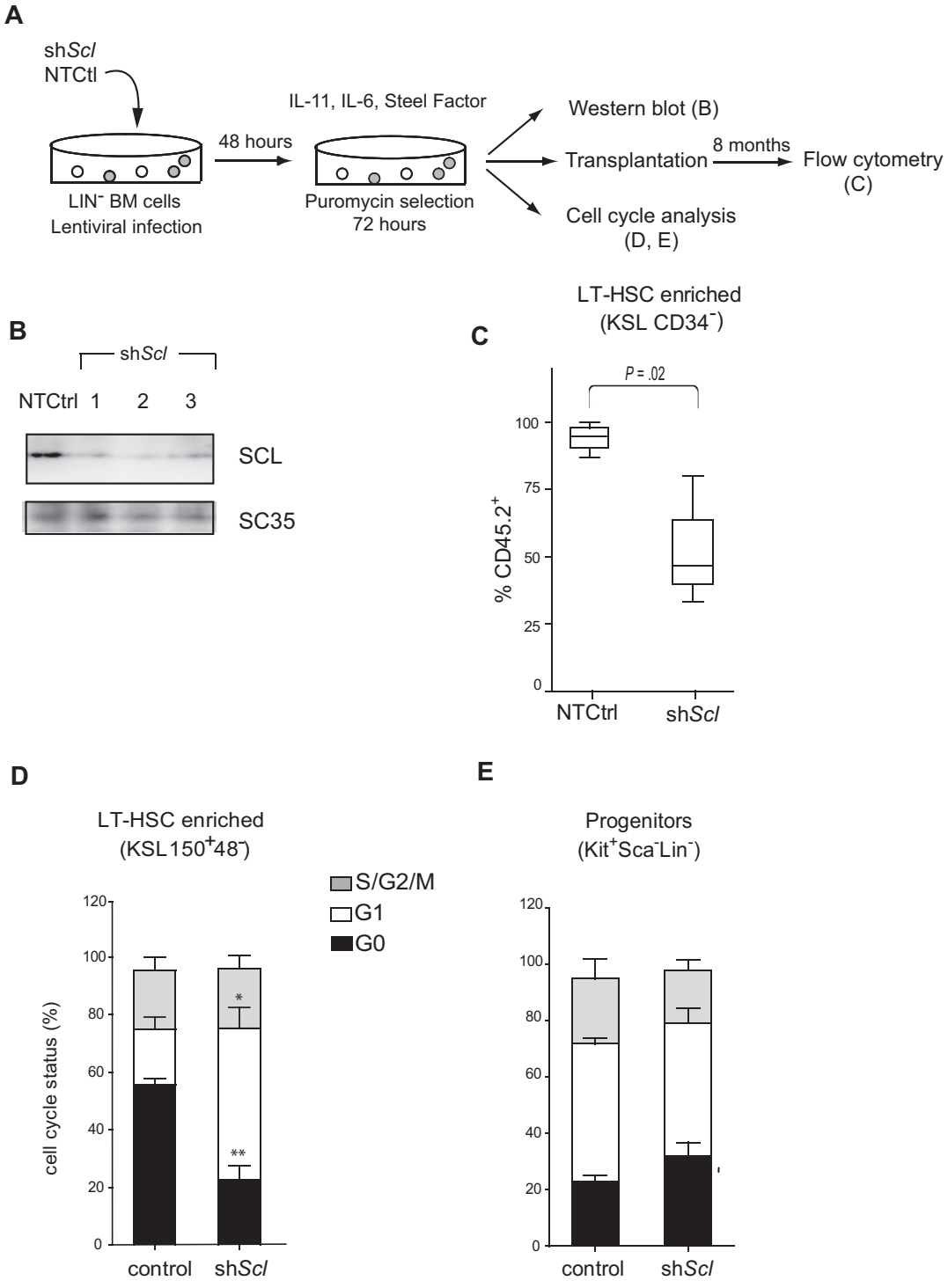

Figure 4. *Scl*-directed shRNAs impair reconstitution in vivo and facilitate the G_0-G_1 transition of LT-HSCs in culture. (A) Schematic representation of shRNA lentiviral delivery in lineage-depleted bone marrow cells. Data shown are representative of 2 independent experiments. (B) Western blot of nuclear extracts from lineage-depleted bone marrow cells after the delivery of 3 different *Scl*-directed shRNAs or a nontargeting control. SC35 was used as loading control. (C) After gene delivery and puromycin selection, cells ($CD45.2^+$) were transplanted into irradiated hosts ($CD45.1^+$). The bone marrow was analyzed 8 months later for reconstitution ($CD45.2^+$) within the HSC population (KSL $CD34^-$). Box plots illustrate pooled data from control cells expressing the empty vector or a nontargeting shRNA (NTCtrl) or from cells expressing *Scl*-directed shRNAs illustrated in panel B (sh*Scl*). (D-E) Cell-cycle analyses of shRNA-infected cells were performed using Hoechst 33342 and Pyronin Y. Stacked columns represent the relative proportion of cells in G_0, G_1, and $S/G_2/M$ phases within the LT-HSCs (D) and progenitor populations (E). Data represent the mean ± SEM of pooled data for controls or sh*Scl*-expressing cells as in panel C (*$P < .01$, **$P < .001$).

elevated in adult compared with fetal HSCs in 3 independent reports.[25,37,41] Our observations indicate that SCL specifically controls LT-HSC quiescence in the adult.

