The critical regulator of embryonic hematopoiesis, SCL, is vital in the adult for megakaryopoiesis, erythropoiesis, and lineage choice in CFU-S_{12}

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Gene targeting studies have shown that the transcription factor SCL is critically important for embryonic hematopoiesis, but the early lethality of SCL null mice has precluded the genetic analysis of its function in the adult. We have now generated a conditional knockout of SCL by using Cre/Lox technology and an IFN-inducible Cre transgenic mouse. Deletion of SCL in adult mice perturbed megakaryopoiesis and erythropoiesis with the loss of early progenitor cells in both lineages. This led to a blunted response to the hematopoietic stress induced by polyinosinic-polycytidylic acid, with a persistently low platelet count and hematocrit compared with controls. In contrast, progenitors of granulocyte and macrophage lineages were not affected, even in the setting of stress. Immature progenitor cells (day 12 colony-forming unit spleen) with multilineage capacity were still present in the SCL null bone marrow, but these progenitors had lost the capacity to generate erythroid and megakaryocyte cells, and colonies were composed of only myeloid cells. These results suggest that SCL is critical for megakaryopoiesis and erythropoiesis, but is dispensable for production of myeloid cells during adult hematopoiesis.

SCL, a basic helix–loop–helix transcription factor, initially identified in a subset of human T-cell leukemias with multilineage characteristics, has also been implicated in the earliest stages of hematopoiesis in the mouse embryo and in mature hematopoietic cells (reviewed in ref. 1). SCL is expressed in early murine myeloid, megakaryocytic, erythroid, and mast cells and in human $CD34^+$ cells, suggesting roles in each of these lineages and in the stem cell compartment. In erythroid cell lines, induction of erythroid differentiation is accompanied by increased expression of SCL, whereas myeloid differentiation is associated with decreased SCL expression. Overexpression of SCL in human $CD34^+$ cells resulted in increased numbers of erythroid and megakaryocyte progenitors [BFU-E and Mk-CFC (colony-forming cells), respectively] and an increase in the size of erythroid colonies (2, 3). A "lacZ–knock-in" at the SCL locus showed that SCL-expressing (β-galactosidase$^+$) cells mark committed progenitors of all lineages, including B- and T-lymphoid lineages and also day 12 colony-forming unit spleen (CFU-S_{12}) (4).

Gene ablation studies of SCL in mice revealed its critical importance for hematopoieis (5, 6). Embryos lacking SCL die at embryonic day (E) 9.5–10.5 with a complete absence of blood. Expression of SCL under the GATA1 promoter in these mice rescued yolk sac erythropoiesis, but embryos still died at E9.5 because of defective angiogenesis (7). Expression of SCL controlled by its own "stem cell" enhancer element partially rescued yolk sac hematopoiesis as myeloid progenitors (granulocyte/macrophage CFC) were present. However, the $SCL^{-/-}$ embryos still died at E9.5 with complete anemia (8). These data suggest important roles for SCL in endothelial and erythroid cells in the context of embryonic hematopoiesis.

The embryonic lethality of SCL deletion has precluded analyses of the role of SCL in adult hematopoiesis. *In vitro* and *in vivo* defects in $SCL^{-/-}$ embryonic stem (ES) cells suggest essential functions for the protein in definitive/adult hematopoiesis. $SCL^{-/-}$ ES cells contribute to all tissues in chimeric animals with the exception of the bone marrow and spleen (9, 10). In addition, $SCL^{-/-}$ ES cells are unable to form definitive hematopoietic cells *in vitro*. However, the possibility cannot be excluded that these effects are solely caused by loss of a critical primordial hematopoietic stem cell, and do not truly reflect the role of SCL in adult hematopoiesis.

To address the role of SCL in adult hematopoiesis, we generated a conditional-SCL-null mouse strain by using IFN-inducible Cre/LoxP-mediated mutagenesis. Our results suggest a critical role for SCL in committed megakaryocyte and erythroid progenitors and in the multilineage potential of CFU-S_{12}.

Materials and Methods

Construction of the SCL–loxP Targeting Vector. The SCL–loxP targeting vector was based on pLoxPNeoNTRTK, a gift from P. Humbert (Peter MacCallum Cancer Institute, Melbourne). This vector contains a bicistronic expression cassette with the neomycin-phosphotransferase and the thymidine kinase genes under control of the PGK-promoter. The 5′-arm of the construct was generated by ligating a 3.5-kb *Not*I/*Hin*dIII (blunted) fragment spanning exons 4 and 5 and part of intron 5 of the SCL gene into the *Not*I/*Bam*HI (blunted) sites in pLoxPNeoNTRTK. The 3′ arm was derived from a region of the SCL gene that abuts the 5′ arm and spans the remaining part of intron 5 and exon 6. A single loxP site was introduced at the unique *Xba*I site in the 3′-UTR in exon 6 and was subcloned as a ≈5.5-kb dAG-filled *Bam*HI fragment into the dTC-filled *Xho*I site of pLoxPNeoNTRTK. The resulting vector was linearized with *Not*I for electroporation. Mice containing the targeted allele were obtained as previously described. Female SCL-loxPNeoNTRTK targeted mice were mated with male EIIA-Cre mice (11), and tail DNA of offspring was analyzed by Southern blot using *Eco*RI digest and probe B, which discriminated all possible products of the mosaic deletion. Mosaic offspring were mated with C57BL/6 mice to segregate alleles and remove EIIA-Cre.

