

## Selective rescue of early haematopoietic progenitors in *Scl*<sup>−/−</sup> mice by expressing *Sc* under the control of a stem cell enhancer

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### SUMMARY

The stem cell leukaemia gene (*Scl*) encodes a basic helix-loop-helix transcription factor with a pivotal role in both haematopoiesis and endothelial development. During mouse development, *Scl* is first expressed in extra-embryonic mesoderm, and is required for the generation of all haematopoietic lineages and normal yolk sac angiogenesis. Ectopic expression of *Scl* during zebrafish development specifies haemangioblast formation from early mesoderm. These results suggest that SCL is essential for establishing the transcriptional programme responsible for the formation of haematopoietic stem cells and have focused attention on the transcriptional regulation of *Scl* itself. Previous studies have identified a panel of *Scl* enhancers each of which directed expression to a subdomain of the normal *Scl* expression pattern. Among them, a 3' enhancer directed expression during development to vascular endothelium and haematopoietic progenitors but not to Ter119<sup>+</sup> erythroid cells. The

expression in haematopoietic stem cells, however, remained undetermined. We demonstrate that this 3' enhancer directs *lacZ* expression in transgenic mice to most foetal and adult long-term repopulating haematopoietic stem cells, and therefore functions as a stem cell enhancer. Consistent with these results, expression in *Scl*<sup>−/−</sup> embryos of exogenous *Scl* driven by the stem cell enhancer rescued the formation of early haematopoietic progenitors and also resulted in normal yolk sac angiogenesis. By contrast, erythropoiesis remained markedly deficient in rescued embryos. This observation is consistent with the inactivity of the stem cell enhancer in erythroid cells and reveals an essential role for SCL during erythroid differentiation in vivo.

Key words: *Scl* gene, Stem cell enhancer, Haematopoietic Stem cells, Mouse

### INTRODUCTION

All blood cell types are derived from haematopoietic stem cells (HSC), characterised by their capacity upon transplantation to give rise to long-term, multilineage haematopoietic engraftment. Adult murine bone marrow has been the most frequent source from which HSCs have been isolated and characterised. Several different approaches have been used, including gradient centrifugation (Visser et al., 1984), resistance to cytotoxic drugs such as 5-fluorouracil (Lerner and Harrison, 1990) and fluorescence-activated cell sorting (FACS), using specific dyes (Goodell et al., 1996; Kim et al., 1998) or antibodies to cell surface antigens (Muller-Sieburg et al., 1986; Spangrude et al., 1988). Although there are no monoclonal antibodies that exclusively recognise HSCs, several groups have shown that HSCs express a number of antigens, including c-Kit, CD34, Sca1 and Thy1, but lack a panel of mature haematopoietic lineage markers (Lin<sup>−</sup>) (Morel et al., 1996; Okada et al., 1991; Osawa et al., 1996; Uchida and Weissman, 1992). Remarkably, HSC-enriched fractions from bone marrow not only have the capacity to

reconstitute blood, but they can also give rise to neurones (Eglitis and Mezey, 1997), endothelium (Asahara et al., 1997), muscle cells (Ferrari et al., 1998; Gussoni et al., 1999) and hepatocytes (Lagasse et al., 2000) when located in an appropriate environment. Although not yet formally demonstrated in some cases, it has been suggested that HSCs may be capable of regenerating these different tissues, thus considerably broadening the potential clinical utility of haematopoietic stem cells.

During mouse embryogenesis, the first morphological evidence of haematopoietic activity occurs at day 7.5 of gestation (E7.5) in the yolk sac blood islands derived from the extra-embryonic mesoderm. By E8, the yolk sac contains haemoglobin producing erythroid cells together with multipotent erythroid/myeloid progenitors (Cumano et al., 1996; Palis et al., 1999). Around the same developmental time and preceding the onset of blood circulation, the intra-embryonic para-aortic splanchnopleura region contains multipotent lymphoid/erythroid/myeloid progenitors (Cumano et al., 1996). These results suggest that haematopoietic cells in the mouse arise independently in the yolk sac and para-aortic

splanchnopleura, a model analogous to the situation in birds (Dieterlen-Lievre, 1975).

It has been assumed that HSCs are also present at early stages of development, but no functional demonstration is available before E9. Thus, HSC activity is first detected in yolk sac and para-aortic splanchnopleura around E9, within the c-Kit<sup>+</sup>CD34<sup>+</sup> population (Weissman et al., 1978; Yoder et al., 1997). These cells are capable of long-term reconstitution if transplanted into foetal or new born recipients but do not engraft adult recipients, suggesting that they lack a homing activity for adult bone marrow. c-Kit<sup>+</sup>CD34<sup>+</sup> HSCs capable of long-term reconstitution in adult recipient mice are detected from day 10 of gestation in the aorta, gonads and mesonephros (AGM) region, a derivative of the para-aortic splanchnopleura region (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Muller et al., 1994; Sanchez et al., 1996) and are found subsequently in the foetal liver and adult bone marrow (Jordan et al., 1990; Morrison et al., 1995; Spangrude et al., 1988). The lineage relationship between multipotent progenitors/HSCs arising in the murine yolk sac and AGM remains unclear in the mouse. However lineage tracing studies in *Xenopus* have recently shown that the yolk sac (ventral blood islands) and intraembryonic (dorsolateral plate) blood components derive from distinct blastomeres in 32-cell embryos, strongly suggesting that the first adult HSCs arise independently of yolk sac haematopoiesis (Ciau-Uitz et al., 2000).

Although the mechanisms that control HSC differentiation and self-renewal are largely unknown, loss-of-function and gain-of-function studies have demonstrated a pivotal role for transcription factors. Yolk sac, foetal liver or adult haematopoiesis can be differentially affected by the loss of a particular transcription factor indicating intrinsic differences in the transcriptional regulation of haematopoiesis in different sites (Mucenski et al., 1991; Tsai et al., 1994; Wang et al., 1998; Wang et al., 1996). Among the regulatory molecules crucial for the proper development of embryonic and adult haematopoiesis is the bHLH transcription factor encoded by the stem cell leukaemia gene, *Scl* (*Tal1* – Mouse Genome Informatics) (Begley and Green, 1999). *Scl* is expressed in blood cells, endothelium and the central nervous system (Drake et al., 1997; Drake and Fleming, 2000; Green et al., 1991; Green et al., 1992; Hwang et al., 1993; Kallianpur et al., 1994; Silver and Palis, 1997; Sinclair et al., 1999; Visvader et al., 1991). Within the haematopoietic cells, SCL is found in multipotent progenitors, as well as in the erythroid, megakaryocytic and mast cell lineages (Cross et al., 1994; Green et al., 1992; Visvader et al., 1991), and is expressed at all sites of haematopoiesis in the mouse embryo, including the yolk sac, paraortic splanchnopleura, AGM region and foetal liver (Elefanty et al., 1999; Silver and Palis, 1997).

SCL is essential for normal yolk sac and adult haematopoiesis (Porcher et al., 1996; Robb et al., 1996; Robb et al., 1995; Shivdasani et al., 1995). It also plays an important role in endothelial development. *Scl*<sup>-/-</sup> embryos exhibit defective yolk sac angiogenesis, which appears to reflect an intrinsic defect in *Scl*<sup>-/-</sup> endothelial cells (Visvader et al., 1998). In addition, two lines of evidence suggest a crucial role for SCL in the development of haemangioblasts, common progenitors of both blood and endothelium. First, ectopic expression of *Scl* during zebrafish development specifies haemangioblast formation from lateral mesoderm at the

expense of other mesodermal derivatives (Gering et al., 1998), and can also partially rescue the blood and endothelial defects of *cloche* mutants (Liao et al., 1998). Second, *Scl*<sup>-/-</sup> ES cells fail to differentiate in vitro to form blast cell colonies that are thought to arise from haemangioblasts (Faloon et al., 2000; Robertson et al., 2000).

There is considerable interest in identifying a regulatory element that targets expression to HSCs. Characterisation of such an enhancer would generate important insights into the transcriptional programmes responsible for haematopoiesis, and would also provide a powerful tool for experimental manipulation of these processes in vivo. Regulatory elements from the *Ly6E.1* gene (*Ly6a* – Mouse Genome Informatics) (Miles et al., 1997) and the *H2K* gene (Domen et al., 2000) have been used to target adult HSCs. However, the expression of *Ly6E.1* in HSC is mouse strain dependent (Codias et al., 1989; Spangrude et al., 1988), and the *H2K* gene is widely expressed in most tissues. Moreover an expression cassette capable of targeting adult, foetal and embryonic HSCs has not been described.