In vitro cytokine stimulation triggers HSC proliferation and increases the proportion of cells in $S/G_2/M$ (Figure 6A-B). We therefore assessed the cell-cycle status of $Scl^{+/-}$ HSCs after 24 hours of cytokine stimulation and found a 2-fold increase in the G_1 fraction compared with $Scl^{+/+}$ HSCs, associated with a concomitant decrease in the G_0 fraction. It is noteworthy that $Scl^{+/-}$ cells that were stimulated for 24 hours with cytokines (Figure 6B right

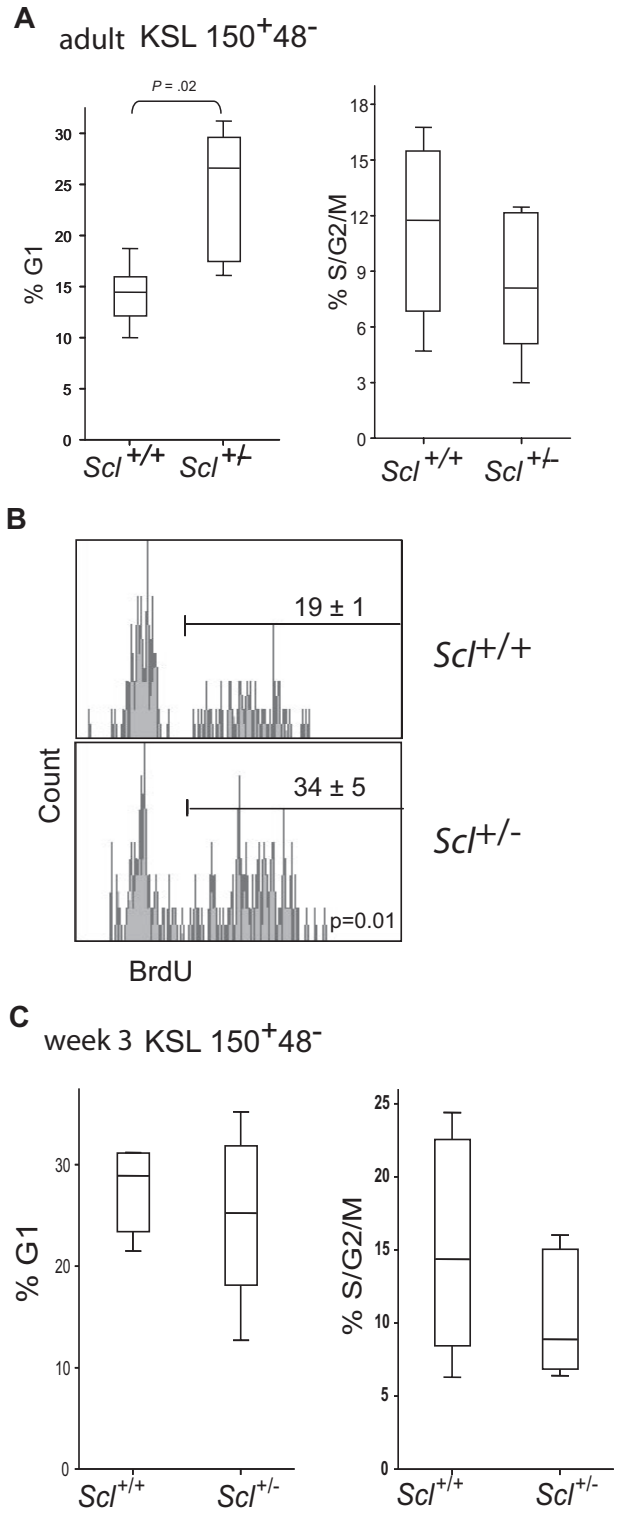

Figure 5. *Scl* gene dosage and the cell-cycle status of LT-HSCs. (A) The cell-cycle status of adult LT-HSCs was assessed within the Kit$^+$Sca1$^+$Lin$^-$CD150$^+$CD48$^-$ population by Hoechst 33342 and Pyronin Y staining. Box plots represent pooled data from 6 experiments with *Scl*$^{+/+}$ and *Scl*$^{+/-}$ bone marrow cells at steady state, together with the medians and extreme values of the 2 distributions, illustrating the percentages of cells in G_1 (left panel) and S-G_2M (right panel). (B) BrdU incorporation in LT-HSCs was analyzed within the Kit$^+$Sca1$^+$Lin$^-$CD150$^+$CD48$^-$ population. Representative histograms are shown from *Scl*$^{+/+}$ and *Scl*$^{+/-}$ bone marrows. Numbers shown are the mean ± SEM of 3 experiments. (C) The cell-cycle status of perinatal LT-HSCs (week 3) from *Scl*$^{+/+}$ and *Scl*$^{+/-}$ bone marrows was assessed by flow cytometry, as in panel A. The 2 distributions do not differ significantly.

panel) more closely resemble *Scl* knockdown cells (Figure 4D) that were exposed to cytokines for 5 days in culture because only 27% and 25% to 35% of KSL 150$^+$48$^-$, respectively, are in G_0. Therefore, cell-cycle analysis at this early time point reveals that decreasing SCL levels facilitates the G_0-G_1 transition and cell-cycle progression when HSCs are stimulated by cytokine. In contrast, the cell-cycle state of adult progenitors (Kit$^+$Sca1$^-$Lin$^-$) or mature cells (Lin$^+$) was not significantly affected by *Scl* gene dosage (Figure 6C; supplemental Figure 3B), in good agreement with lower *Scl* expression level in these populations compared with HSCs (Figure 1C-D). Furthermore, we did not observe differences in cytokine-induced cell-cycle progression when *Scl*$^{+/+}$ and *Scl*$^{+/-}$ LT-HSCs from 3-week-old mice were compared (Figure 6D). We therefore conclude that decreasing *Scl* gene dosage facilitates the G_0-G_1 transition in adult LT-HSCs in vivo and in vitro.

