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# The *scl* gene product is required for the generation of all hematopoietic lineages in the adult mouse

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**Homozygosity for a null mutation in the *scl* gene causes mid-gestational embryonic lethality in the mouse due to failure of development of primitive hematopoiesis. Whilst this observation established the role of the *scl* gene product in primitive hematopoiesis, the death of the *scl* null embryos precluded analysis of the role of *scl* in later hematopoietic development. To address this question, we created embryonic stem cell lines with a homozygous null mutation of the *scl* gene ($scl^{-/-}$) and used these lines to derive chimeric mice. Analysis of the chimeric mice demonstrates that the $scl^{-/-}$ embryonic stem cells make a substantial contribution to all non-hematopoietic tissues but do not contribute to any hematopoietic lineage. These observations reveal a crucial role for the *scl* gene product in definitive hematopoiesis. In addition, *in vitro* differentiation assays with $scl^{-/-}$ embryonic stem cells showed that the *scl* gene product was also required for formation of hematopoietic cells in this system.**

*Keywords*: chimera/gene targeting/hematopoiesis/*scl*/*tal*-1

## Introduction

The *scl* (*tal*-1, *tcl*5) gene encodes a helix–loop–helix transcription factor which is aberrantly expressed in the majority of cases of pediatric T-cell acute lymphoblastic leukemia (T-ALL) (Bash *et al.*, 1995). It was first identified as one of the partners involved in a rare recurrent reciprocal translocation between chromosomes 1 and 14 t(1;14)(p32; q11) (Begley *et al.*, 1989; Finger *et al.*, 1989; Chen *et al.*, 1990). More frequently, *scl* expression is ectopically activated in T-ALL via a submicroscopic interstitial deletion which juxtaposes the coding region of the gene with the promoter elements of the *sil* gene, which lies ~90 kb upstream of *scl* (Aplan *et al.*, 1990; Bernard *et al.*, 1990; Brown *et al.*, 1990). Recently, *scl* expression was documented in an additional group of T-ALL samples in the absence of detectable gene rearrangement (Bash *et al.*, 1995). The mechanism by which *scl* expression is activated in these cases has not been established.

In non-malignant adult human and murine tissues, expression of the *scl* gene was shown to be restricted largely to the hematopoietic system, being found in erythroid, megakaryocytic, mast and early myeloid cells and in committed progenitor ($CD34^+/CD38^+$) cells (Begley *et al.*, 1989; Green *et al.*, 1991b; Visvader *et al.*, 1991; Mouthon *et al.*, 1993). Scl protein was detectable in the murine embryo as early as E7.5, and by E8.5 it localized to yolk sac blood progenitors and endothelial cells. Expression was also seen in the developing nervous and skeletal systems and in vascular endothelium (Green *et al.*, 1992; Kallianpur *et al.*, 1994). By analogy with other members of the basic helix–loop–helix family of transcription factors (reviewed in Jan and Jan, 1993; Kadesch, 1993), it seemed likely that the *scl* gene product was involved in regulating cell type determination and differentiation, and the expression pattern of *scl* suggested that it may play a role in lineage-specific commitment and differentiation within the hematopoietic system. Initial support for this notion arose from gene delivery experiments which demonstrated a role for *scl* in erythroid proliferation and differentiation (Green *et al.*, 1991a; Aplan *et al.*, 1992; Hoang *et al.*, 1996). In addition, erythropoietin (Epo) stimulation of a lymphoid cell line (Ba/F3) in which an Epo receptor was installed activated transcription of *scl* and was followed by the accumulation of α- and β-globin chains (Chiba *et al.*, 1993). Furthermore, *scl* overexpression in M1 cells, a murine myeloid leukemic cell line which undergoes monocytic differentiation in response to cytokine induction, and in the bipotential cell line TF-1, also implicated *scl* in monocytic differentiation (Tanigawa *et al.*, 1993; Hoang *et al.*, 1996).

Creation of mice homozygous for a null mutation of the *scl* gene ($scl^{-/-}$) provided strong evidence for a role for *scl* in hematopoietic commitment. The $scl^{-/-}$ embryos died around embryonic day 9 due to failure of primitive hematopoiesis (Robb *et al.*, 1995; Shivdasani *et al.*, 1995). Blood islands were not present in the yolk sacs of $scl^{-/-}$ embryos and hematopoietic colonies were absent when $scl^{-/-}$ yolk sacs were cultured *in vitro*. This complete block in hematopoietic development led to the prediction that *scl* acted at a step during hematopoietic differentiation which lay upstream of other transcription factors, such as GATA-1 and GATA-2, known to be important in control of gene expression and development of hematopoietic cells (Orkin, 1992). Experiments with GATA-1 null ES cells had shown it was essential for the terminal differentiation of the primitive and definitive erythroid lineages (Pevny *et al.*, 1991, 1995), whilst a global reduction in embryonic erythropoiesis in GATA-2 null embryos suggested a key role in proliferation or survival of early hematopoietic progenitor cells (Tsai *et al.*, 1994). The $scl^{-/-}$ phenotype was similar to that observed in embryos homozygous for a null mutation of the *LMO2* gene (*rbtn2*) (Warren *et al.*, 1994). *LMO2* encodes a LIM domain nuclear protein which, like *scl*, is involved in a recurrent translocation in T-ALL (Boehm *et al.*, 1991; Royer-Pokora