We have therefore performed a systematic survey of the promoters and chromatin structure of the murine *Scl* gene and identified several regulatory elements active in transient and stable transfection assays (Bockamp et al., 1998; Bockamp et al., 1997; Bockamp et al., 1995; Gottgens et al., 1997). Analysis of reporter constructs in transgenic mice subsequently revealed a panel of spatially distinct enhancers, each of which directs expression to a subdomain of the normal *Scl* expression pattern (Gottgens et al., 2000; Sanchez et al., 1999; Sinclair et al., 1999). A 3' enhancer reporter construct was active in haematopoietic progenitors and vascular endothelium during development (Sanchez et al., 1999) but did not direct expression to most mature haematopoietic cells, including erythroid cells. We demonstrate that an *Scl* 3' enhancer construct directs expression to long term repopulating HSCs in foetal liver and adult bone marrow. Furthermore, in *Scl*<sup>-/-</sup> embryos expression of exogenous *Scl* under control of the 3' enhancer selectively rescued the formation of early haematopoietic progenitors and yolk sac angiogenesis, but failed to rescue significant levels of erythropoiesis, thus revealing a requirement for SCL during erythroid differentiation.

## MATERIALS AND METHODS

### Mice

Transgenic mice carrying the +6E5/*lacZ*/3' En constructs have been described previously (Sanchez et al., 1999). Lines 2262 and 2257 were used in the long-term reconstitution experiments. Embryos were obtained from heterozygous transgenic males crossed with (CBA×C57Bl/6) F<sub>1</sub> females.

Transgenic mice carrying the +6E5/*Scl-lacZ*/3' En construct (Tg<sup>+</sup> mice) were obtained as described by pronuclear injection of fertilised eggs derived from (CBA×C57Bl/6)F<sub>1</sub> × (CBA×C57Bl/6)F<sub>1</sub> crosses. Five independent lines were generated and bred with *Scl*<sup>+/-</sup> heterozygous mice kindly provided by L. Robb and C. G. Begley (Robb et al., 1995) to obtain transgene positive *Scl*<sup>+/-</sup> mice (Tg<sup>+/-</sup>/*Scl*<sup>+/-</sup>). These were intercrossed for maintenance of the transgenic lines. Embryos were derived from crosses between Tg<sup>+/-</sup>/*Scl*<sup>+/-</sup> or Tg<sup>+/-</sup>/*Scl*<sup>+/-</sup> and Tg<sup>-/-</sup>/*Scl*<sup>+/-</sup> mice.

### Transgenic constructs

A unique *NcoI* restriction site was engineered upstream of the murine

SCL open reading frame. The picornaviral internal ribosome-entry site (IRES) from the encephalomyocarditis virus (EMCV) was inserted downstream to the SCL stop codon (Mountford et al., 1994). An *NcoI* fragment containing the *Scf*-IRES-coding sequence was subcloned upstream of the *lacZ* gene in the +6E5/*lacZ* plasmid (Sanchez et al., 1999). A 5.5 kb fragment containing the 3' enhancer was then inserted downstream of the *lacZ* gene. The final 13 kb +6E5/*Scf-lacZ*/3' En insert was released by *XhoI*/*SalI* digestion and introduced into fertilised eggs by pronuclear injection. To identify transgenic founders *lacZ* PCR analysis was performed as previously described (Sanchez et al., 1999).

### Reconstitution analysis

Adult bone marrow and E11-E12 foetal liver cells were stained for FDG and FACS sorted as previously published (Sanchez et al., 1999). Sorted cells were counted, and appropriate cell number suspended in a final volume of 0.5 ml phosphate-buffered saline (PBS) for intravenous injection into the tail vein of 3- to 5-month-old irradiated females (CBA×C57Bl/6)F<sub>1</sub> mice. Transgenic donor cells were co-injected with non transgenic 2×10<sup>5</sup> splenic cells for radioprotection. On the day of the transfer, the recipients were exposed to a split dose of total 950 RADS from an X-ray source. Mice were maintained on antibiotic water containing 0.16% neomycin sulphate (Sigma) a week before and 4 weeks after irradiation. Transplanted animals were bled from the tail vein at 1-2 months and 5-10 months post-transplantation to monitor reconstitution. Donor cell contribution was assessed semiquantitatively by PCR analysis of recipient peripheral blood DNA using *lacZ* and myogenin primers as previously described (Sanchez et al., 1996). Negative results were confirmed by Southern hybridisation using a *lacZ* probe.

### Multilineage analysis of donor cell contribution

To test the contribution of donor HSCs to different haematopoietic lineages long-term transplanted mice were sacrificed at 5-12 months post-transplant. Genomic DNA was isolated from tissues or FACS-sorted cells and analysed by PCR (Sanchez et al., 1996).

### Cytospin preparation and X-gal staining from the AGM region

Embryos obtained from crosses from transgenic males (line 2262) and (CBA×C57Bl/6)F<sub>1</sub> females were removed after 11-12 days of gestation and the livers and AGM regions from individual embryos dissected out. To identify transgenic embryos, the livers were mechanically homogenised in PBS, cell suspensions stained for FDG and analysed in the flow cytometer. Cells from individual AGM regions were prepared by incubation at 37°C for 1 hour in 0.04% in collagenase (Sigma) in L-15 medium (Gibco) supplemented with 5% foetal calf serum and followed by mechanical dispersion (Sanchez et al., 1996). Cell suspensions were incubated with Fc-blocking monoclonal antibody (CD16/CD32) followed by anti CD34-biotin (RAM34) and c-Kit-PE (2B8) antibodies and then streptavidin-FITC. AGM cells from transgenic embryos were sorted, and cytopsin preparations fixed for 5 minutes in 1% paraformaldehyde, 0.4% glutaraldehyde in PBS. Preparations were washed in PBS and stained overnight for X-gal. After Neutral Red counterstaining and ethanol dehydration, preparations were mounted in DPX (Sigma). All antibodies were purchased from PharMingen.

### β-galactosidase detection in whole embryos and sections

X-gal staining was performed to detect β-galactosidase activity as previously described (Sanchez et al., 1999) with the following modifications. Founder embryos were stained for 24 hours at 37°C and embryos from established lines 48 hours at room temperature, washed in PBS and stored in fixative (1% paraformaldehyde, 0.4% glutaraldehyde in PBS). For wax sections, embryos were dehydrated in serial dilutions of ethanol, wax embedded and sectioned. For cryostat sectioning, X-gal-stained embryos were washed in PBS,

incubated overnight in 25% sucrose solution, immersed in OCT embedding matrix (CellPath) and frozen on dry ice. Cryostat sections (8 μm) were counterstained with Neutral Red, dehydrated, immersed in HistoClear and mounted in EUKITT compound (Agar Scientific, UK). The embryo head was removed to obtain DNA for *lacZ* and *Scf* genotyping and the rest of the embryo was used for staining (Miles et al., 1997; Robb et al., 1995).

### Fluorescence-activated cell-sorting (FACS) analysis of yolk sac cells

Individual yolk sacs were removed from E9 embryos derived from crosses between Tg<sup>+/-</sup>*Scf*<sup>+/-</sup> or Tg<sup>+/+</sup>*Scf*<sup>+/-</sup> and Tg<sup>-/-</sup>*Scf*<sup>+/-</sup> mice (Tg line 2708). Cells from individual yolk sacs were prepared by incubation at 37°C for 30 minutes in 0.1% in collagenase (Sigma) in L-15 medium (Gibco) supplemented with 20% foetal calf serum and followed by mechanical dispersion (Yoder et al., 1997). The rest of the body was used for *lacZ* and *Scf* PCR genotyping. Individual cells were counted under the microscope. No cells with typical round blood morphology were detected in the Tg<sup>-/-</sup>*Scf*<sup>+/-</sup> yolk sacs, instead the cells were large and vacuolated. Cells were incubated with anti-Fcγ receptor antibody (2.4G2) to reduce the nonspecific binding followed by incubation with the indicated antibodies and Streptavidin-PE (PharMingen, San Diego) as the secondary reagent in PBS 5% foetal calf serum. Antibody incubations were performed at 4°C for 30 minutes. The monoclonal antibodies were from PharMingen (San Diego, CA) and included PE-conjugated anti-Ter119, FITC-conjugated anti-c-Kit (2B8 and 3C1) and biotin-conjugated anti-CD34 (RAM34). Rat PE-conjugated IgG2a and FITC-conjugated IgG2b isotype controls were used.

### In vitro colony assays for yolk sac progenitors

These were performed essentially as described (Sanchez et al., 1999). E9 embryos were derived from crosses of male Tg<sup>+/-</sup>*Scf*<sup>+/-</sup> or Tg<sup>+/+</sup>*Scf*<sup>+/-</sup> with female Tg<sup>-/-</sup>*Scf*<sup>+/-</sup> mice (Tg line 2708). To avoid maternal blood contamination, anaemic embryos were separated and transferred to multiple washes in PBS 5% FCS before removal of the yolk sac. Individual yolk sacs were mechanically disaggregated by sequential passage through a 30 gauge needle in D-MEM medium (GIBCO) and the rest of the body used for *lacZ* and *Scf* PCR genotyping. Cells from each yolk sac were plated in cytokine (SCF, IL3, IL6, erythropoietin)-supplemented Methocult GF-M3434 (StemCell Technologies, Vancouver, Canada) for erythroid and myeloid colony formation. Cell suspensions from each yolk sac were split into two plates (Nunc, 4 cm plates) and the total number of colonies scored after 7 days. Erythroid colonies were assessed by staining for haemoglobin with 2,7-diaminofluorene (Sigma) (Kaiho and Mizuno, 1985). Individual colonies from two experiments were picked, genomic DNA obtained by protein kinase treatment and PCR performed for *lacZ*/myogenin and *Scf* as indicated. For cytopsin preparations colonies were harvested from plates, spun onto slides and stained for May-Grunwald-Giemsa. The number of colonies analysed were as follows: a total of 28 colonies derived from three Tg<sup>+/-</sup>*Scf*<sup>+/-</sup> embryos, 20 colonies from two Tg<sup>-/-</sup>*Scf*<sup>+/-</sup> embryos and 16 colonies from Tg<sup>+/-</sup>*Scf*<sup>+/-</sup> embryos.

