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Absence of yolk sac hematopoiesis from mice with a targeted disruption of the *scl* gene

(*tal-1* gene/gene targeting/homologous recombination)

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ABSTRACT **The *scl* gene encodes a basic-helix–loop–helix transcription factor which was identified through its involvement in chromosomal translocations in T-cell leukemia. To elucidate its physiological role, *scl* was targeted in embryonic stem cells. Mice heterozygous for the *scl* null mutation were intercrossed and their offspring were genotyped. Homozygous mutant ($scl^{-/-}$) pups were not detected in newborn litters, and analysis at earlier time points demonstrated that $scl^{-/-}$ embryos were dying around embryonic day 9.5. The $scl^{-/-}$ embryos were pale, edematous, and markedly growth retarded after embryonic day 8.75. Histological studies showed complete absence of recognizable hematopoiesis in the yolk sac of these embryos. Early organogenesis appeared to be otherwise normal. Culture of yolk sac cells of wild-type, heterozygous, and homozygous littermates confirmed the absence of hematopoietic cells in $scl^{-/-}$ yolk sacs. Reverse transcription PCR was used to examine the transcripts of several genes implicated in early hematopoiesis. Transcripts of GATA-1 and PU.1 transcription factors were absent from RNA from $scl^{-/-}$ yolk sacs and embryos. These results implicate *scl* as a crucial regulator of early hematopoeisis.**

The *scl* (*SCL*) gene, also known as *tal-1*, was identified through its involvement in a t(1;14)(p32;q11) chromosomal translocation in a human leukemic cell line (1). This translocation juxtaposed the *scl* locus with regulatory elements in the T-cell receptor δ locus and caused the gene to be aberrantly expressed in the leukemic cells. Overexpression of *scl* in T-cell acute lymphoblastic leukemia (T-ALL) was also shown to occur as a result of a site-specific deletion on chromosome 1 which deleted ≈90 kb of DNA encompassing the *sil* locus (2–4). The *sil/scl* deletion is the most commonly identified chromosomal abnormality in T-ALL (5, 6).

The *scl* gene encodes a transcription factor with a basic-helix–loop–helix (b-HLH) motif. *scl* expression is seen predominantly within the hematopoietic system (1, 7, 9, 10) and is also detected in embryonic brain and spinal cord from embryonic day 9.5 (E9.5), in the developing skeletal system, and in intra- and extra-embryonic and adult endothelium (9, 11, 12).

Several members of the b-HLH family of transcription factors participate in cell-type determination and differentiation (reviewed in ref. 13). By analogy, it seems likely that *scl* is involved in regulating hematopoiesis-specific gene expression and developmental processes. *In vitro* gene delivery experiments provide support for this idea. Such studies have implicated *scl* in proliferation and differentiation events in erythroleukemia cell lines (14, 15). Enforced *scl* expression in M1 cells implicated *scl* in monocytic differentiation and suggested a role for the gene product in the cellular differentiative response to interleukin 6, leukemia inhibitory factor, and oncostatin M signaling pathways (16).

To further define the function of *scl* in normal hematopoiesis we used embryonic stem (ES) cell technology to generate mice with a null mutation of the *scl* gene.

MATERIALS AND METHODS

Construction of the Targeting Vectors. To create targeting vector 582 a blunt-ended 6-kb *Sac* I genomic *scl* fragment containing exons 4 and 5 (17) was subcloned into the *Xba* I site of pKJ1 (18), which contains the neomycin-resistance gene (*neo*) with the phosphoglycerate kinase (*pgk*) promoter and *pgk* polyadenylylation signals. The *Eco*RI–*Hin*dIII insert of pKJtk (18) containing the thymidine kinase (*tk*) gene with the *pgk* promoter and polyadenylylation signals (*pgktk*) was ligated into the *Sma* I site at the 5′ end of the construct. A *Sac* I–*Eco*RI 3′ *scl* genomic fragment was then inserted into a *Hin*dIII site in the 3′ polylinker. Targeting vector 607 was constructed as follows: a 5-kb *Bam*HI genomic fragment, containing exon 6 of the *scl* locus, was subcloned into pBluescript SK (+) (Stratagene). This plasmid was digested with *Sma* I and *Bgl* II, and the *Sma* I–*Bgl* II fragment from pRMM (19), comprising the hygromycin-resistance gene (*hph*), *pgk* promoter, and polyadenylylation signal, was inserted. A 2-kb *Bgl* II 3′ *scl* genomic fragment was ligated into the *Bgl* II site. *pgktk* was ligated into the *Bam*HI site in the 3′ polylinker. A 3.8-kb *Not* I *scl* genomic fragment was inserted into a *Cla* I site in the 5′ polylinker (see Fig. 1). *scl* genomic fragments used in the construction of both targeting vectors were from the 129Sv strain, with the exception of the 3′ fragment of 582, which was BALB/c-derived.