Induction of SCL Deletion by MX1-Cre. Inducible deletion of SCL was achieved by using the MX1-Cre strain of mice (12). $SCL^{loxP/loxP}$ homozygotes were mated with $SCL^{LacZ/WT}$MX1-

Abbreviations: PI-PC, polyinosinic-polycytidylic acid; CFU-S, colony-forming unit spleen; ES, embryonic stem; E*n*, embryonic day *n*.

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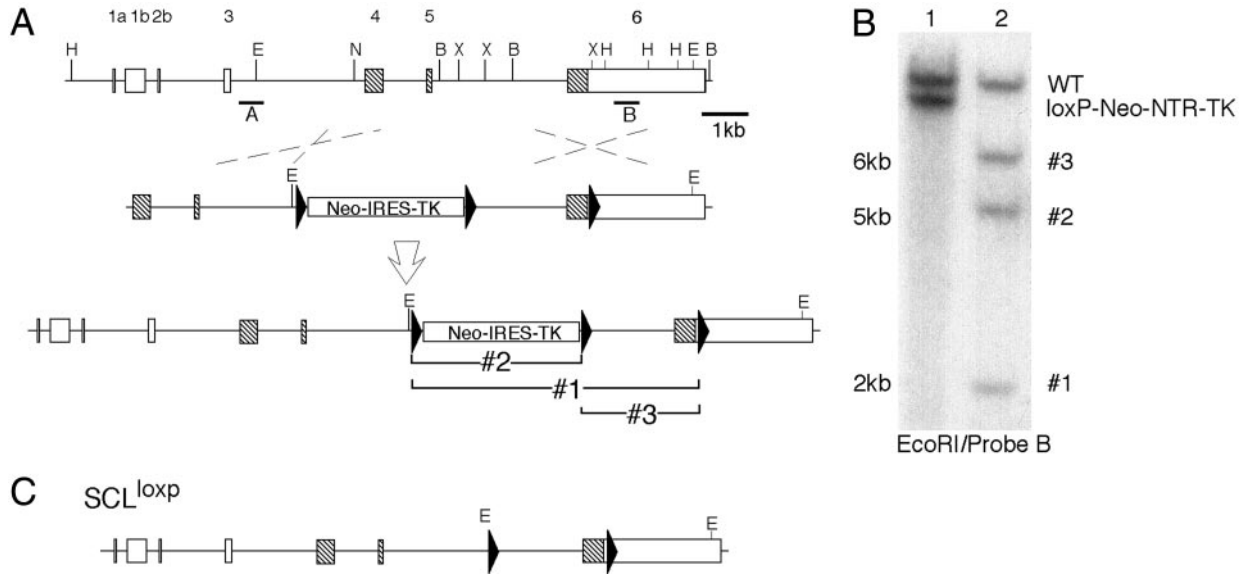

Fig. 1. Targeting of the SCL locus with loxP sites. (*A*) Map of the SCL locus showing positions of noncoding (boxed) and coding (boxed and hatched) exons, the targeting construct, pLoxSCLNeoTK (*Middle*) and the SCL locus after recombination (*Bottom*). pLoxSCLNeoTK has Neo-IRES-TK cassette flanked by loxP sites (triangles); a third loxP site is in the noncoding region of exon 6. Probe B used for Southern blot analyses is marked. Possible Cre-mediated deletions at the loxP sites are shown as 1, 2, and 3. (*B*) Gene targeting of the SCL locus and mosaic deletion at the SCL locus after Cre-mediated recombination. Lane 1 is a Southern blot of the targeted locus with probe B. The wild-type (WT) and targeted (loxP) alleles migrate at 10 and 8.8 kb, respectively. Lane 2 is Southern blot of *Eco*RI-digested DNA hybridized with probe B. The three deleted alleles, 1, 2, and 3, relate to the possible deletions shown in *A*. (*C*) Map of the SCL locus after removal of the loxP/Neo-TK cassette by Cre-mediated recombination. This allele is referred to as SCL^{loxP} throughout.

$Cre^{+/-}$ mice. Each of the four possible genotypes was equally represented in litters, and $SCL^{loxP/LacZ}Cre^{+}$ and $SCL^{loxP/WT}Cre^{+}$ were selected as experimental and control animals, respectively. Animals (6–8 weeks of age) were injected i.p. with 300 μg of polyinosinic-polycytidylic acid (PI-PC) (Sigma) dissolved in normal saline on experimental days 0, 2, and 4, and were killed on day 6.

Megakaryocyte Counts. Megakaryocyte counts are shown as numbers of megakaryocytes in 15 adjacent ×400 fields of view ± SD.

Analysis of Platelet Production by *in Vivo* Biotinylation. Platelet biotinylation was performed as described (13), with animals injected with biotin-*N*-hydroxysuccinimide (Sigma) 3 days after the first PI-PC treatment. The proportion of biotinylated platelets was measured on days 3 and 6. Megakaryocyte ploidy was measured according to the procedure of Arnold *et al.* (14).