SCL controls Cdkn1a and Id1 gene expression in HSCs

We next assessed whether SCL levels affect the expression levels of known cell-cycle genes and stem cell regulators in purified KSL 150$^+$48$^-$ populations. By PCR array (Figure 7A), we observed that most genes were not affected in *Scl*$^{+/-}$ cells compared with *Scl*$^{+/+}$ cells, except for the *Scl* mRNAs, which were 2-fold decreased as expected. *Pten* mRNA levels were also decreased. We therefore measured by quantitative RT-PCR the expression levels of genes that are known to control HSCs quiescence: transcription factors encoding genes, ie, *Mef*, *Gfi1*, *JunB*, *E2a*, and *Id1*, as well as CDK inhibitor genes, ie, *Cdkn2c* (p18^{Ink4c}) and *Cdkn1a* (p21$^{waf1/cip}$), and *Pten*.[8-10,12,13,43,44] In purified LT-HSCs (Figure 7B), *Mef*, *Gfi1, E2a*, and *JunB* as well as *Cdkn2c* expression levels were not significantly affected by decreased *Scl* gene dosage, suggesting that these genes are not downstream of *Scl*. In contrast, *Cdkn1a* and *Id1* were reproducibly decreased by 10- and 3-fold, respectively, whereas *Pten* showed a more modest decrease. Sequence analysis of the *Id1, Cdkn1a,* and *Cdkn2c* proximal promoters reveals the presence of consensus E box binding sites for the E47-SCL heterodimer[42] (supplemental Figure 4A), whereas *Mef* proximal promoter contains nonconsensus E boxes. We designed PCR primers in the vicinity of these E boxes (Figure 7C) for chromatin immunoprecipitation of purified Lin$^-$ cells. *Cdkn1a* and *Id1* promoter sequences were specifically immunoprecipitated by the SCL antibody compared with control immunoglobulins, whereas *Mef* promoter sequences and *β-actin* control sequences were not (Figure 7D). Despite the presence of a potential TAL1 binding site in the *Cdkn2c* promoter, SCL binding was below the limit of detection of the assay, possibly because the sequence is of lower affinity (CAGTTG[42]) or because of an unfavorable context. These results indicate that the SCL protein specifically occupies the *Cdkn1a* and *Id1* loci in immature hematopoietic cells. Finally, we show that the SCL complex up-regulates the *Cdkn1a* minimal promoter, whereas E2A has little activity on its own (supplemental Figure 4B). We therefore conclude that the *Cdkn1a* and *Id1* genes are direct SCL targets and their expression is sensitive to *Scl* gene dosage.

Discussion

In the present study, we show that SCL controls HSC long-term competence and restrains the G_0-G_1 transition in these cells. Furthermore, SCL directly regulates the expression of 2 genes involved in HSCs quiescence, *Id1* and the cell-cycle regulator *Cdkn1a*.