Isolation of Recombinant ES Cell Clones, Production of Chimeric Mice, and Identification of Heterozygotes. The ES cell line W9.5 (20) was maintained and electroporation was carried out as previously described (21). For vector 582, selection with G418 (350 μg/ml) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU; 0.2 μM) was begun 24 hr after electroporation and continued for 10 days. ES cells electroporated with vector 607 were cultured on STO feeder cells transfected with a *hph* expression vector and were selected with hygromycin B (175 μg/ml) and FIAU (0.2 μM) for 5 days. Surviving colonies were expanded on feeder layers in 48-well dishes, and homologous recombinants were identified by Southern analysis of DNA from pools of six clones, using probe A or B (see Fig. 1). Positive pools were reanalyzed and clones with a targeted mutation of *scl* were injected into (C57BL/6 × C57BL/10)F_2 blastocysts obtained from females 3.5 days post coitus.

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Abbreviations: T-ALL, T-cell acute lymphoblastic leukemia; RT-PCR, reverse transcription PCR; ES, embryonic stem; b-HLH basic-helix–loop–helix; E*n*, embryonic day *n*; FCS, fetal calf serum; HPRT, hypoxanthine phosphoribosyltransferase; AFP, α-fetoprotein.

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Chimeras were mated to C57BL/6 mice to produce heterozygotes, which were identified by Southern analysis of tail DNA.

PCR Genotyping of Embryos and Yolk Sacs. Yolk sacs and embryos were obtained from timed matings of heterozygous intercrosses. Embryos and fetal membranes were dissected free of maternal tissues in phosphate-buffered saline medium (PB1; ref. 23) with 20% fetal calf serum (FCS), washed, and stored at −70°C. Embryo samples were boiled in 200 μl of water for 10 min and digested with proteinase K (100 μg/ml) at 55°C for 2 hr. Yolk sacs were digested at 55°C for 2 hr in 100 μl of lysis buffer (50 mM KCl/10 mM Tris·HCl, pH 8.3/2.0 mM $MgCl_2$/0.1 mg of gelatin per ml/0.45% Nonidet P-40/0.45% Tween 20/100 μg of proteinase K per ml). Samples were heated at 95°C for 10 min and 1 μl was amplified in a PCR mixture containing *scl* primers A and D (5′-GTTTTGGTCTAGAGTTTGTGAGCC-3′ and 5′-GCATGCTCAAGGCTGCTGACTTGG-3′) and *neo* primer B (5′-ATATTGCTGAAGAGCTTGGCGGC-3′) for lines derived with vector 582 or *pgk* poly(A) primer C (5′-GAAGGGTGAGAACAGAGTACCTAC-3′) for lines derived with vector 607 (see Fig. 1*A*). Reaction conditions were 96°C for 30 sec, 65°C for 50 sec, and 72°C for 3 min for 35 cycles in a 50-μl mixture containing 200 ng of each primer, 0.2 mM each dNTP, 1× PCR buffer (Boehringer Mannheim buffer for 582 typing and Invitrogen buffer with 2.0 mM $MgCl_2$ for 607 typing). Hybridization of Southern blots of tail DNA to *neo* or *hph* probes, as appropriate, showed only a single integration of the targeting vector in each of the lines studied (not shown).