### Immunohistochemistry

Whole-mount staining of embryos and yolk sacs with anti-PCAM1 monoclonal antibody (MEC13.3, PharMingen, San Diego, CA) was performed as described previously (Schlaeger et al., 1995).

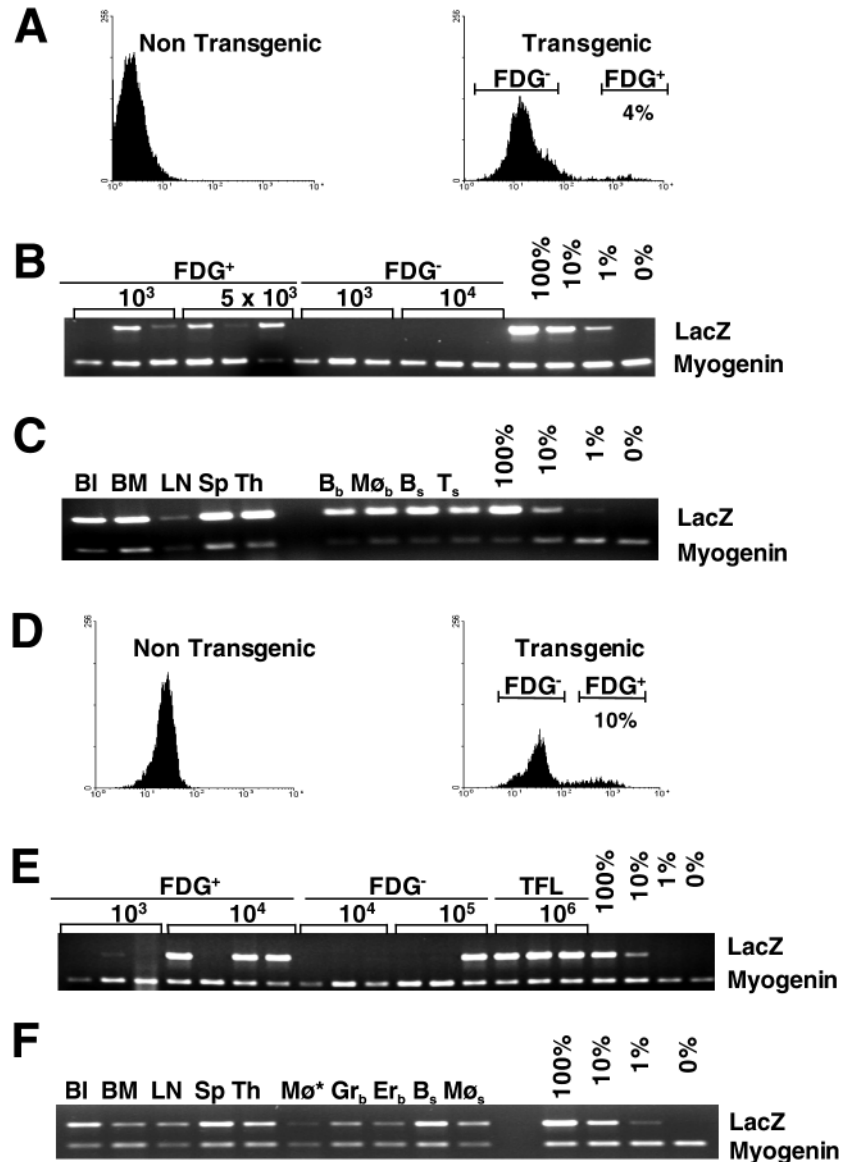
## RESULTS

### The *Scf* 3' enhancer construct targets *lacZ* expression to long-term reconstituting haematopoietic stem cells in adult bone marrow

We have previously identified an *Scf* 3' enhancer construct



**Fig. 1.** The *Scl* 3' enhancer construct targets *lacZ* expression in transgenic mice to long-term reconstituting haematopoietic stem cells from adult bone marrow and foetal liver. (A) FACS analysis of cell suspensions from non transgenic and transgenic adult bone marrow for the expression of *lacZ* by FDG staining on gated live cells (PI-negative cells). Histograms for FDG expression in one representative experiment are shown. Cells included in the sorted windows were designated as FDG<sup>-</sup> and FDG<sup>+</sup>. (B) Ethidium bromide-stained gels of peripheral blood PCR analysis for the presence of the transgene (*lacZ*) together with an internal control (myogenin). PCR products are shown from a representative experiment performed 5 months post-transplantation with bone marrow cells. Lanes correspond to individual animals transplanted with the indicated number of FDG<sup>+</sup> or FDG<sup>-</sup> cells. Peripheral blood from transgenic and non-transgenic mice was mixed to provide 100%, 10%, 1% and 0% *lacZ* controls, allowing semi-quantitative evaluation of reconstitution levels. (C) Multilineage analysis in one recipient mouse 12 months post-transplant with FDG<sup>+</sup> bone marrow cells. (D) FACS analysis of cell suspensions from non transgenic and transgenic foetal liver for the expression of *lacZ* by FDG staining on gated live cells (PI-negative cells). Histograms for FDG expression in one representative experiment from E12 liver cells are shown. (E) *lacZ*-PCR analysis of peripheral blood 4 months post-transplantation with foetal liver cells. (F) Multilineage analysis of a recipient mouse 6 months post transplant with foetal liver FDG<sup>+</sup> cells. B<sub>b</sub>, B lymphocytes B220<sup>+</sup> from bone marrow; B<sub>s</sub>, B lymphocytes B220<sup>+</sup> from spleen; B<sub>i</sub>, blood; BM, bone marrow; E<sub>b</sub>, erythrocytes Ter119<sup>+</sup> from bone marrow; G<sub>b</sub>, granulocytes Gr-1<sup>+</sup> from bone marrow; LN, lymph nodes; M<sub>o</sub><sup>\*</sup>, peritoneal cells; M<sub>o</sub><sub>b</sub>, macrophages CD11b<sup>+</sup> from bone marrow; M<sub>o</sub><sub>s</sub>, macrophages CD11b<sup>+</sup> from spleen; Sp, spleen; Th, thymus; T<sub>s</sub>, T lymphocytes CD4<sup>+</sup>CD8<sup>+</sup> from spleen; TFL, total foetal liver. The 2262 transgenic line was used in the experiments shown.



(+6E5/*lacZ*/3'En) that directs *lacZ* expression to blood and endothelium throughout mouse development (Sanchez et al., 1999). The *lacZ*-positive cells expressed c-Kit and CD34, both associated with haematopoietic progenitors, and were also highly enriched for myeloid and erythroid colony forming cells. However the colony assays do not identify haematopoietic stem cells (Trevisan and Iscove, 1995). It was therefore of considerable interest to determine whether the 3' enhancer construct targeted expression to haematopoietic stem cells.

In order to address this issue, we performed long term reconstitution experiments using irradiated adult mice as recipients. Bone marrow cell suspensions were obtained from individual adult +6E5/*lacZ*/3'En transgenic mice and were stained with the fluorescent  $\beta$ -galactosidase substrate, FDG. As shown in Fig. 1A, two sorting regions were set according to the relative levels of *lacZ* expression. Varying numbers of sorted FDG<sup>+</sup> and FDG<sup>-</sup> cells were transferred intravenously into irradiated female recipient mice with  $2 \times 10^5$  non-transgenic female splenocytes to provide short-term

radioprotection (Sanchez et al., 1996). Donor cell engraftment was examined initially at 1.5-2 months and subsequently at more than 5 months post-transplantation. Recipient peripheral blood DNA was analysed by PCR for the donor *lacZ* transgene. Short-term engraftment was observed in animals transferred with either FDG<sup>+</sup> or FDG<sup>-</sup> cell fractions; however, long-term engraftment was detected only in recipients transplanted with FDG<sup>+</sup> cells (Table 1). As shown for one representative experiment in Fig. 1B, as few as  $10^3$  FDG<sup>+</sup> donor bone marrow cells produced engraftment in two out of three recipients 5 months post-transplant, while no engraftment was detected at this time point in animals transplanted with FDG<sup>-</sup> cells. The cumulative results from several experiments demonstrated that *lacZ* transgene was readily detected by PCR more than 5 months post-transplant in almost half of recipient mice (11 out of 26) transferred with  $10^3$  to  $10^4$  FDG<sup>+</sup> cells. By contrast, the donor *lacZ* transgene was not detected in any of 28 recipients transplanted with up to  $10^6$  FDG<sup>-</sup> cells (Table 1). These data suggest that the vast majority of HSCs were in the FDG<sup>+</sup> fraction. To further assess the self-renewal capacity of the