Semisolid Culture of Bone Marrow and Spleen CFCs and CFU-S_{12} Assays from SCL-Deleted Mice. Cultures were performed as described by Metcalf (15) and stained for acetylcholinesterase (megakaryocytes), followed by Luxol Fast Blue (eosinophils) and hematoxylin/eosin. Methylcellulose cultures were enumerated *in situ* by using a dissection microscope after staining for hemoglobinized erythroid colonies by using 2,7-diaminofluorene. CFU-S_{12} assays were performed as described by Till and McCulloch (16), and colonies were stained for actelycholinesterase followed by hematoxylin/eosin counterstaining, or immunostained for erythroid cells with anti-Ter119 (for late erythroid cells) and detected with anti-rat horseradish peroxidase. Sections were counterstained with nuclear fast red.

Results

Generation of the Floxed-SCL Strain. The SCL locus was targeted by homologous recombination by using a vector that introduced a single loxP site into the 3′-UTR in exon 6 and a loxP-flanked Neo-IRES-TK selection cassette into intron 6 (Fig. 1*A*). The construct was designed in such a way that Cre-mediated recombination would result in deletion of the entire coding region of exon 6 which has been shown to be essential for SCL function (5). Neomycin-resistant ES cell clones were verified for homologous recombination by Southern blot (Fig. 1*B*, lane 1). Two independent clones (276 and 280) were used to generate lines carrying the targeted allele. Our intention was to generate an SCL allele containing loxP sites in positions that would not affect SCL gene function. This entailed removal of the floxed-Neo-IRES-TK cassette by crossing with the EIIA-Cre mouse strain (11). Given the design of the SCL targeting construct, three deletional events were possible (shown as 1, 2, and 3 in Fig. 1*A*). Each event was observed in F_1 animals from the cross with EIIA-Cre mice (Fig. 1*B*, lane 2). All three deletional events were transmitted to the F_2 generation, and the desired type *#2* deletion (subsequently referred to as SCL^{loxP}) (Fig. 1*C*) was bred to homozygosity.

We initially confirmed that deletion of the loxP flanked region of SCL generated an SCL-null allele. To achieve this, heterozygous animals carrying one type 1 deleted allele of SCL (Fig. 1*A*) and one wild-type SCL allele ($SCL^{Type\#1/WT}$) were crossed with $SCL^{LacZ/WT}$ animals (the LacZ knock-in generates a nonfunctional SCL allele; ref. 4). Embryos were harvested at E9.5–E10.5. As anticipated, ≈25% of embryos showed the typical SCL-null phenotype. The embryos were pale in appearance, and there was no evidence of blood in the yolk sac or embryo (data not shown).

Induced Deletion of SCL Is Efficient in Hematopoietic Organs and Leads to Loss of the SCL Transcript. The MX1-Cre transgenic mouse strain was used to induce deletion of SCL *in vivo* (12). The MX1-Cre transgene is responsive to IFN-α/β and is rapidly induced in multiple tissues after injection of either IFN-α/β or the double-stranded RNA analogue PI-PC (12). $SCL^{loxP/LacZ}Cre^{+}$ and control $SCL^{loxP/WT}Cre^{+}$ animals were subjected to three injections of PI-PC over 4 days and killed after 6 days.

At day 6, the efficiency of deletion in hematopoietic organs (denoted SCL^{Δ}) was highest in bone marrow (>90%) and spleen

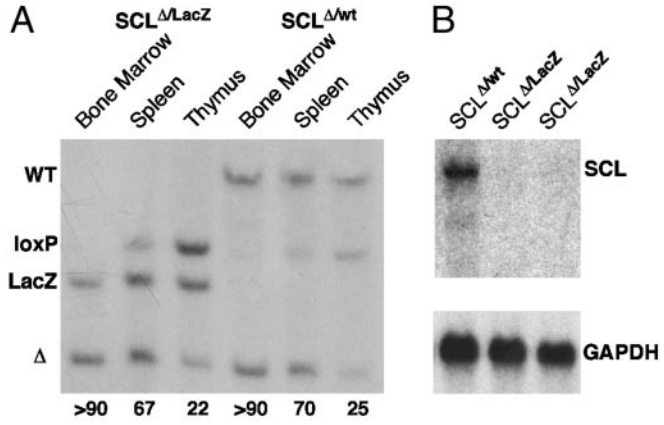

Fig. 2. MX1-Cre-mediated deletion of the floxed SCL allele in hematopoietic tissues leads to loss of SCL expression. Animals were injected on days 0, 2, and 4 with 300 μg of PI-PC. On day 6, organs were harvested and genomic DNA was prepared. (*A*) Representative Southern blot of genomic DNA from hematopoietic organs after PI-PC treatment. Genotypes of animals and tissues are shown above, alleles of SCL are marked: SCL^{wt} (WT), SCL^{loxP} (undeleted), SCL^{LacZ} (LacZ), and SCL^{Δ} (deleted). (*B*) Northern blot analysis of total bone marrow RNA probed with a probe to SCL (*Upper*) and GAPDH (*Lower*). Genotypes of animals are shown above.