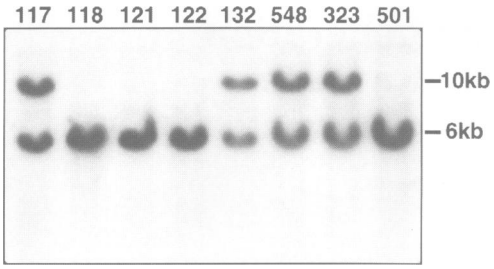


**Fig. 1.** Southern analysis of $scl^{-/-}$ and control $scl^{+/-}$ ES cells. DNA was prepared from $scl^{+/-}$ control ES cell lines (117, 1323, 548, 323) and $scl^{-/-}$ ES cell lines (118, 121, 122, 501) digested with *Eco*RI and electrophoresed through a 0.8% agarose gel, transferred to nylon membrane and probed with probe B (Robb *et al.*, 1995). Bands corresponding to the endogenous (10 kb) and targeted (6 kb) alleles are indicated.

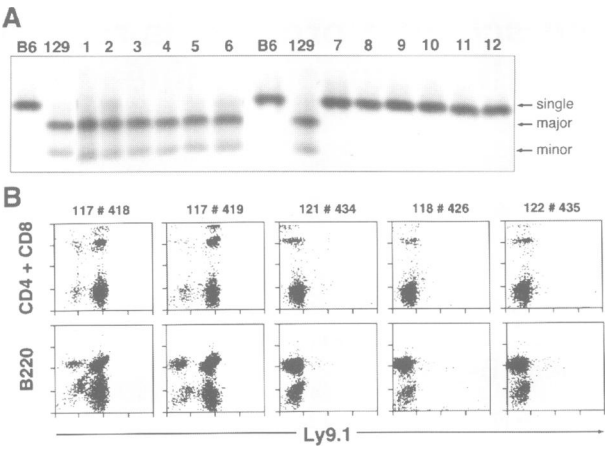


**Fig. 2.** Failure of $scl^{-/-}$ ES cells to contribute to peripheral blood erythrocytes and lymphocytes. (**A**) Absence of ES cell-derived β-globin isoforms in $scl^{-/-}$ chimeras. Blood proteins from adult $scl^{+/-}$ chimeras (1–6) and $scl^{-/-}$ chimeras (7–12), C57BL/6 (B6) and 129 mice were separated by cellulose acetate electrophoresis and stained as in Materials and methods. The Hb major and Hb minor forms of β-globin produced by the $Hbb^{d}$ haplotype of strain 129 Sv (ES cell-derived) and the Hb single ($Hbb^{s}$ haplotype) of strain C57BL/6 are indicated. (**B**) Absence of ES cell-derived lymphocytes in $scl^{-/-}$ chimeras. Peripheral blood mononuclear cells from $scl^{+/-}$ chimeras (117#418, 117#419) and $scl^{-/-}$ chimeras (121#434, 118#426, 122#435) were stained for expression of Ly9.1 and B220 or CD4 and CD8 and analysed by two-color flow cytometry. In the same experiment, four additional $scl^{+/-}$ and three additional $scl^{-/-}$ chimeras were analyzed with similar results.

*et al.*, 1991). In rare cases of T-ALL, both *scl* and *LMO2* have been shown to be activated aberrantly in the leukemic cells (Wadman *et al.*, 1994). The Scl and LMO2 proteins have been demonstrated to interact physically within the nucleus of cells of the erythroid lineage (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994), and the phenotypic similarity of the *scl* and *LMO2* null embryos supports the hypothesis that this protein–protein interaction is of functional relevance and may play a role in the establishment of the primitive hematopoietic system.

The mid-gestational embryonic lethality of $scl^{-/-}$ embryos prevented analysis of the role of *scl* in definitive hematopoiesis. To address this question, we have generated embryonic stem (ES) cell lines with a homozygous null mutation of the *scl* gene ($scl^{-/-}$ ES cells) and used them to create chimeric mice. Analysis of the contribution of the $scl^{-/-}$ cells to hematopoietic and other tissues of the chimeras revealed the *scl* gene product is also required for definitive murine hematopoiesis. In addition, *in vitro* differentiation assays revealed that primitive and definitive hematopoietic cells were absent in $scl^{-/-}$ embryoid bodies.

