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# SCL specifies hematopoietic mesoderm in *Xenopus* embryos

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## SUMMARY

**Targeted gene disruption experiments in the mouse have demonstrated an absolute requirement for several transcription factors for the development of hematopoietic progenitors during embryogenesis. Disruption of the basic helix-loop-helix gene SCL (stem cell leukemia) causes a block early in the hematopoietic program with defects in all hematopoietic lineages. To understand how SCL participates in the organogenesis of blood, we have isolated cDNAs encoding *Xenopus* SCL and characterized the function of SCL during embryogenesis. We demonstrate that SCL is expressed in ventral mesoderm early in embryogenesis. SCL expression is induced by BMP-4, and a dominant negative BMP-4 receptor inhibits SCL expression in the ventral region of the embryo. Expression of SCL in either bFGF-treated animal pole explants or dorsal marginal zone explants leads to the expression of globin protein. Furthermore, over-expression of SCL does not alter normal dorsal-ventral patterning in the embryo, indicating that SCL acts to specify mesoderm to a hematopoietic fate after inductive and patterning events have occurred. We propose that SCL is both necessary and sufficient to specify hematopoietic mesoderm, and that it has a similar role in specifying hematopoietic cell fate as MyoD has in specifying muscle cell fate.**

Key words: *Xenopus*, SCL, Hematopoiesis Mesoderm, Cell fate

## INTRODUCTION

The term hematopoiesis describes the induction, proliferation and differentiation of ventral mesoderm to form blood (Zon, 1995). During vertebrate development there are two distinct phases of hematopoiesis, termed primitive and definitive. Primitive hematopoiesis occurs first and produces predominantly erythrocytes (red blood cells). Primitive erythrocytes are produced exclusively in the ventral blood island (VBI) of *Xenopus* (the functional equivalent of the visceral yolk sac of higher vertebrates) and are morphologically and biochemically distinct from definitive erythrocytes. Definitive blood cells, representing all blood cell lineages, are formed later in development and arise predominantly from the dorsal or AGM (aorta-gonads-mesonephros) region (Kau and Turpen, 1983; Cumano et al., 1996; Medvinsky and Dzierzak, 1996; Turpen et al., 1997). Recent studies have shown that both primitive and definitive hematopoietic cell populations are derived from a common pool of mesoderm cells on the ventral axis of the early gastrula (Turpen et al., 1997).

The induction of the blood program is thought to be initiated by soluble growth factors such as bone morphogenetic protein (BMP-4). Ectopic expression of BMP-4 in the developing *Xenopus* embryo leads to ventralization characterized by lack of notochord, decreased muscle, and increased blood formation. Animal pole ectoderm explanted from BMP-4 loaded embryos expresses globin mRNA, suggesting that BMP-4 can directly induce hematopoietic stem cells (Dale et al., 1992; Jones et al., 1992). Injection of a dominant negative BMP-4 receptor results in dorsalized embryos with decreased blood formation (Graff et al., 1994; Maeno et al., 1994; Suzuki et al., 1994). Several targets of BMP-4 signaling have been identified, including Egr-1, GATA-1, GATA-2, Mix.1, Msx-1, Msx-2, PV-1, Smad1, Vox-1, Xbr-1, Xom, Xvent-1 and Xvent-2 (Vainio et al., 1993; Gawantka et al., 1995; Re'em-Kalma et al., 1995; Ault et al., 1996; Chen et al., 1996; Ladher et al., 1996; Lagna et al., 1996; Liu et al., 1996; Maeno et al., 1996; Mead et al., 1996; Onichtchouk et al., 1996; Papalopulu and Kintner, 1996; Schmidt et al., 1996; Maeda et al., 1997; Wilson et al., 1997; Xu et al., 1997). The homeodomain protein Mix.1 is induced by BMP-4, and expression of a Mix.1 dominant negative construct can partially rescue BMP-4 induced ventralization (Mead et al., 1996). Genes acting downstream of Mix.1 are likely to participate in the specification of hematopoietic mesoderm.

The zinc finger transcription factors GATA-1 and GATA-2 are early markers of hematopoietic mesoderm (Kelley et al., 1994). GATA-1 expression is restricted to hematopoietic cells during embryogenesis. In *Xenopus*, GATA-1 can be detected by in situ hybridization in the late neurula (stage 25). GATA-1 expression is initially detected by RT-PCR at mid-gastrula (stage 11), and expression increases in ventral mesoderm as blood islands form (Kelley et al., 1994). GATA-2 is present as a maternal RNA (at a very low level) and is expressed as a zygotic gene at stage 11 (Zon et al., 1991). GATA-2 is more

widely expressed along the ventral axis than GATA-1 and is also expressed in the ventral sensorial ectoderm and lateral mesoderm (Kelley et al., 1994 ; Walmsley et al., 1994). Animal pole explants express GATA-1 and GATA-2 mRNA, and the timing of expression coincides with the expression in the whole embryo (Kelley et al, 1994). BMP-4 induces the expression of GATA-1 and GATA-2, suggesting that these genes function downstream of events that affect ventral mesoderm induction (Maeno et al., 1996; Zhang and Evans, 1996).

SCL (stem cell leukemia), a basic helix-loop-helix transcription factor, is an early marker of hematopoietic cells in the vertebrate embryo. The human SCL gene was originally identified as a result of a chromosomal translocation in T cell leukemias (Begley et al., 1989a,b; Aplan et al., 1990a; Bernard et al., 1991; Chen et al., 1990a,b; Begley et al., 1991; Visvader et al., 1991a). SCL binds DNA as a heterodimer with E12 or E47 (to an E-box site; CANNTG). Other proteins also interact with SCL (Hsu et al., 1991). The lim-domain protein LMO-2 (TTG-2/rhombotin-2) has been shown to heterodimerize with SCL, and the dimerization regulates the ability of SCL to transactivate minimal reporter constructs (Wadman et al., 1994). During normal hematopoiesis, SCL is expressed in erythroid cells, mast cells, megakaryocytes, early CD34+ hematopoietic progenitors, but not T-cells. (Aplan et al., 1990b; Green et al., 1991; Visvader et al., 1991b). This is similar to the lineage-restricted expression pattern of GATA-1 and GATA-2. SCL may have a role in erythroid maturation since the level of SCL increases about 10-fold in murine erythroleukaemia cells induced to differentiate by treatment with DMSO (Green et al., 1991; Begley et al., 1989a; Visvader et al., 1991b). The 5′ regulatory region of the SCL gene contains two functional promoters. It is unclear whether the two promoters are differentially utilized in distinct tissues. The proximal SCL promoter contains a GATA site in promoter Ia at –34 to –31 (Aplan et al., 1990b; Lecointe et al., 1994; Bockamp et al., 1995) and studies have shown that GATA-1 and GATA-2 can each transactivate this SCL promoter in heterologous cells, suggesting that SCL is an early developmental target of GATA-1 or GATA-2 activation (Aplan et al., 1992).

