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Impaired *In Vitro* Erythropoiesis following Deletion of the *Scl* (*Tal1*) +40 Enhancer Is Largely Compensated for *In Vivo* despite a Significant Reduction in Expression

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The *Scl* (*Tal1*) gene encodes a helix-loop-helix transcription factor essential for hematopoietic stem cell and erythroid development. The *Scl* +40 enhancer is situated downstream of *Map17*, the 3′ flanking gene of *Scl*, and is active in transgenic mice during primitive and definitive erythropoiesis. To analyze the *in vivo* function of the *Scl* +40 enhancer within the *Scl*/*Map17* transcriptional domain, we deleted this element in the germ line. $Scl^{\Delta 40/\Delta 40}$ mice were viable with reduced numbers of erythroid CFU in both bone marrow and spleen yet displayed a normal response to stress hematopoiesis. Analysis of $Scl^{\Delta 40/\Delta 40}$ embryonic stem (ES) cells revealed impaired erythroid differentiation, which was accompanied by a failure to upregulate *Scl* when erythropoiesis was initiated. *Map17* expression was also reduced in hematopoietic tissues and differentiating ES cells, and the *Scl* +40 element was able to enhance activity of the *Map17* promoter. However, only *Scl* but not *Map17* could rescue the $Scl^{\Delta 40/\Delta 40}$ ES phenotype. Together, these data demonstrate that the *Scl* +40 enhancer is an erythroid cell-specific enhancer that regulates the expression of both *Scl* and *Map17*. Moreover, deletion of the +40 enhancer causes a novel erythroid phenotype, which can be rescued by ectopic expression of *Scl* but not *Map17*.

The basic helix-loop-helix transcription factor Scl (Tal1) is a key regulator of hematopoiesis (1–4), with additional important roles in the development of the vascular system (5) and central nervous system (CNS) (6). During development, *Scl* is expressed in sites of embryonic and fetal hematopoiesis, vascular endothelium, and specific regions of the CNS (7). *Scl* expression is maintained in adult endothelium and CNS (8); however, its expression in the adult hematopoietic system is restricted to hematopoietic stem cells (HSCs) and progenitors as well as the erythroid, megakaryocytic, and mast cell lineages (9, 10).

Scl null embryos die between days 8.5 and 10.5 of gestation due to the complete absence of primitive hematopoiesis (3, 4). *Scl* null embryonic stem (ES) cells fail to contribute to any hematopoietic lineages in chimeric mice (2) and are unable to generate hematopoietic colonies *in vitro* (1). Further studies in ES cells have uncovered that *Scl* is not required for the development of the hemangioblast but is essential for the formation of hemogenic endothelial cells (11). In the adult, *Scl* is not necessary for HSC maintenance (12–14), but loss of *Scl* severely affects the erythroid, megakaryocytic, and mast cell lineages (13, 15). *Scl*-deleted mice are anemic, display enlarged spleens, and show a shift toward more immature erythroid progenitors in bone marrow and spleen. While these animals have normal numbers of erythroid CFU (CFU-e), erythroid burst-forming units (BFU-e) were undetectable and the numbers of megakaryocyte-erythrocyte progenitors (MEP) were increased (16).

Scl is situated in a single transcriptional domain together with *Map17* (17, 18). *Map17*, also known as *Pdzk1ip1*, encodes a transmembrane protein expressed in kidney cells (19), keratinocytes (20), early hematopoietic progenitors (21), and human adult reticulocytes (22). Not much is known about *Map17* function; however, aberrant expression is observed in carcinomas arising from kidney, colon, lung, and breast (23). We have previously shown that Map17 has a role in zebrafish erythropoiesis, as morpholino-mediated knockdown caused a reduction in the number of circulating erythrocytes (24).

Systematic dissection of *cis*-regulatory mechanisms operating at the murine *Scl* locus has identified multiple *cis*-regulatory elements, each of which directs expression to a subdomain of the normal *Scl* expression pattern in transgenic mice (17, 25–29). One of these regulatory elements is the *Scl* +40 region, an enhancer situated 40 kb downstream of *Scl* exon 1a. We have shown previously that a 3.7-kb DNA fragment containing this element [*Scl* +40(3.7)] drives expression of a linked LacZ reporter gene to midbrain and primitive erythropoiesis *in vivo* (17), and extending this fragment to 5.0 kb [*Scl* +40(5.0)] resulted in additional expression of LacZ in definitive erythropoiesis (27).

To further analyze the function of the +40 enhancer *in vivo*, we have deleted the *Scl* +40 region in ES cells and generated $Scl^{\Delta 40/\Delta 40}$ knockout (KO) mice. Analysis of the knockout mice showed that the *Scl* +40 enhancer is functionally important for erythropoiesis since $Scl^{\Delta 40/\Delta 40}$ mice have reduced numbers of CFU-e in both bone marrow and spleen and in fetal liver during embryonic development. An erythroid defect was also observed during *in vitro* differentiation of $Scl^{\Delta 40/\Delta 40}$ ES cells and was accompanied by the failure of *Scl* upregulation at the later stages of erythroid differentiation. We also showed that the *Scl* +40 enhancer regulates

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Map17 expression but only ectopic expression of *Scl* can rescue the erythroid phenotype $Scl^{\Delta 40/\Delta 40}$ ES cells.

MATERIALS AND METHODS

Generation of knockout ES cells. A 15-kb region spanning the *Scl* +40 enhancer (nucleotides [nt] 5758 to 20460 relative to Al731651) was retrieved onto the PL253 vector from bacterial artificial chromosome clone 175N02 using a recombineering protocol previously described (30). A LoxP-PGK-*Neo*-poly(A)-LoxP cassette was used to substitute the *Scl* +40(4.2) genomic region (nt 11716 to 15984 relative to Al731651). This generated a targeting vector containing 5′ and 3′ homologous regions of 6 kb and 4.7 kb, respectively, flanking the LoxP-PGK-*Neo*-poly(A)-LoxP cassette. Details of the cloning strategy are available on request.

AB2.2 ES cells were electroporated with the targeting construct and selected with 200 μg/ml of G418 (Sigma-Aldrich) and 2 μM ganciclovir (Sigma-Aldrich). Individual clones were picked, expanded, and genotyped by Southern blotting using internal 5′ (nt 8249 to 8845 relative to Al731651) and external 3′ (nt 21156 to 21833 relative to Al731651) probes. Positive clones (*Scl* +40/*Neo*′) were transiently transfected with a PGK-*Cre* construct for deletion of the *Neo* cassette. Clones were picked, expanded, and genotyped by Southern blotting. Clones in which the *Neo* cassette was deleted ($Scl^{\Delta 40/WT}$) were used for the generation of homozygous $Scl^{\Delta 40/\Delta 40}$ ES cells. An identical protocol was used for targeting of the second allele, with the generation of $Scl^{\Delta 40/Neo}$ clones and subsequent deletion of the *Neo* cassette to generate $Scl^{\Delta 40/\Delta 40}$ ES clones.

Generation of knockout mice. Two independent $Scl^{Neo/WT}$ ES cell clones were injected into albino C57BL/6 blastocysts, and chimeras were bred with C57BL/6 mice to produce heterozygous $Scl^{Neo/WT}$ mice. These mice were further bred with CMV-Cre transgenic mice (31) to remove the *Neo* cassette and generate $Scl^{\Delta 40/WT}$ mice. Mice used in the experiments presented were backcrossed to C57BL/6 mice for at least 3 generations. The $Scl^{\Delta 40/\Delta 40}$ embryonic phenotype was analyzed at embryonic day 10.5 (E10.5), E12.5, and E14.5. Results shown here are from E12.5 embryos unless stated otherwise. The $Scl^{\Delta 40/\Delta 40}$ adult phenotype was analyzed in 6-, 12-, and 48-week-old mice. Results shown here are for 12-week-old animals unless stated otherwise. All mice were kept in specific-pathogen-free conditions, and all procedures were performed under the project license approved by the United Kingdom Home Office.

Peripheral blood analysis, fluorescence-activated cell sorting, colony assays, and stress hematopoiesis. Peripheral blood was taken from tail vein into EDTA-coated tubes, and automated total and differential blood cell counts were determined using the ABC blood counter (Woodley). For analysis of mature hematopoietic lineages, single-cell suspensions from bone marrow, spleen, and fetal liver were stained with Gr-1 and Mac-1 for myeloid analysis, B220 for B-cell analysis, CD41 for megakaryocytic analysis, and CD71 and Ter119 for erythroid analysis (BD Biosciences). The concentration of erythropoietin (Epo) was measured using Quantikine mouse/rat Epo immunoassay (R&D systems).

For analysis of early hematopoietic progenitors, single-cell suspensions were lineage depleted using the MACS lineage cell depletion kit (Miltenyi Biotec), and depleted cells were stained for Sca1, cKit, CD34, FcγRIII, and interleukin-7R (IL-7R). For analysis of hematopoietic lineages during ES cell differentiation, cells were stained for cKit and Ter119. Populations of increasing erythroid maturity were defined as described previously (32). Flow cytometric analysis was performed with a CyAn ADP analyzer (Beckman Coulter). Cell sorting was performed with a MoFlo cell sorter (Beckman Coulter). Data were analyzed using FlowJo software (TreeStar). Granulocytes/macrophage CFU (CFU-GM), BFU-e, and CFU-e were grown using the methylcellulose-based medium kit Cameo-4 (HemoGenix), and megakaryocyte CFU (CFU-MK) were grown using collagen-based medium Megacult-C (Stem Cell Technologies) according to the manufacturers' instructions.