(60–70%) and less efficient in thymus (25%), in keeping with several other published reports using MX1-Cre (12, 17, 18) (Fig. 2*A*). Other organs had less efficient deletion, with the exception of liver, which was also deleted to >90% (data not shown). Control experiments using the GT-ROSA reporter mice indicated that MX1-Cre-mediated deletion occurred in >90% of megakaryocytes present in the spleen (data not shown). Loss of SCL expression was demonstrated by Northern blot of whole bone marrow RNA hybridized with an SCL-specific probe (Fig. 2*B*). Six days after initiation of deletion expression of SCL RNA was undetectable by Northern blot compared with PI-PC treated controls.

At 6 Days, Deletion of SCL Has Specific Hematopoietic Consequences. We hypothesized that the effects of SCL deletion may be rapidly diluted by compensatory mechanisms by nondeleted cells. We therefore focused the analysis of the hematologic effects of SCL deletion at day 6, the earliest time point at which there was a high level of deletion in the bone marrow and loss of SCL expression. One problem associated with analysis of mice at this early time point was the nonspecific hematologic effects of PI-PC administration, that affected white cells, platelets, and red cells. A single injection of PI-PC resulted in a rapid fall in leukocyte counts to $1.26 \pm 0.3 \times 10^9$ per liter (normal 6.5×10^9 per liter) (Fig. 3*A*), and platelet counts to $250 \pm 84 \times 10^9$ liter (normal $1{,}100 \times 10^9$ per liter) (Fig. 3*B*) at day 1 and 3, respectively, in both control and experimental animals. After 6 days (and three injections), increased megakaryocytopoiesis in the spleen (see below) had normalized the platelet count in control animals, but not in the SCL-deleted mice (Fig. 3*B*). Similarly, the leukocyte count had normalized in both controls and experimental animals by this time point (Fig. 3*A*). A 25% reduction in the hematocrit at day 6 was also observed as a nonspecific effect of PI-PC injection in both groups. Again, the control animals were able to normalize this defect 1 week later, whereas the SCL-deleted mice were still anemic (Fig. 3*C*) (see below).

$SCL^{\Delta/LacZ}$ mice exhibited lower (50%) total bone marrow cellularity compared with $SCL^{\Delta/WT}$ controls. Control treated animals exhibited an increase in cellularity 6 days after PI-PC injections. The difference in cellularity was presumably caused by a lack of this response to PI-PC treatment in the SCL-deleted animals. Flushed bone marrow samples from $SCL^{\Delta/LacZ}$ animals appeared pale in comparison to controls (data not shown). This was associated with a 4- to 5-fold reduction in Ter-119-positive cells in the spleen when analyzed by fluorescence-activated cell sorting, though Ter-119 cell numbers in the bone marrow were not consistently different (see below). Differential cell counts of nonerythroid cells were not different between groups (data not shown).

Megakaryocytes Are Absent from the Spleens of Mice with the SCL Deletion. Recovery from acute thrombocytopenia in mice generally depends on increased bone marrow and spleen megakaryocyte numbers and size. We therefore examined these tissues from $SCL^{\Delta/LacZ}$ and $SCL^{\Delta/WT}$ animals. These studies showed equivalent numbers of megakaryocytes in the bone marrow at day 6 (134 ± 40, $n = 6$ $SCL^{\Delta/LacZ}$; compare with 106 ± 26; $n = 5$ $SCL^{\Delta/WT}$) (Fig. 4*A*). However, megakaryocytes were virtually absent in the spleen of $SCL^{\Delta/LacZ}$ animals compared with the controls (0.7 ± 1.1 $n = 6$ $SCL^{\Delta/LacZ}$ compare with 6.2 ± 2.5 $n = 6$ $SCL^{\Delta/WT}$) (Fig. 4*B*). The appearance of the megakaryocytes in bone marrows of $SCL^{\Delta/LacZ}$ mice were strikingly different to the controls (Fig. 4*A*). Megakaryocytes from SCL-deleted marrows were larger (≈2-fold increase in diameter) and showed dysplastic features with hyperlobulated nuclei. Comparison of megakaryocyte ploidy between the two groups of mice did not reveal any significant differences (data not shown).

To determine whether the megakaryocytes in the deleted bone marrow retained the ability to shed platelets, we measured *de novo* platelet production by *in vivo* biotinylation. Animals were injected with biotin on the day between the second and

