RESEARCH COMMUNICATION

SCL/Tal-1 transcription factor acts downstream of *cloche* to specify hematopoietic and vascular progenitors in zebrafish

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SCL/Tal-1 is a transcription factor necessary for hematopoietic stem cell differentiation. Although SCL is also expressed in endothelial and neural progenitors, SCL function in these cells remains unknown. In the zebrafish mutant *cloche* (*clo*), SCL expression is nearly abolished in hematopoietic and vascular tissues. Correspondingly, it was shown previously that *clo* fails to differentiate blood and angioblasts. Genetic analysis demonstrates that the *clo* mutation is not linked to the SCL locus. Forced expression of SCL in *clo* embryos rescues the blood and vascular defects, suggesting that SCL acts downstream of *clo* to specify hematopoietic and vascular differentiation.

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SCL (or Tal-1) is a basic helix-loop-helix (bHLH) transcription factor identified by its involvement in chromosomal translocations or upstream deletions associated with acute lymphocytic leukemia (Begley et al. 1989; Finger et al. 1989; Chen et al. 1990). During normal development the SCL gene is expressed in blood precursors (Green et al. 1991), endothelial progenitors (Kallianpur et al. 1994; Drake et al. 1997), and the brain (Green et al. 1992). Targeted disruption of the SCL gene in ES cells has established an essential role in hematopoiesis, acting at the stem cell level to specify all blood lineages (for review, see Green 1996; Shivdasani and Orkin 1996). Because the SCL^{-/-} mice die during early embryogenesis, this precludes direct analysis of possible SCL function in other tissues where SCL is expressed, such as the endothelium and the brain.

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Previous studies demonstrate an intimate relationship between blood and vascular development. Throughout vertebrate ontogeny, hematopoietic and vascular tissues arise in anatomical proximity (Sabin 1920; Pardanaud et al. 1987; Pardanaud et al. 1996). Blood and endothelial progenitors coexpress some molecular markers, such as *CD34*, *c-kit*, and *flk-1* (Yamaguchi et al. 1993; Bernex et al. 1996; Kabrun et al. 1997; Shalaby et al. 1997; Wood et al. 1997). Targeted disruption of some genes, such as *flk-1* and *CBF*, result in combined vascular and hematopoietic defects (Shalaby et al. 1995; Okuda et al. 1996; Wang et al. 1996). Furthermore, a zebrafish mutant *cloche* (*clo*) affects both blood and endothelial differentiation (Stainier et al. 1995). Because of the loss of endocardium, the *clo* mutant has enlarged cardiac chambers evident at 26 hr post-fertilization (hpf) (Liao et al. 1997). *clo* homozygotes have near undetectable expression of *GATA-1* and *flk-1*, and complete loss of *tie-1* (Liao et al. 1997). *GATA-1* is a marker for differentiated red blood cells, whereas *flk-1* and *tie-1* are markers for endothelial cells (for review, see Mustonen and Alitalo 1995; Shivdasani and Orkin 1996). These and other studies suggest that interactions between hematopoietic and vascular tissue during early embryogenesis contribute to the proper differentiation of both tissues.

Here, we identify zebrafish SCL and examine its expression in hematopoietic and vascular progenitors during zebrafish embryogenesis.

Results

SCL is highly conserved across vertebrate species

A zebrafish SCL cDNA was identified (*SCLa2.1*) by hybridization using the bHLH domain of murine and *Xenopus* SCL cDNAs. Conceptual translation of *SCLa2.1* sequence reveals that SCL is highly conserved across vertebrates (Fig. 1), where the bHLH domain (amino acids 188 to 243) is identical in zebrafish, *Xenopus*, chick, mouse, and human. Zebrafish SCL is most homologous to the chicken peptide (36% identity). Such high conservation of SCL among vertebrates implies a shared function during vertebrate development.