Mice were injected intraperitoneally at day 0 and day 1 with 12 μl/g body weight of 0.4% phenylhydrazine (PHZ) in phosphate-buffered saline (PBS) (Sigma) and analyzed at day 5.

Expression analysis. Total RNA was isolated from tissues using Trireagent (Sigma-Aldrich) and from sorted cell populations using the ZR RNA MicroPrep kit (Zymo Research) according to the manufacturers' instructions. An identical quantity of total RNA (0.1 to 1 μg) was used to prepare cDNA [with either oligo(dT) or random hexamer primers] with the cDNA synthesis kit (Bioline) following the manufacturer's instructions. Real-time quantitative PCR was performed using the Brilliant SYBR green qPCR master mix (Stratagene). The following primers were used: *Scl* F, CATGTTCACCAACAACAACCG, and R, GGTGTGAGGACCATC AGAAATCTC; *Map17* F, GTCCTTGTTGCAATCGTCTTC, and R, GAG GAGTATCTGCCATCCATTC; β-*actin* F, TCCTGGCCTCACTGTCCA, and R, GTCCGCCTAGAAGCACTTGC. *Gata1* F, CAACAGTATGGAG GGAATTCCT, and R, GTGTCCAAGAACGTGTTGTTGC; *Beta major* F, ATGCCAAAGTGAAGGCCCAT, and R, CCCAGCACAATCACGAT CAT. Expression was normalized to β-*actin*.

***In vitro* differentiation of ES cells.** For embryoid body generation, ES cells were seeded at 2×10^3 cell/ml in Iscove's modified Dulbecco's medium (IMDM; HyClone) supplemented with 15% fetal calf serum (FCS) (HyClone), 2 mM L-glutamine (PAA), 300 μg/ml transferrin (Sigma), 4×10^{-4} M monothioglycerol (MTG) (Sigma), 50 μg/ml ascorbic acid (Sigma), and 5% protein-free hybridoma medium II (PFHM-II) and allowed to differentiate up to 8 days (33). BFU-e colonies were grown by seeding 1.5×10^5 cells in 1.5 ml M3434 medium (Stem Cell Technologies). Colonies were counted after 14 days in culture.

F0 transgenic mouse assays and luciferase reporter assays. All LacZ reporter constructs were generated by substitution of the luciferase gene with the *lacZ* gene. F0 transgenic mice were generated as described previously (27). Luciferase assays were performed as described previously (34). *Map17*P-Luc was generated by cloning a PCR-amplified 350-bp fragment containing the *Map17* promoter into pGL2 vector (Stratagene). *Map17*P-Luc-+40 was generated by substitution of the simian virus 40 (SV40) promoter in SV40-Luc-+40(5.0) by the 350-bp *Map17* promoter. Results were normalized to *Map17*P-Luc.

RESULTS

Deletion of the *Scl* +40 enhancer. To define the function of the *Scl* +40 enhancer *in vivo*, we elected to delete this element in ES cells. Since the region corresponding to the *Scl* +40(5.0) included the poly(A) tail of the *Map17* gene, we decided to delete a 4.2-kb fragment corresponding to the remaining sequence of the *Scl* +40(5.0) (Fig. 1A). To confirm that the deleted region was indeed a functional *Scl* +40 enhancer, the 4.2-kb fragment was inserted downstream of the SV40 promoter-LacZ cassette, and enhancer activity of the resultant construct was analyzed in transgenic embryos. The *Scl* +40(4.2) fragment drove LacZ expression to both primitive and definitive erythropoiesis and midbrain in E12.5 transgenic embryos, similarly to the *Scl* +40(5.0) fragment (Fig. 1B). The transgene therefore recapitulates the endogenous Scl and Map17 expression previously reported by us using *in situ* hybridization (24, 28).

Satisfied that the *Scl* +40(4.2) fragment is a bona fide Scl +40 enhancer, we proceeded with its deletion in ES cells. A targeting construct was generated using recombineering technology to replace the *Scl* +40(4.2) sequence by a LoxP-PGK-*Neo*-LoxP cassette (30), and this construct was used to target ES cells by homologous recombination (Fig. 1C). Correct targeting was confirmed by Southern blotting, and two independent clones were used to generate $Scl^{\Delta 40/\Delta 40}$ ES cells and mice for further analysis (Fig. 1D).

$Scl^{\Delta 40/\Delta 40}$ mice are viable and have normal blood counts. Two independent clones of correctly targeted ES cells were used for the generation of $Scl^{Neo/WT}$ mice. These mice were further bred with CMV-*Cre* transgenic mice (31) to remove the *Neo* cassette and generate $Scl^{\Delta 40/WT}$ mice, which were interbred to generate

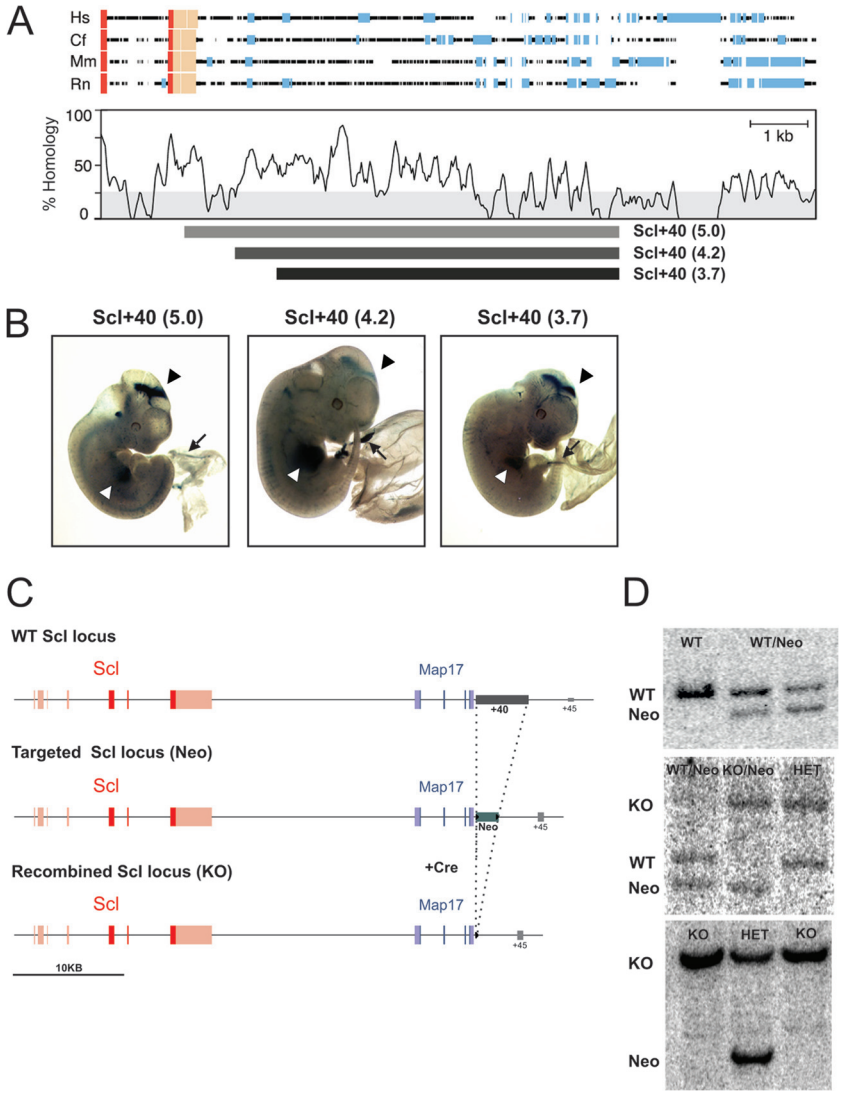

FIG 1 Generation of $Scl^{\Delta 40/\Delta 40}$ mice and ES cells. (A) Schematic representation of a four-way sequence alignment of the region downstream of *Map17* in human (Hs), dog (Cf), mouse (Mm), and rat (Rn), showing peaks of sequence homology (modified from Delabesse et al. [17]). Red boxes, coding exons; beige boxes, untranslated exons; blue boxes, repeat sequences. The relative positions of the various +40 fragments incorporated into luciferase and *lacZ* reporter constructs are shown below the graph. (B) Representative E11.5 to E12.5 SV40-LacZ-*Scl* +40(5.0), SV40-LacZ-*Scl* +40(4.2), and SV40-LacZ-*Scl* +40(3.7) transgenic embryos stained for LacZ, showing expression in circulating blood (black arrow), fetal liver (white arrowhead), and midbrain (black arrowhead). (C) Schematic representation of the *Scl* locus with the *Scl* exons depicted in red and *Map17* exons in blue. (Top) Structure of the WT locus, with the location of the *Scl* +40 enhancer indicated in gray. (Middle) Targeted *Scl* locus, where the *Scl* +40 enhancer was replaced by a LoxP-PGK-*Neo*-p(A)-LoxP cassette, indicated in green. (Bottom) Recombined *Scl* locus where the *Neo* cassette was removed by Cre-mediated recombination. (D) Southern blots used for genotyping of $Scl^{Neo/WT}$ (top), $Scl^{\Delta 40/WT}$ and $Scl^{\Delta 40/Neo}$ (middle), and $Scl^{\Delta 40/\Delta 40}$ (bottom) ES cells.

homozygous $Scl^{\Delta 40/\Delta 40}$ mice. $Scl^{\Delta 40/\Delta 40}$ mice were born at normal Mendelian ratios and developed normally.