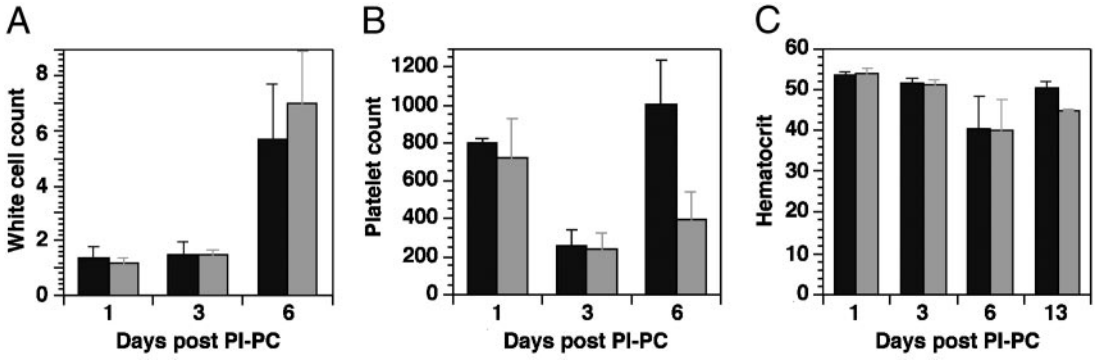

Fig. 3. Effects of PI-PC treatment on peripheral blood counts in $SCL^{\Delta/lacZ}$ and $SCL^{\Delta/WT}$ mice. (*A*) White cell counts ($\times 10^3$ cells/μl) at days 1, 3, and 6 are shown for $SCL^{\Delta/LacZ}$ (light bars) and $SCL^{\Delta/WT}$(dark bars). (*B*) Platelet counts ($\times 10^9$ cells per liter) at days 1, 3, and 6 are shown for $SCL^{\Delta/LacZ}$ (light bars) and $SCL^{\Delta/WT}$(dark bars). (*C*) Hematocrit (%) at days 1, 3, 6, and 13 are shown for $SCL^{\Delta/LacZ}$ (light bars) and $SCL^{\Delta/WT}$(dark bars). Values are shown as mean ± SD for $n = 4$ (days 1, 3, and 13) or $n \geq 12$ (day 6) animals.

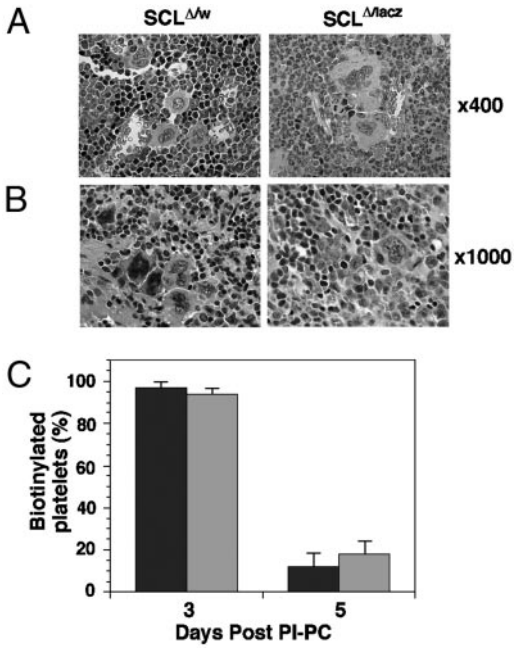

Fig. 4. Perturbed megakaryopoiesis in SCL$\Delta^{/LacZ}$ bone marrow and spleen at day 6. (*A*) Bone marrow samples from SCL-deleted and control mice. Sternums were sectioned and stained with hematoxylin and eosin. Representative medium power fields from SCL$^{\Delta/LacZ}$ and SCL$^{\Delta/WT}$ mice are shown. (*B*) Spleen samples from SCL-deleted and control mice. Spleens were sectioned and stained with hematoxylin and eosin. Representative high power fields from SCL$^{\Delta/LacZ}$ and SCL$^{\Delta/WT}$ mice are shown. (*C*) *De novo* platelet production in SCL$^{\Delta/LacZ}$ (light bars) and SCL$^{\Delta/WT}$ (dark bars) mice. On experimental day 3, the peripheral blood was biotinylated by i.v. injection of *N*-hydroxysuccinimide-biotin. Animals ($n = 4$) were bled on that day and 2 days later, and the percentage of biotinylated platelets was measured by flow cytometry. The mean percentage ± SD of biotinylated platelets on either day is presented.

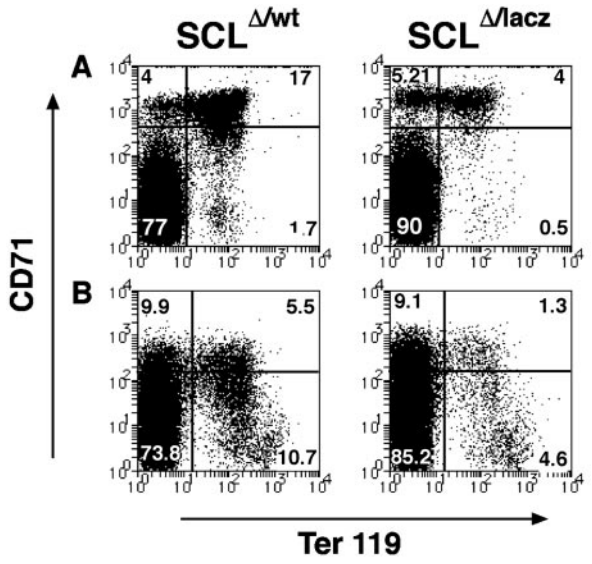

Fig. 5. Perturbed erythropoiesis in bone marrow and spleen of SCL-deleted mice at day 6. Ter119/CD71 analysis of bone marrow (*A*) and spleen cells (*B*) from SCL$^{\Delta/WT}$ and SCL$^{\Delta/LacZ}$ mice. Single cell suspensions of bone marrow and spleen cells were immunostained with Ter119 and CD71 antibodies. Live cells were analyzed by flow cytometry, and erythrocytes were excluded by gating out cells with low forward scatter.

third PI-PC injection. Nonbiotinylated platelets appeared in the circulation of SCL$^{\Delta/LacZ}$ animals after 2 days, indicating that deletion of SCL did not completely halt platelet production (Fig. 4*C*). Therefore, deletion of SCL in megakaryocytes does not prevent platelet production in response to thrombocytopenia.