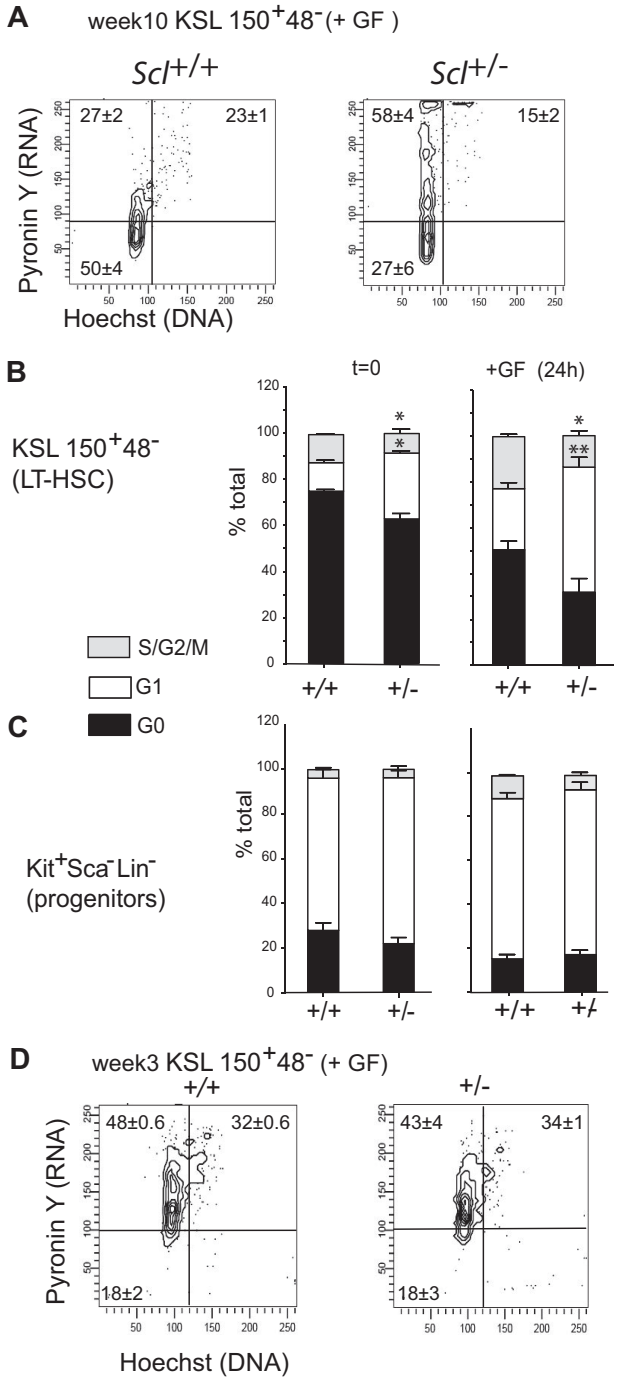

Figure 6. *Scl* gene dosage and LT-HSC cell cycle in response to cytokine stimulation. (A) $Scl^{+/+}$ and $Scl^{+/-}$ lineage-depleted bone marrow cells harvested from adult mice were stimulated with Steel Factor, IL-11, and IL-6 for 24 hours before cell-cycle analysis. The percentages shown in each quadrant are the mean ± SEM of 3 experiments with representative contour plots. (B-C) Stacked columns represent the relative proportion of cells in G_0, G_1, and S-G_2M within LT-HSCs (B) or progenitors (C) from $Scl^{+/+}$ and $Scl^{+/-}$ mice (mean ± SEM of 3 experiments). The left panels represent cells at steady state before culture; the right panels represent growth factor (GF) stimulated cells after 24 hours in culture (*P = .02-.03; **P = .005). (D) Cell-cycle analysis of LT-HSCs from 3-week-old mice after 24 hours of cytokine stimulation. The percentages shown in each quadrant are the mean ± SEM of 3 experiments with representative contour plots.

SCL regulates the G_0-G_1 progression of LT-HSCs

Using a highly purified population of LT-HSCs, we show that *Scl* expression is higher in cells with an LT-HSC phenotype compared with ST-HSCs and progenitors. This might appear to differ from data reported in human cells, in which *SCL* was shown to be lower in the most primitive $CD34^+CD38^-$ cells compared with $CD34^+CD38^+$ cells.[39] However, markers are not yet available to purify human HSCs to the same degree as the murine KSL 150^+48^- because the $CD34^+CD38^-$ population in human contains SCID-repopulating cells at a frequency of 1 of 617 only.[45]

When cells with an LT-HSC phenotype were further purified into G_0 and G_1/S fractions, we found that *Scl* and *Cdkn1a* expression is higher in purified G_0 cells. Furthermore, a 2-fold decrease in SCL protein levels, caused by either *Scl* haplodeficiency or RNA interference, is revealed by a facilitation of G_0/G_1 progression in vivo or in vitro in LT-HSCs without affecting non–stem cell populations. Together, these observations suggest that high *Scl* levels in murine LT-HSCs maintain these cells in quiescence during steady state. This activity of SCL was more prominent when LT-HSCs were stimulated with cytokines in vitro, or triggered into cycling by BrdU administration in vivo. Furthermore, reducing *Scl* gene dosage also impairs HSC long-term competitive potential as discussed in the next section.

SCL controls HSCs function under proliferative stress

In the adult, HSCs must achieve a balance between quiescence and rapid cell-cycle entry on conditions of high proliferative demand, to fulfill immediate requirements for mature hematopoietic cells without compromising the maintenance of the stem cell pool. HSCs that are normally quiescent are triggered into cycle after transplantation in vivo[6] as these cells expand and occupy the bone marrow niche in irradiated hosts.[46] Our studies uncover an important role for SCL when HSCs are subject to an intense proliferative demand, ie, when transplanted under limiting conditions (~1-5 CRU per mouse) or in long-term transplantation assays.

Despite the proliferation imposed by transplantation, $Scl^{+/-}$ HSCs transplanted at saturating doses did not exhibit detectable functional deficits when reconstitution was assessed with lineage markers 4 months after transplantation, in good agreement with published results.[20,21,47] However, when transplanted at limiting doses, $Scl^{+/-}$ HSCs show on average a 4-fold competitive disadvantage, possibly caused by extensive proliferation. Furthermore, when reconstitution was assessed at 4 months in the primitive $Kit^+Sca1^+Lin^-$ population or at 8 months in lineage-positive cells, $Scl^{+/-}$ cells were 2- to 5-fold less competitive than $Scl^{+/+}$ HSCs. This stem cell deficit also carries through in secondary transplantations. Although HSC doubling time after transplantation has not been clearly established, our observations are consistent with the possibility that adult HSCs divide infrequently, perhaps every 145 days,[3,48] unless forced into proliferation by transplantation at a limiting dose of CRU. We therefore propose that our assay conditions may reveal haploinsufficiency in other stem cell regulators.