We checked the expression levels of *Scl* in the hematopoietic organs. *Scl* expression was severely reduced in both bone marrow and spleen (Fig. 2A). To confirm that *Map17* expression was not affected due to the proximity of the *Scl* +40 deletion to the *Map17* poly(A) site, we analyzed the expression of *Map17* in kidney, where the *Scl* +40 enhancer is not active (17). *Map17* was expressed at normal levels in $Scl^{\Delta 40/\Delta 40}$ kidneys, indicating that its poly(A) is intact (Fig. 2B). However, the expression of *Map17* was severely reduced in both bone marrow and spleen (Fig. 2B), suggesting that *Map17* may be regulated by the *Scl* +40 enhancer in hematopoietic cells.

Analysis of peripheral blood parameters in mice of different ages (6, 12, and 48 weeks) showed no statistically significant difference between $Scl^{WT/WT}$ and $Scl^{\Delta 40/\Delta 40}$ mice (Table 1). Peripheral blood parameters can be within the normal range despite defects in hematopoietic progenitors within the bone marrow (35, 36). We therefore performed a more detailed analysis of adult hematopoietic organs by quantifying the different hematopoietic lineages in the bone marrow and spleen by flow cytometry. No difference was observed in the percentages of bone marrow granulocytes ($Gr1^+$ $Mac1^+$), B lymphocytes ($B220^+$), and megakaryocytes ($CD41^+$ FSC^{high}) between $Scl^{WT/WT}$ and $Scl^{\Delta 40/\Delta 40}$ mice (Fig. 2C). More-detailed analysis of late erythroid progenitors using the CD71 and TER119 antibodies also failed to reveal any

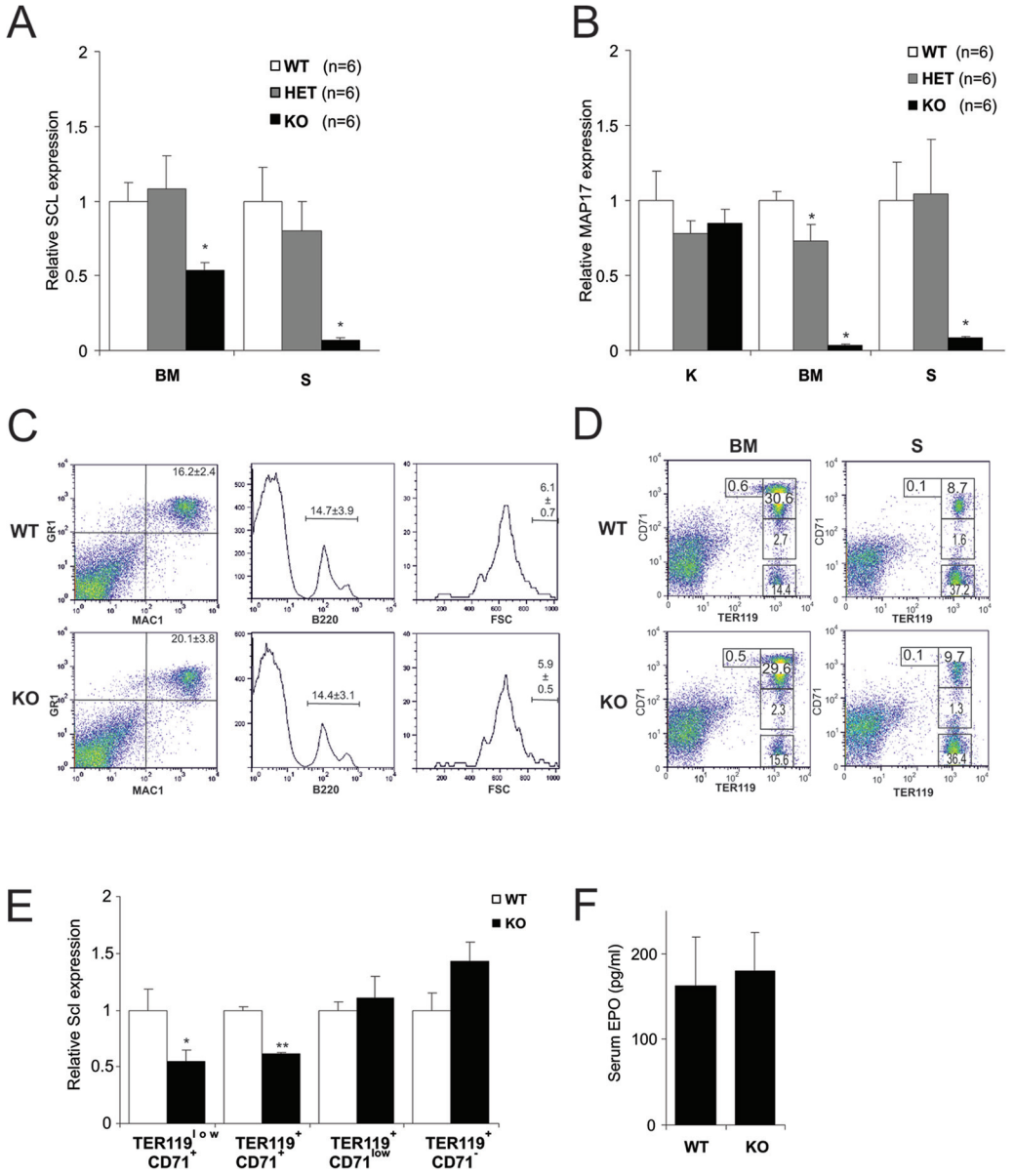

FIG 2 Mature hematopoietic lineages are normal in $Scl^{\Delta 40/\Delta 40}$ mice. (A and B) Relative *Scl* (A) and *Map17* (B) expression in bone marrow (BM), spleen (S), and kidney (S). *, $P \leq 0.01$. (C) Representative flow cytometry plots of bone marrow staining for granulocytes ($Mac1^+$ $Gr1^+$), B cells ($B220^+$), and megakaryocytes ($CD41^+$ FSC^{high}). (D) Terminal erythroid differentiation is normal in $Scl^{\Delta 40/\Delta 40}$ mice. Dot plots show CD71 and TER119 staining for the different stages of terminal erythroid differentiation in bone marrow and spleen. No difference was observed in early erythroid progenitors $TER119^{low}$ $CD71^+$, during terminal erythroid differentiation ($TER119^+$ $CD71^+$ and $TER119^+$ $CD71^{low}$), or in erythrocytes ($TER119^+$ $CD71^-$). Numbers represent averages for at least 5 independent animals. (E) Relative Scl expression in erythroid progenitor populations purified from bone marrow. *, $P \leq 0.05$; **, $P \leq 0.005$. (F) Concentration of serum erythropoietin (pg/ml).WT, $Scl^{WT/WT}$; HET, $Scl^{WT/\Delta 40}$; KO, $Scl^{\Delta 40/\Delta 40}$.

defect in terminal erythroid differentiation in both bone marrow and spleen, consistent with the normal peripheral blood parameters (Fig. 2D). To analyze *Scl* expression levels in $Scl^{\Delta 40/\Delta 40}$ mice during terminal erythroid differentiation, the populations described above were purified from bone marrow and used for RNA isolation. Levels of *Scl* were significantly reduced in $Scl^{\Delta 40/\Delta 40}$ mice at the earlier stages of differentiation ($TER119^{low}$ $CD71^+$ and $TER119^+$ $CD71^+$) but increased at later stages of differentiation ($TER119^+$ $CD71^{low}$ and $TER119^+$ $CD71^-$) (Fig. 2E). These results are consistent with the fact that blood counts are normal but suggest a defect during earlier stages of differentiation.

A possible explanation for the phenotype observed in early progenitors but not in terminal differentiated erythrocytes can be compensatory mechanisms via cytokines and growth factors. Therefore, we measured the concentration of erythropoietin in serum of $Scl^{\Delta 40/\Delta 40}$ mice. Although we observed a 10% increase in Epo concentration in $Scl^{\Delta 40/\Delta 40}$ mice, the variation between samples was high, and therefore the results were not statistically significant (Fig. 2F).