## Results

### *Generation of homozygous $scl^{-/-}$ and control ES cell lines*

Four $scl^{-/-}$ ES cell lines were generated by inactivating the functional allele of the *scl* locus in the *scl* heterozygous ($scl^{+/-}$) ES cell line 93 by homologous recombination with a second targeting construct (Robb *et al.*, 1995). In line 93, the portion of the *scl* gene encoding the basic DNA binding and helix–loop–helix region was replaced with a neomycin resistance gene cassette. Targeting construct 607 (Robb *et al.*, 1995), which has a hygromycin resistance cassette in place of this region and also contains the thymidine kinase cassette to facilitate negative selection, was electroporated into line 93 ES cells. ES cells which were resistant to hygromycin, neomycin and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) 5-iodouracil were expanded and screened by Southern analysis for homologous recombination of the second construct into the *scl* locus (Figure 1). Further Southern analysis with additional probes showed that a single copy of each targeting vector was present in each of the four $scl^{-/-}$ lines and each line was shown to have a normal karyotype (not shown).

In order to provide appropriate control lines, four $scl^{+/-}$ clones which survived the second round of electroporation and antibiotic selection, having non-homologously integrated targeting construct 607, were randomly selected and expanded. $scl^{-/-}$ cells were injected into C57BL/6J blastocysts, and six chimeric offspring, ranging in coat color chimerism from 25 to 90%, were generated from three of the $scl^{-/-}$ lines. Six chimeras created with the control $scl^{+/-}$ ES cell lines, ranging in coat color chimerism from 35 to 100%, were also analyzed. To validate further the $scl^{-/-}$ and control ES cell lines, chimeras derived from each cell line were mated and shown to be able to contribute to the germline.

### *Contribution of $scl^{-/-}$ ES cells to hematopoietic and non-hematopoietic tissues*

Peripheral blood samples from the $scl^{-/-}$ and $scl^{+/-}$ chimeras were assayed for the presence of the Hb major and Hb minor forms of β-globin produced by the $Hbb^{d}$ haplotype of strain 129Sv (ES cell derived) and the Hb single ($Hbb^{s}$ haplotype) of the host C57BL/6 (B6) blastocyst. The red blood cell fraction from the $scl^{-/-}$ chimeras showed no detectable contribution from the $scl^{-/-}$ ES cells. In contrast, the peripheral blood of $scl^{+/-}$ control chimeras was predominantly of the ES cell-derived β-globin form (Figure 2A). 129Sv ES cells are homozygous for the a allele of glucose phosphate isomerase (*GPI-1s*$^{a}$, encoding GPI-1A) whilst the B6 host cells are homozygous for the *GPI-1s*$^{b}$ allele (encoding GPI-1B). GPI isoenzyme analysis of whole blood, white cell and platelet fractions demonstrated that these cell fractions were of host blastocyst type (GPI-1B) in the $scl^{-/-}$ chimeras but largely of ES cell type (GPI-1A) in control chimeras (not shown).

**Table I.** Bone marrow progenitor cell assays from $scl^{-/-}$ and control chimeric mice

| Chimera No. (ES cell genotype) | % Coat color chimerism | Erythroid colonies[a] | | Granulocyte–macrophage, eosinophil, megakaryocyte colonies[a] | |
|---|---|---|---|---|---|
| | | –G418 | +G418 | –G418 | +G418 |
| 117#415 (+/–) | 100 | 46 | 33 ± 9 | 313 ± 24 | 258 ± 11 |
| 117#416 (+/–) | 90 | 38 | 35 ± 1 | 289 ± 22 | 252 ± 5 |
| 548#418 (+/–) | 80 | 48 | 47 ± 1 | 331 ± 16 | 282 ± 29 |
| 121#434 (–/–) | 90 | 68 | 0 | 239 ± 1 | 0 |
| 118#425 (–/–) | 90 | 49 | 0 | 146 ± 4 | 0 |
| 118#426 (–/–) | 85 | 58 | 0 | 268 ± 14 | 0 |
| 122#436 (–/–) | 70 | 38 | 0 | 256 | 0 |
| 122#435 (–/–) | 65 | 43 ± 3 | 0 | 282 ± 10 | 0 |
| 122#438 (–/–) | 25 | 15 ± 6 | 0 | 189 ± 42 | 0 |

[a]Colony number per $10^5$ cells, shown as the mean and standard deviation of duplicate or quadruplicate cultures.

Peripheral blood, bone marrow, thymus and spleen cells from the 12 chimeras were stained with Ly 9.1 and B220 or Thy1 or, for peripheral blood analysis, CD4 plus CD8, and analyzed by two-color flow cytometry. The antigen detected by Ly9.1 is present on lymphoid cells of the 129Sv (ES cell) strain but absent on B6 (host) strain (Durda *et al.*, 1979; Ledbetter *et al.*, 1979). In the $scl^{+/-}$ control chimeras, T and B lymphocytes were predominantly of 129Sv origin in all tissues studied, but ES cell-derived lymphoid cells were not detected in any tissues from the $scl^{-/-}$ chimeras (Figure 2B).