Yolk Sac Culture. Yolk sacs, in PB1 with 20% FCS, were passed through 26- and 30-gauge needles to obtain a single-cell suspension. The cells were plated in duplicate 1.25-ml cultures containing 1.5% methylcellulose in Iscove's modified Eagle's medium (IMEM), 20% selected FCS, 75 μM 2-mercaptoethanol, 8% pokeweed mitogen-stimulated spleen-conditioned medium, murine stem cell factor (100 ng/ml), murine granulocyte–macrophage colony-stimulating factor (CSF) (10 ng/ml), human erythropoietin (2 international units/ml), and human granulocyte CSF (10 ng/ml). Cultures were incubated in a fully humidified atmosphere of 5% CO_2/95% air for 7–15 days. Individual colonies were dispersed on slides and stained with May-Grunwald/Giemsa stain.

Histological Analysis. Specimens were fixed in 4% paraformaldehyde in phosphate-buffered saline, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

Reverse Transcription (RT)-PCR. Total RNA was prepared from single yolk sacs or embryos by using Trizol (GIBCO). Oligo(dT)-primed cDNAs were prepared in a 20-μl volume from ≈50 ng of total RNA. One microliter of cDNA was then used for PCR amplification in 20-μl reaction mixtures containing 1× PCR buffer (Boehringer Mannheim), 0.1 mM each dNTP, and 0.5 unit of *Taq* DNA polymerase with 100 ng of each exon-specific primer. *scl* primers were as described for genotyping. Primers for hypoxanthine phosphoribosyltransferase (HPRT), α-fetoprotein (AFP), Brachyury, and βH1 were as described by Keller *et al.* (24). GATA-2 primers were as in Weiss *et al.* (25) and PU.1 primers as in Wulf *et al.* (26). The primers used for rbtn2 and GATA-1 were rbtn2, 5′-ACCATGTCCTCGGCCATCGAAAGGA-3′/5′-TAGATGATCCCATTGATCTTGGT-3′; GATA-1, 5′-GGAATTCGGGCCCCTTGTGAGGCCAGAGAG-3′/5′-CGGGGTACCTCACGCTCCAGCCAGATTCGACCC-3′. Reaction conditions were 96°C for 30 sec, 55°C for 30 sec, 72°C for 1 min for 25 cycles except for *scl*, GATA-1, GATA-2, and PU.1, for which annealing was at 60°C and for 35 cycles. PCR products were separated on 3% agarose gels, trans-