$Scl^{\Delta 40/\Delta 40}$ mice have reduced numbers of CFU-e and CFU-MK progenitors. Hematopoietic progenitors in the bone marrow and spleen were also quantified using standard methyl-

TABLE 1 $Scl^{\Delta 40/\Delta 40}$ mice have normal hematological parameters[a]

Genotype	WBC ($10^3/\mu l$)	RBC ($10^3/\mu l$)	Hgb (g/liter)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/liter)	PLT ($10^3/\mu l$)	MPV (fl)
$Scl^{WT/WT}$	10.0 ± 2.34	9.9 ± 0.69	159.2 ± 43.04	54.3 ± 2.28	53.5 ± 2.07	16.9 ± 1.28	315.4 ± 13.95	1,015.2 ± 147.25	5.0 ± 0.20
$Scl^{WT/\Delta 40}$	9.8 ± 2.74	9.7 ± 1.24	162.0 ± 34.58	52.4 ± 6.41	53.3 ± 2.36	16.9 ± 1.37	290.4 ± 87.31	971.6 ± 265.53	5.0 ± 0.195
$Scl^{\Delta 40/\Delta 40}$	12.3 ± 3.07	10.6 ± 0.68	172.8 ± 6.93	56.3 ± 2.88	53.5 ± 2.95	16.4 ± 1.09	307.5 ± 10.24	923.4 ± 307.36	5.1 ± 0.29

[a] Data shown are for 12-week-old animals. Similar results were obtained for 6- and 48-week-old animals (not shown). WBC, leukocyte concentration; RBC, erythrocyte concentration; Hgb, hemoglobin concentration; Hct, hematocrit level; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin amount; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet count; MPV, mean platelet volume.

cellulose-based colony-forming methods. No difference was observed in the number of granulocyte/macrophage CFU (CFU-GM) (Fig. 3A). Megakaryocyte CFU (CFU-MK) were reduced in $Scl^{\Delta 40/\Delta 40}$ spleen but not in bone marrow (Fig. 3B). A reduction of erythroid CFU (CFU-e) (Fig. 3C) was observed in the bone marrow and spleen, but no such difference was observed in the earlier erythroid progenitor erythroid burst-forming units (BFU-e) (Fig. 3D).

Earlier hematopoietic stem/progenitor cells were quantified by flow cytometry using standard combinations of cell markers. No differences were observed in the Lin^- $Sca1^+$ Kit^+ (LSK) stem cell-enriched population, common myeloid progenitor (CMP; Lin^- $Sca1^-$ Kit^+ $CD34^+$ $Fc\gamma R^-$), myeloid-erythroid progenitor (MEP; Lin^- $Sca1^-$ Kit^+ $CD34^-$ $Fc\gamma R^-$), granulocyte-macrophage progenitor (GMP; Lin^- $Sca1^-$ Kit^+ $CD34^+$ $Fc\gamma R^+$), and common lymphocyte progenitor (CLP; IL-$7R^+$ $cKit^+$ $Sca1^+$) populations (Fig. 3E). Moreover, *Scl* expression was unaltered in $Scl^{\Delta 40/\Delta 40}$ LSK, CMP, MEP, and GMP (Fig. 3F). *Map17* expression was unaltered in the LSK population (Fig. 3G) and undetectable in the CMP, MEP, and GMP populations of both $Scl^{WT/WT}$ and $Scl^{\Delta 40/\Delta 40}$ mice. Expression of *Scl* and *Map17* in CLP was not analyzed. Taken together, these data show that expression changes are accompanied by functional defects in specific progenitor subsets in $Scl^{\Delta 40/\Delta 40}$ mice.

$Scl^{\Delta 40/\Delta 40}$ mice show a normal response to stress erythropoiesis. Given the specific defects under homeostatic conditions, we next asked how the hematopoietic system of $Scl^{\Delta 40/\Delta 40}$ mice responds under stress conditions. To test the ability of $Scl^{\Delta 40/\Delta 40}$ mice to respond to hematopoietic stress, we injected $Scl^{\Delta 40/\Delta 40}$ mice and $Scl^{WT/WT}$ littermate controls with 0.4% phenylhydrazine (PHZ) and analyzed mice at day 5 postinjection. No differences were observed in blood parameters (Table 2) or in spleen weight or cellularity of the bone marrow and spleen (data not shown). Analysis of terminal erythroid differentiation in bone marrow and spleen also revealed no difference between $Scl^{\Delta 40/\Delta 40}$ and $Scl^{WT/WT}$ controls (Fig. 4A). CFU-e (Fig. 4B) and BFU-e (Fig. 4C) numbers were also similar in $Scl^{\Delta 40/\Delta 40}$ and $Scl^{WT/WT}$ mice after PHZ treatment, which was a surprising result given the difference in CFU-e numbers observed in $Scl^{\Delta 40/\Delta 40}$ mice under nonstress conditions (see previous section).

***$Scl^{\Delta 40/\Delta 40}$* embryos display reduced numbers of CFU-e progenitors.** Since mild hematopoietic phenotypes in adult mice are sometimes exacerbated during early embryonic development (37, 38), we next analyzed $Scl^{\Delta 40/\Delta 40}$ embryos during development. $Scl^{WT/WT}$ and $Scl^{\Delta 40/\Delta 40}$ embryos (E10.5, E12.5, and E14.5) were morphologically indistinguishable (not shown), and analysis of the erythroid lineage by flow cytometry using CD71 and TER119 markers showed no difference in terminal erythroid differentiation at E12.5 (Fig. 4D) and E14.5 (not shown). Similarly to the observations in adult tissues, a reduction in CFU-e numbers was observed in $Scl^{\Delta 40/\Delta 40}$ fetal livers (Fig. 4E) but the BFU-e numbers were not significantly different (Fig. 4F).

Analyses of *Scl* and *Map17* expression levels showed a significant reduction in *Scl* expression at E12.5 (Fig. 4G) in $Scl^{\Delta 40/\Delta 40}$ fetal livers, but a recovery in Scl expression levels was observed at E14.5 (not shown). *Map17* expression was almost absent in $Scl^{\Delta 40/\Delta 40}$ fetal livers at both E12.5 and E14.5 (Fig. 4G and data not shown). No significant difference in Scl expression was observed when expression was analyzed in the embryo head, where the *Scl* +40 enhancer is functional in mid- and hindbrain (not shown). The erythroid phenotype in fetal liver is therefore consistent with the adult Scl phenotype. The reduced *Scl* expression levels at E12.5 and a recovery of Scl expression levels observed at later stages of development can partially explain the mild phenotype observed.

Erythroid differentiation is impaired in $Scl^{\Delta 40/\Delta 40}$ ES cells. Two independently targeted ES clones were used for the generation of $Scl^{\Delta 40/\Delta 40}$ ES cells (Fig. 1D). $Scl^{Neo/WT}$ ES cells were transiently transfected with a PGK-*Cre* construct to remove the floxed *Neo* cassette and generate $Scl^{\Delta 40/WT}$ ES cells. The second *Scl* allele, containing an intact *Scl* +40 enhancer, was then deleted using the same targeting vector to generate $Scl^{\Delta 40/Neo}$ ES, and PGK-*Cre* was again used to remove the *Neo* cassette and generate $Scl^{\Delta 40/\Delta 40}$ ES cells. $Scl^{\Delta 40/\Delta 40}$ ES cells are indistinguishable from $Scl^{WT/WT}$ ES cells when cultured under self-renewal conditions with serum and leukemia inhibitory factor (LIF). To investigate the differentiation potential of these ES cells, embryoid bodies (EBs) were generated from $Scl^{WT/WT}$ and $Scl^{\Delta 40/\Delta 40}$ ES cells, and samples were collected for analysis at days 4, 6, and 8 of differentiation. These time points were selected because *Scl* expression initiates in EBs at day 3.75, at day 6 only primitive erythropoiesis is observed, and by day 8 definitive hematopoiesis is present.

$Scl^{\Delta 40/\Delta 40}$ ES cells formed EBs similarly to $Scl^{WT/WT}$ ES cells, but the $Scl^{\Delta 40/\Delta 40}$ EBs were pale compared to the $Scl^{WT/WT}$ controls (Fig. 5A). Detailed flow cytometric analysis of relevant hematopoietic markers in day 8 EBs revealed a significant reduction in the percentage of $TER119^+$ cells and an increase of $cKit^+$ cells at day 8 (Fig. 5B). To evaluate the differentiation potential of the erythroid lineage in more detail, EBs were disrupted at both day 6 and day 8 and BFU-e were quantified by methylcellulose-based colony assays. BFU-e numbers were variable, and no statistically significant difference between $Scl^{WT/WT}$ and $Scl^{\Delta 40/\Delta 40}$ cells was obtained. However, while BFU-e obtained from $Scl^{\Delta 40/\Delta 40}$ ES cells were hemoglobinized, the clones were significantly smaller and very compact, a characteristic of primitive BFU-e (data not shown). These observations, together with the pale appearance of the EBs, indicated a defect in the differentiation of erythroid cells from $Scl^{\Delta 40/\Delta 40}$ ES cells.