SCL expression delineates hematopoietic and endothelial progenitors

To examine patterns of SCL expression during zebrafish embryogenesis, RNA in situ analysis was carried out on whole embryos at various time points (Fig. 2). SCL transcripts are detected as early as the 1 somite stage in the anterior and posterior regions of the lateral plate mesoderm (data not shown). By 5 somites (12 hpf) SCL is expressed in the anterior (Fig. 2A), dorsal (Fig. 2B), and posterior (Fig. 2C) lateral plate mesoderm. As the embryo develops, SCL expression expands rostrally and caudally. The anterior lateral mesoderm cells contribute to form bilateral cranial vascular plexi (arrowhead, Fig. 2E); dorsal cells contribute to the dorsal aorta primordium. The posterior cells begin to fuse by 20 somites (arrowhead

Figure 1. Zebrafish *SCL* peptide sequence compared with that of *Xenopus*, chick, mouse, and human (amino acids identical across species is in dark blue, with decreasing similarity in lighter shades).

with asterisk, Fig. 2D) and form the intermediate cell mass (ICM) by 24 hpf (arrowhead, Fig. 2G). The ICM contributes to both hematopoiesis and vasculogenesis (Detrich et al. 1995; Pardanaud et al. 1996).

Although *SCL* expression is similar to that of *flk-1* during early zebrafish development, there are two significant differences. *SCL* transcripts are not detected in the rostral bilateral *flk-1*-positive cells at 20 somites, which represent the precardiac angioblasts that form the endocardium (Stainier et al. 1993; Liao et al. 1997). Instead, a more caudal set of bilateral stripes are *SCL* positive, representing those lateral mesoderm cells that do not converge to form the ICM (arrowhead, Fig. 2D). These bilateral cells persist dorsally to 24 hpf (arrowhead, Fig. 2F), and are located immediately lateral to the dorsal aorta primordium. Furthermore, *SCL* expression in the ICM differs from that of *GATA-1* (Detrich et al. 1995). Although both *SCL* and *GATA-1* transcripts are detected at high levels in the ICM, *SCL* expression extends more posteriorly (arrowhead, Fig. 2G).

As circulation commences ~26 hpf, *SCL*-positive blood cells begin to leave the ICM and enter the primitive vascular network. Circulating blood is evident in the cardinal vein (arrowhead, Fig. 2H). However, it is not clear whether the blood progenitors in the posterior ICM

enter circulation (arrowhead with asterisk, Fig. 2I). By 2 days postfertilization (dpf), *SCL* expression in vascular primordia subsides and hematopoietic expression is reduced to a few circulating cells (arrowhead, Fig. 2I). By 3 dpf, *SCL* expression is most intense in the developing brain but can also be detected weakly in the posterior tail (arrowhead, Fig. 2J). At the same time, *c-myb* transcripts are detected more strongly in this posterior tail region (Fig. 2K); *c-myb* is associated with definitive hematopoiesis in mice (Mucenski et al. 1991). *SCL* expression in the posterior tail region becomes evident by 4 dpf (Fig. 2L). Interestingly, *SCL* expression is also localized to the heart (Fig. 2L). Transverse histological section of the heart reveals that both cardiac chambers are filled with *SCL*-positive cells (arrowhead, Fig. 2N). *SCL*-positive cells are also consistently observed within the dorsal aorta (arrowhead, Fig. 2O) and axial vein (arrowhead with asterisk, Fig. 2O) throughout the embryo. We suggest that these *SCL*-positive cells in the heart lumen represent pooled blood in the fixed day 4 embryo. However, the posterior tail *SCL* expression is not restricted to the vessel (Fig. 2P). Here, *SCL* transcripts are detected strongly in a group of cells ventral to the axial vein [ventral vein region (VVR)], bound anteriorly by the end of the pronephric duct, and posteriorly by the end of notochord. By 5 dpf, *SCL* expression in the VVR subsides.

The zebrafish *clo* mutation abrogates *SCL* expression

RNA in situ analysis in the *clo* mutant reveals that *SCL* expression is abrogated in the lateral plate mesoderm at 5-somites (Fig. 3A). By 24 hpf, *SCL* transcripts are detected in the hindbrain and spinal neurons (Fig. 3B). However, the *clo* mutant fails to express *SCL* in any of the angioblast populations described previously (cf. Fig. 3B with Fig. 2, E, F, and G). Previous work suggests that most endothelial cells are deleted by the *clo* mutation, where only a small population in the posterior tail is spared (Stainier et al. 1995; Liao et al. 1997). Moreover, the hematopoietic progenitors fail to form in the embryo. At 24 hpf, very few cells (5–10) with *SCL* transcripts can be detected in the ICM (arrow, Figure 3B). These rare hematopoietic precursors can differentiate and give rise to a very small number of *GATA-1*-positive cells, which remain in the tail (Liao et al. 1997). These *GATA-1*-positive blood cells persist into 36 hpf (data not shown) but are not detected with *GATA-1* (Fig. 4) or *SCL* (Fig. 3C) in situ later at 2 dpf. *SCL* expression in the developing brain appears unperturbed by *clo*.