To determine the contribution of $scl^{-/-}$ ES cells to adult hematopoietic progenitor cells, semi-solid cultures of bone marrow cells from the chimeras were performed. G418 was added to duplicate cultures to select for $scl^{+/-}$ or $scl^{-/-}$ ES cell-derived progenitor cells, as host-derived progenitor cells did not carry the neomycin resistance gene. Although erythroid, granulocyte–macrophage and megakaryocytic colonies were abundant in unselected cultures and in G418-treated cultures from $scl^{+/-}$ chimeras, no colonies arose in G418-containing cultures of the $scl^{-/-}$ chimera bone marrow (Table I), demonstrating that the $scl^{-/-}$ ES cells were unable to give rise to progenitor cells of any hematopoietic lineage. Mast cell cultures, with and without the addition of G418, were established from bone marrow samples from control and $scl^{-/-}$chimeras. Mast cells were readily obtained in unselected cultures and G418-resistant mast cells were cultured from the bone marrow of control animals but not from $scl^{-/-}$ cells, indicating that the $scl^{-/-}$ ES cells were also unable to contribute to this hematopoietic lineage. In addition, G418-resistant colonies were not obtained in cultures of fetal livers from two E12.5 $scl^{-/-}$ chimeras which had been shown, by GPI analysis, to be >50% ES cell-derived (not shown).

To determine the overall contribution of the $scl^{-/-}$ and control ES cells to the chimeras, GPI analysis was performed on tissues. All the control chimeras had high ES cell contributions to all tissues tested but, whilst the ES cell contribution was substantial in non-hematopoietic tissues of the $scl^{-/-}$ chimeras, there was no detectable ES cell contribution to blood or spleen. This is illustrated in Figure 3 which shows the contribution of $scl^{-/-}$ ES cells to various tissues from three $scl^{-/-}$ chimeras, as a percentage of the GPI-1A isoform. The GPI assay is not sensitive enough to detect an isoform when present in amounts of 5% or less (L.Robb, unpublished observation) so a small contribution of the $scl^{-/-}$ ES cells to parenchymal

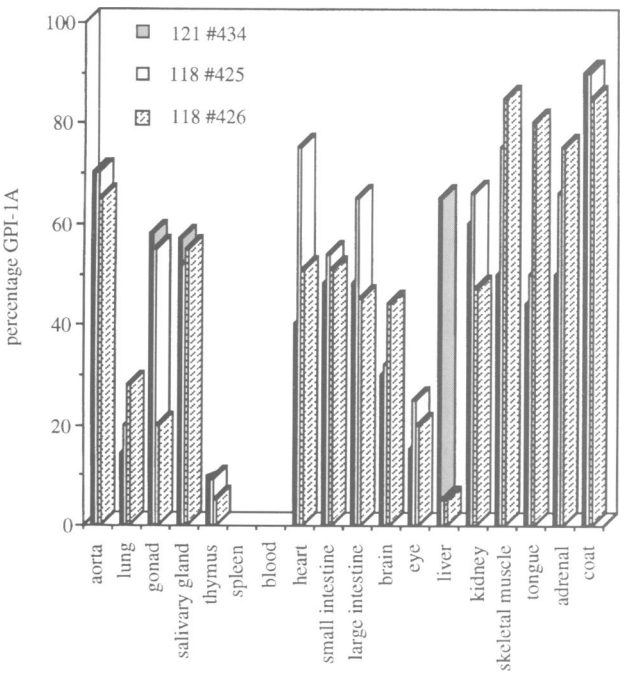


**Fig. 3.** $scl^{-/-}$ ES cell contribution to chimeric tissues. Percentage of GPI isotype 1A in tissues from $scl^{-/-}$ chimeras. Homogenates of chimeric tissues were separated by cellulose acetate electrophoresis and stained for GPI activity as described in Materials and methods. The percentage of each isotype was quantified by densitometry using Imagequant software. The contribution to the coat was judged by the amount of agouti versus black fur. Results are shown for three $scl^{-/-}$ chimeras (121#434, 118#425, 118#426).

or other low abundance tissues within the spleen cannot be excluded. Gross morphological and histological examination of the control and $scl^{-/-}$ chimeras did not reveal any abnormalities.