Targeted gene disruption experiments have highlighted a requirement for both SCL and LMO-2 during normal erythroid maturation (Warren et al., 1994; Orkin, 1995; Robb et al., 1995; Shivdasani et al., 1995; Green, 1996; Porcher et al., 1996; Robb et al., 1996; Elefanty et al., 1997). Both the SCL and LMO-2 deficient mice show defects in hematopoiesis. Other studies utilizing dominant negative SCL constructs have demonstrated a requirement for the factor in the spontaneous differentiation of erythroid cell lines (Aplan et al., 1992). Despite the observation that SCL is required for normal hematopoiesis, the mechanism by which SCL regulates blood formation early in development remains to be determined.

We have isolated the *Xenopus* homologs of SCL and demonstrated that SCL is an early marker of blood formation on the ventral axis. Untreated animal pole explants did not express SCL, suggesting that the regulation of SCL expression is different from that of GATA-1 and GATA-2. Over-expression of SCL was not sufficient to induce hematopoiesis in animal pole ectoderm. However, in the presence of a mesoderm inducer such as bFGF, SCL did direct mesoderm to a hematopoietic fate. Ectopic expression of SCL on the dorsal axis lead to globin protein expression in dorsal marginal zone explants, but did not interfere with the formation of dorsal structures. This indicates that SCL acts downstream of embryonic patterning events to direct blood formation in mesodermal cells.

## MATERIALS AND METHODS

### Embryological methods

Wild-type and albino *Xenopus* embryos were harvested, dejellied and cultured as described by Newport and Kirschner (1982). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Animal pole explants were dissected at stage 7-8. Recombinant human basic fibroblast growth factor (Boehringer Mannheim Biochemicals) was used at a final concentration of 50 ng/ml. Activin (kindly provided by Genentech) was used at a dose of 100 pM final concentration for 1 hour in standard culture conditions (0.5× MMR pH 7.6, 0.5 mg/ml bovine serum albumin and penicillin G (100 U/ml; Sigma) and streptomycin (100 μg/ml; Sigma)). After treatment, the explants were transferred to fresh culture medium without added growth factors and cultured at 21ºC until they reached the desired stage. Marginal zone explants were dissected at stage 10.25 as described by Kelley et al. (1994). Ten to 12 marginal zone explants or animal pole explants were harvested for each experimental group.

### Cloning and sequencing of the *Xenopus* SCL cDNA

A randomly primed cDNA library in λZAP-II made from *Xenopus* tadpole red blood cells was screened using a $^{32}$P-labeled (random primed) murine SCL cDNA probe. Positive cDNA clones were sequenced by the dideoxynucleotide chain termination method and sequence analysis was performed using the Wisconsin GCG computing package. Gene Inspector (Textco, Inc.) was used to generate the peptide sequence alignment diagram.

### Generation of ΔSCL

A mutant of the full length *Xenopus* SCL (*SCL*) gene lacking the DNA-binding basic domain was prepared by PCR overlap mutagenesis (Ho et al., 1989). Primers used to generate this mutant were ΔBF 5′-GAAGGTCCCCAGCCAAAACAGCAGAACGTA-AACGGG-3′ and ΔBR 3′-CCCGTTTACGTTCTGCTGTTTTGGCT-GGGGACCTTC-3′. This mutant will not bind DNA.

### In vitro transcription and micro-injection

Full length SCL and the truncated mutant, SCLΔB, were subcloned into the *Xenopus* RNA expression vector pGEM-HE which contains 5′- and 3′-untranslated sequence from *Xenopus* adult β-globin mRNA. Synthetic mRNA was prepared from linearized plasmid DNA using mMessage mMachine in vitro transcription kits (Ambion). RNA yield was quantitated spectrophotometrically. The integrity of the in vitro transcribed RNA was determined by agarose gel electrophoresis in the presence of formaldehyde. Injection of *Xenopus* embryos was as previously described (Smith and Harland, 1992).

### Whole embryo in situ analysis and immunohistochemistry

In situ analysis using labeled probes was performed as described by Harland (1991); Hemmati-Brivanlou et al. (1990a). Sense and antisense SCL RNA probes were in vitro transcribed in the presence of digoxigenin-labeled rUTP (Boehringer Mannheim Biochemicals). Whole embryo immunohistochemistry was performed, using a monoclonal antibody (L4-27) directed against tadpole α-globin at 1/40 (v/v), as described (Hemmati-Brivanlou et al., 1990b).

### RT-PCR assay

RNA extractions, first strand cDNA synthesis and PCR analysis was performed as previously described (Kelley et al., 1994). PCR primer sets and conditions for EF-1α and αT3 globin were as described (Kelley et al., 1994). PCR conditions were determined for each primer set to ensure that amplification was in the exponential range. PCR primers for SCL were forward; 5′-CACCGAGACCCCCAGCTGAAC-3′ and reverse; 5′-GTCGTCTTGCATTTGCCCCGT-3′.

### Western blot analysis

Embryonic explants and sibling whole embryos were homogenized in SDS-PAGE sample buffer containing 2-mercaptoethanol and denatured by heating at 100°C for three minutes. Samples were loaded on a 15% (w/v) polyacrylamide-SDS gel. Proteins were transferred to nitrocellulose and probed using standard procedures (Kelley et al., 1994). The anti-tadpole α-globin monoclonal antibody (L5-41) was used at a 1/10,000 dilution. Secondary, alkaline phosphatase-conjugated goat anti-mouse FAB was used according to manufacturers specifications (Promega). Western blots were developed with BM Purple alkaline phosphatase substrate (Boehringer Mannheim Biochemicals).