Quiescence and self-renewal

An essential hallmark of LT-HSCs is their capacity for sustained self-renewal. Indeed, ST-HSCs are also capable of undergoing self-renewal divisions but are unable to maintain hematopoiesis in the long-term. Sustainability is therefore a true stem cell characteristic. Self-renewal divisions can lead to expansion, as observed during ontogeny when the stem pool increases in size, or to maintenance in the adult, preserving HSC numbers and functions

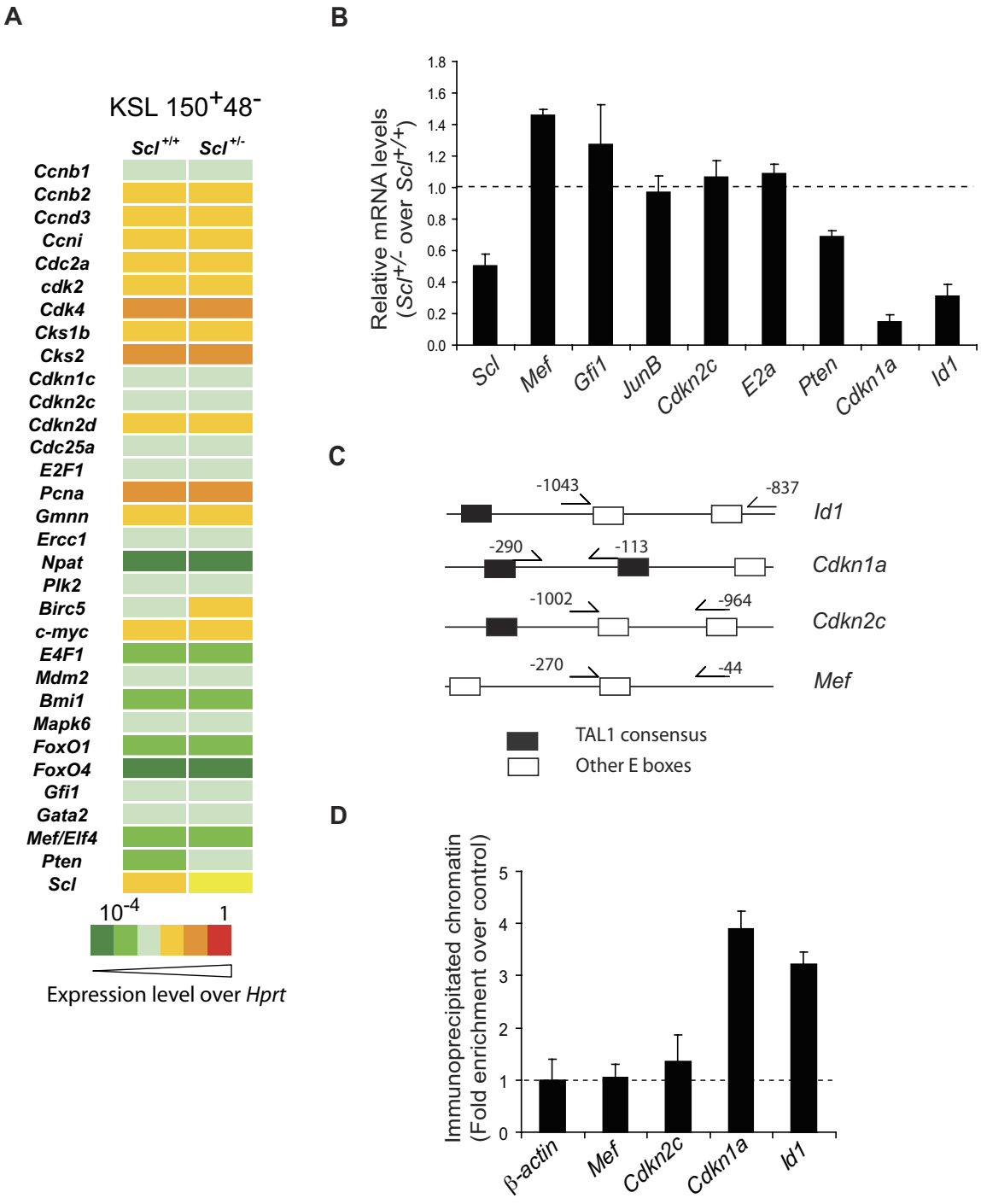

Figure 7. *Cdkn1a* and *Id1* are direct target genes of SCL in immature hematopoietic cells. (A) Cells with an LT-HSC phenotype were purified by flow cytometry from *Scl*$^{+/+}$ and *Scl*$^{+/-}$ bone marrows. After reverse transcription, gene expression was assessed by quantitative PCR array for the indicated cell cycle–related genes and stem cell genes. Data shown are representative of 2 independent experiments. (B) Gene expression in LT-HSCs from *Scl*$^{+/+}$ and *Scl*$^{+/-}$ bone marrows was determined by quantitative RT-PCR. mRNA levels of the indicated genes were first normalized using *Hprt* as an internal control and second compared with expression levels in the *Scl*$^{+/+}$ LT-HSC population, which was set as 1 (illustrated here as the dotted line; mean ± SD of 4 replicates after normalization; 2 experiments). (C) Diagram illustrating the positions of primers used for ChIP in panel D for the indicated murine genes. Note the presence of E boxes in the vicinity of the primers. Black box represents TAL1 E boxes (supplemental Figure 4A)[42]; open box, other E boxes. (D) Chromatin extracts from lineage-depleted bone marrow cells were immunoprecipitated with an anti-murine SCL antibody or species-matched IgG (control, shown as dotted line). Immunoprecipitated promoter sequences were analyzed by quantitative PCR (mean ± SD of 4 replicates; 2 experiments). β-*actin* downstream sequences serve as negative controls for PCR amplification.

throughout the entire life span of the organism. Very few transcriptional regulators are known to be involved in this long-term maintenance, and our observations indicate an important role for *Scl* gene dosage in this process.