### *In vitro differentiation of $scl^{-/-}$ and control ES cells*

The $scl^{-/-}$ lines and doubly electroporated $scl^{+/-}$ control lines were studied in a two-step differentiation assay (Keller *et al.*, 1993). Whilst ~15% of primary embryoid bodies (EB) from control lines were visibly hemoglobinized, $scl^{-/-}$ EB were not hemoglobinized, an observation confirmed by diaminofluorene (DAF) staining. To determine whether the $scl^{-/-}$ EB harbored hematopoietic progenitor cells, EB were disaggregated and replated in

**Table II.** *In vitro* differentiation of $scl^{-/-}$ ES cell lines

| Cell line (genotype) | Visibly hemoglobinized embryoid bodies | | Primitive erythroid colonies | | Granulocyte–macrophage colonies | |
|---|---|---|---|---|---|---|
| | 1st plating | 2nd plating[a] | Expt 1[b] | Expt 2[b] | Expt 1[b] | Expt 2[b] |
| W9.5 (+/+) | 13.4% | 52 ± 3 | 1216 ± 221 | 337 ± 8 | 3 ± 1 | ND |
| 548 (+/–) | ND | 20 ± 2 | 698 ± 54 | 76 ± 6 | 35 ± 4 | 118 |
| 117 (+/–) | 13.1% | 18 ± 2 | 220 ± 74 | 81 ± 5 | 4 ± 1 | 11 |
| 118 (–/–) | 0 | 0 | 0 | 0 | 0 | 0 |
| 501 (–/–) | 0 | 0 | 0 | 0 | 0 | 0 |

[a]Number of hemoglobinized secondary EB (per $10^5$ cells replated from day 6 primary EB) (mean and standard deviation of triplicate cultures).
[b]Colony number per $10^5$ cells, shown as the mean and standard deviation of triplicate cultures. For enumeration of primitive erythroid colonies, primary EB were stimulated with SCF and Epo (Expt 1) or no stimulus (Expt 2) and were harvested at 6 days and replated with SCF and Epo. For enumeration of granulocyte–macrophage colonies, primary EB were stimulated with SCF, interleukin-3 and M-CSF, harvested after 10 days (Expt 1) or 21 days (Expt 2) and replated in the same stimuli.
ND: not done.

secondary methylcellulose cultures. Although EB from control lines replated after 6 days in primary culture with stem cell factor (SCF) and Epo developed numerous secondary EB and primitive erythroid colonies which stained positively with DAF, no erythroid colonies or hemoglobinized secondary EB were ever observed in secondary replatings of $scl^{-/-}$ primary EB (Table II). Consistent with the failure of the $scl^{-/-}$ ES cells to contribute to the myeloid lineages in chimeras, no granulocyte–macrophage colonies were found in secondary replatings of $scl^{-/-}$ EB in methylcellulose in the presence of hematopoietic growth factors known to stimulate myeloid colony formation (Table II).

Expression of a panel of genes involved in hematopoiesis was compared in EB formed from W9.5 parental ES cells, two control $scl^{+/-}$ and three $scl^{-/-}$ lines (Figure 4 and data not shown). Day 8 EB from primary differentiation cultures stimulated with SCF and Epo were pooled and RNA prepared. To compare the levels of gene expression in EB from the different genotypes, PCR reactions were performed using equivalent amounts of cDNA (quantified using primers for HPRT as described in Materials and methods) in 5-fold serial dilutions. As anticipated, *scl* expression was not detected in $scl^{-/-}$ EBs. The expression levels of the genes studied were not significantly different in control and $scl^{+/-}$ lines, with the exception of the globin genes, which were reduced in one of the two $scl^{+/-}$ lines, reflecting clonal heterogeneity. Consistent with our morphological observations, expression of embryonic and adult β-globin genes (ε, βH1 and $\beta_{maj}$ globin) was absent in differentiated EB from all three $scl^{-/-}$ lines. However, we did see a consistent, albeit low, level of α-globin expression in $scl^{-/-}$ EB. The significance of this finding is unclear, since, using the same samples, we were unable to detect simultaneous expression of ζ-globin, the embryonic α-globin cluster gene (Figure 4).

We compared the expression profiles of a number of hematopoietic transcription factors as possible indicators of early hematopoietic commitment in the $scl^{-/-}$ EB. Transcripts from NF-E2, EKLF, GATA-1, PU.1 and c-*myb* genes were detected in $scl^{-/-}$ EB. The latter four genes were expressed at levels markedly lower than that observed in wild-type and $scl^{+/-}$ control lines. LMO2, the LIM domain protein which interacts with Scl (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994), was expressed equally in control and $scl^{-/-}$ EBs, suggesting that its expression pattern is not restricted to hematopoietic committed cells (Figure 4).