SCL Is Required for Normal Maturation of Erythroid Cells. Although the hematocrits of control and SCL-deleted mice were identical at day 6, our preliminary fluorescence-activated cell sorting data from bone marrow suggested differences in the erythroid compartment between the two groups. To extend this observation, we examined the immunophenotype of erythroid cells from bone marrow and spleens of SCL-deleted and control animals at day 6, costaining for Ter-119 and CD71 (Fig. 5). This combination of cell surface markers can be used to discriminate several stages of erythroblast maturation (19). The frequency of Ter-119 positive cells was reduced 4-fold in spleens of SCL-deleted mice. This reduction was restricted to the more mature Ter-119 bright/CD71 bright and CD71 negative fractions. In contrast, the relative and absolute numbers of the most immature erythroid cell defined by the immunophenotype Ter-119 dull/CD71 bright was relatively preserved. The accumulation of erythroid cells in the less differentiated compartment was observed in both bone marrow and spleens of SCL-deleted mice.

SCL-Deleted Bone Marrows and Spleens Are Devoid of Mk-CFC and BFU-E After 6 Days. SCL has previously been shown to be expressed in progenitor cells of all lineages including BFU-E and Mk-CFC (4). To assess the effects of SCL deletion on the progenitors colony-formation assays in response to a range of cytokine combinations in semisolid media were performed (Tables 1 and 2). Stimulation with Multi-CSF (IL3) alone or IL3, SCF and Epo in agar revealed a dramatic reduction in Mk-CFC (Table 1). The difference in Mk-CFC was more marked in spleen, consistent with the differences in megakaryocyte numbers in the spleen sections compared with bone marrow. Occasional Mk-CFC were grown from the SCL-deleted samples but PCR genotyping revealed that these always arose from non-deleted cells (data not shown).

BFU-E were also absent from spleen and bone marrow of SCL$^{\Delta/LacZ}$ animals (Table 2). This finding was consistent with the pallor of flushed marrow samples and the reduction in Ter-119-positive cells seen in the SCL-deleted mice. To exclude the possibility that BFU-E were present but poorly hemoglobinized, colonies were picked manually, spread onto glass slides, and stained. From >50 colonies grown from SCL$^{\Delta/LacZ}$ bone marrows and spleens, none contained morphologically identifiable erythroid cells (data not shown).

The frequency and behavior of myeloid CFCs [G-CFC, granulocyte/macrophage (GM)-CFC, and M-CFC] was not significantly affected by removal of SCL (Table 1). There was no difference in the size or number of myeloid colonies in response to individual or combinations of cytokines. Although SCL is not expressed in mature myeloid cells, it is expressed in myeloid progenitors (4). It was therefore surprising that behavior of myeloid progenitors was not affected by loss of SCL. To ensure this was not caused by the selective failure of SCL deletion in myeloid progenitor cells, individual myeloid colonies were picked and genotyped for deletion by PCR. From 39 picked colonies, 38 were confirmed to be deleted for SCL (data not shown). The proportion of deleted GM-colonies reflected the overall degree of deletion in the bone marrow or spleen. Therefore, SCL was not required for the generation of either committed myeloid progenitors cells or their mature progeny.

Loss of SCL Leads to Reduced Numbers of CFU-S_{12} with No Erythroid or Megakaryocytic Component. To determine whether the block in Mk-CFC and BFU-E could be traced to a defect in a more

Table 1. *In vitro* myeloid colony-forming potential of $SCL^{\Delta/LacZ}$ and $SCL^{\Delta/wt}$ mice