## RESULTS

### Isolation of *Xenopus* SCL

The basic helix-loop-helix (bHLH) domain of murine SCL (Begley et al., 1991) was used to screen a randomly primed *Xenopus* larval peripheral blood cDNA library in the λZAP-II vector (Stratagene). 500,000 plaques were screened, a total of 13 positives were isolated and plaque purified to obtain single cDNA clones. The cDNA inserts were rescued with helper-phage, and the excised plasmids were sequenced. Two independent cDNAs were obtained of the full-length clone. Sequence differences in other partial cDNAs that were obtained are likely to represent the presence of another allele based on the pseudo-tetraploid nature of *Xenopus laevis* genome.

The predicted *Xenopus* SCL amino acid sequence is similar to that of the chicken (82% similarity), mouse (71%) and human (71%) homologues (Fig. 1) (Begley et al., 1989a,b; Aplan et al., 1990b; Chen et al., 1990a,b; Begley et al., 1991). The basic helix-loop-helix domain is highly conserved between these species (100% identity between frog, chicken, mouse and human; Fig. 1). More differences are noted in the N terminus, in particular, *Xenopus* SCL has a repeat (consensus: ($^{T}/_{S}$)ELCR($^{A}/_{P}$)P) which is represented eight times in the frog protein. The significance of this repeat is not known.

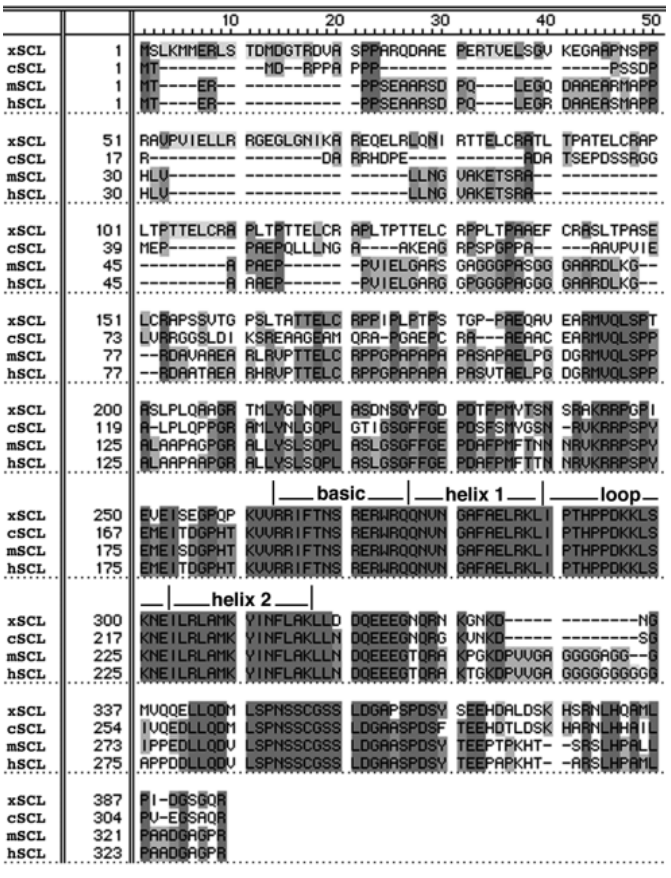


**Fig. 1.** *Xenopus* SCL is very similar to the SCL proteins found in higher vertebrates. *Xenopus* (xSCL), chicken (cSCL), mouse (mSCL) and human (hSCL) SCL amino acid sequences were aligned using Gene Inspector (Textco Inc., NH). The alignment highlights the extensive sequence identity shared by these vertebrate counterparts in the DNA-binding region which is labeled 'basic-helix 1-loop-helix 2′. The nucleotide sequence of the *Xenopus* SCL gene has been submitted to GenBank; accession no. AF060151.

### Expression pattern of SCL

RT-PCR analysis was used to detect SCL RNA during *Xenopus* development. As demonstrated in Fig. 2, SCL mRNA is detected at stage 12/13 of embryogenesis, closely following the initial expression of the GATA-binding proteins, GATA-1 and GATA-2, during gastrula stages (Zon et al., 1991). SCL expression increases as the tadpole develops. SCL expression is first detected by whole embryo in situ analysis at stage 15 in a small interspersed patch of cells in the ventral-most region of the embryo (Fig. 3a-l). This is the region which later gives rise to the ventral blood island (VBI) and is functionally equivalent to mammalian yolk sac blood islands (Kau and Turpen, 1983; Turpen et al., 1997). By late neurula stages, the ventral expression of SCL extends rostral in an almost rectangular shape, excluding a small inner area in the region of the liver anlage (Fig. 3c). As development proceeds, SCL expression expands laterally and caudally, forming a V-shaped pattern characteristic of the developing VBI (Fig. 3g,h,i). Double staining for SCL and GATA-2 demonstrates that SCL expression is limited to a subset of GATA-2-staining cells on the ventral axis during neurula and early tailbud stages (Fig. 3n,o). GATA-2 expression spans the entire ventral and ventral-lateral axis of the neurula (Fig. 3n, turquoise staining). As determined by morphology and hematopoietic molecular

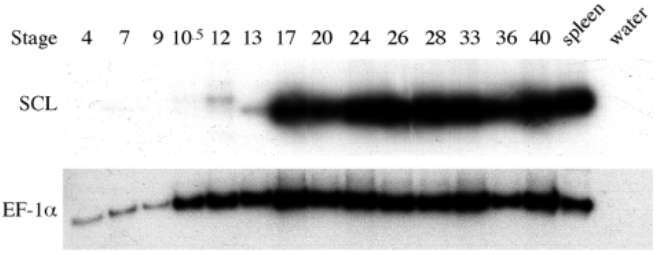


**Fig. 2.** SCL expression during *Xenopus* development analysed using reverse transcriptase-PCR (RT-PCR). RNA was prepared from embryos harvested at the developmental stages indicated (Nieuwkoop and Faber, 1967). Radiolabeled SCL and EF-1α sequences were amplified from first strand cDNA and separated on polyacrylamide gels. SCL transcripts are first expressed at early neurula stages (stage 13). Expression increases as development proceeds and remains at a high level throughout the life span of the frog. The SCL transcript is abundant in adult spleen, a site of hematopoiesis in *Xenopus*.