**Table 1. Long-term haematopoietic reconstitution by *lacZ*<sup>+</sup> cells from +6E5/*lacZ*/3'En transgenic mice**

Donor tissue	Cell type	Cells per recipient	1-2 months post-transplant (reconstituted recipient/total recipients)	4-11 months post-transplant (reconstituted recipient/total recipients)
Adult bone marrow	FDG <sup>+</sup>	10 <sup>3</sup>	10/15	4/15
		0.5-1×10 <sup>4</sup>	8/11	7/11
	FDG <sup>-</sup>	10 <sup>3</sup>	0/3	0/3
		10 <sup>4</sup>	1/14	0/14
		10 <sup>5</sup>	1/8	0/8
		10 <sup>6</sup>	1/3	0/3
	Total	10 <sup>5</sup>	3/3	2/3
		10 <sup>6</sup>	7/7	7/7
E11-12 fetal liver	FDG <sup>+</sup>	10 <sup>3</sup>	3/18	2/18
		0.5-1×10 <sup>4</sup>	11/20	11/20
	FDG <sup>-</sup>	10 <sup>3</sup>	0/4	0/4
		10 <sup>4</sup>	0/17	0/8
		10 <sup>5</sup>	4/16	4/16
		10 <sup>6</sup>	3/3	3/3
	Total	10 <sup>5</sup>	3/3	3/3
		10 <sup>6</sup>	3/3	3/3

The FDG<sup>+</sup> and FDG<sup>-</sup> cell populations from transgenic adult bone marrow and foetal liver were sorted as indicated in Fig. 1. Different numbers of sorted cells were transferred into irradiated recipients. To determine donor engraftment, peripheral blood DNA from recipient mice was analysed by PCR for the donor marker *lacZ* at 1-2 months and 4-11 months post-transplant. Data are from five independent experiments in which bone marrow was transferred from transgenic line 2262 and five independent experiments in which foetal liver was transferred from transgenic lines 2257 (three experiments) and 2262 (two experiments).

FDG<sup>+</sup> donor cells, secondary reconstitution experiments were carried out. Total bone marrow cells from a recipient mouse 5 months post-transplant were transferred into four irradiated mice (10<sup>6</sup> cells per mouse). Seven months after the second transplant, all secondary recipients exhibited high levels of reconstitution by cells carrying the *lacZ* transgene, thus demonstrating that cells in the original FDG<sup>+</sup> population were capable of self-renewal (data not shown).

To determine whether transplantation of FDG<sup>+</sup> bone marrow cells resulted in complete, multilineage haematopoietic engraftment, we examined seven primary recipients more than 5 months post-transplantation. Haematopoietic tissues and

purified cells from individual lineages were analysed by semi-quantitative PCR for the presence of the *lacZ* transgene (Fig. 1C). As shown in Table 2, in four out of seven animals that showed high levels of donor-derived peripheral blood, all the haematopoietic tissues contained 10%-100% donor-derived cells. High levels of donor signal were also observed within individual lineages. In three recipients (Table 2, recipient numbers 5, 6 and 7), the peripheral blood *lacZ* signal was consistently lower after 5-10 months than at the 1-2 month time point, and these three mice showed some variation in the level of repopulation of individual tissues and cell lineages. Taken together, these data demonstrate that the +6E5/*lacZ*/3'En

**Table 2. Multilineage engraftment of mice transplanted with *lacZ*<sup>+</sup> cells from +6E5/*lacZ*/3'En transgenic mice**

Donor cells	Recipient identification number	Donor signal after 1-2 months	Donor signal after 5-12 months										
		Bl	Bl	BM	LN	Sp	Th	B	T	Mφ	Ma	Gr	Ery
Adult bone marrow	1	+++	+++	+++	+++	+++	+++	ND	ND	+++*	ND**	ND	ND
	2	+++	+++	++	+++	+++	++	+++	+++	+++	ND	ND	ND
	3	+++	+++	+++	++	+++	+++	+++	+++	+++	ND	ND	ND
	4	+++	+++	++	++	+++	+++	++	++	+	ND	ND	ND
	5	+++	++	++	-	+++	+++	+++	+++	++	ND	ND	ND
	6	+++	++	-	ND	++	+	+	++	+	ND	ND	ND
	7	+++	++	-	-	++	-	+	+	-	++	ND	ND
Fetal liver	1	+++	+++	+++	+++	+++	+++	+++	ND	+++	ND	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	ND	+++	ND	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	ND	+++	ND	++	+
	4	+++	+++	++	+	++	+++	++	ND	++	ND	+++	ND
	5	+++	+++	++	+++	+++	-	+++	ND	+	ND	+++	-
	6	+++	+++	+	+	+++	++	+++	+++	-	++	ND	ND
	7	++	++	+	+++	+++	++	+++	ND	-	ND	++	-
	8	++	+	+	++	+++	+	+	+	+	++	ND	ND

Genomic DNA samples from each individual recipient tissues together with 100%, 10%, 1% and 0% dilutions of the *lacZ* genomic DNA control were simultaneously subjected to *lacZ* and myogenin PCR amplification as shown in Fig. 1. Scoring of ethidium bromide stained gels: -, no signal; +, signal lower than the 10% but higher than 1% control; ++, signal lower than 100% but higher than 10% control; +++, signal very strong, similar to 100%). In several experiments the semi-quantitative analysis of the donor signal was confirmed by blotting the PCR gels and hybridisation with myogenin and *lacZ* probes. Bl, Blood; BM, bone marrow; B, splenic B lymphocytes B220<sup>+</sup>; Ery, bone marrow erythrocytes Ter119<sup>+</sup>; Gr, bone marrow granulocytes Gr1<sup>+</sup>; LN, lymph nodes; Mφ, bone marrow and splenic macrophages CD11b<sup>+</sup>; Ma, cultured splenic mast cells; Sp, spleen; T, splenic T lymphocytes CD4<sup>+</sup>CD8<sup>+</sup>; Th, thymus.

\*In recipient number 1, peritoneal cells were analyzed. \*\*ND, not done.

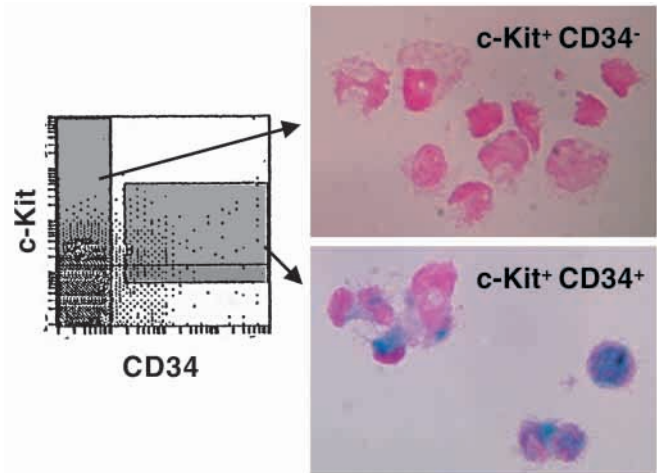
construct targets the vast majority of bone marrow HSCs capable of long-term multilineage reconstitution.

### The *Scl* 3' enhancer construct targets *lacZ* expression to foetal haematopoietic stem cells

Adult and foetal HSCs do not have identical characteristics and several functional and phenotypic differences have been previously described. Thus, most murine foetal liver HSCs are known to express c-Kit, CD34, Mac1 and AA4.1 (Jordan et al., 1995; Morrison et al., 1995; Sanchez et al., 1996), whereas adult bone marrow HSCs do not normally express Mac-1 or AA4.1 antigens (Rebel et al., 1996; Trevisan and Iscove, 1995). Functionally foetal liver HSCs can respond to the foetal thymic environment by producing V $\gamma$ 3 and V $\gamma$ 4 T cells, whereas adult HSCs have only a very limited capacity to differentiate in this way (Ikuta et al., 1990). Furthermore, the Ly-6E.1/*lacZ* transgene is expressed in adult bone marrow HSCs but not in foetal liver HSCs (Miles et al., 1997).

We therefore analysed whether the *Scl* 3' enhancer construct was capable of directing *lacZ* expression to HSCs present during foetal development, as well as to adult bone marrow HSCs. The embryonic AGM region is the first location during development where HSCs are detected and these cells have been shown to be c-Kit<sup>+</sup>CD34<sup>+</sup> (Sanchez et al., 1996; Yoder et al., 1997). Previously we have shown that the 3' enhancer construct directed prominent *lacZ* expression to the endothelium of the dorsal aorta and to haematopoietic cells in the lumen of the vessel (Sanchez et al., 1999). In the AGM from E11 embryos, flow cytometric analysis of *lacZ* expression by FDG staining is not feasible. Therefore, to address whether the *Scl* 3' enhancer was expressed in c-Kit<sup>+</sup>CD34<sup>+</sup> population, cell suspensions were obtained after collagenase treatment, costained with anti-c-Kit and anti-CD34 antibodies, and purified by FACS sorting. Cytospin preparations of c-Kit<sup>+</sup>CD34<sup>-</sup> and c-Kit<sup>+</sup>CD34<sup>+</sup> populations were prepared and stained for *lacZ* expression. The majority (70%) of c-Kit<sup>+</sup>CD34<sup>+</sup> cells expressed *lacZ* compared with less than 1% of the c-Kit<sup>+</sup>CD34<sup>-</sup> cells (Fig. 2). These results suggest that HSCs from the AGM region are targeted by the *Scl* 3' enhancer construct.