Expression time course analysis of the EBs revealed that *Scl* is

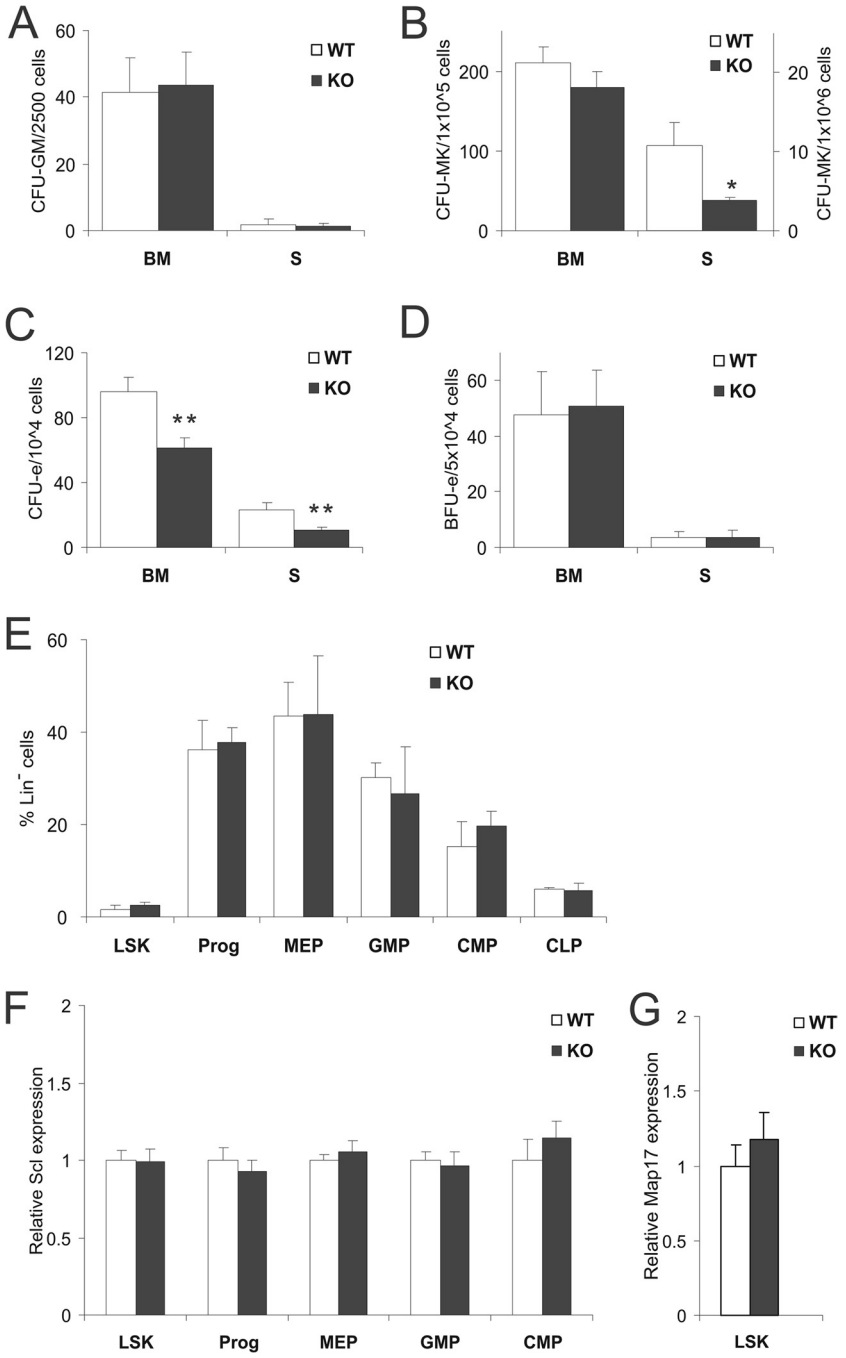

FIG 3 CFU-e numbers are reduced in $Scl^{\Delta 40/\Delta 40}$ bone marrow (BM) and spleen (S). (A to D) Histograms showing numbers of CFU-GM (A), CFU-MK(B), CFU-e (C), and BFU-e (D) in bone marrow and spleen. *, $P \leq 0.05$; **, $P \leq 0.01$. (E) Histogram showing percentages of hematopoietic stem and progenitor cells in lineage-negative bone marrow cells. (F) Relative expression of *Scl* in early stem and progenitor cells. (G) Relative expression of *Scl* in Lin^- $Sca1^+$ Kit^+ cells. WT, $Scl^{WT/WT}$; KO, $Scl^{\Delta 40/\Delta 40}$.

expressed at normal levels at day 4 but fails to be upregulated at later days (Fig. 5C). Since *Scl* upregulation is essential for terminal erythroid differentiation, we also analyzed the expression of several other genes known to be involved in this process. Similarly to *Scl*, *Gata1* levels failed to be upregulated from day 6 to day 8 (Fig. 5C). Moreover, there was no induction of *Beta-major*, the beta-globin gene expressed in the definitive erythroid lineage, in $Scl^{\Delta 40/\Delta 40}$ EBs after day 6. Due to the genomic location of the *Scl* +40 enhancer, we also checked the expression of the *Map17* gene, situated immediately upstream of the +40 enhancer. Similarly to *Scl*, *Map17* was not expressed in ES cells and was induced by day 4, with its expression remaining low until day 6 but being upregulated thereafter. Expression of *Map17* in $Scl^{\Delta 40/\Delta 40}$ EBs was already reduced by day 4 and remained absent after day 6, the time its expression is upregulated in $Scl^{WT/WT}$ EBs (Fig. 5C).

Together, these results indicate that deletion of the *Scl* +40

TABLE 2 $Scl^{\Delta 40/\Delta 40}$ mice show normal response to stress erythropoiesis[a]

Time after PHZ addition (days)	Genotype	RBC ($10^3/\mu l$)	Hgb (g/liter)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/liter)	PLT ($10^3/\mu l$)	MPV (fl)
0	$Scl^{WT/WT}$	9.24 ± 1.84	147.7 ± 22.90	50.2 ± 8.39	55.0 ± 2.00	16.1 ± 0.75	294.3 ± 3.78	1,308.3 ± 235.97	5.3 ± 0.45
	$Scl^{\Delta 40/\Delta 40}$	10.42 ± 0.50	158.67 ± 3.21	53.9 ± 2.14	51.7 ± 2.08	15.3 ± 0.59	294.7 ± 6.35	1,148.3 ± 439.03	5.5 ± 0.66
5	$Scl^{WT/WT}$	4.45 ± 0.50	117.67 ± 29.29	30.0 ± 2.00	69.3 ± 10.78	26.3 ± 4.25	393.0 ± 127.23	1,505.0 ± 192.59	6.07 ± 0.45
	$Scl^{\Delta 40/\Delta 40}$	4.32 ± 0.46	123.67 ± 19.03	29.6 ± 0.52	69.3 ± 8.50	28.6 ± 3.12	418.6 ± 71.50	1,719.7 ± 324.22	6.33 ± 0.50

[a] RBC, erythrocyte concentration; Hgb, hemoglobin concentration; Hct, hematocrit level; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin amount; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet count; MPV, mean platelet volume.

enhancer prevents the upregulation of *Scl* and *Map17* during terminal erythroid differentiation. This is accompanied by a reduction in the percentage of mature erythrocytes in day 8 EBs.

The erythroid differentiation phenotype of $Scl^{\Delta 40/\Delta 40}$ ES cells is rescued by restoration of *Scl* expression. To confirm that the observed phenotype was indeed due to the defect in *Scl* expression, we transduced the $Scl^{\Delta 40/\Delta 40}$ ES cells with a retrovirus containing a