markers (such as globin and GATA-1), the VBI matures in an anterior to posterior wave (Kelley et al., 1994 and references therein). Consistent with this, SCL expression extends posteriorly in the VBI as development proceeds. SCL expression also extends rostral to the presumptive VBI in two lines that cross at the midline in the region of the developing heart (Fig. 3f,i). This results in a patch of cells anterior to the presumptive VBI that do not express SCL and may represent the liver anlage. The cells that form the two lines of SCL expression rostral to the VBI do not express GATA-1 or globin (Kelley et al., 1994). The anterior pattern of SCL expression is strikingly similar to that of flk-1, a marker of vascular endothelial precursors (Cleaver et al., 1997), and thus may represent a population of cells with vasculogenic potential. In support of this notion, SCL RNA and protein have been detected in avian and mammalian vascular endothelial precursors and angioblasts (Hwang et al., 1993; Kallianpur et al., 1994; Drake et al., 1997). Recent studies in zebrafish have shown that ectopic expression of SCL will partially rescue both the hematopoietic and vasculogenic defects in the *cloche* mutant (Liao et al., 1998). Furthermore, transgenic rescue of hematopoietic defects of *SCL–/–* mouse embryos has revealed a requirement for SCL in the remodeling of yolk sac vessels (Visvader et al., 1998). SCL expression (at stage 26) is broader than that of GATA-1 (Kelley et al., 1994). This may indicate that cells in the most ventral region of the VBI are committed to becoming primitive erythrocytes, while cells situated more laterally are maintained as multipotential progenitors which will later contribute to definitive hematopoiesis (Turpen et al., 1997). SCL expression intensifies as the ventral blood island forms (Fig. 3k). After circulation begins, hematopoietic cells leave the ventral blood island and SCL expression is gradually diminished in this region (Fig. 3l,m).

SCL is also expressed in the dorsal mesentery (AGM) of the embryo adjacent to the pronephric ducts in a similar pattern to GATA-2 (Kelley et al., 1994) and GATA-3 (Kelley, 1995) (Fig. 3j). SCL staining in this region is first detected at stage 25. GATA-3 is expressed in and adjacent to the pronephric ducts (Kelley and Zon, unpublished data). The expression of hematopoietic transcription factors in this region may reflect the initiation of the dorsal (definitive) hematopoietic program in these cells. Transplantation studies have demonstrated that hematopoietic progenitors reside in this location (Kau and Turpen, 1983; Turpen et al., 1997). As development proceeds, expression of SCL