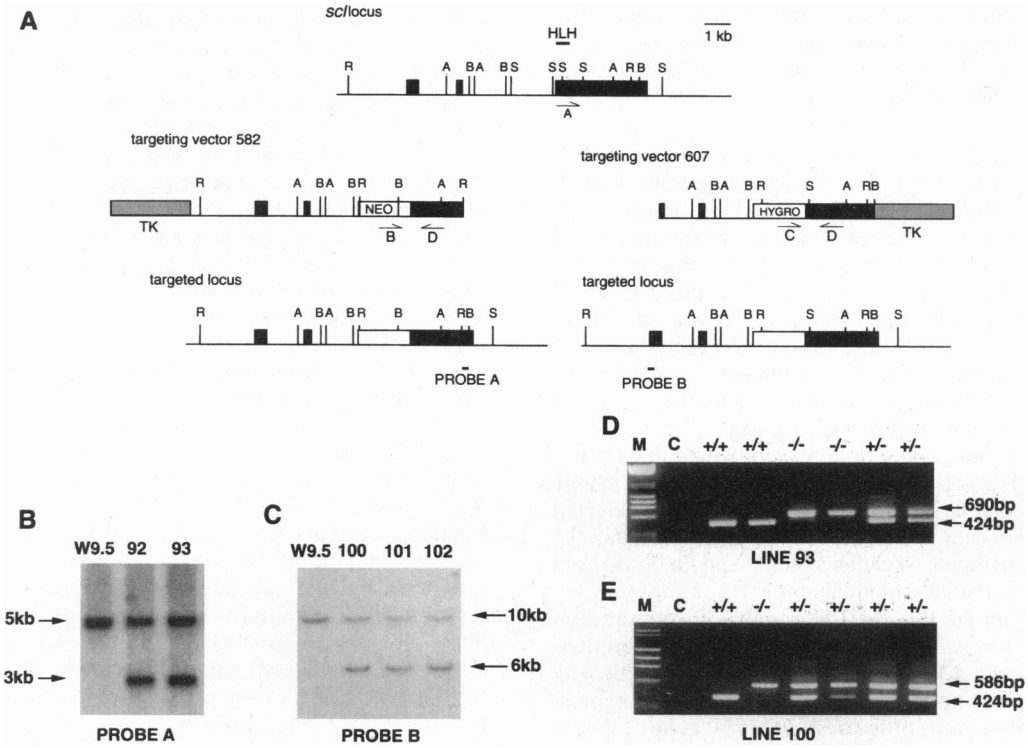

FIG. 1. *scl* gene targeting strategy, targeted ES cell lines, and genotyping of offspring from heterozygous $scl^{+/-}$ intercrosses. (*A*) At the top is shown a portion of the *scl* genomic locus. Targeting constructs 582 and 607 with the resulting targeted loci are depicted below. Probes A and B, used for screening for targeted ES cell clones by Southern analysis, are indicated, as are oligonucleotide primers A–D, used for genotyping embryo and yolk sac samples. A, *Apa* I site; R, *Eco*RI site; B, *Bam*HI site; S, *Sac* I site; HLH, helix–loop–helix domain. (*B* and *C*) Southern blot analysis of DNA from wild-type and targeted ES cell lines targeted with construct 582 (*B*) and 607 (*C*). Genomic DNA was prepared from wild-type ES cells (W9.5) and targeted clones (92 and 93 in *B* and 100, 101, and 102 in *C*) digested with *Bam*HI (*B*) or *Eco*RI (*C*) and probed as in *A*. Sizes of germ-line (5 kb, 10 kb) and targeted (3 kb, 6 kb) alleles are indicated. (*D*) Genotyping of E8.5 embryos from matings of $scl^{+/-} \times scl^{+/-}$ mice of the 93 and 100 lines. Embryo samples were prepared and PCR was performed as described in the text. Sizes of the PCR products are indicated. M, DNA size markers; C, PCR control (no added DNA); +/+, wild-type; +/−, heterozygous; −/−, homozygous.

ferred to nylon membranes, and hybridized with ^{32}P-labeled internal oligonucleotides.

RESULTS

Targeting of *scl* in ES Cells. Two targeting vectors (582 and 607) were used to disrupt the *scl* gene. The targeting strategy was similar for both vectors, each of which deleted the b-HLH domain of *scl* (Fig. 1*A*). This region was replaced by the bacterial *neo* gene in 582 or the *hph* gene in 607. Both vectors contained a flanking *tk* expression cassette. The antibiotic-resistance genes were under the control of the *pgk* promoter and had a *pgk* polyadenylylation signal. The vectors were introduced into W9.5 ES cells by electroporation, and homologous recombination between vector 582 and the endogenous *scl* locus was identified by Southern analysis of *Bam*HI-digested ES cell DNA using the 5′ flanking probe A. For vector 607, ES cell DNA was digested with *Eco*RI and hybridized with probe B (Fig. 1 *A–C*). The structure of the targeted allele was verified by Southern analysis using a 3′ (vector 582) or 5′ (vector 607) flanking probe and *neo* or *hph* probes (data not shown). Clones with the targeted *scl* mutation were identified at a frequency of 1 in 250 doubly selected clones in several experiments with vector 582 and at 1 in 9 in a single electroporation with vector 607. Chimeras generated from two 582- and three 607-targeted lines were mated to C57BL/6 mice. Chimeras from all ES cell lines transmitted the targeted mutation to their offspring.

Homozygous Disruption of *scl* Causes Embryonic Lethality with Failure of Yolk Sac Hematopoiesis. Mice heterozygous for the *scl* mutation did not display an obvious phenotype. Homozygous mutant mice were not recovered from crosses between heterozygous mice. Therefore embryos from timed matings of heterozygous mice were genotyped by using a PCR-based strategy (Fig. 1 *A* and *C*). At E10.5, wild-type ($scl^{+/+}$), heterozygous ($scl^{+/-}$), and homozygous mutant ($scl^{-/-}$) embryos were present in a mendelian ratio (Table 1). By E11.5, however, only a small number of resorption sites containing necrotic remnants of $scl^{-/-}$ embryos were found.