HSCs with the ability to reconstitute adult irradiated mice appear in the foetal liver around day 11 of gestation (Muller et al., 1994; Sanchez et al., 1996). To investigate whether the foetal liver FDG<sup>+</sup> fraction contained HSCs, we carried out long-term reconstitution assays. Studies were performed using transgenic lines 2262 and 2257, previously shown to contain 17% and 5%, respectively, FDG<sup>+</sup> cells in E11 foetal liver (Sanchez et al., 1999). At least two factors are likely to contribute to variability between lines in the proportion of FDG<sup>+</sup> foetal liver cells. First, both stable and variegating position effects are recognised to influence the level of transgene expression (Martin and Whitelaw, 1996) and in this context it is relevant to note that the *Scl* 3' enhancer does not have LCR activity (Sanchez et al., 1999). Second, there is likely to be some degree of biological variation between individual mice in the proportion of foetal liver cells that are early progenitors and in which the 3' enhancer is active. Cells were sorted according to windows shown in Fig. 1D. Donor engraftment was observed 4-5 months post-transplantation when as few as 10<sup>3</sup> FDG<sup>+</sup> cells were transferred and high levels of donor cells were detected in more than 50% of recipients (11/20) which received 0.5-1.0×10<sup>4</sup> FDG<sup>+</sup> cells (Fig. 1E, Table



**Fig. 2.** The *Scl* 3' enhancer construct targets *lacZ* expression to c-Kit<sup>+</sup>CD34<sup>+</sup> cells from the AGM region. Cell suspensions from the AGM region of +6E5/*lacZ*/3'En transgenic E11 embryos were stained with biotinyl-conjugated anti-CD34 and PE-conjugated anti-c-Kit antibodies followed by streptavidin-FITC. Cells were sorted according to indicated windows. After sorting, cytospin preparations were stained for  $\beta$ -galactosidase. In the representative experiment shown, approximately 70% of the c-Kit<sup>+</sup>CD34<sup>+</sup> cells were *lacZ*<sup>+</sup>, compared with fewer than 1% of the c-Kit<sup>+</sup>CD34<sup>-</sup> population. Dead cells were excluded by PI staining.

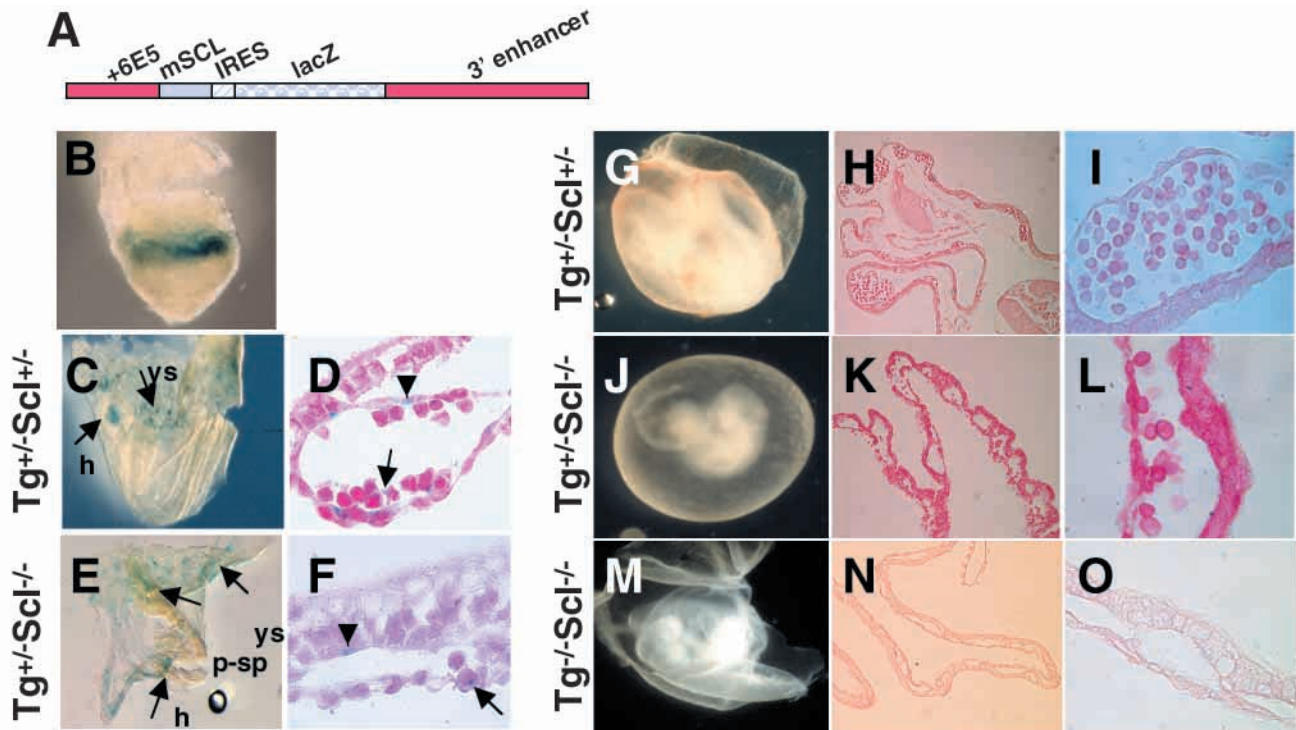
1). By contrast, long-term reconstitution by FDG<sup>-</sup> cells was observed only when 10<sup>5</sup> cells were used. The cumulative results of five experiments are shown in Table 1 and demonstrate that the majority of long-term repopulating cells were present in the FDG<sup>+</sup> fraction. In these experiments, long-term reconstitution by FDG<sup>-</sup> cells may well reflect contamination by FDG<sup>+</sup> cells. FACS analysis of sorted populations showed that the purity of the foetal liver cell fractions was between 80-85% compared with 95% for bone marrow. This relatively low purity is due to the intrinsic properties of foetal liver cells stained with FDG, which result in impaired discrimination between FDG<sup>+</sup> and FDG<sup>-</sup> populations. Attempts to re-sort the populations failed, as FDG staining is sensitive to duration of incubation and exposure to light.

Multilineage reconstitution was also addressed in recipients engrafted with FDG<sup>+</sup> foetal liver (Fig. 1F). In most recipients the level of donor cells in the peripheral blood was similar at 1-2 months and after 5 months post-transplant (Table 2). There was donor engraftment in all haematopoietic tissues analysed, except for one thymus. Within individual mice the proportion of FDG<sup>+</sup> cells varied between individual cell lineages, with an absence of detectable donor-derived erythroid cells and macrophages in two recipients. These data demonstrate that most of the HSCs in mid-gestation foetal liver are within the FDG<sup>+</sup> fraction and that these cells can yield multilineage repopulation.

### Rescue of early haematopoiesis in *Scl*<sup>-/-</sup> embryos by expression of SCL protein under the control of the *Scl* 3' enhancer

The *Scl* 3' enhancer provides a powerful tool for exploring HSC biology in vivo by permitting expression of exogenous





**Fig. 3.** Expression of exogenous *Scl* under control of the *Scl* 3' enhancer rescues haematopoiesis in *Scl*<sup>-/-</sup> embryos. (A) The +6E5/*Scl*-*lacZ*/3'En transgene. (B) X-gal staining of E7 embryo carrying the +6E5/*Scl*-*lacZ*/3'En transgene showing *lacZ* expression in the extra-embryonic mesoderm. (C-F) E8 embryos of the indicated genotype analysed by whole-mount (C, E) or cryostat sections (D, F) for *lacZ* staining. Note the presence of small numbers of round haematopoietic cells in blood islands of rescued yolk sacs (F). h, heart; p-sp, para-aortic splanchnopleura; ys, yolk sac; arrows, *lacZ*<sup>+</sup> blood cells; arrowheads, *lacZ*<sup>+</sup> endothelial cells. (G-O) E9 embryos of the indicated genotypes. Unfixed Tg<sup>+/+</sup>*Scl*<sup>-/-</sup> embryos were anaemic (compare G with J and M). Histological sections of wax-embedded X-gal-stained embryos (H, I, K, L, N, O) demonstrated the presence of haematopoietic cells in rescued embryos (K, L). Embryos are from transgenic line 2708 and were genotyped by PCR.

genes to be targeted to the HSC compartment. *Scl* mutant mice die by day 9 of gestation and exhibit a complete absence of blood cell development thought to reflect a failure of HSC formation or differentiation. We reasoned that if precursors of HSCs and HSCs themselves are targeted by the *Scl* 3' enhancer at early developmental stages, we should be able to rescue blood development in *Scl*<sup>-/-</sup> embryos by expression of exogenous SCL protein under control of the 3' enhancer.