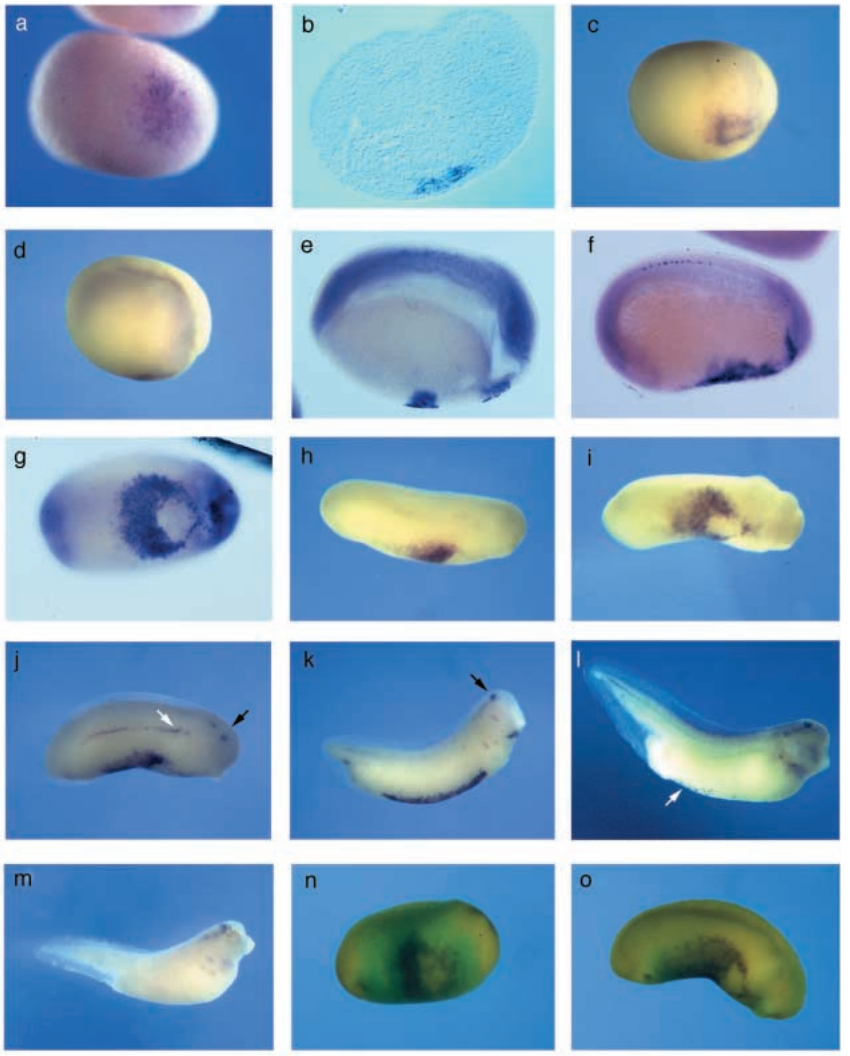


**Fig. 3.** Whole embryo in situ analysis of SCL expression. (a) Ventral view. SCL is first detected at stage 15 in the ventral region of the embryo. (b) Cross section of a stage 16 embryo indicates that SCL staining is present in ventral mesoderm and ectoderm. (c and d) Ventral and lateral view, respectively, of a stage 20 embryo. SCL staining increases as development proceeds. (e-g) Embryos cleared with benzyl alcohol/benzyl benzoate (1:2 v/v) to reveal staining within the embryo. (e) Stage 22. (f and g) Stage 24. (f) Staining is evident in individual spinal neurons. (g) SCL staining demarcates the ventral blood island (VBI) and extends rostrally in two stripes that cross at the mid-line. These stripes may represent vascular endothelial precursors. Staining is absent from the region of the developing liver which demarcates the anterior boundary of the ventral blood island. (h,i) Lateral and ventral-lateral views, respectively, of a stage 25 embryo. SCL staining extends caudally as the embryo grows and the VBI increases in size. (j) Stage 27; SCL staining is evident in the AGM (aorta-gonads-mesonephros) region (white arrow) and most likely indicates the development of a population of definitive hematopoietic stem cells in this region. SCL staining in neural tissue of the brain is evident at this stage (black arrow). (k) At stage 32, SCL staining in the VBI is very intense. Although neural staining (particularly at the midbrain-hindbrain boundary; arrow) is still evident, staining in the AGM region has diminished. (l) Stage 37/38; as circulation begins, blood cells leave the VBI and enter circulation and SCL staining in this region decreases. Note that the most posterior cells in the VBI are still present (white arrow). (m) Stage 40; all SCL positive cells in the VBI have entered circulation. (n,o) Double staining for SCL (purple) and GATA-2 (turquoise). (n) Double stained embryo at stage 24; SCL expression is limited to a subset of GATA-2-expressing cells on the ventral axis of the embryo. (o) Stage 25; SCL expression persists but GATA-2 expression decreases as the cells within the VBI differentiate (see text for details).

(Fig. 3k) and GATA-2 (Kelley et al., 1994) decreases in the dorsal mesentery. GATA-3 expression continues and marks the migration of presumptive hematopoietic stem cells to the region of the ducts of Cuvier (Kelley and Zon, unpublished data).

SCL expression in neural tissue has been reported in the mouse (Green et al., 1992). During late neurula stages in *Xenopus*, SCL expression is detected in the central nervous system (Fig. 3f,j-m). This includes individual spinal neurons and cells in the midbrain-hindbrain boundary. SCL expression in neural tissue is similar to that of GATA-2 and GATA-3 which are also expressed in the central nervous system (Kelley et al., 1994; Walmsley et al., 1994; Kelley and Zon, unpublished data).

### BMP-4 induces expression of SCL

Animal pole explant assays were used to investigate the induction of SCL expression in primitive ectoderm. Animal caps were cultured in the presence or absence of growth factors and SCL expression was assayed by RT-PCR analysis. SCL mRNA was not expressed in untreated animal pole explants during the culture period (Fig. 4a). This is in sharp contrast to GATA-1 and GATA-2, which are expressed in these primitive ectodermal cells (Kelley et al., 1994; Zhang and Evans, 1996) and indicates that the regulation of SCL expression is distinct from that of GATA-1 and -2. Treatment of animal pole explants with activin (100 pM) did not induce SCL expression. Treatment with bFGF (at 50 ng/ml) induced a barely detectable level of SCL. Animal pole explants harvested from embryos injected with BMP-4 RNA (1 ng) expressed abundant SCL mRNA. Over-expression of BMP-4 in the whole embryo leads to severe axial abnormalities and the resulting ventralized embryos have excessive blood formation (Dale et al., 1992; Jones et al., 1992; data not shown). Mix.1, a PAX class homeodomain protein, is a target of BMP-4 signaling and over-expression of Mix.1 results in a phenotype very similar to that of BMP-4 ventralized embryos (Mead et al., 1996). To determine the effect of ectopic BMP-4 signaling on SCL expression we performed in situ hybridization on BMP-4- or Mix.1-injected embryos. Injection of either BMP-4 or Mix.1 mRNA resulted in expanded SCL expression in the ventralized

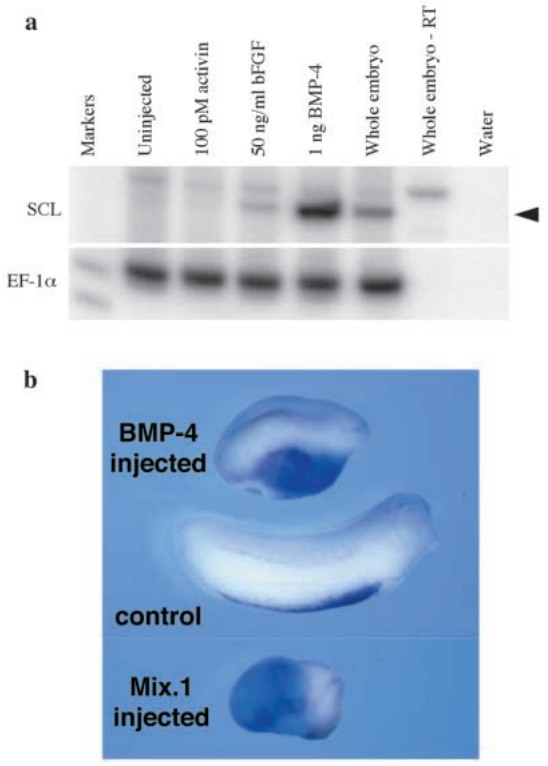


**Fig. 4.** SCL expression responds to BMP-4 signaling. (a) BMP-4 induces SCL expression in primitive ectoderm. Embryos were injected with BMP-4 mRNA (1 ng per embryo in the animal pole region). Animal pole explants were removed at stage 8 and treated with soluble growth factors (activin at 100 pM (Genentech); human basic FGF at 50 ng/ml; Gibco BRL) and cultured to mid-neurula stage (stage 19). Sibling whole embryo was used as a positive control. Whole embryo −RT (without reverse transcriptase) was used as a negative control. SCL and EF-1α sequences were amplified from first strand cDNA, separated on polyacrylamide gels and visualized by autoradiography. (b) Ectopic expression for BMP-4 or Mix.1 induces SCL expression in the whole embryo. Embryos were injected with either BMP-4 or Mix.1 mRNA (500 pg per blastomere at the two cell stage) and cultured to sibling tailbud stage. SCL expression was assayed by whole-mount in situ hybridization. Expression of either BMP-4 or Mix.1 caused ventralization and increased expression of SCL.