Because *SCL* expression is severely affected by the *clo* defect, it was important to ascertain whether the *SCL* locus represents the *clo* mutation. A single-strand length polymorphism (SSLP) was defined in the *SCL* cDNA. In-

Figure 2. *SCL* expression in vascular and hematopoietic progenitors during early development. *SCL* RNA in situ hybridization at 5-somites stage (A–C), 18-somites stage (D), 24 hpf (E–G), 26 hpf (H), 2 dpf (I), 3 dpf (J), 4 dpf (K,L), and 5 dpf (M). Transverse histological sections (6 μ m) from 4 dpf embryos are shown in N–P, corresponding to labeled lines in L. All are in situ done with *SCL*, except K, which is with *c-myb*. All are viewed laterally, with anterior to the left and dorsal up, except A which is an anterior view, B and D, which are dorsal views, and C, which is a posterior view. (See text for details.)

dividual *clo*^{m39+} and *clo*^{m39–} haploids were generated and genotyped. The *SCL* SSCP randomly segregated between the wild-type and mutant haploids, thus excluding *SCL* as a candidate gene for *clo*. When an AB/DAR mapping panel was genotyped with this SSCP, the *SCL* locus was localized to linkage group 22 (Fig. 3E) of the Oregon zebrafish genetic map.

Forced expression of *SCL* in *clo* rescues both the hematopoietic and vascular defects

To determine whether *SCL* can rescue the *clo* defect, we expressed the full-length *SCLa2.1* cDNA (*cmv-SCLa2.1*) in embryos microinjected at the one- to four-cell stage. Embryos were scored and fixed at 2 dpf, when *GATA-1* (Fig. 4B) and *tie-1* (Fig. 4E) transcripts are absent in *clo*^{–/–} embryos, and *flk-1* expression is undetectable in the head and trunk (mut, Fig. 4D). Because CMV promoter expression is unrestricted, *cmv-SCLa2.1*-injected embryos exhibit ectopic expression of *SCL* at high levels persisting into 2 dpf (Fig. 4A).

For microinjection experiments, embryos were obtained from either adult *clo* heterozygote mating pairs or

wild-type mating pairs as control. The embryos were scored at 2 dpf, separated with respect to wild-type or mutant enlarged heart phenotype, and fixed for in situ analysis. *SCL* forced expression in embryos had no effect on normal development (Fig. 4A). When *SCL* is expressed in *clo*^{–/–} embryos, red blood cells are evident in the trunk of the live 2 dpf embryos, whereas none are seen in the uninjected *clo*^{–/–} (data not shown). These red cells are better demonstrated by in situ for *GATA-1* (Fig. 4B). Alternatively, *o*-dianisidine staining for complexed hemoglobin specifically demonstrates differentiated erythrocytes in the *clo* wild type and rescued *clo*^{–/–} embryos (Fig. 4C). The number of red cells rescued by injection appears to be lower than that observed in uninjected *clo* wild-type embryos, suggesting that the rescue of hematopoietic defect is incomplete. *SCL* expression also rescued the expression of *flk-1* and *tie-1* in the injected *clo*^{–/–} embryos (Fig. 4D,E). The rescue of *flk-1* expression in the cranial (Fig. 4D, arrowhead in rsc1, rsc2) and trunk vasculature (Fig. 4D, arrow with asterisks in rsc2) is mosaic among the injected embryos, and near complete rescues have been observed. Rescue of *tie-1* expression is partial and is restricted to vasculature of the posterior tail (Fig. 4E, arrowhead). The pattern of *tie-1* expression in the rescued *clo*^{–/–} embryos also appears disorganized, lacking discrete sprouting of intersomitic vessels that are present in *clo* wild type (compare rsc and wt in Fig. 4E). Of the 502 embryos (381 *clo*^{+/–}, 121 *clo*^{–/–}) injected with *cmv-SCLa2.1* and analyzed by RNA in situ for molecular rescue, 42/121 were rescued (35%). Of the 473 uninjected embryos (349 *clo*^{+/–}, 124 *clo*^{–/–})

similarly analyzed, none were rescued. Control injection of *cmv-GFP* into 75 embryos (55 *clo*^{+/–}, 21 *clo*^{–/–}) also failed to rescue the molecular markers in the mutants. Collectively, these experiments suggest that *SCL* acts downstream of *clo* to specify hematopoietic and angioblasts precursor formation.