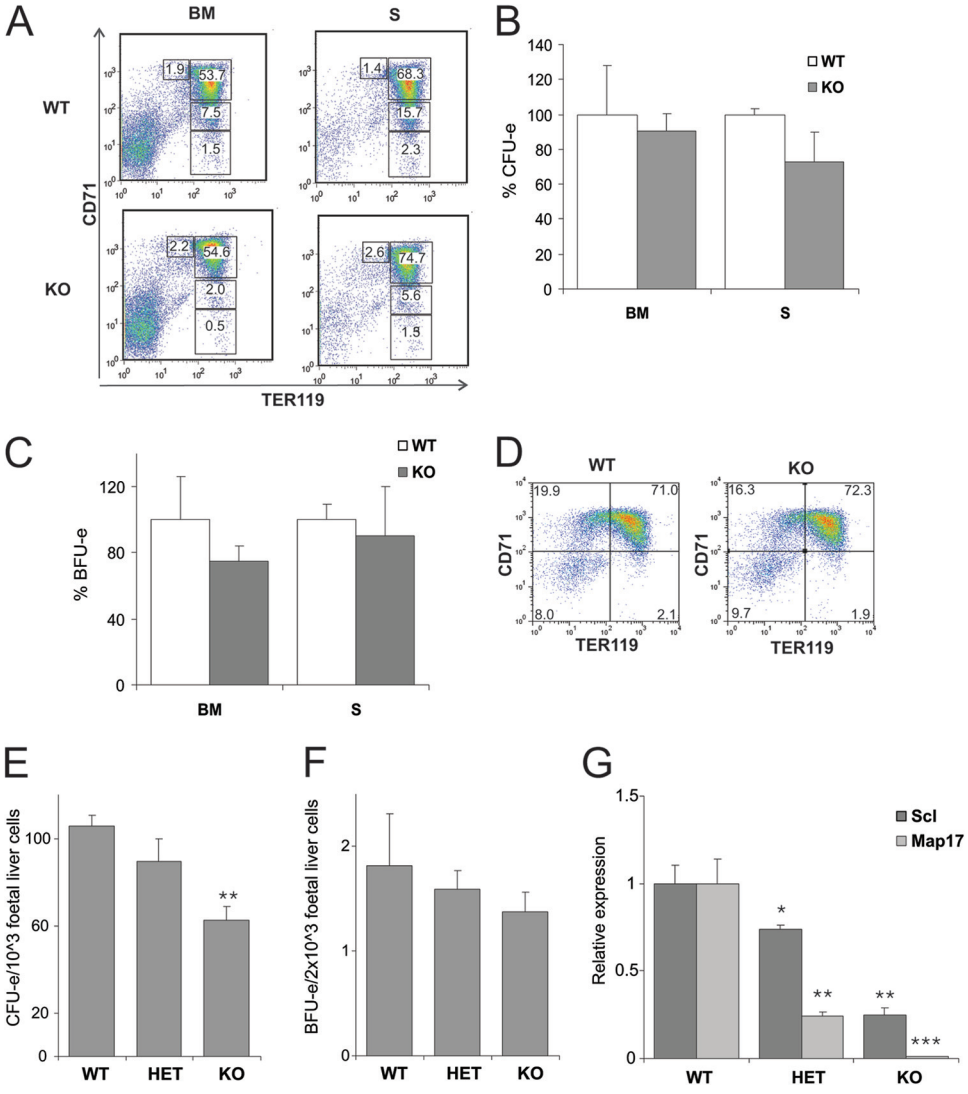

FIG 4 Response to stress hematopoiesis is normal, but CFU-e numbers are reduced in fetal liver of $Scl^{\Delta 40/\Delta 40}$ mice. (A) Dot plots showing representative staining of erythroid cells in bone marrow (BM) and spleen (S) 5 days after induction of stress hematopoiesis by PHZ. Values indicate averages for 4 animals. (B and C) CFU-e (B) numbers are reduced, but BFU-e (C) numbers are unaltered in $Scl^{\Delta 40/\Delta 40}$ mice. Results are shown as percentages of colonies compared to $Scl^{WT/WT}$ mice. (D) Dot plots showing representative CD71/TER119 profile of E12.5 fetal liver cells. (E and F) CFU-e (E) and BFU-e (F) counts in E12.5 fetal livers. **, $P \leq 0.005$. (G) Expression analysis of *Scl* and *Map17* in E12.5 fetal livers. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. WT, $Scl^{WT/WT}$; HET, $Scl^{WT/\Delta 40}$; KO, $Scl^{\Delta 40/\Delta 40}$.

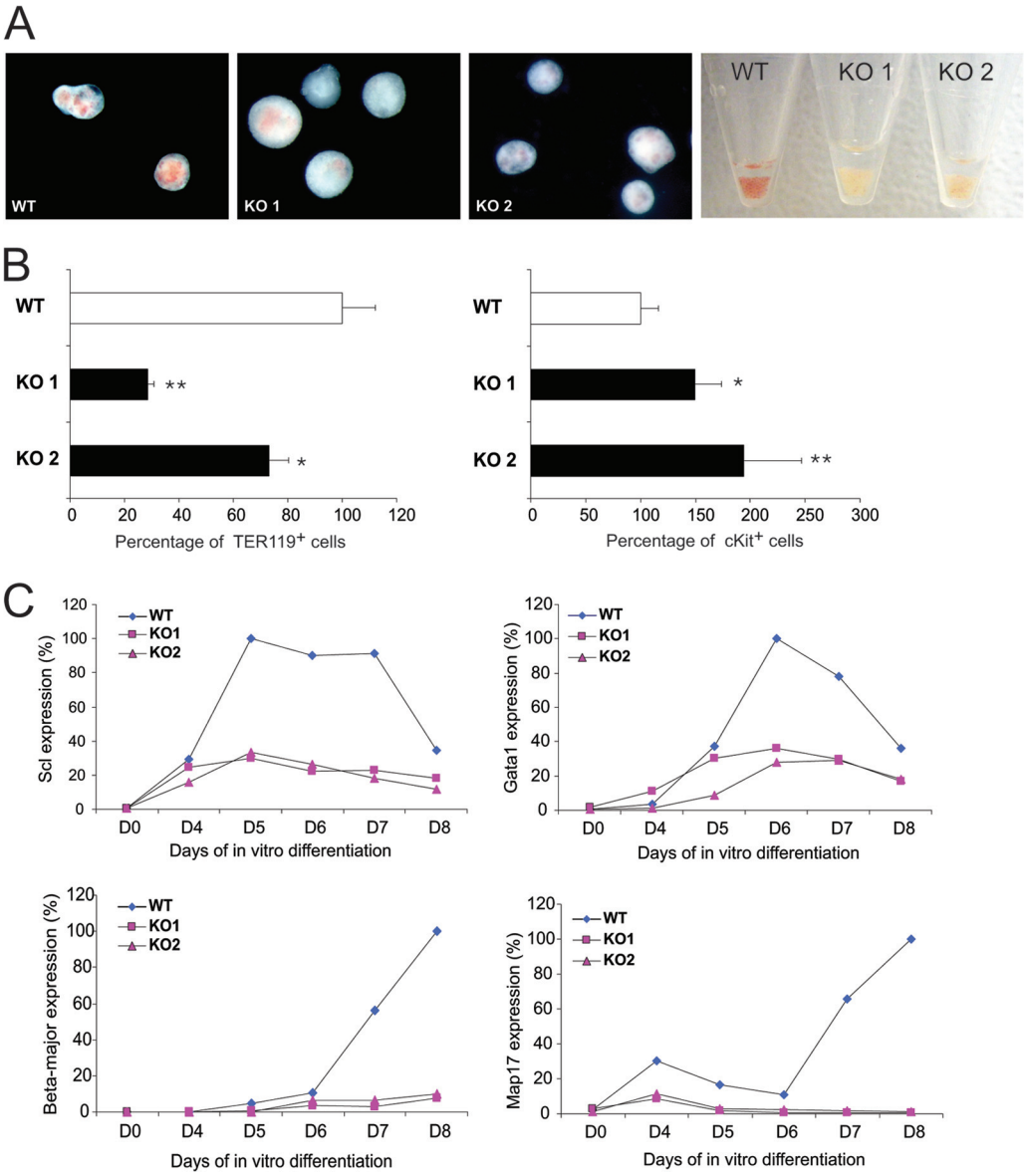

FIG 5 Analysis of $Scl^{\Delta 40/\Delta 40}$ ES cells reveals an erythroid maturation defect. (A) Representative photographs of EBs generated from $Scl^{WT/WT}$ (WT) and 2 independent $Scl^{\Delta 40/\Delta 40}$ ES clones (KO 1 and KO 2) as well as pelleted EBs (right). (B) Histograms showing the percentages of TER119-positive (left) and cKit-positive (right) cells in day 8 EBs. *, $P \leq 0.05$; **, $P \leq 0.01$. (C) Expression of *Scl* (top left), *Gata1* (top right), *Beta-major* (bottom left), and *Map17* (bottom right) during EB differentiation. WT, $Scl^{WT/WT}$; KO, $Scl^{\Delta 40/\Delta 40}$.

Flag-tagged cDNA of *Scl*, which has been previously used to rescue the phenotype of *Scl* knockout (KO) ES cells (1, 39). Expression of Flag-*Scl* in $Scl^{\Delta 40/\Delta 40}$ ES cells was able to rescue the erythroid phenotype during ES differentiation. In the presence of Flag-*Scl*, hemoglobinization was significantly restored in $Scl^{\Delta 40/\Delta 40}$ EBs (Fig. 6A). More-detailed analysis showed that the percentage of $TER119^+$ cells was increased while the percentage of $cKit^+$ progenitors was decreased, indicating a restoration of terminal erythroid differentiation (Fig. 6B). Moreover, expression time course analysis confirmed that *Scl* was indeed overexpressed in the transduced $Scl^{\Delta 40/\Delta 40}$ EBs (Fig. 6C). *Scl* overexpression had no effect on *Map17* expression, which remained unaltered in both $Scl^{WT/WT}$ and $Scl^{\Delta 40/\Delta 40}$ ES cells expressing Flag-*Scl*.