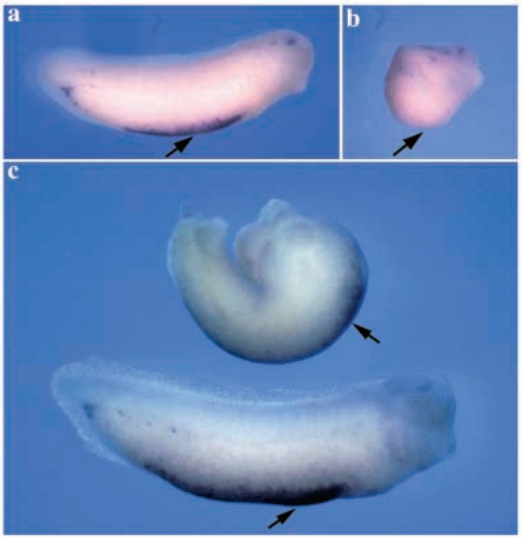


**Fig. 5.** Ventral expression of SCL is blocked by a dominant negative BMP receptor but not by a dominant negative FGF receptor. (a,b) Expression of a dominant negative BMP receptor blocks SCL expression on the ventral axis. Embryos were injected at the two cell stage with 1 ng dnTFRII. Injected and uninjected sibling embryos were fixed at stage 32 and stained for SCL expression by in situ hybridization. SCL expression on the ventral axis (arrows) is ablated by injection of dominant negative BMP receptor mRNA. (c) Expression of a dominant negative FGF receptor does not block ventral expression of SCL. Embryos were injected with mRNA encoding dominant negative FGF receptor (XFD; upper embryo; *n*=73) or a truncated mutant of XFD lacking part of the ligand binding domain (D50; lower embryo; *n*=51) (2.5 ng per blastomere at the two cell stage; Amaya et al., 1991). Embryos were fixed at the tailbud stage and analyzed for SCL expression by in situ hybridization. Although trunk and tail abnormalities exist, the SCL expression on the ventral axis (arrows) was not affected by over-expression of the dominant negative FGF receptor.

embryos (Fig. 4b). These data suggest that SCL is a target of the BMP-4 signaling cascade.

To evaluate the effects of blocking BMP-4 signaling on SCL expression in the context of the whole embryo, a dominant negative BMP-4 receptor (dnTFRII; Maeno et al., 1994; Suzuki et al., 1994) was injected at the one or two cell stage. Whole embryo in situ hybridization analysis for SCL expression was performed at stage 32 (Fig. 5a,b). Expression of the dominant negative BMP-4 receptor lead to dorsalized embryos (Graff et al., 1994; Maeno et al., 1994; Suzuki et al., 1994). SCL expression in the ventral region of these embryos was completely ablated (*n*=12; Fig. 5b). SCL expression in neural tissue on the dorsal side of the embryo persisted. Animal pole explants treated with bFGF express low levels of SCL (Fig. 4a). To determine whether expression of a dominant negative FGF receptor (XFD) effects SCL expression we injected embryos with XFD mRNA and with a control construct, D50 (Amaya et al., 1991). Embryos were cultured to tailbud stage and examined for SCL expression by in situ hybridization. The axial abnormalities in XFD-injected embryos were consistent with published data; however, expression of this dominant negative FGF receptor did not block the expression of SCL in these animals (*n*=73). The ablation of SCL expression on the ventral axis by a dominant negative BMP-receptor confirms that SCL activation in ventral mesoderm requires BMP signaling.

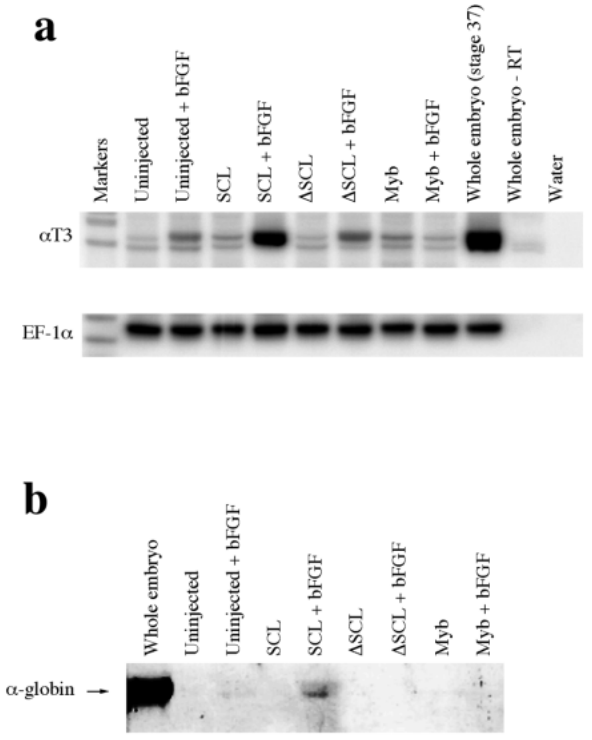


**Fig. 6.** SCL induces globin mRNA expression in animal pole explants treated with bFGF. (a) SCL-loaded animal pole explants treated with bFGF express globin mRNA. Embryos at the one cell stage were injected with 1 ng of either full length SCL, truncated SCL (ΔSCL; a mutant construct lacking the basic domain which will not bind DNA) or *Xenopus* c-myb. Animal caps were explanted at stage 8 and cultured with or without human bFGF (50 ng/ml) to control stage 36/37. RNA prepared from explants and whole embryos was analyzed by RT-PCR for tadpole α-globin (αT3). Levels of EF-1α were determined as an RNA recovery control. (b) SCL-loaded animal pole explants treated with bFGF express globin protein. Western blot analysis for globin protein was performed on animal pole explants injected and treated as described above. Primary monoclonal antibody was anti-tadpole α-globin L4-51.