We therefore replaced the *lacZ* cDNA in the +6E5/*lacZ*/3'En construct with a bicistronic cassette containing the mouse *Scl* cDNA together with *lacZ* to form the +6E5/*Scl*-*lacZ*/3'En construct (Fig. 3A). After microinjection of the construct, transgene expression was assessed in midgestation founder embryos by whole-mount X-gal staining. Four transgenic embryos were analysed, three of which displayed a weak X-gal signal in the liver and vasculature detectable only after 24 hour incubation at 37°C, thus demonstrating that the +6E5/*Scl*-*lacZ*/3'En construct was functional in similar locations to the +6E5/*lacZ*/3'En construct (data not shown).

Five independent +6E5/*Scl*-*lacZ*/3'En transgenic lines (Tg) were generated and crossed with the *Scl*<sup>+/+</sup> mice to obtain the transgene in an *Scl* mutant background. Tg<sup>+/+</sup>*Scl*<sup>+/+</sup> mice were subsequently interbred and the resultant embryos analysed for *lacZ* expression by RT-PCR and X-gal staining. Midgestation foetal livers from embryos derived from all five transgenic lines contained *lacZ* transcripts by RT-PCR but X-gal staining was almost undetectable at this stage. X-gal signal was evident in

E7 and E8 embryos in only two of the five lines (lines 2708 and C7L). Subsequent detailed X-gal analysis was restricted to these two transgenic lines. The level of staining was weak (only clearly detectable after 48 hours) and was similar to weakly expressing +6E5/*lacZ*/3'En transgenic lines (Lines 220 and 2137) (Sanchez et al., 1999), with staining in the extra-embryonic region of E7 embryos and in the endothelium and blood cells within yolk sac blood islands of E8 embryos (Fig. 3B-D). By E9, the X-gal staining was very weak and it was almost undetectable by E11 (data not shown).

Tg<sup>+/+</sup>*Scl*<sup>-/-</sup> embryos were generated by crossing Tg<sup>+/+</sup>*Scl*<sup>+/+</sup> or Tg<sup>+/+</sup>*Scl*<sup>+/+</sup> with Tg<sup>-/-</sup>*Scl*<sup>+/+</sup> mice from line 2708. The level and pattern of X-gal staining in E8 Tg<sup>+/+</sup>*Scl*<sup>-/-</sup> embryos was similar to Tg<sup>+/+</sup>*Scl*<sup>+/+</sup> embryos (Fig. 3, compare C with E). Sections of Tg<sup>+/+</sup>*Scl*<sup>-/-</sup> yolk sacs showed occasional round presumptive blood cells attached to the endothelial inner surface of blood islands and X-gal staining was observed in blood cells as well as in endothelial cells (Fig. 3F). The number of blood cells was reduced compared with the Tg<sup>+/+</sup>*Scl*<sup>+/+</sup> control (Fig. 3, compare D with F).

E9 Tg<sup>+/+</sup>*Scl*<sup>-/-</sup> embryos were markedly anaemic and died at a time similar to *Scl*<sup>-/-</sup> embryos (Robb et al., 1995; Shivdasani et al., 1995) (Fig. 3, compare G, J and M). However, morphological evidence of yolk sac haematopoiesis was clearly observed in yolk sac sections of E9 Tg<sup>+/+</sup>*Scl*<sup>-/-</sup> embryos, although the number of blood cells in the blood islands was still less than in controls (Fig. 3, compare H with

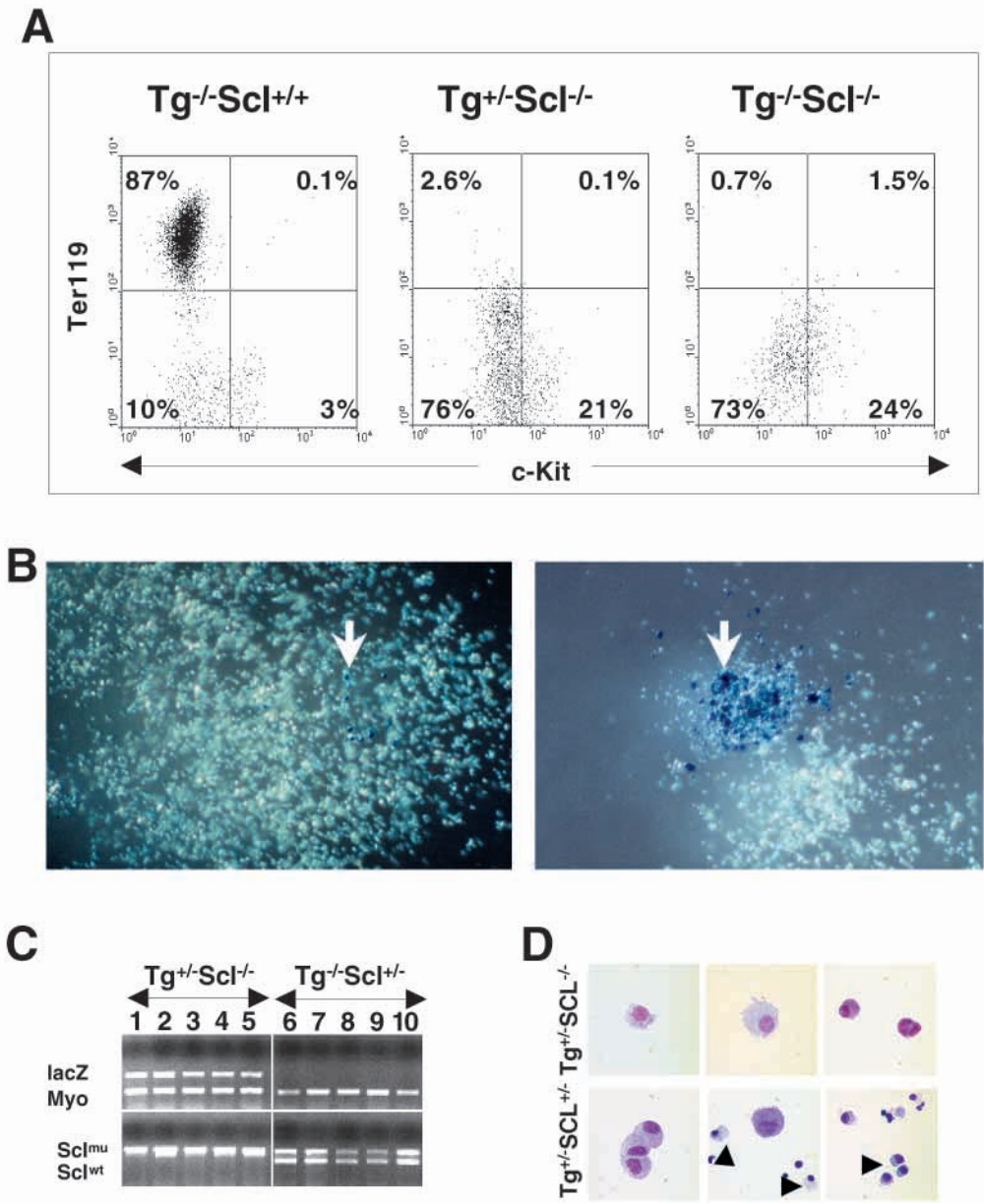
K and I with L). As previously reported, blood cells were not seen in yolk sacs from  $Tg^{-/-}Scl^{-/-}$  embryos (Fig. 3N,O) (Robb et al., 1995; Shivdasani et al., 1995). These data demonstrate that placing exogenous *Scl* under control of the *Scl* 3' enhancer results in partial rescue of early haematopoiesis. The *Scl* 3' enhancer therefore directs expression to cells in which *Scl* exerts a non-redundant function essential for establishing early haematopoiesis.

Flow cytometry was used to characterise the haematopoietic cells in the rescued embryos (Fig. 4A; Table 3). The mean number of cells recovered per yolk sac was  $161 \pm 81 \times 10^3$  from  $Tg^{-/-}Scl^{+/+}$  wild-type embryos, compared with  $2.3 \pm 0.9 \times 10^3$  for  $Tg^{-/-}Scl^{-/-}$  embryos and to  $29 \pm 15 \times 10^3$  from rescued  $Tg^{+/-}Scl^{-/-}$  embryos (Table 3). Yolk sac cells were analysed for the expression of c-Kit, CD34 (both markers of haematopoietic progenitors) and Ter119 (an erythroid marker). As shown in Fig. 4A and Table 3,  $Tg^{-/-}Scl^{-/-}$  yolk sacs contained very few cells positive for c-Kit, CD34 or Ter119. By contrast, rescued

yolk sacs contained approximately 30-fold more cells positive for each marker. These data are consistent with the morphological evidence that rescued yolk sacs contain blood cells not seen in *Scl* null yolk sacs.