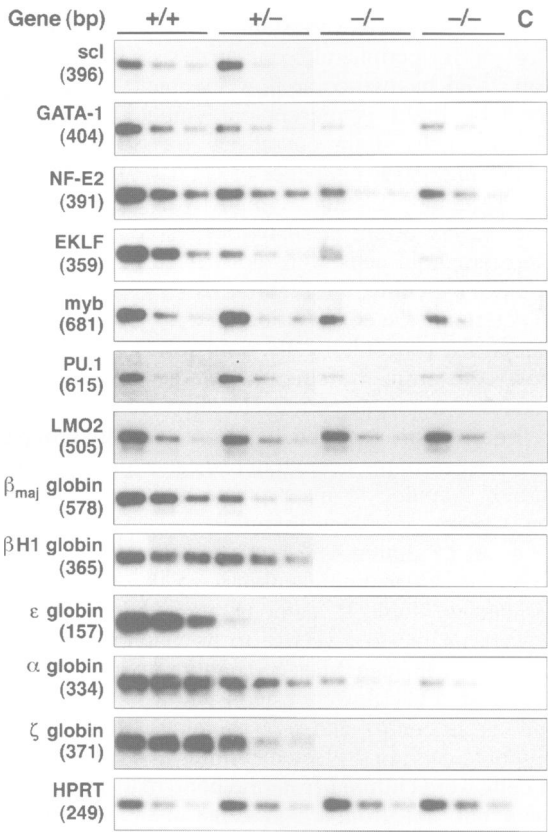


**Fig. 4.** Expression of hematopoietic genes in parental, control, $scl^{+/-}$ and $scl^{-/-}$ ES cell-derived embryoid bodies. PCR analysis of three 5-fold serially diluted cDNA samples from d8 embryoid bodies derived from parental (W9.5), $scl^{+/-}$ (+/–) and $scl^{-/-}$ (–/–) ES cell lines grown in methylcellulose cultures stimulated with SCF and Epo. Twenty five-fold less cDNA was loaded in the HPRT lanes and water was used as a negative control. For each gene studied, non-adjacent lanes from the same autoradiograph of the same blot are shown.

## Discussion

Gene targeting experiments in mice have revealed key roles for several transcription factors in hematopoietic commitment and differentiation and have led to the notion that a hierarchy of transcription factors controls gene

expression at key stages during this process. Because of the complete absence of blood formation in $scl^{-/-}$ embryos, *scl*, together with *LMO2*, is thought to act very early during hematopoietic development. The interpretation of the *scl* gene targeting experiments is, however, limited by death of the mutant mice at the earliest time point at which the product of the gene being studied is essential for a vital function. This prevents analysis of the role of the gene product at later time points during development. The present work demonstrates that *scl* is not only required for primitive hematopoiesis, but also for definitive hematopoiesis. To show this, we assessed the contribution of $scl^{-/-}$ ES cells to hematopoietic cells in the adult chimeric mouse. This strategy has been used successfully by others to circumvent embryonic lethality of a null phenotype and assess the role of a gene product in adult hematopoiesis (e.g. Tsai *et al.*, 1994; Williams *et al.*, 1994). Using a variety of tools to distinguish between ES cell-derived and host blastocyst-derived hematopoietic cells, we showed that mast cells, lymphoid cells, erythrocytes, platelets and hematopoietic progenitor cells of lineages were host derived in the $scl^{-/-}$ chimeras. Repeated manipulation and subcloning of ES cells is well recognized to limit their pluripotency. Therefore, we derived control ES cell lines which had undergone the same manipulations as the $scl^{-/-}$ lines and examined chimeras created with the control lines. This analysis confirmed the ability of the doubly manipulated $scl^{+/-}$ control lines to contribute to all tissues and all hematopoietic compartments. We showed, using GPI analysis, that the $scl^{-/-}$ cells made substantial contributions to all the non-hematopoietic tissues tested and, by breeding the chimeras, that $scl^{-/-}$ ES cell-derived gonads were able to participate in spermatogenesis.

Previous studies have shown *scl* expression in erythroid, megakaryocytic, mast and early myeloid cells and in committed progenitor ($CD34^+/CD38^+$) cells but not in primary lymphocytes, macrophage lines, most B cell lines, or T cell lines other than those established from T-ALL cells in which *scl* was dysregulated (Begley *et al.*, 1989; Green *et al.*, 1991b; Visvader *et al.*, 1991; Mouthon *et al.*, 1993). The failure of the $scl^{-/-}$ ES cells to contribute to any hematopoietic lineage in the adult mouse, including lymphoid cells, together with the absence of primitive hematopoietic progenitor cells in the $scl^{-/-}$ embryo, suggests it has a role in very early hematopoietic development and that the requirement for the *scl* gene product in this process is cell autonomous. Whether *scl* is a key regulatory molecule during the specification of ventral mesoderm to a hematopoietic cell fate or has a role in maintenance or expansion of the hematopoietic stem cell is not known.

*In vitro* differentiation experiments using ES cells bearing homozygous null mutations of particular genes enable dissection of the contribution of the gene product to growth and differentiation of primitive and definitive erythropoiesis and to the granulocyte–macrophage, mast cell and megakaryocytic lineages. When $scl^{-/-}$ ES cells were differentiated in a two-step culture system, hematopoietic colonies were not observed, whereas erythroid and granulocyte–macrophage colonies were readily obtained in experiments using control and parental ES cell lines. By day 8 of primary culture, hemoglobinized EB were clearly detectable in control cultures, and so this time point was chosen for the analysis of hematopoietic gene expression in EB from the two control lines, the parental line and three $scl^{-/-}$ lines. In the parental and control ES cell lines, the expression patterns of the genes studied were similar to those obtained in previous studies (Keller *et al.*, 1993). In the $scl^{-/-}$ EB, expression of βH1, $\beta_{maj}$ and ζ-globin and ε-globin was not detected. Intriguingly, a low level of expression of α-globin was seen (50-fold less than the parental line). As previously noted for $scl^{-/-}$ yolk sacs, *LMO2* gene expression was present at comparable levels in the $scl^{-/-}$ and control EB. Given the absence of detectable hematopoietic cells in these tissues, these results suggest that *LMO2* may be expressed in other tissues during early embryonic development.