## SCL specifies hematopoietic mesoderm

Gene disruption experiments in the mouse have defined SCL as a critical factor for the generation of all hematopoietic lineages (Robb et al., 1995; Shivdasani et al., 1995; Porcher et al., 1996; Robb et al., 1996; Elefanty et al., 1997). In the absence of SCL, no hematopoietic progenitors are formed, and embryos null for SCL die early in development. Since SCL is expressed in the region of the early neurula that will form the VBI, we speculated that SCL was involved in the commitment of ventral mesoderm to hematopoiesis. To test this hypothesis, SCL or control mRNAs were injected into the animal pole of embryos at the one cell stage. Animal caps (embryonic ectoderm) were dissected at stage 8 and cultured with or without human basic fibroblast growth factor (bFGF) and grown until control stage 36. Messenger RNA prepared from the animal pole explants was analyzed by RT-PCR for globin expression. As demonstrated in Fig. 6a, SCL induced globin mRNA in the presence of basic bFGF but not when cultured without the growth factor. The presence of globin protein was demonstrated by western blot in SCL-injected animal cap cells that had been treated with bFGF; control explants did not express globin protein (Fig. 6b). These data indicate that SCL alone does not induce hematopoietic cells from embryonic ectoderm, but is capable of specifying bFGF-induced mesoderm to a hematopoietic fate. The mutant SCL protein (ΔSCL), lacking the DNA-binding basic domain, was not capable of directing mesoderm to a hematopoietic fate. Likewise the hematopoietic specific transcription factor c-myb did not induce globin mRNA synthesis. c-myb is believed to play a role in definitive hematopoiesis and thus may act later in development than the initial requirement for SCL in the hematopoietic program (Mucenski et al., 1991).

To examine whether SCL is capable of specifying dorsal-type mesoderm to a hematopoietic fate we utilized a marginal zone explant assay. Embryos at the two cell stage were injected with SCL mRNA and cultured to stage $10^+$, at which time dorsal and ventral marginal zones were dissected. The explants and sibling controls were cultured to tadpole stage 36/37 and assayed for globin protein by whole-mount immunohistochemistry or western blot analysis. Ectopic expression of SCL on the dorsal axis led to the production of globin protein in the dorsal marginal zone (DMZ) explant (21/22 DMZ explants expressed globin; Fig. 7). Control DMZ explants did not express globin (0/18 explants). The presence of globin protein in SCL-loaded dorsal marginal zone explants was confirmed by western blot analysis (data not shown). Over-expression of ventralizing agents such as Mix.1 (Mead et al., 1996) and BMP-4 (data not shown) also lead to the expression of globin in DMZ explants. Unlike Mix.1 and BMP-4, ectopic expression of SCL did not grossly perturb the normal dorsal-anterior patterning of the developing embryo. Similar to whole embryo studies, dorsal marginal zone explants expressing ectopic Mix.1 or BMP-4 adopted a ventral pattern and lacked characteristic dorsal structures such as the cement gland and

eye (Mead et al., 1996). In contrast, SCL-loaded DMZs maintained these dorsal structures yet expressed globin protein (Fig. 7c). Taken together with the timing of expression, these data indicate that SCL specifies hematopoietic stem cells after dorsal/ventral patterning events have occurred. Interestingly, sibling whole embryos injected with SCL, cultured to the same stage as DMZ explants, did not produce ectopic globin protein as detected by whole embryo immunohistochemistry (data not shown). This suggests that there are repressive signals present in the whole embryo that limit the activity of SCL.

## DISCUSSION

### SCL is a marker of early hematopoietic mesoderm

SCL is expressed on the ventral axis of the embryo in a region that will give rise to hematopoietic tissue. The expression of GATA-2 overlaps that of SCL but appears to be more diffuse, including the ventral sensorial ectoderm and mesoderm (Kelley et al., 1994; Walmsley et al., 1994). SCL expression is thus present in a subset of GATA-2 expressing cells along the ventral axis of the embryo. SCL is not detected in uninduced animal pole explants, where GATA-1 and -2 are expressed, indicating that these transcription factors are differentially regulated during development. Our recent transplantation studies demonstrate that the ventral and lateral cells of the early neurula, which express GATA-2, have the potential to contribute to hematopoiesis when transplanted to a permissive environment (VBI). However, at the time that SCL is expressed, the ventral cells are irreversibly committed to primitive hematopoiesis (Turpen et al., 1997). Targeted gene disruption experiments in mice have shown that both GATA-2 and SCL are essential for normal hematopoiesis but have distinct roles in stem cell biology. SCL is required for the formation of multipotential progenitors (Porcher et al., 1996) and GATA-2 is required for the proliferation of these progenitors (Tsai et al., 1994; Tsai and Orkin, 1997) (Fig. 7). Understanding their patterns of expression during embryogenesis and the regulation of their expression will have significant impact on the understanding of how the blood program is initiated.

Early in development, SCL is expressed in a ‘salt and pepper’ pattern, suggesting that there are cells within the zone of expression that do not express SCL. This may indicate a difference in the commitment of the cells in this region to the hematopoietic program. As development proceeds to late neurula stages, SCL expression is detected throughout the ventral blood island region. By tailbud stages, SCL expression extends in anterior stripes that cross at the midline. This anterior pattern may reflect SCL expression in vascular progenitor cells. The excluded region between the VBI and cardiac mesoderm may be a result of underlying hepatic endoderm.

### A role for SCL and other hematopoietic transcription factors during dorsal hematopoiesis

SCL, GATA-2 and GATA-3 are expressed in the dorsal mesentery of the tailbud stage embryo (Kelley, 1995; Turpen et al., 1997). This region, surrounding the pronephric duct, has been shown to contain hematopoietic stem cells that are fated to definitive hematopoiesis (Kau and Turpen, 1983). We propose that expression of SCL and the GATA-binding transcription factors may be indicative of hematopoietic precursor populations within this region. The expression of SCL and GATA-2 in the AGM region decreases as development proceeds, suggesting that hematopoietic stem cell development is suppressed. This may represent a mechanism by which the hematopoietic stem cells in this region can be maintained in an undifferentiated state to be used later in development. Thus, SCL expression anticipates the formation of blood cells in both ventral and dorsal sites of hematopoiesis.

### Regulation of SCL and GATA expression

Given the complexity of hematopoietic stem cell activation and the overlapping expression patterns of the transcription factors known to be required for hematopoiesis, it remains unclear how SCL and the GATA genes regulate and coordinate their expression and biological effects. It is possible that transactivation of *cis*-elements within the SCL and GATA genes by each of these DNA-binding proteins accounts for their apparent coordinate regulation. For instance, it is possible that SCL is a direct target of the GATA-binding proteins during early development. GATA-1, -2 and -3 are expressed during mid-gastrula stages in *Xenopus* earlier than SCL initiation (Zon et al., 1991; Kelley, 1995). The proximal promoter of the SCL gene has a GATA motif that is required for efficient transcription (Aplan et al., 1992). However, high levels of SCL expression precede the major activation of the GATA binding proteins in the VBI; GATA-1 is not evident in the VBI by in situ hybridization until stage 25 (Kelley et al., 1994). Furthermore, inactivation of the SCL gene in mice leads to a hematopoietic progenitor cell defect that is earlier than that detected for the GATA-1 and GATA-2 mutant mice, and mouse embryonic stem cells null for SCL express wild-type levels of GATA-2 and CD34 (a stem cell marker) but no GATA-1 (Elefanty et al., 1997). It is also possible that a cell-specific stabilization of transcriptional activity accounts for the coordinate regulation of SCL and GATA-binding proteins. In support of this, recent evidence indicates that SCL, E47, Ldb1/NL1, LMO2 and GATA-1 associate to form a large transcriptional complex that may regulate hematopoietic targets (Wadman et al., 1997).