The phenotype of $scl^{-/-}$ embryos was studied in litters from intercrosses of $scl^{+/-}$ mice from all five lines. DNA extracted from fetal membranes or a portion of the tail was used to genotype each embryo. At E7.5 and E8.5, no differences were observed between $scl^{+/+}$, $scl^{+/-}$, or $scl^{-/-}$ embryos. However, by E8.75–E9.0 the yolk sac of $scl^{-/-}$ embryos was markedly paler than that of littermates and blood islands and major vitelline vessels were not seen (Fig. 2*A*). Cardiac contraction was observed in the $scl^{-/-}$ embryos, and approximately two-thirds completed axial rotation. Few progressed beyond 18 somites (range 8–20). Erythroid cells were not seen and the embryos were pale, growth retarded, and edematous with dilated pericardial sacs (Fig. 2 *B* and *C*). Fusion of the allantois to the chorion occurred normally in the mutant embryos.

Table 1. Genotyping of the progeny of crosses of heterozygous *scl* mutant mice

Age	No. of pups typed	*scl* genotype		
		+/+	+/−	−/−
E8.5	79	20	39	20
E9.5	69	15	37	17
E10.5	17	4	9	4
E11.5	25	8	15	2*
E15.5	24	7	17	0
28 days	128	45	83	0

Genotyping was performed by PCR. +/+, Wild type; +/−, heterozygotes; −/−, homozygotes.
*Resorption sites with small embryonic remnants only.

Histological examination of E7.5 $scl^{-/-}$ yolk sac mesoderm and endoderm and the embryonic tissues did not reveal any abnormalities. By E8.0, early blood island formation was present in $scl^{+/+}$ and $scl^{+/-}$ yolk sacs but was not seen in yolk sacs from $scl^{-/-}$ littermates. $scl^{-/-}$ yolk sacs appeared to consist only of a vascular endothelial and an endodermal cell layer. In sections through the intact decidua, the endothelium was flattened against the endoderm, but when the yolk sac was dissected free, empty capillary spaces devoid of hematopoietic cells were apparent (Fig. 2 *D, E, G,* and *H*). After E8.75, $scl^{-/-}$ embryos were increasingly retarded and extensive cell death was seen. Organogenesis, including the formation of the heart, neural tube, vascular endoderm, notocord, primitive gut, somites, and allantois, although retarded, appeared normal. Erythrocytes were absent from embryonic vessels (Fig. 2 *F* and *I*). Overall, the most striking abnormality seen in $scl^{-/-}$ embryos was the failure of development of yolk sac hematopoiesis.

Absence of Hematopoietic Colony Formation in Cultures of *scl*-Null Yolk Sacs. The *in vitro* hematopoietic differentiation capacity of $scl^{-/-}$ yolk sacs was tested by culturing individual yolk sacs of each conceptus in timed litters from heterozygous intercrosses. At 7 days colonies were counted and scored as erythroid or nonerythroid on the basis of morphology and visible hemoglobinization. Cultures of all yolk sacs from six E8.5 and six E9.5 litters were performed. As shown in Fig. 3*A*, colony numbers in cultures of $scl^{+/+}$ and $scl^{+/-}$ yolk sacs were similar, but cultures from $scl^{-/-}$ yolk sacs did not contain hematopoietic colonies. Representative colonies from $scl^{+/+}$ and $scl^{+/-}$ yolk sac cultures were stained and examined. In hemoglobinized colonies, the cells were predominantly maturing erythroblasts. Nonhemoglobinized colonies contained maturing neutrophils and macrophages (Fig. 3 *B–D*). Rare cell aggregates were seen in the $scl^{-/-}$ cultures at 10–15 days. Cells within these aggregates had the morphological appearance of yolk sac endoderm cells (Fig. 3*E*).