Relative to  $Tg^{-/-}Scl^{+/+}$  wild-type yolk sacs, rescued yolk sacs contained an approximately sevenfold increase in the percentage of c-Kit<sup>+</sup> cells (21% compared with 3.1%; Fig. 4A). However calculation of absolute cell numbers showed that rescued and wild-type yolk sacs contained similar absolute numbers of c-Kit<sup>+</sup> cells ( $9.2 \times 10^3$  and  $9.8 \times 10^3$  respectively) and CD34<sup>+</sup> cells ( $7.9 \times 10^3$  and  $7.0 \times 10^3$ , respectively) (Table 3). However, the absolute number of Ter119<sup>+</sup> erythroid cells present in rescued yolk sacs was approximately 20-fold lower than in wild-type  $Tg^{-/-}Scl^{+/+}$  yolk sacs. These data demonstrate that the lower total number of cells present in rescued yolk sacs largely reflects a loss of committed Ter119<sup>+</sup> erythroid cells, whereas the number of early progenitors appears to be normal.

**Fig. 4.** Myeloid and erythroid colony forming cells are obtained from cultures of  $Tg^{+/-}Scl^{-/-}$  yolk sacs. (A) FACS analysis of yolk sacs from E9 embryos. Cell suspensions from individual yolk sacs were stained with the indicated antibodies and body remnants used for *lacZ/Scl* PCR genotyping. Double staining was performed using FITC-conjugated c-Kit with PE-conjugated Ter119. Quadrants were established according to values obtained using cells stained with isotype controls in the same experiment. (B) Two erythroid colonies containing diaminofluorene positive haemoglobinised cells (arrows) from  $Tg^{+/-}Scl^{-/-}$  embryos are shown. (C) PCR analysis of individual colonies from yolk sacs of the indicated genotypes. (D) May-Grunwald-Giemsa staining of cytospin preparation of yolk sac haematopoietic colonies obtained from embryos of the indicated genotype. Myeloid cells were seen in colonies from both  $Tg^{+/-}Scl^{-/-}$  and  $Tg^{+/-}Scl^{+/+}$  embryos. Normoblasts (arrows) were only seen in colonies from  $Tg^{+/-}Scl^{-/-}$ .





**Table 3. Quantification of cell populations from rescued yolk sacs**

Genotype of embryos	Cell number ( $\times 10^3$ ) per yolk sac			
	Total	c-Kit <sup>+</sup>	Ter119 <sup>+</sup>	CD34 <sup>+</sup>
Tg <sup>-/-</sup> Scl <sup>+/+</sup>	161±81 (n=15)	9.8±5 (n=6)	110±65 (n=4)	7±3 (n=5)
Tg <sup>-/-</sup> Scl <sup>-/-</sup>	2.3±0.9 (n=3)	0.3±0.1 (n=3)	0.02±0 (n=3)	0.22±0.12 (n=3)
Tg <sup>+/-</sup> Scl <sup>-/-</sup>	29±15 (n=5)	9.2±4 (n=5)	5±2 (n=3)	7.9±7.6 (n=3)
Tg <sup>+/-</sup> Scl <sup>+/+</sup>	150±60 (n=4)	10±4.6 (n=4)	86 (n=1)	11.8±9.6 (n=4)
Tg <sup>+/+</sup> Scl <sup>+/+</sup>	88±44 (n=11)	6.6±4 (n=4)	58 (n=1)	8±8.8 (n=5)

Numbers represent the mean absolute number of cells per yolk sac in each population. Cell suspensions were stained for the indicated antigens and analysed by FACS to determine the percentage of each population (see Fig. 4). The total cell number for each yolk sac was then calculated from the total cell count obtained for that yolk sac. Data are from six independent experiments. *n*, number of embryos analysed. ND, not determined.

Colony assays were then used to assess functional haematopoietic progenitor activity in yolk sacs from rescued and wild-type embryos. Haematopoietic colonies were obtained from rescued Tg<sup>+/-</sup>Scl<sup>-/-</sup> embryos but not from Tg<sup>-/-</sup>Scl<sup>-/-</sup> embryos (Fig. 4B; Table 4). PCR analysis confirmed the *lacZ* and *Scl* genotype of individual colonies and excluded the possibility of contamination by *Scl*<sup>+/-</sup> or *Scl*<sup>+/+</sup> progenitors (Fig. 4C). The number of myeloid colonies obtained from Tg<sup>+/-</sup>Scl<sup>-/-</sup> yolk sacs was not significantly different from the number of myeloid colonies obtained from Tg<sup>+/-</sup>Scl<sup>+/+</sup> or Tg<sup>+/+</sup>Scl<sup>+/+</sup> yolk sacs. By contrast, the number of erythroid colonies from Tg<sup>+/-</sup>Scl<sup>-/-</sup> yolk sacs was much lower than for the other genotypes (Table 4). Cytospin preparations confirmed the presence of myeloid cells together with cells at late stages of erythroid differentiation in colonies derived from Tg<sup>+/-</sup>Scl<sup>+/+</sup> yolk sacs, whereas terminally differentiated erythroblasts were not seen in colonies from rescued Tg<sup>+/-</sup>Scl<sup>-/-</sup> yolk sacs (Fig. 4D). We therefore conclude that expression of *Scl* under control of the 3' enhancer construct rescued early HSC development and permitted myeloid differentiation, but did not support full erythroid maturation. These results are consistent with our previous observation that the 3' enhancer construct is not active in Ter119-positive erythroid cells (Sanchez et al., 1999) and reveal an essential requirement for SCL during erythroid differentiation in vivo.

#### Yolk sac angiogenesis is rescued in Tg<sup>+/-</sup>Scl<sup>-/-</sup> embryos

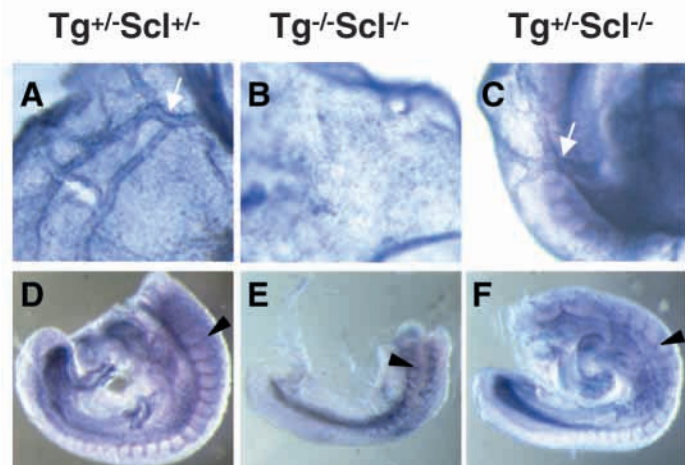
In addition to its central role in haematopoiesis SCL is also essential for normal endothelial development. In particular, *Scl*<sup>-/-</sup> embryos exhibit defective yolk sac angiogenesis, which appears to reflect an intrinsic defect in *Scl*<sup>-/-</sup> endothelial cells (Visvader et al., 1998). The 3' enhancer directs *lacZ* expression to a population of cells in the E7.5 extra-embryonic mesoderm and subsequently to both endothelial cells and haematopoietic progenitors during embryonic development (Sanchez et al., 1999). We therefore examined whether the yolk sac blood vessel defect in the *Scl*<sup>-/-</sup> embryos could be rescued by expression of SCL protein under the 3' enhancer.

**Table 4. Colony-forming cells in yolk sac from rescued embryos**

Genotype of embryos	Myeloid colonies per yolk sac	Erythroid colonies per yolk sac
Tg <sup>-/-</sup> Scl <sup>+/+</sup> (n=10)	74±27	32±22
Tg <sup>-/-</sup> Scl <sup>+/+</sup> (n=15)	91±37	30±15
Tg <sup>-/-</sup> Scl <sup>-/-</sup> (n=6)	0	0
Tg <sup>+/-</sup> Scl <sup>-/-</sup> (n=7)	122±48	6±6
Tg <sup>+/+</sup> Scl <sup>-/-</sup> (n=10)	70±32	21±15
Tg <sup>+/+</sup> Scl <sup>+/+</sup> (n=12)	66±41	20±17

Yolk sacs from E9-E9.5 embryos were cultured in Methocult plates. The colonies were counted after 7 days. Detection of erythroid cells was performed by staining of the plates with diaminofluorene. Data from five independent experiments. *n*, number of embryos analysed.

Vitelline vessels were not evident macroscopically in the yolk sacs of Tg<sup>+/-</sup>Scl<sup>-/-</sup> E9 embryos. However the embryos were markedly anaemic, making yolk sac vessels difficult to visualise. We therefore stained whole embryos with an antibody to PECAM, a marker of endothelial cells. The vasculature of Tg<sup>+/-</sup>Scl<sup>+/+</sup> embryos appeared normal and a prominent network of vitelline vessels was evident (Fig. 5A,D). As previously reported (Visvader et al., 1998) large vitelline vessels were absent in Tg<sup>-/-</sup>Scl<sup>-/-</sup> yolk sacs (Fig. 5B), and intraembryonic vessels appeared poorly developed (Fig. 5E). By contrast, large vitelline vessels were readily apparent in Tg<sup>+/-</sup>Scl<sup>-/-</sup> yolk sacs and intra-embryonic vessels also appeared normal (Fig. 5C,F). These data demonstrate that defective yolk sac and embryonic angiogenesis seen in *Scl*<sup>-/-</sup> embryos can be rescued by expression of *Scl* under control of the *Scl* 3' enhancer. The fact that rescued embryos still die by E9-E10 presumably reflects their inability to sustain normal erythropoiesis.