### Specification and organogenesis

During development, early events regulate the induction and patterning of mesoderm along the dorsal-ventral axis of the embryo. Following these early events, mechanisms must exist that allow for the further specification of particular types of tissue during the process of organogenesis. Our studies indicate that over-expression of SCL does not perturb the normal axis of the embryo or alter the pattern of dorsal tissues within a dorsal marginal zone explant. This was surprising given our previous studies with ventralizing factors such as BMP-4 and Mix.1. Over-expression of these genes leads to a decrease in dorsal tissues in whole embryos and dorsal marginal zone explants. We conclude that SCL has the ability to specify hematopoietic tissue from dorsal or ventral mesoderm within the embryo. As BMP-4 has a role in the formation of ventral mesoderm, and SCL lies downstream of this growth factor in a BMP-4 responsive cascade, SCL is likely to function as a transcription factor for the initiation and specification of the blood program.

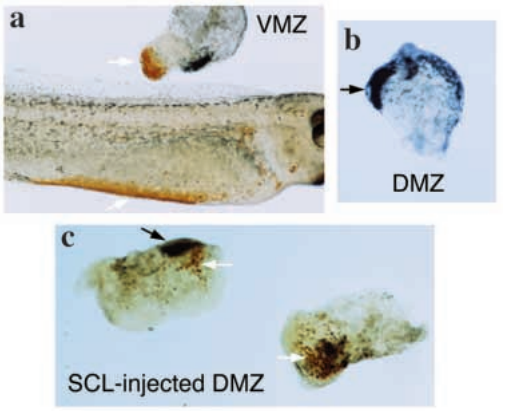


**Fig. 7.** Over-expression of SCL in dorsal mesoderm explants leads to ectopic globin production. Both blastomeres of embryos at the two cell stage were injected with 500 pg SCL, and cultured to stage 10.25 when marginal zone explants were prepared. Whole-mount immunohistochemistry for tadpole α-globin protein was performed when embryos and explants had reached stage 35/36. (a) Sibling, uninjected ventral marginal zone (VMZ; 18/18 explants were globin positive) and whole embryo. White arrows point to globin-staining cells in the VMZ and VBI of the tadpole. (b) Uninjected dorsal marginal zone (DMZ; 0% globin positive, *n*=18). Note the presence of dorsal anterior structures such as eye and cement gland (black arrow). (c) SCL-loaded DMZ explants had abundant blood (white arrows; 21/22 DMZ explants were globin positive). Dorsal anterior structures such as eye and cement gland (black arrow) persist.

SCL is a bHLH transcription factor with structural similarities to MyoD. Our data suggest that SCL plays a similar functional role in cell fate determination as MyoD. Over-expression studies indicate that bHLH proteins are capable of redirecting cell fate in the embryo. Ectopic expression of MyoD in fibroblast cell lines can convert them to myoblasts (Davis et al., 1987). MyoD can induce muscle directly in animal cap experiments and can convert ventral mesoderm to a muscle fate (Hopwood and Gurdon, 1990; Ludolph et al., 1994). Co-expression of the heterodimeric partner *Xenopus* E12 with MyoD augments the activation of muscle specific markers (Rashbass et al., 1992). Micro-injection of mRNA encoding chimeras of MyoD and SCL will be used to determine which domains in these proteins are essential for the specification of particular tissues.

Development of the hematopoietic system begins on the ventral side of the *Xenopus* embryo. Ventral mesoderm is induced and patterned early in development by inductive signals such as BMP-4. Subsequently, a subset of ventral mesoderm is specified to become hematopoietic stem cells. These cells then proliferate and differentiate into the various lineages of the hematopoietic system (Fig. 8). Our studies are complementary to the gene targeting experiments with SCL in mice and recent studies in zebrafish. Targeted disruption of the SCL gene demonstrates that it is required for normal hematopoietic progenitor formation during development. In *Xenopus*, we have shown that over-expression of SCL directly specifies hematopoietic stem cells in both FGF-treated ectoderm and dorsal mesoderm explants. Taken together with the expression data, this suggests that SCL acts in early ventral mesoderm to specify hematopoietic stem cells.

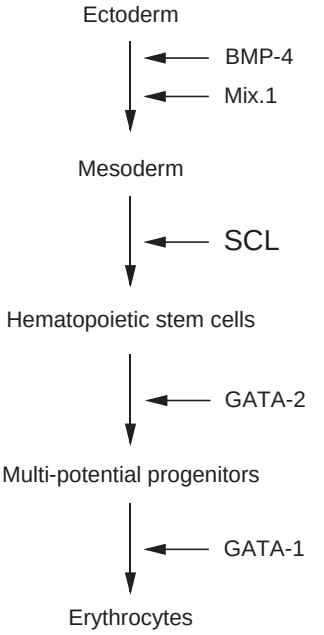


**Fig. 8.** Schematic model of hematopoiesis in vertebrates. The action of mesoderm inducing factors such as BMP-4 leads to the production of ventral mesoderm. Mix.1 is a target of BMP-4 signaling in ventral mesoderm (Mead et al., 1996). A subset of ventral mesoderm cells express SCL and develop into hematopoietic (and probably vasculogenic) stem cells. Stem cells give rise to a population of multipotential progenitors capable of differentiating into all blood lineages. GATA-2 is required for the maintenance and proliferation of this pool of progenitor cells (Tsai et al., 1994). Terminal differentiation of erythrocytes requires GATA-1 (Pevny et al., 1991; Fujiwara et al., 1996).

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