During development, the dramatic expansion of HSCs in the fetal liver is associated with a high cycling state,[37,49] controlled in part by Sox17.[50] Between week 3 and 4 after birth, HSCs then switch from this proliferative state to a quiescence state, associated

with an increase in *Scl* and *Gata-2* expression.[25,37,41] It remains to be determined whether these genes control the fetal-to-adult switch, although our analysis of perinatal HSCs indicates that *Scl* does not control their cell-cycle state. Contrary to the proliferative state of fetal HSCs, the increased cell cycle within the adult LT-HSC population in $Scl^{+/-}$ mice is not associated with expansion but with accelerated exhaustion of the stem cell pool as discussed underneath.

Stemness in many systems is associated with a low cycling state and long-term activity.[2,51] For example, HSC defects caused by *Id1* deficiency[9,52] are reminiscent of those observed here with *Scl* deficiency. ID proteins heterodimerize with bHLH factors and prevent their DNA binding capacity. ID1, however, does not heterodimerize with SCL[53] and therefore is unlikely to inhibit SCL activity. Rather, ID1[9,52] and SCL (the present study) have comparable functional impacts on HSC cell cycle and long-term competence. Furthermore, we show that the *Id1* gene is a direct target of SCL in LT-HSCs. There are nonetheless some differences, as $Scl^{+/-}$ HSCs do not exhibit accelerated differentiation, as reported for $Id1^{-/-}$ HSCs. This difference is currently unresolved but could be the result of the 30% residual levels of Id1 in $Scl^{+/-}$ cells or to the contribution of other SCL target genes, such as *Cdkn1a*. Alternatively, SCL is part of a subfamily of bHLH transcription factors that includes LYL1[54] and TAL2,[22] which may compensate for decreased *Scl* gene dosage in HSCs.

Cdkn1a deficiency is also associated with an early exhaustion of the stem cell pool,[8] and we show here that *Cdkn1a* is a direct SCL target gene in Lin^- cells. Transcription activation by SCL depends on its capacity to nucleate a multiprotein complex on DNA.[17] Depending on the context, the same target gene can be activated or repressed by SCL.[55] In the absence of appropriate partners as in the lymphoid lineages, ectopic SCL expression inhibits E-protein activity[31,40,55] and the expression of E2A target genes, such as *Cdkn1a*.[56] However, in the presence of appropriate SCL partners as in erythroid cells, ie, E2A, LMO2, LDB1, and GATA proteins, SCL activates transcription[17,57] and enhances *Cdkn1a* expression.[16] Our results indicate that, in LT-HSCs, SCL enhances *Cdkn1a* expression, suggesting the presence of appropriate SCL partners in these cells.

Similar to *Scl* and *Id1* deficiency, HSCs from $Egr1^{-/-}$,[58] $Pten^{-/-}$,[43] $Foxo1^{-/-}$, $3^{-/-}$, and $4^{-/-}$[59,60] mice also exhibit increased cell cycle and defective maintenance of the HSC pool. Contrary to the above, *Mef* facilitates G_1 progression and *Mef* deletion is associated with enhanced HSC function.[12] Therefore, quiescence and long-term HSC function appear to be controlled by the same transcriptional network. In this circuitry, our observations are consistent with the view that *Scl* acts in parallel to or downstream of *Gfi1*, *Foxo3* and *4*, and *Mef* and upstream of *Id1*, *Cdkn1a*, and possibly *Pten* to actively maintain HSCs in G_0, restrain G_1 progression, and sustain long-term HSC competence. One possible mechanism linking quiescence control and long-term HSCs maintenance involves the regulation of early G_1, a sensitive period of the cell cycle during which HSCs could potentially either be exposed to stimuli that cause an early exhaustion,[2] or exhibit decreased marrow homing capacity.[46,58] Together, these observations suggest that HSC quiescence preserves HSC activity[3] and enhances HSC self-renewal.[2]

In conclusion, we show that *Scl* gene dosage restricts G_1 entry in dormant HSCs and preserves HSC activity under conditions of proliferative demand.

Acknowledgments

The authors thank Richard Martin for help with the colony replating experiment, Danièle Gagné for flow cytometry, Pierre Chagnon for the quantitative PCR array, and Véronique Mercille and Véronique Litalien for the mouse colony.

This work was supported in part by the Canadian Cancer Society Research Institute (T.H., G.S., N.N.I.), the Canadian Institutes of Health Research (T.H., G.S.), the Cancer Research Society Inc (T.H.), the Canadian Research Chair program (T.H., G.S.), and by Fonds de la Recherche en Santé du Québec (studentship) (J.L.) and the Cole foundation (studentship) (S.R.-S., S.B.). The infrastructure is supported in part by the Institute of Research in Immunology and Cancer (Fonds de la Recherche en Santé du Québec group grant).

Authorship

Contribution: J.L. and S.H. performed the research, analyzed the data, and wrote the paper; S.R.-S., S.B., and A.H. performed the research, analyzed the data, and revised the manuscript; N.N.I. and G.S. participated in designing the research and revised the manuscript; and T.H. designed the research, analyzed the data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Trang Hoang, University of Montreal, PO Box 6128, Downtown Station, Montreal, QC H3C 2J7, Canada; e-mail: trang.hoang@umontreal.ca.