**Fig. 5.** Anti-PECAM1 antibody staining of E9.5 embryos. Large branching vitelline vessels in the yolk sac (white arrows) are prominent in wild type Tg<sup>+/+</sup>Scl<sup>+/+</sup> embryos (A) as well as in rescued Tg<sup>+/-</sup>Scl<sup>-/-</sup> embryos (C) but were not observed in mutant Tg<sup>-/-</sup>Scl<sup>-/-</sup> yolk sacs (B). Inter-somatic vessels (arrowhead) are also well formed in both wild-type (D) and rescued embryos (F) but not in the mutant embryo (E). The head of the embryos were used to determine the embryo genotype.

## DISCUSSION

### The *Scl* 3' enhancer targets haematopoietic stem cells

Haematopoietic stem cells are crucial for the maintenance of haematopoiesis throughout development but their transcriptional programmes remain poorly understood. We demonstrate that an enhancer downstream of the *Scl* gene is able to target reporter gene expression to adult and embryonic long term repopulating HSCs. *lacZ*-expressing cells from E11-12 foetal liver or from adult bone marrow were both able to give rise to long term multi-lineage haematopoietic reconstitution of adult recipient mice. *lacZ* was also expressed in c-Kit<sup>+</sup>CD34<sup>+</sup> cells from E11 AGM, suggesting that HSCs in this region were also targeted.

There has been considerable interest in identifying stem cell enhancers, both to express exogenous genes in HSCs and thereby to manipulate HSC behaviour in vivo, and also to provide molecular insights into the transcriptional programme of HSCs. However, very few candidate HSC enhancers have been identified. The CD34 gene is flanked by two enhancers that are active in haematopoietic cell lines (May and Enver, 1995) but it is not known whether these elements target HSCs in vivo. A 14 kb genomic cassette containing the Ly-6E.1 gene directs *lacZ* expression to HSCs from adult bone marrow (Miles et al., 1997); however, the Ly-6E.1 cassette is not expressed in foetal liver or yolk sac progenitors and no individual enhancer has been shown to be sufficient to target HSCs. The H2K promotor/enhancer in combination with the Moloney MuLV enhancer will direct expression to HSCs. However, this construct is widely expressed in many cell types and the relative contribution of the H2K and retroviral enhancers to HSC expression is not clear (Domen et al., 2000).

The *Scl* 3' enhancer therefore provides a unique tool for manipulation of HSCs in vivo. Moreover, recent reports suggest that progenitors present in bone marrow may have a wider biological potential than previously realised, and be capable of generating hepatocytes (Lagasse et al., 2000; Petersen et al., 1999), neurones (Eglitis and Mezey, 1997) and muscle (Ferrari et al., 1998; Gussoni et al., 1999). It may therefore be possible to use the 3' enhancer to examine the molecular basis for stem cell plasticity.

Our results, together with previous data, also allow a description of the pattern of activity displayed by the *Scl* 3' enhancer. It is active in the region of the extra-embryonic mesoderm of E7.5 embryos that gives rise to the yolk sac, and it subsequently directs *lacZ* expression to endothelial and blood cells within yolk sac blood islands of E8 embryos (Sanchez et al., 1999). Within the embryo proper, the enhancer is active in endothelial cells and also in haematopoietic progenitors at diverse sites and times, including E8 para-aortic splanchnopleura, E11 AGM region and E11 foetal liver (Sanchez et al., 1999). In adult mice, the 3' enhancer is active in the vast majority of HSCs and progenitors present in bone marrow (Sanchez et al., 1999) and preliminary evidence suggests widespread expression in adult endothelial cells (L. G., unpublished).

The SCL protein is essential for the development of embryonic and adult blood in mice (Porcher et al., 1996; Robb et al., 1996), and is essential for the development of

haemangioblasts from murine ES cells (Robertson et al., 2000). Furthermore ectopic expression of SCL specifies the formation of haemangioblasts from lateral mesoderm in zebrafish embryos (Gering et al., 1998), and also partially rescues the haematopoietic and endothelial defects of zebrafish *cloche* mutants. Taken together with these observations, the pattern of *Scl* 3' enhancer activity therefore suggests a model in which the 3' enhancer functions as a nodal point for the integration of signals responsible for establishing the transcriptional programme for blood and blood vessel development. Interestingly both endothelial and haematopoietic activity are retained in a 600 bp minimal enhancer fragment (B. Göttgens, unpublished). Identification of the factors that regulate this element should illuminate the molecular mechanisms controlling the origin of blood and endothelium.

### Expression of *Scl* under control of the 3' enhancer rescues early haematopoietic progenitors and yolk sac angiogenesis in *Scl*<sup>-/-</sup> embryos

In *Scl*<sup>-/-</sup> mice, embryonic lethality results from a failure of HSC formation and functions together with a defect in yolk sac angiogenesis (Porcher et al., 1996; Robb et al., 1996; Visvader et al., 1998). We have demonstrated that the *Scl* 3' enhancer is active in adult and embryonic HSCs and is also expressed in yolk sac and embryonic endothelial cells (Sanchez et al., 1999). We therefore elected to study whether an SCL cDNA driven by the 3' enhancer would be able to rescue haematopoiesis and yolk sac angiogenesis in *Scl*<sup>-/-</sup> embryos.

Our results show that expressing *Scl* under the control of the 3' enhancer rescues the formation of early haematopoietic progenitors. Normal numbers of c-Kit<sup>+</sup> progenitors and myeloid colony-forming cells were found in the yolk sacs of rescued embryos. Interestingly, erythropoiesis remained defective with rescued yolk sacs containing markedly reduced numbers of both Ter119<sup>+</sup> erythroid cells and erythroid colony-forming cells compared with wild-type yolk sacs. The majority of erythroid cells in E9 yolk sacs result from a wave of primitive erythropoiesis, whereas most yolk sac erythroid colony-forming cells at E9 represent definitive erythroid progenitors (Palis et al., 1999). Our data therefore suggest that both primitive and definitive erythropoiesis are deficient in rescued yolk sacs. The resultant severe anaemia is likely to be the main reason that rescued embryos still die at the same time as *Scl*<sup>-/-</sup> embryos.

It is informative to compare our results with a previous study in which haematopoiesis in *Scl*<sup>-/-</sup> embryos was partially rescued by *Scl* under the control of heterologous regulatory elements from the *Gata1* gene (Visvader et al., 1998). Yolk sac angiogenesis remained abnormal, consistent with the fact that *Gata1* is not expressed in the endothelial lineage, and a three- to fivefold reduction was observed in both erythroid and myeloid colony numbers, suggesting only partial rescue of the HSC compartment. By contrast, our results suggest that *Scl* driven by the 3' enhancer is capable of fully rescuing the stem cell compartment but is unable to support erythroid differentiation.

At least two distinct mechanisms can be envisaged. Failure to rescue normal levels of erythropoiesis could reflect an effect of abnormal levels of SCL protein in HSCs, resulting in reduced erythroid but not myeloid commitment. Indeed, relatively small alterations in the level of an individual

transcription factor can have dramatic effects on lineage commitment (Heyworth et al., 1999; Kulesa et al., 1995). Alternatively the 3' enhancer construct may lack a distinct SCL erythroid enhancer which normally maintains SCL transcription during erythroid differentiation. These two mechanisms are not mutually exclusive, and rigorous discrimination between them would require fate mapping the progeny of single HSCs. However, the concept of a separate erythroid enhancer is supported by our previous observation that the 3' enhancer construct does not direct *lacZ* expression to cells expressing the erythroid marker Ter119 (Kina et al., 2000; Sanchez et al., 1999), whereas *lacZ* 'knocked into' the *Scf* locus is expressed in differentiated erythroid cells (Elefanti et al., 1999). Whatever the precise mechanism, the failure to rescue erythropoiesis demonstrates a requirement for SCL during commitment to or differentiation along the erythroid lineage in vivo. A distinct role for SCL during erythroid differentiation, in addition to its role in HSC formation/function, would accord well with the previous suggestion that SCL acts as a positive regulator of erythroid differentiation in vitro (Aplan et al., 1992; Elwood et al., 1998; Valtieri et al., 1998) and with the observation that full rescue of erythropoiesis from *Scf*<sup>-/-</sup> ES cells requires DNA binding by SCL (Porcher et al., 1999).

In addition to rescuing the generation of early haematopoietic progenitors, SCL under control of the 3' enhancer construct also rescued normal development of yolk sac vitelline vessels in *Scf*<sup>-/-</sup> embryos. This is likely to reflect correction of a block in the ability of endothelial cells to undergo angiogenic differentiation. However, the 3' enhancer construct is probably expressed in haemangioblasts as well as in their endothelial progeny (Sanchez et al., 1999). Our data can not therefore exclude the possibility that it is endothelial commitment from haemangioblasts that is being rescued, and that the endothelial cells seen in *Scf*<sup>-/-</sup> yolk sacs arise by a distinct developmental route (Pardanaud et al., 1996).

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