Expression of EKLF and GATA-1, transcription factors, which are known to play a role in erythroid development, and of PU.1 and c-*myb*, which have been implicated in myeloid development, was markedly reduced, although not absent, in the $scl^{-/-}$ EB. To study this further, we compared expression of these genes in day 4, day 6 and day 8 EB from parental, two control $scl^{+/-}$ and three $scl^{-/-}$ lines. This demonstrated comparable low level expression of these genes in EB from all lines at day 4 and day 6 of culture. However, unlike control EB, expression did not increase over the following 48 h in $scl^{-/-}$ EB. The low level expression of hematopoietic regulator genes in the $scl^{-/-}$ EB could reflect the presence of an early hematopoietic progenitor cell in the $scl^{-/-}$ cultures which is unable to survive, or proliferate, in the absence of *scl*. Alternatively, these genes may be expressed in other, non-hematopoietic cell types within the EB. Expression of NF-E2, GATA-1, c-*myb* and PU.1 was not seen in the yolk sacs of E9.5 $scl^{-/-}$ embryos (Robb *et al.*, 1995; Shivdasani *et al.*, 1995; L.Robb, unpublished observations). This difference between expression in $scl^{-/-}$ yolk sacs and $scl^{-/-}$ EB embryos may be due to non-equivalence in terms of the stage of hematopoiesis represented by the two samples.

Recent observations have signaled a need for caution in the interpretation of phenotypes observed in gene targeting experiments due to the potential confounding influences of altered expression of nearby genes (Olsen *et al.*, 1996). Whilst it seems unlikely that the phenotype observed in the *scl* gene targeting studies is due to alteration of the expression of a neighboring gene, this remains formally possible. To at least partially address this, experiments designed to complement the defect and to introduce subtle alterations into the *scl* locus are required.

The *scl* gene targeting studies and the *in vivo* experiments using $scl^{-/-}$ ES cells presented here establish a crucial role for the *scl* gene product in primitive hematopoiesis and in definitive hematopoiesis at a stage prior to lineage commitment. The results of the *in vitro* differentiation of $scl^{-/-}$ ES cells concur with the *in vivo* observations. This system will provide a useful assay in which to conduct rescue experiments and to study the regulators and targets of the *scl* gene product.

## Materials and methods

### *Generation of $scl^{-/-}$ ES cell lines and chimeras*

$scl^{-/-}$ ES cell lines were generated by electroporation of the $scl^{+/-}$ ES cell line 93 with the linearized targeting vector 607 (Robb *et al.*, 1995).

Selection and identification of clones which had homologously integrated the targeting vector into the wild-type *scl* allele was as described previously, except that surviving clones were screened individually (Robb *et al.*, 1995). Chimeric mice were generated by injection of seven $scl^{-/-}$ or control cells into C57BL6/J blastocysts obtained from females 3.5 days post-coitus. Preliminary experiments showed that the number of $scl^{-/-}$ cells injected was critical, as injection of 10 or more cells caused most of the chimeric offspring to die between E9 and E10, with a phenotype essentially similar to that observed in $scl^{-/-}$ embryos.

### Hemoglobin electrophoresis

Mice were anesthetized and heparinized blood obtained from the retro-orbital plexus. Packed red blood cells were lysed and cellulose acetate electrophoresis of cystamine-modified hemoglobins was performed as described (Whitney, 1978).

### GPI assay

Separation and detection of GPI isoenzymes was carried out as described (Nagy and Rossant, 1993). Blood samples were fractionated (Pevny *et al.*, 1995) and tissue samples were diluted in water and disrupted with a Dounce homogenizer (Wheaton) and repeated cycles of freezing and thawing. Samples were diluted in Tris–glycine running buffer (25 mM Tris, 200 nM glycine, pH 8.5) so that comparable amounts of GPI activity were applied to each lane. Triplicate samples were applied to Titan III Zip Zone cellulose acetate plates (Helena Laboratories), electrophoresed in running buffer in a Zip Zone Chamber at 200 V for 35 min at room temperature and stained as described (Nagy and Rossant, 1993). Quantification of the amount of GPI-1A and GPI-1B isoforms present in individual tissues samples was determined by densitometry using with ImageQuant software (Molecular Dynamics).