The *Scl* +40 enhancer is shared by *Scl* and *Map17* genes. To confirm that the *Scl* +40 enhancer is a hematopoietic cell-specific enhancer not only of the *Scl* gene but also of *Map17*, constructs were generated wherein the luciferase reporter gene was placed under the control of the *Map17* promoter. Addition of the *Scl* +40(5.0) enhancer downstream of the luciferase reporter gene resulted in enhanced expression in a cell-type-dependent matter (Fig. 7A). No enhanced activity was detected in the BW5147 T-cell line, in which the *Scl* +40 enhancer is known to be inactive. A moderate increase in luciferase activity was detected in the 416B hematopoietic progenitor cell line, with much more substantial enhancement in the F4N erythroid cell line, thus once more demonstrating that the *Scl* +40 enhancer is mostly erythroid cell specific. Only a very minor increase in luciferase activity was detected in the MDCT kidney cell line, where *Map17* is highly expressed,

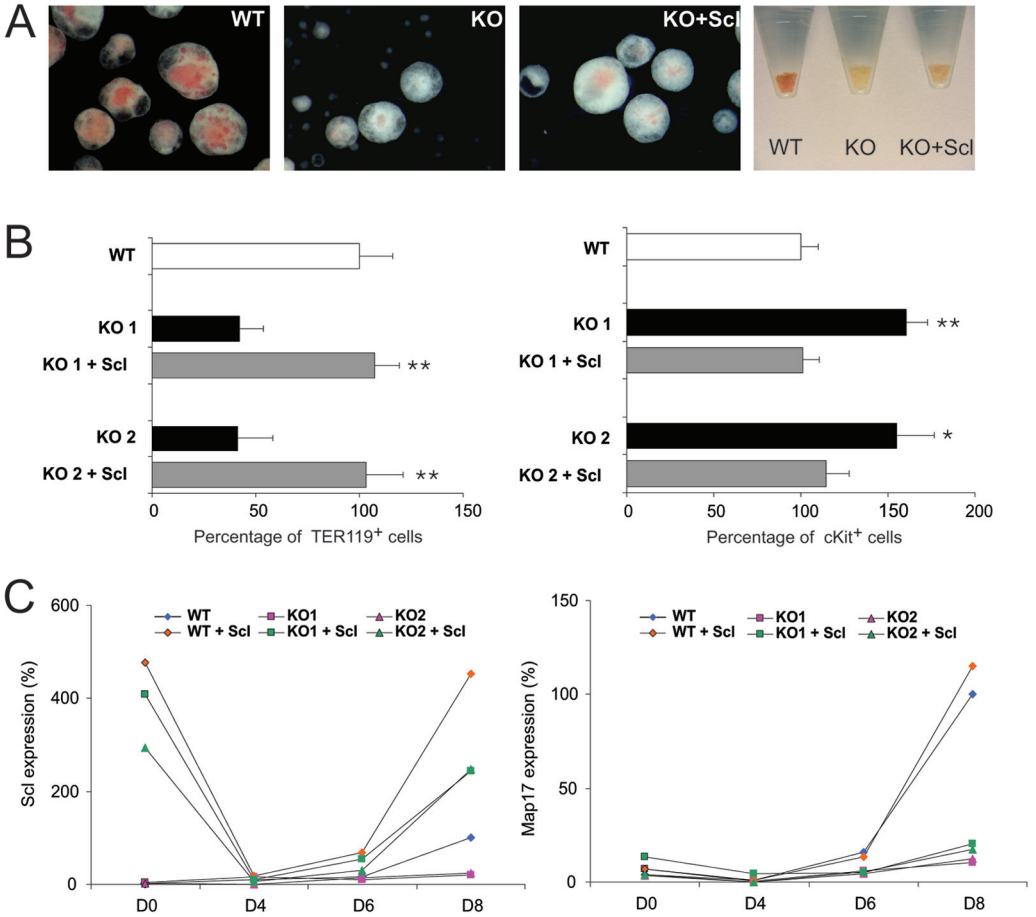

FIG 6 Rescue of the $Scl^{\Delta 40/\Delta 40}$ ES cells phenotype by Flag-*Scl*. (A) Representative photographs of EBs generated from $Scl^{WT/WT}$ (WT), $Scl^{\Delta 40/\Delta 40}$ (KO), and $Scl^{\Delta 40/\Delta 40}$ mice overexpressing Flag-*Scl* (KO + Scl) and pelleted EBs (right). (B) Histograms showing the percentages of Ter119-positive (left) and cKit-positive (right) cells in day 8 EBs. *, $P \leq 0.05$; **, $P \leq 0.01$ (compared to KO mice). (C) Expression of *Scl* (left) and *Map17* (right) during embryoid body differentiation. Data are normalized to the expression at day 8 in $Scl^{WT/WT}$ EBs. WT, $Scl^{WT/WT}$; KO, $Scl^{\Delta 40/\Delta 40}$.

thus suggesting that the +40 enhancer is not active in kidney, consistent with the lack of any loss of *Map17* expression in the kidney in $Scl^{\Delta 40/\Delta 40}$ mice.

The fact that expression of both *Scl* and *Map17* is altered in $Scl^{\Delta 40/\Delta 40}$ EBs raised the question as to which gene is responsible for the observed phenotype. To address this question, we infected the $Scl^{\Delta 40/\Delta 40}$ ES cells with a retrovirus containing a Flag-tagged cDNA for *Map17*. Overexpression of Flag-*Map17* in $Scl^{\Delta 40/\Delta 40}$ ES cells did not have a significant impact on hemoglobinization of the $Scl^{\Delta 40/\Delta 40}$ EBs; however, an increased generation of EBs was observed, indicating a potential function for *Map17* in the early stages of EB formation and/or differentiation (Fig. 7B). *Map17* overexpression had no effect on the erythroid differentiation phenotype of the $Scl^{\Delta 40/\Delta 40}$ ES cells, with the percentage of $TER119^+$ erythroid cells or $cKit^+$ progenitors remaining unaltered (Fig. 7C). Expression analysis of transduced EBs during *in vitro* differentiation confirmed that *Map17* was overexpressed (Fig. 7D). *Map17* overexpression had no effect on *Scl* expression, which remained unaltered in both $Scl^{WT/WT}$ and $Scl^{\Delta 40/\Delta 40}$ ES cells expressing Flag-*Map17*.

Together, these data clearly demonstrate that the *Scl* +40 enhancer regulates *Map17* expression but reduced *Map17* expression is not the cause of the erythroid phenotype observed in $Scl^{\Delta 40/\Delta 40}$ ES cells.

DISCUSSION

Concerted research efforts over the past 20 years have established the *Scl* gene as a paradigm gene locus for the study of hematopoietic transcriptional control mechanisms (17, 18, 25, 26, 40–48). We have shown previously that the *Scl* +40 enhancer can drive expression of a linked reporter gene in the erythroid lineage in transgenic assays (17, 27). By deleting this element in the germ line, we now confirm that this enhancer indeed is important for the accurate spatiotemporal expression of *Scl* in the erythroid lineage. Deletion of the *Scl* +40 enhancer caused defects in the erythroid lineage, both *in vitro* and *in vivo*. No difference in Scl expression was detected in the brain, presumably because neuronal expression of Scl is also driven by the promoter (28). Furthermore, we show that the +40 enhancer regulates the expression of *Map17* as well as *Scl* in hematopoietic cells but only *Scl* is able to rescue the erythroid defect in ES cell differentiation assays.

The data obtained from analysis of $Scl^{\Delta 40/\Delta 40}$ mice and ES cells confirm that indeed the *Scl* +40 enhancer, in the hematopoietic system, is specific for the erythroid lineage. Deletion of this ele-