Gene Expression in $scl^{-/-}$ Yolk Sacs and Embryos. Total RNA was prepared from E9.5 yolk sacs and embryos. RT-PCR analysis of gene expression was performed using at least three specimens of each genotype, which were tested in triplicate. HPRT expression was used to quantify the amount of cDNA amplified. *scl* expression was not detected in $scl^{-/-}$ yolk sacs or embryos, confirming the null mutation. Transcripts for embryonic globin βH1, GATA-1, and PU.1 were absent, whilst those of rbtn2 and GATA-2 were unaltered in $scl^{-/-}$ yolk sacs or embryos (Fig. 4).

DISCUSSION

This study demonstrates a crucial role for the scl protein in formation of yolk sac hematopoietic cells. In the developing embryo, mesoderm of the visceral yolk sac differentiates into hematopoietic cells and endothelial cells of the vitelline vessels. The yolk sac remains the primary hematopoietic organ until day 12 (27, 28). In embryos with a null mutation of *scl*, yolk sac hematopoietic cells were not present and hematopoietic colonies did not form *in vitro* in optimally stimulated cultures of $scl^{-/-}$ yolk sac cells. These findings are similar to those reported by Shivdasani *et al.* (29).

The early period of organogenesis in murine development is critically dependent on the maintenance of a functional blood circulation in the yolk sac. The absence of hematopoiesis in $scl^{-/-}$ mice caused growth retardation after E8.75 and death by E10.5. Neurulation and development of the intraembryonic vascular system appeared normal but death at E10.5 prevented analysis of the later role of *scl*. Although endothelial capillary spaces were present in $scl^{-/-}$ yolk sacs, large vitelline vessels did not form. The fusion of capillary spaces to form large vitelline vessels may be induced, at least in part, by mechanical stresses after the onset of circulation (30). The absence of these