	Bl		G		GM		M		Eo		Meg	
	$SCL^{\Delta/wt}$	$SCL^{\Delta/LacZ}$	$SCL^{\Delta/wt}$	$SCL^{\Delta/LacZ}$	$SCL^{\Delta/wt}$	$SCL^{\Delta/LacZ}$	$SCL^{\Delta/wt}$	$SCL^{\Delta/LacZ}$	$SCL^{\Delta/wt}$	$SCL^{\Delta/LacZ}$	$SCL^{\Delta/wt}$	$SCL^{\Delta/LacZ}$
WBM												
GM-CSF	0.3 ± 0.5	0 ± 0	18.5 ± 5.7	20.4 ± 6.4	9.0 ± 5.0	16.0 ± 13	43.5 ± 31.0	50.4 ± 18.0	3.5 ± 1.8	2.0 ± 1.4	0 ± 0	0 ± 0
G-CSF	0 ± 0	0 ± 0	13.8 ± 8.8	10.6 ± 3.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
M-CSF	0 ± 0	0 ± 0	2.3 ± 2.1	1.4 ± 1.1	5.0 ± 3.0	3.2 ± 1.5	59.3 ± 21.5	68.4 ± 35.6	0 ± 0	0 ± 0	0 ± 0	0 ± 0
IL3	5.2 ± 2.6	6.4 ± 3.4	25.5 ± 11.3	24 ± 8.8	12.2 ± 4.8	10.6 ± 6.6	18.8 ± 11.4	19.4 ± 4.5	1.3 ± 1.5	2.0 ± 1.7	8.0 ± 2.8	0 ± 0
SCF	6.2 ± 1.8	5.0 ± 1.4	20.0 ± 11.8	20.6 ± 3.8	0.2 ± 0.4	0.2 ± 0.4	0 ± 0	0 ± 0	0.3 ± 0.8	0 ± 0	0 ± 0	0 ± 0
G-CSF/SCF	10.8 ± 5.6	8.0 ± 1.4	32.5 ± 16.4	33.2 ± 1.6	5.3 ± 8.6	0.2 ± 0.4	1.3 ± 1.4	0.6 ± 0.6	0.3 ± 0.5	1.3 ± 0.5	0 ± 0	0 ± 0
IL3/SCF/Epo	6.4 ± 4.0	11.6 ± 4.3	36.2 ± 16.6	36.0 ± 13.1	18.5 ± 12.1	17.0 ± 5.7	19.8 ± 8.0	26.2 ± 2.5	2.5 ± 1.5	2.8 ± 3.0	13.2 ± 7.8	0.8 ± 1.0
Spleen												
IL3	3.5 ± 1.4	2.2 ± 1.9	12.8 ± 11.5	11.6 ± 6.7	4.8 ± 3.7	3.2 ± 2.4	8.8 ± 9.4	6.6 ± 4.0	1.0 ± 1.1	0.4 ± 0.5	7.2 ± 5.2	0.2 ± 0.4
IL3/SCF/Epo	2.0 ± 2.7	8.2 ± 1.8	19.0 ± 11.4	14.0 ± 6.3	11.0 ± 9.1	4.2 ± 2.8	9.0 ± 6.7	13.0 ± 5.7	0.5 ± 0.5	0.2 ± 0.4	30.5 ± 15.8	0.4 ± 0.5

Numbers of progenitor cells present in samples of 25,000 whole bone marrow (WBM) and 50,000 spleen cells in response to single or multiple cytokines. Cells from marrows and spleens of day 6 PI-PC-treated mice were plated in agar in presence of various cytokine stimuli (shown at left). These were fixed, dried, and stained 7 days later. Colony classifications are blast (Bl), granulocyte (G), granulocyte/macrophage mixed (GM), macrophage (M), eosinophil (Eo), and megakaryocyte (Meg) ($n \geq 5$).

Table 2. Representation of erythroid progenitors (BFU-E) in marrows and spleens of $SCL^{\Delta/LacZ}$ and $SCL^{\Delta/wt}$ mice at day 7 (BFU-E)

	$SCL^{\Delta/wt}$		$SCL^{\Delta/LacZ}$	
	Total CFC	Percent BFU-E	Total CFC	Percent BFU-E
WBM	34.8 ± 16.3	16.3 ± 12.4	23.4 ± 9.9	0 ± 0
Spleen	16.2 ± 14.4	30.8 ± 14.4	17.1 ± 8.8	0 ± 0

Mean total number of CFC and mean percentage BFU-E at day 7 of culture are represented ($n \geq 5$).

immature progenitor cell, $CFU\text{-}S_{12}$ experiments were performed. Bone marrow cells from PI-PC-treated control and experimental mice were injected into lethally irradiated recipients. The spleens of recipient mice were examined 12 days later. The numbers of obvious $CFU\text{-}S_{12}$ colonies were reduced 2-fold in $SCL^{\Delta/LacZ}$ bone marrow (17 ± 16 colonies per 10^5 cells $SCL^{\Delta/LacZ}$; compare with 35 ± 7 colonies per 10^5 $SCL^{\Delta/WT}$). In addition, colonies that did form were smaller and paler than those derived from control bone marrow (Fig. 6*A*). Colonies ($n \geq 10$) were picked and analyzed by Southern blot (Fig. 6*B*) or PCR (data not shown) to determine their SCL genotype. The small pale colonies derived from the whole bone marrow of $SCL^{\Delta/LacZ}$ mice were without exception SCL-null. Histological examination of these colonies revealed a complete absence of erythroid cells and megakaryocytes (≥40 colonies, four donor animals), which was confirmed by immunohistochemical staining of the colonies with anti-Ter-119, and megakarocyte-specific acetylcholinesterase (data not shown). This result was further confirmation of the loss of Mk-CFC and BFU-E observed *in vitro*.

Discussion

These experiments directly examine the role of SCL in the adult bone marrow, expanding on the work of our group and others in cell lines, and genetic rescue experiments in the embryo. Our results clearly demonstrate the importance of SCL at distinct stages of adult hematopoiesis, in select lineages. These findings differ from our predictions based on SCL chimeric animals and SCL-null ES cell differentiation experiments, which suggested loss of SCL would result in a profound defect in all hematopoietic cells. We have observed effects of SCL deletion in only erythroid and megakaryocyte lineages. The overall numbers of myeloid progenitors and $CFU\text{-}S_{12}$ were unaffected or only mildly altered by SCL-deletion. However, the multilineage capacity of $CFU\text{-}S_{12}$ was severely curtailed.