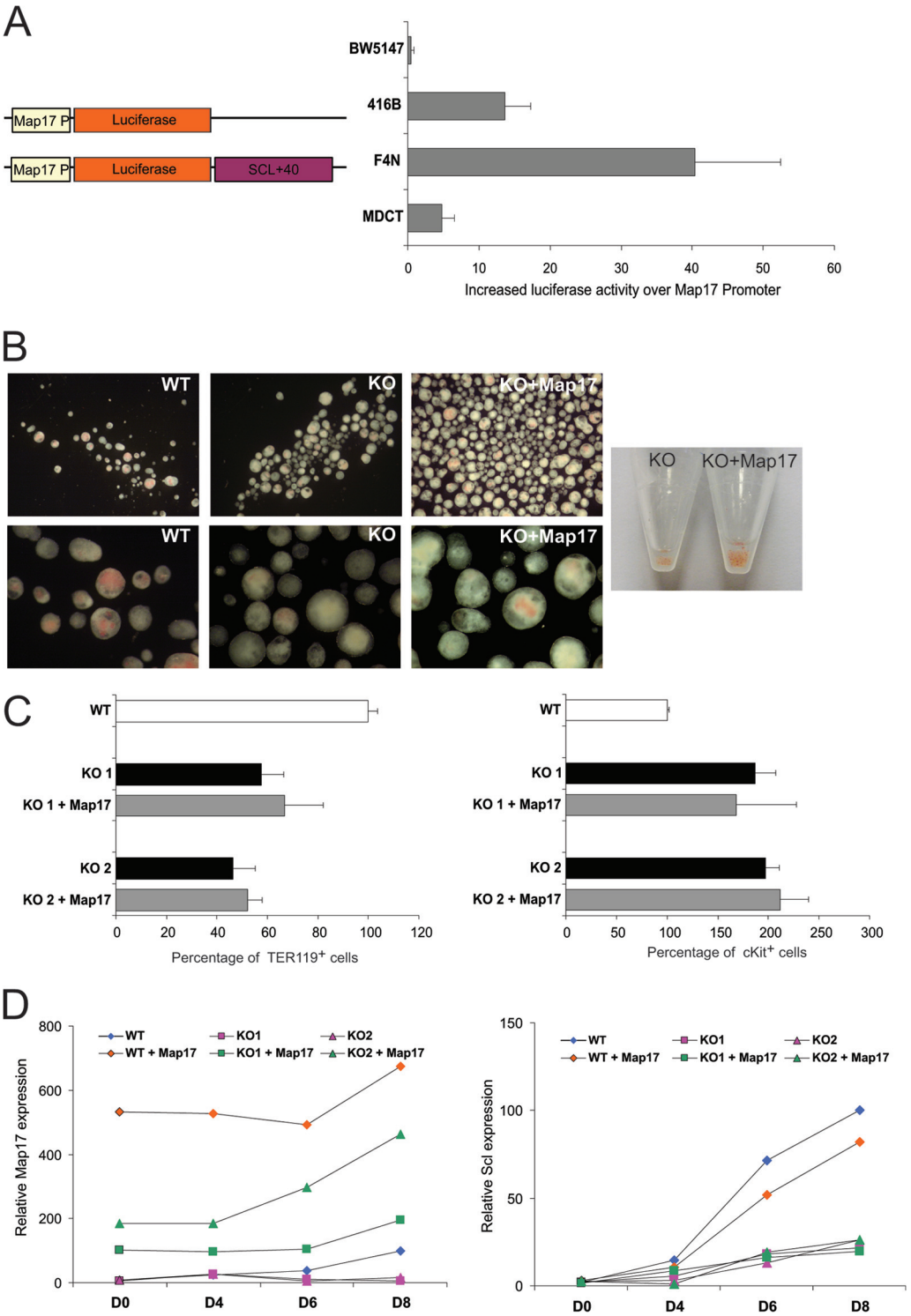

FIG 7 The *Scl* +40 element can enhance the *Map17* promoter, but *Map17* cannot rescue the $Scl^{\Delta 40/\Delta 40}$ ES cell phenotype. (A) (Left) Schematic representation of constructs used for luciferase assays. (Right) Luciferase activity in BW5147, 416B, F4N, and MDCT cell lines. Data are normalized to *Map17*P-Luc. (B) Representative photographs of EBs generated from $Scl^{WT/WT}$, $Scl^{\Delta 40/\Delta 40}$, and $Scl^{\Delta 40/\Delta 40}$ mice overexpressing Flag-*Map17* and pelleted EBs (right). Magnification, ×1.5 (top) or ×5 (bottom). (C) Histograms showing the percentages of TER119-positive (left) and cKit-positive (right) cells in day 8 EBs. (D) Graphs showing expression of *Map17* (left) and *Scl* (right) during embryoid body differentiation. Data are normalized to the expression at day 8 in $Scl^{WT/WT}$ EBs. WT, $Scl^{WT/WT}$; KO, $Scl^{\Delta 40/\Delta 40}$.

ment leads to reduced *Scl* expression in whole bone marrow and spleen, containing large numbers of erythroid cells, and specifically in the erythroid lineage, with the highest reduction observed in the progenitor populations containing BFU-e, CFU-e, and proerythroblasts ($Ter119^{low}$ $CD71^+$ and $Ter119^+$ $CD71^+$). This is also confirmed by a reduction in CFU-e numbers in bone marrow spleen and fetal liver. No difference was observed in early progenitors like LSK, CMP, and MEP, where *Scl* is known to be functionally important (1–4, 12–14, 16). Furthermore, expression analysis of $Scl^{\Delta 40/\Delta 40}$ ES cells during *in vitro* differentiation also showed that *Scl* expression is normal during early stages of differentiation corresponding to the hemangioblast (day 4) but fails to be upregulated in later stages, when erythroid differentiation occurs. These observations contrast with the absence of differentiation defects in $Scl^{+/-}$ ES cells (2, 7, 49). Although little is known about the function of *Map17* in the hematopoietic system, reports have indicated that *Map17* is highly expressed in long-term repopulating HSCs (21) and is upregulated in leukemic stem cells (50), suggesting functional importance in HSCs. Our observation of normal *Scl* expression levels in HSCs and early hematopoietic progenitors are not unexpected, since we have shown previously that alternative regulatory elements such as the *Scl* +19 and −4 enhancers are active in the progenitor compartment (18, 25, 26, 28). Similarly, we have shown recently that *Map17* can be regulated via the *Scl* +19 enhancer in early hematopoietic cells (24), thus providing a potential explanation for normal *Map17* levels in immature cells.

$Scl^{\Delta 40/\Delta 40}$ mice are mostly normal, showing only a transient defect in erythroid differentiation at the level of the CFU-e, with no phenotype observed at earlier or later stages of erythroid differentiation, both in adults and during embryonic development. This mild phenotype is surprisingly different from the ES cell phenotype, whereby EBs derived from $Scl^{\Delta 40/\Delta 40}$ ES cells showed reduced hemoglobinization and reduced numbers or $TER119^+$ cells. A potential explanation for the different phenotypes could lie in the increased complexity of the *in vivo* whole-animal setting, where, for example, the erythropoietin (Epo) concentrations as well as other growth factors in the bone marrow can be adjusted to stimulate erythroid differentiation (51, 52). In an attempt to understand if that is the case, we measured the concentration of Epo in the sera of $Scl^{\Delta 40/\Delta 40}$ mice. Although we observed a slight increase in the Epo concentration, it failed to reach statistical significance. These data do not, however, dismiss the possibility that increased levels of Epo or other growth factors could be the explanation for the mild phenotype. The fact that the induction of stress erythropoiesis by PHZ did not result in an exacerbated phenotype further emphasizes that such regulation exists *in vivo*.

The $Scl^{\Delta 40/\Delta 40}$ phenotype is also mild in comparison to the phenotype observed when the *Scl* gene is deleted in adult mice (13). Mice with *Scl* deletion are anemic, display enlarged spleens, and show a shift toward more immature erythroid progenitors in bone marrow and spleen (16), while the $Scl^{\Delta 40/\Delta 40}$ mice show only a reduction in CFU-e numbers. In contrast, *Scl* heterozygous mice display a defect in the repopulation capacity of short-term (ST)-HSCs but no defect in erythropoiesis was reported (12, 53). We have, however, recently reported a similar ST-HSC phenotype upon the deletion of the Scl stem cell enhancer, thus emphasizing functional differences between the various Scl enhancers (36). The mild phenotype observed in $Scl^{\Delta 40/\Delta 40}$ mice is, however, similar to what is observed in adult mice homozygous for a mutant *Scl* protein with a nonfunctional DNA binding domain (37). The comparatively mild phenotype in our $Scl^{\Delta 40/\Delta 40}$ mice may be a reflection of the fact that *Scl* is still normally expressed in earlier progenitors and also by possible compensation from the other *Scl* enhancers still functional in these animals. Analysis of the *Scl* locus in $Scl^{\Delta 40/\Delta 40}$ mice by chromatin immunoprecipitation (data not shown) did not suggest a compensatory mechanism by any previously described regulatory element (Scl-4, Promoter, and Scl +19). However, considering that the *in vivo* phenotype is restricted to a particular stage of erythroid differentiation, it is possible that the effect is diluted in the tissues analyzed by the presence of cells with normal Scl expression. It is also possible that such compensation may be driven by an as-yet-unidentified regulatory element. Due to our extensive study of the Scl/Map17 locus, it is unlikely that such an element resides within the locus; however, the existence of a shadow enhancer situated in a more distant location cannot be excluded (54). Shadow enhancers that can drive expression in patterns similar to that of the primary enhancer have been identified in *Drosophila*, where they confer robustness to the expression pattern of developmentally important genes (55). Of notice, mild phenotypes upon deletion of regulatory elements are a common occurrence and have been frequently reported before (36, 56–59).

Our data strongly indicate that the *Scl* +40 enhancer can regulate the expression of *Map17* in the erythroid lineage. The +40 element enhances the expression of the *Map17* promoter in luciferase reporter assays in erythroid cells and to a lower extent in hematopoietic progenitors but not in kidney cells. This connection between the +40 element and *Map17* is reinforced by the fact that in $Scl^{\Delta 40/\Delta 40}$ ES cells *Map17* is downregulated. The +40 enhancer contains two functionally important *Gata*/E-box motifs that are bound in erythroid cells by *Scl* and *Gata1* (25). Our results are therefore consistent with a model whereby binding of *Scl* to the +40 E-box contributes to *Map17* expression in the erythroid lineage. Furthermore, we showed that ectopic expression of *Map17* does not alter endogenous *Scl* expression and argue against the possibility of Map17 protein regulating *Scl*.

Rescue of the $Scl^{\Delta 40/\Delta 40}$ ES phenotype by ectopic expression of Flag-*Scl* without upregulation of *Map17* expression suggests that *Map17* expression is not necessary for terminal erythroid differentiation, which would also be consistent with the observed failure of *Map17* ectopic expression to rescue the phenotype in $Scl^{\Delta 40/\Delta 40}$ ES cells. Surprisingly, ectopic overexpression of *Map17* led to an increase in the number of EBs, presumably through an expansion of multipotent cells during the earliest stages of differentiation. This suggests that *Map17* may perform important functions during the early stages of EB differentiation, which future experiments will need to confirm through comprehensive loss- and gain-of-function studies during early embryo development. It is, however, attractive to speculate that the elevated expression of *Map17* seen in many cancers may be related to the apparent increase in cells with EB formation ability.

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