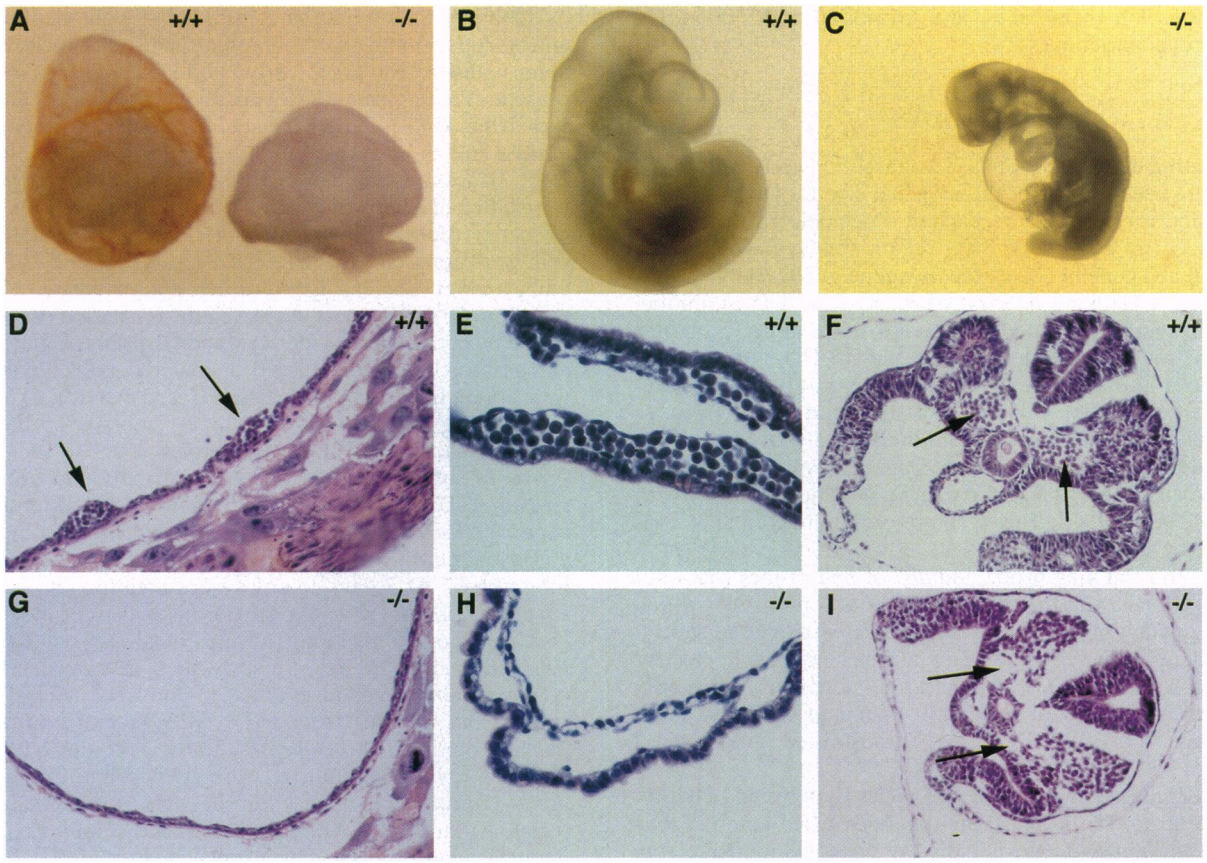

FIG. 2. Appearance and histology of *scl* null embryos. (*A*) E9.5 yolk sacs from wild-type (+/+) and *scl* null mice (−/−). (×10.) (*B* and *C*) E9.5 wild-type and *scl* null embryos. (×25.) (*D* and *G*) Section through the decidua of wild-type and $scl^{-/-}$ E9.0 embryos demonstrating the flattened yolk sac endoderm and absence of blood islands (arrows) in the mutant. (×90.) (*E* and *H*) Yolk sac of wild-type and $scl^{-/-}$ E9.0 embryos. (×180.) (*F* and *I*) Caudal transverse section through wild-type and $scl^{-/-}$ embryos. Blood is present in the dorsal aortae (arrows) of the wild-type embryo but is absent from the $scl^{-/-}$ embryo. The space between the somites and neural tube is artefactual. (×27.)

vessels in the yolk sacs of $scl^{-/-}$ embryos may be due to their developmental failure.

The phenotype of $scl^{-/-}$ embryos is similar to that seen in embryos homozygous for a null mutation of *rbtn2*. Erythroid cells failed to form in the $rbtn2^{-/-}$ yolk sac, and the embryos died by E10.5 (31). *rbtn2* encodes a LIM-domain transcription factor and, like *scl*, is involved in a recurrent translocation in T-ALL (32). Complex formation between rbtn2 and scl has been observed *in vivo* (33, 34). The demonstration of a critical role for both these genes in primitive erythroid development suggests that this complex may have a role in specification of this lineage. In cultures of $rbtn2^{-/-}$ yolk sacs, however, macrophage colonies were present. The absence of all hematopoietic colony types in cultures of $scl^{-/-}$ yolk sacs suggests that *scl* may have an earlier role in the hierarchy of hematopoietic cell differentiation.

Members of the GATA family of genes, which encode zinc finger transcription factors, are expressed in hematopoietic cells very early in development (35). GATA-1 and *scl* expression are strikingly similar (8–10). Moreover, activation of the *scl* promoter by GATA-1 has been demonstrated (15, 36). GATA-1-null ES cells failed to generate erythroid cells in chimeric mice (37, 38), and erythroid colonies formed by GATA-1-null ES cells *in vitro* died prior to hemoglobinization (25). Other hematopoietic lineages were normal (37, 38). Mice bearing a null mutation for GATA-2 died between E10 and E11 and had quantitative defects in yolk sac and fetal liver hematopoiesis. Yolk sac cultures from GATA-$2^{-/-}$ embryos showed a reduction of all colony types, and GATA-$2^{-/-}$ ES cells failed to contribute to later stages of hematopoiesis (39). The hematopoietic defect in $scl^{-/-}$ embryos appears earlier than that