References

1. Coulombel L. Identification of hematopoietic stem/progenitor cells: strength and drawbacks of functional assays. *Oncogene.* 2004;23(43):7210-7222.
2. Orford KW, Scadden DT. Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. *Nat Rev Genet.* 2008;9(2):115-128.
3. 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(6):1118-1129.
4. Harrison DE, Lerner CP. Most primitive hematopoietic stem cells are stimulated to cycle rapidly after treatment with 5-fluorouracil. *Blood.* 1991;78(5):1237-1240.
5. Trevisan M, Yan XQ, Iscove NN. Cycle initiation and colony formation in culture by murine marrow cells with long-term reconstituting potential in vivo. *Blood.* 1996;88(11):4149-4158.
6. Nilsson SK, Dooner MS, Quesenberry PJ. Synchronized cell-cycle induction of engrafting long-term repopulating stem cells. *Blood.* 1997;90(11):4646-4650.
7. Wilson A, Trumpp A. Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol.* 2006;6(2):93-106.
8. Cheng T, Rodrigues N, Shen H, et al. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science.* 2000;287(5459):1804-1808.
9. Jankovic V, Ciarrocchi A, Boccuni P, DeBlasio T, Benezra R, Nimer SD. Id1 restrains myeloid commitment, maintaining the self-renewal capacity of hematopoietic stem cells. *Proc Natl Acad Sci U S A.* 2007;104(4):1260-1265.
10. Hock H, Hamblen MJ, Rooke HM, et al. Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. *Nature.* 2004;431(7011):1002-1007.
11. Zeng H, Yucel R, Kosan C, Klein-Hitpass L, Moroy T. Transcription factor Gfi1 regulates self-renewal and engraftment of hematopoietic stem cells. *EMBO J.* 2004;23(20):4116-4125.
12. Lacorazza HD, Yamada T, Liu Y, et al. The transcription factor MEF/ELF4 regulates the quiescence of primitive hematopoietic cells. *Cancer Cell.* 2006;9(3):175-187.
13. Passegue E, Wagers AJ. Regulating quiescence: new insights into hematopoietic stem cell biology. *Dev Cell.* 2006;10(4):415-417.
14. Gregory CJ, Eaves AC. Three stages of erythropoietic progenitor cell differentiation distinguished

by a number of physical and biologic properties. *Blood.* 1978;51(3):527-537.