### Flow cytometry

Cell suspensions were stained with monoclonal antibodies that had been prepared and conjugated to fluorochromes as described (Strasser *et al.*, 1991) and were analyzed on a fluorescence-activated cell sorter (FACScan, Becton-Dickinson).

### Bone marrow progenitor cell assays

A total of 100 000 bone marrow cells or 30 000 fetal liver cells were assayed in semi-solid agar cultures (for granulocyte–macrophage colonies) or methylcellulose cultures (for erythroid colonies) (Metcalf, 1984). After 7 days of culture, colonies were scored using a dissecting microscope. Colony formation was stimulated by purified recombinant bacterially synthesized growth factors at the following final concentrations: murine granulocyte–macrophage colony-stimulating factor (GM-CSF) (10 ng/ml), murine stem cell factor (SCF) (100 ng/ml), human granulocyte CSF (10 ng/ml), human Epo (2 U/ml) and 8% pokeweed mitogen (PWM)-stimulated spleen conditioned medium. In parallel experiments, 1.5 mg/ml G418 was added to the cultures. The G418 concentration used to select bone marrow cells carrying a single copy of the neomycin resistance gene under the control of the phosphoglyceraldehyde dehydrogenase promoter was determined in preliminary experiments by titrating the dose of G418 against wild-type C57BL/6J bone marrow and bone marrow from $scl^{+/-}$ heterozygous mice derived from line 93. Similar experiments were performed using wild-type and $scl^{+/-}$ fetal liver cells. For mast cell cultures, $5–10\times10^6$ bone marrow cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum and 10% PWM-stimulated spleen conditioned medium with and without the addition of 1.5 mg/ml G418. The medium was changed weekly and, after 4 weeks, cytospins of the cells were stained with May–Grunwald–Giemsa and examined by microscopy.

### In vitro differentiation of ES cells

Parental (W9.5), $scl^{+/-}$ and $scl^{-/-}$ cell lines were weaned off feeders and maintained in leukemia inhibitory factor (LIF). Primary EB were allowed to form in 1% methylcellulose cultures with 100 ng/ml SCF and 2 U/ml rhEpo without LIF. Hemoglobinization of primary embryoid bodies was scored by eye using an inverted microscope and was confirmed by staining with diaminofluorene (Kaiho and Mizuno, 1985) (minimum 100 embryoid bodies examined). At intervals from 6 to 21 days, EB were harvested, dispersed in collagenase and replated in 1% methylcellulose with purified recombinant factors as indicated in Table II. Seven days later, hemoglobinized erythroid colonies were enumerated using an inverted microscope (×40 and ×100) and a dissecting microscope for granulocyte–macrophage colonies. Colony morphology was confirmed by examination after spreading on glass slides and staining with May–Grunwald–Giemsa.

### Isolation of RNA, cDNA synthesis and RT–PCR

Total RNA was isolated from pooled day 8 EB using Trizol (BRL). Samples were DNase-treated (Boehringer Mannheim) and oligo(dT)-primed cDNA synthesized from 3 µg of total RNA in 60 µl. One µl of 5-fold serial dilutions of each sample were PCR amplified with primers for hypoxanthine phosphoribosyltransferase (HPRT) (Keller *et al.*, 1993), and a portion of the product was separated by agarose gel electrophoresis, transferred to GeneScreen Plus (Dupont) and hybridized with an internal oligonucleotide. The intensity of the resulting signal was measured using a PhosphoImager (Molecular Dynamics) and ImageQuant software. Subsequently, 30 cycles of PCR amplification were performed using a comparable amount of cDNA, serially diluted, in 20 µl reaction mixtures as described previously (Robb *et al.*, 1995) except that TaqStart Antibody (Clontech) was included in the reaction mixture. Primers for EKLF, ζ-globin and α-globin (Weiss *et al.*, 1994), LMO2 and PU.1 (Robb *et al.*, 1995) and HPRT, c-*myb*, βH1 and $\beta_{maj}$ globin (Keller *et al.*, 1993) were as published previously. Amplification of the other genes used the following forward (f) and reverse (r) primer pairs: SCL f (5′-TAT GAG ATG GAG ATT TCT GAT G-3′) and r (5′-GCT CCT CTG TGT AAC TGT CC-3′); GATA-1 f [5′-GGA ATT CGG GCC CCT TGT GAG GCC AGA-3′ (this includes a 5′ *Eco*RI site not relevant to the current experiments)] and r [5′-CGG GGT ACC TCA CGC TCC AGC CAG ATT CGA CCC-3′ (this includes a 5′ *Asp*718 site not relevant to the current experiments)]; ε-globin f (5′-GGA GAG TCC ATT AAG AAC CTA GAC AA-3′) and r (5′-CTG TGA ATT CAT TGC CGA AGT GAC-3′) and NF-E2 f (5′-GAG CCC TGG CCA TGA AGA TTC C-3′) and r (5′-CAC CAT CAG CAG CCT GTT GCA G-3′).

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