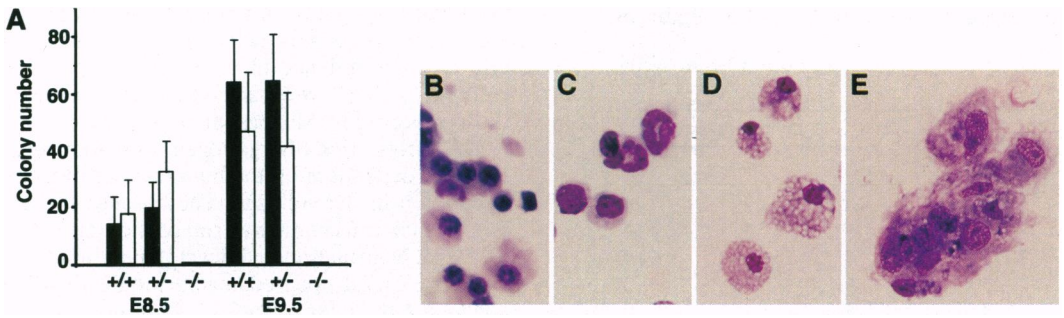

FIG. 3. Culture of yolk sacs from wild-type and *scl* null mice. (*A*) Numbers of erythroid (black bars) and nonerythroid (open bars) colonies present at 7 days in cultures of yolk sac cells from E8.5 and E9.5 wild-type (+/+), heterozygous (+/−), and *scl* null (−/−) mice. Standard deviations are shown. (*B–D*) Morphology of cells from 7-day cultures of wild-type yolk sac cells, stained with May-Grunwald/Giemsa stain: *B*, erythroid; *C*, neutrophilic; and *D*, macrophage. (*E*) Endodermal-type cells from a rare cell aggregate from an $scl^{-/-}$ yolk sac culture. (*B–E*, ×360.)

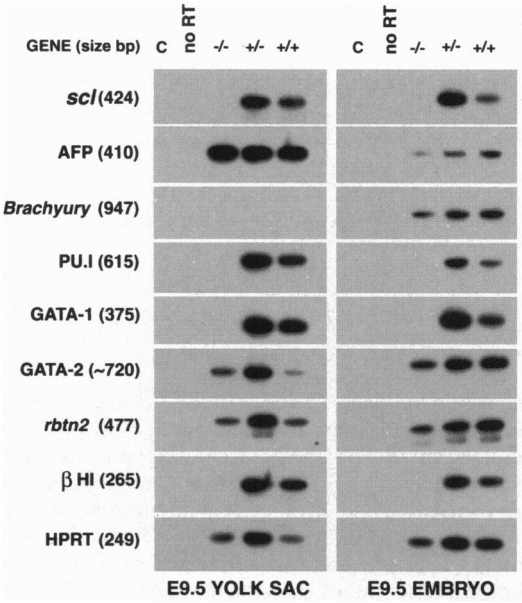

FIG. 4. Expression of genes in wild-type, heterozygous, and *scl* null E9.5 yolk sacs and embryos. RT-PCR was performed on RNA extracted from wild-type (+/+), heterozygous (+/−), and *scl*-null (−/−) yolk sacs and embryos. The amplified product and its size are indicated. C, product from a mock PCR (no cDNA added); no RT, product from PCR with an RNA sample but with reverse transcriptase omitted.

seen with either GATA-1-null ES cells or GATA-$2^{-/-}$ embryos, suggesting that the *scl* gene product may act upstream of the GATA transcription factors during yolk sac hematopoiesis.

We examined $scl^{-/-}$ yolk sacs and embryos for expression of several genes believed to play a role in early hematopoiesis. As expected from the phenotype, expression of βH1 (encoding embryonic z globin) was absent from $scl^{-/-}$ samples. Expression of GATA-1 was also absent from E9.5 $scl^{-/-}$ yolk sacs and embryos, further suggesting that *scl* may be acting upstream of GATA-1. Expression of GATA-2 and *rbtn2* was detected, but additional studies are required to establish the cell types in which these genes are expressed. PU.1, a transcription factor implicated in myeloid and lymphoid lineage commitment during fetal development (8), was not expressed in the $scl^{-/-}$ samples.

While the complex interrelationships of the multiple transcription factors believed to direct hematopoietic commitment and differentiation remain to be elucidated, gene targeting experiments have defined a role for *scl* in the earliest steps of commitment to the hematopoietic lineage. This is analogous to the role of other b-HLH transcription factors in developmental commitment processes (e.g., see ref. 22). Delineating the mechanisms of *scl* induction in extra-embryonic mesoderm may offer insights into the origins of the earliest hematopoietic stem cells.

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