15. Iscove NN. The role of erythropoietin in regulation of population size and cell cycling of early and late erythroid precursors in mouse bone marrow. *Cell Tissue Kinet.* 1977;10(4):323-334.
16. Goardon N, Lambert JA, Rodriguez P, et al. ETO2 coordinates cellular proliferation and differentiation during erythropoiesis. *EMBO J.* 2006;25(2):357-366.
17. Lahlil R, Lecuyer E, Herblot S, Hoang T. SCL assembles a multifactorial complex that determines glycophorin A expression. *Mol Cell Biol.* 2004; 24(4):1439-1452.
18. Xu Z, Huang S, Chang LS, Agulnick AD, Brandt SJ. Identification of a TAL1 target gene reveals a positive role for the LIM domain-binding protein Ldb1 in erythroid gene expression and differentiation. *Mol Cell Biol.* 2003;23(21):7585-7599.
19. Lecuyer E, Hoang T. SCL: from the origin of hematopoiesis to stem cells and leukemia. *Exp Hematol.* 2004;32(1):11-24.
20. Curtis DJ, Hall MA, Van Stekelenburg LJ, Robb L, Jane SM, Begley CG. SCL is required for normal function of short-term repopulating hematopoietic stem cells. *Blood.* 2004;103(9):3342-3348.
21. Mikkola HK, Klintman J, Yang H, et al. Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. *Nature.* 2003;421(6922):547-551.
22. Capron C, Lecluse Y, Kaushik AL, et al. The SCL relative LYL-1 is required for fetal and adult hematopoietic stem cell function and B-cell differentiation. *Blood.* 2006;107(12):4678-4686.
23. Brunet de la Grange P, Armstrong F, Duval V, et al. Low SCL/TAL1 expression reveals its major role in adult hematopoietic myeloid progenitors and stem cells. *Blood.* 2006;108(9):2998-3004.
24. Reynaud D, Ravet E, Titeux M, et al. SCL/TAL1 expression level regulates human hematopoietic stem cell self-renewal and engraftment. *Blood.* 2005;106(7):2318-2328.
25. Venezia TA, Merchant AA, Ramos CA, et al. Molecular signatures of proliferation and quiescence in hematopoietic stem cells. *PLoS Biol.* 2004;2(10): e301.
26. Elefanty AG, Begley CG, Metcalf D, Barnett L, Kontgen F, Robb L. Characterization of hematopoietic progenitor cells that express the transcription factor SCL, using a lacZ "knock-in" strategy. *Proc Natl Acad Sci U S A.* 1998;95(20):11897-11902.
27. Kiel MJ, Yilmaz OH, Iwashita T, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell.* 2005; 121(7):1109-1121.
28. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature.* 2000;404(6774): 193-197.
29. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell.* 1997;91(5):661-672.
30. Lecuyer E, Herblot S, Saint-Denis M, et al. The SCL complex regulates c-kit expression in hematopoietic cells through functional interaction with Sp1. *Blood.* 2002;100(7):2430-2440.
31. Herblot S, Steff AM, Hugo P, Aplan PD, Hoang T. SCL and LMO1 alter thymocyte differentiation: inhibition of E2A-HEB function and pre-T alpha chain expression. *Nat Immunol.* 2000;1(2):138-144.
32. Szilvassy SJ, Humphries RK, Lansdorp PM, Eaves AC, Eaves CJ. Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. *Proc Natl Acad Sci U S A.* 1990;87(22):8736-8740.
33. Harrison DE, Jordan CT, Zhong RK, Astle CM. Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculations. *Exp Hematol.* 1993;21(2): 206-219.
34. Ema H, Nakauchi H. Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood.* 2000;95(7):2284-2288.
35. Deneault E, Cellot S, Faubert A, et al. A functional screen to identify novel effectors of hematopoietic stem cell activity. *Cell.* 2009;137(2):369-379.
36. Cheshier SH, Morrison SJ, Liao X, Weissman IL. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc Natl Acad Sci U S A.* 1999;96(6):3120-3125.
37. Bowie MB, Kent DG, Dykstra B, et al. Identification of a new intrinsically timed developmental checkpoint that reprograms key hematopoietic stem cell properties. *Proc Natl Acad Sci U S A.* 2007;104(14):5878-5882.
38. Hoang T, Paradis E, Brady G, et al. Opposing effects of the basic helix-loop-helix transcription factor SCL on erythroid and monocytic differentiation. *Blood.* 1996;87(1):102-111.
39. Zhang Y, Payne KJ, Zhu Y, et al. SCL expression at critical points in human hematopoietic lineage commitment. *Stem Cells.* 2005;23(6):852-860.
40. Herblot S, Aplan PD, Hoang T. Gradient of E2A activity in B-cell development. *Mol Cell Biol.* 2002; 22(3):886-900.
41. Phillips RL, Ernst RE, Brunk B, et al. The genetic program of hematopoietic stem cells. *Science.* 2000;288(5471):1635-1640.
42. Hsu HL, Huang L, Tsan JT, et al. Preferred sequences for DNA recognition by the TAL1 helix-loop-helix proteins. *Mol Cell Biol.* 1994;14(2):1256-1265.
43. Yilmaz OH, Valdez R, Theisen BK, et al. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature.* 2006;441(7092):475-482.
44. Yuan Y, Shen H, Franklin DS, Scadden DT, Cheng T. In vivo self-renewing divisions of haematopoietic stem cells are increased in the absence of the early G_1-phase inhibitor, p18INK4C. *Nat Cell Biol.* 2004;6(5):436-442.
45. Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci U S A.* 1997;94(10):5320-5325.
46. Cashman J, Dykstra B, Clark-Lewis I, Eaves A, Eaves C. Changes in the proliferative activity of human hematopoietic stem cells in NOD/SCID mice and enhancement of their transplantability after in vivo treatment with cell cycle inhibitors. *J Exp Med.* 2002;196(9):1141-1149.
47. Kunisato A, Chiba S, Saito T, et al. Stem cell leukemia protein directs hematopoietic stem cell fate. *Blood.* 2004;103(9):3336-3341.
48. Foudi A, Hochedlinger K, Van Buren D, et al. Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. *Nat Biotechnol.* 2008;27(1):84-90.
49. Lansdorp PM, Dragowska W, Mayani H. Ontogeny-related changes in proliferative potential of human hematopoietic cells. *J Exp Med.* 1993; 178(3):787-791.
50. Kim I, Saunders TL, Morrison SJ. Sox17 dependence distinguishes the transcriptional regulation of fetal from adult hematopoietic stem cells. *Cell.* 2007;130(3):470-483.
51. Cheng T. Cell cycle inhibitors in normal and tumor stem cells. *Oncogene.* 2004;23(43):7256-7266.
52. Perry SS, Zhao Y, Nie L, Cochrane SW, Huang Z, Sun XH. Id1, but not Id3, directs long-term repopulating hematopoietic stem-cell maintenance. *Blood.* 2007;110(7):2351-2360.
53. Langlands K, Yin X, Anand G, Prochownik EV. Differential interactions of Id proteins with basic-helix-loop-helix transcription factors. *J Biol Chem.* 1997;272(32):19785-19793.
54. Souroullas GP, Salmon JM, Sablitzky F, Curtis DJ, Goodell MA. Adult hematopoietic stem and progenitor cells require either Lyl1 or Scl for survival. *Cell Stem Cell.* 2009;4(2):180-186.
55. Hsu HL, Wadman I, Tsan JT, Baer R. Positive and negative transcriptional control by the TAL1 helix-loop-helix protein. *Proc Natl Acad Sci U S A.* 1994;91(13):5947-5951.
56. Prabhu S, Ignatova A, Park ST, Sun XH. Regulation of the expression of cyclin-dependent kinase inhibitor p21 by E2A and Id proteins. *Mol Cell Biol.* 1997;17(10):5888-5896.
57. Wadman IA, Osada H, Grutz GG, et al. The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *EMBO J.* 1997;16(11):3145-3157.
58. Min IM, Pietramaggiori G, Kim FS, Passegue E, Stevenson KE, Wagers AJ. The transcription factor EGR1 controls both the proliferation and localization of hematopoietic stem cells. *Cell Stem Cell.* 2008;2(4):380-391.
59. Tothova Z, Kollipara R, Huntly BJ, et al. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell.* 2007;128(2):325-339.
60. Miyamoto K, Araki KY, Naka K, et al. Foxo3a is essential for maintenance of the hematopoietic stem cell pool. *Cell Stem Cell.* 2007;1(1):101-112.