Fig. 6. $CFU\text{-}S_{12}$ analysis of cells from SCL-deleted bone marrows. Lethally irradiated animals ($n = 6$) were injected i.v. with 40,000 bone marrow cells from PI-PC-treated $SCL^{\Delta/LacZ}$ and $SCL^{\Delta/WT}$ mice. Recipient spleens were harvested 12 days later. (*A*) Images of spleens with $SCL^{\Delta/WT}$ (*Upper*) and $SCL^{\Delta/LacZ}$ (*Lower*) $CFU\text{-}S_{12}$. Mean numbers of $CFU\text{-}S_{12}$ and spleen weights are shown. (*B*) Southern blot of three representative deleted $CFU\text{-}S_{12}$ (1–3). Colonies were dissected away from recipient spleen tissue, and genomic DNA was isolated. DNA was digested with *Eco*RI, electrophoresed, and hybridized with probe B (Fig. 1*A*). This confirmed that $CFU\text{-}S_{12}$ were generated in the absence of a functional SCL allele.

The acute effects of PI-PC administration suggest that these studies examine, in part, the role of SCL in stress hematopoiesis. Control animals developed a marked thrombocytopenia, anemia, and leucopenia, which resolved over a week. In contrast, the SCL-deleted mice failed to completely recover from both the fall in platelet count and hematocrit, but did recover completely from the acute fall in their white cell count. These differences were paralleled by effects of SCL deletion on the respective lineage progenitors. Profound effects of SCL deletion were observed on Mk-CFC and BFU-E and CFU-S_{12} lost the ability to generate erythroid and megakaryocyte populations. However, no effect was observed on myeloid colonies. From this we infer that SCL played a key role in the erythroid and megakaryocyte lineages, which was augmented in the context of hematopoietic stress.

The kinetics of platelet production from the committed progenitor stage has been determined in the setting of acute thrombocytopenia (20). Under those conditions, an increase in numbers of splenic Mk-CFC occurs three to four days before the rebound in platelet count. Taking this time frame into consideration, the blunted platelet recovery in the SCL-deleted mice was likely to be caused by a failure of Mk-CFC to populate the spleen and bone marrow, which normally respond to hematological stress. The dysplastic features of the SCL-null megakaryocytes in the bone marrow were reminiscent of the morphological characteristics of megakaryocytes lacking NF-E2. NF-E2 knockout mice exhibit absolute thrombocytopenia associated with mild dyserythropoiesis (21, 22). However, these animals have normal numbers of Mk-CFC. Although SCL-null megakaryocytes were significantly larger than normal and dysplastic in appearance, these changes could be reactive rather than pathologic. Biotinylation experiments showed that SCL-null megakaryocytes still shed platelets. At this stage, we have no evidence to suggest that SCL is required for megakaryocyte maturation or platelet release.

The erythroid defect of the SCL deletion was manifested by reduced numbers of Ter-119 positive cells and complete loss of BFU-E. The absence of erythroid and megakaryocytic cells in CFU-S_{12} was further confirmation of the vital role for SCL in erythroid and megakaryocyte lineages. In the case of CFU-S_{12} development, it is possible that loss of SCL blocked growth of the common megakaryocytic-erythroid progenitor (MEP). The possibility therefore exists that it was loss of this bipotential cell that was responsible for the subsequent disappearance of Mk-CFC and BFU-E.

Several other reports in the literature describe the effect of loss of transcription factors on erythropoiesis and megakaryopoiesis. Of these, ablation of FOG1 and the knockdown mutation of GATA-1 are most relevant to our findings (23–25). Failed megakaryopoiesis and blocked erythropoiesis are characteristic phenotypes of $FOG1^{-/-}$ and GATA-1 knock-down mice similar to the phenotype in the loss of SCL described here. Loss of FOG1 is associated with complete absence of Mk-CFC and blocked maturation of BFU-E. In contrast, the loss of GATA-1 leads to blocked maturation of megakaryocytes and failed erythropoiesis due to apoptotic death of maturing erythroblasts (26). Thus, loss of SCL appeared most similar to loss of FOG1. Complementation of SCL-null ES cells with SCL mutants suggest that many if not all actions of SCL require the helix–loop–helix region but not the basic DNA-binding region (27). Thus, it is possible that the megakaryocyte and erythroid abnormalities or SCL-deficient progenitors were caused by loss of a multimeric complex that includes FOG1. Biochemical studies using immortalized erythroid lines derived from SCL-conditional mice should be able to address this possibility.

Surprisingly, myeloid lineages were not affected by deletion of SCL. Macrophage, granulocytic, and mixed granulocyte/macrophage progenitors were present in marrow and spleens in equivalent numbers and formed colonies of similar size to controls. In addition, more immature multipotent progenitors as defined by CFU-S_{12} were present at only a 2-fold reduced frequency compared with wild-type controls. Indeed, the frequency of CFU-S_{12} may have been underestimated because many were very small because of the loss of erythroid cells, the predominant cell type in CFU-S_{12}. The ability of myeloid progenitors to grow in the absence of SCL is in contrast with $SCL^{-/-}$ mice and ES cells, which are unable to generate any hematopoietic lineages or progenitors. In the embryo, SCL is required for instructing mesoderm to become hematopoietic tissue. Our findings provide evidence that once hematopoiesis is established, SCL may no longer be required for myeloid progenitors in the adult environment.

Future studies examining the consequences of deletion in steady state hematopoiesis and in earlier progenitors and stem cells will complement our results and define the role of SCL in adult hematopoiesis.

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