Discussion

We found that *SCL* is expressed in early hematopoietic and vascular progenitors during normal development and is nearly absent in the *clo* mutant. Genetic analysis demonstrates that *SCL* is not linked to *clo*. In the 2 dpf *clo* mutant embryos, *GATA-1* and *tie-1* transcripts are not detected; *flk-1* is expressed only in a small number of cells in the posterior tail (Liao et al. 1997) but is absent in the head and mid-trunk. *clo*^{–/–} embryos fail to initiate normal hematopoiesis and vasculogenesis (Stainier et al. 1995; Liao et al. 1997). When *SCL* is expressed in the *clo*^{–/–} embryo, expression of *GATA-1*, *flk-1*, and *tie-1* are restored at 2 dpf. However, the rescue is incomplete, perhaps because of mosaicism of the injected embryos. It is also possible that the *clo* defect cannot be entirely

Figure 3. The zebrafish *clo* mutation affects *SCL* expression in hematopoietic and vascular tissues. *SCL* RNA in situ hybridization at 5-somite stage (A), 24 hpf (B), and 2 dpf (C). SSLP is defined in the 3' UTR of the *SCL* cDNA and is unlinked to *clo* (D). *SCL* maps to the centromere of linkage group 22 (E).

compensated by *SCL* expression, precluding a complete rescue.

These results suggest that *SCL* acts downstream of the *clo* defect and upstream of *flk-1*, *GATA-1*, and *tie-1* to specify hematopoietic and vascular differentiation (Fig. 5). In the *clo* mutant, *SCL*, *flk-1*, and *GATA-1* can be detected in a very small number of cells, and *tie-1* is never detected. Interestingly, those few cells expressing *SCL*, *flk-1*, and *GATA-1* in *clo*^{-/-} are all restricted to the posterior tail of zebrafish, a site that is developmentally analogous to the yolk sac of higher vertebrates, where primitive hematopoiesis occurs (for review, see Zon 1995). In the yolk sac of *SCL*^{-/-} mouse, *flk-1* can be detected by RT-PCR at levels comparable to wild type, and *tie-1* transcript level is reduced twofold (Visvader et al. 1998). Similarly, embryoid bodies derived from in vitro differentiation of ES cells demonstrate *flk-1* expression

(Elefanty et al. 1997). However, analysis of *flk-1* and *tie-1* expression in the embryo proper of *SCL*^{-/-} mouse has not been performed. Hence, the epistatic relationship between *SCL* and *flk-1* in the context of the mouse embryo remains unclear. Our rescue of *flk-1* expression in the *clo*^{-/-} mutant suggests that *SCL* acts upstream or in parallel to *flk-1* in the zebrafish embryo.

Analysis of *SCL* expression during embryogenesis also provides insight into how the hematopoietic and vascular tissues may be specified. In the posterior ICM, *SCL*, *LMO-2*, and *GATA-2* are expressed, but not *GATA-1* (Detrich et al. 1995; M.A. Thompson and L. Zon, unpubl.). In mice, *GATA-2* is involved in hematopoietic progenitor proliferation (Tsai et al. 1994), *LMO-2* in blood progenitor differentiation (Warren et al. 1994), and *GATA-1* in erythroid differentiation (for review, see Shivdasani and Orkin 1996). Morphologically, the hematopoietic cells in the posterior ICM are less differentiated than those of the anterior ICM (Detrich et al. 1995). Collectively, these results suggest that the posterior ICM hematopoietic cells represent a population of hematopoietic progenitors. Although most *SCL*-positive blood cells of the ICM enter circulation, we believe some *SCL*-positive progenitors remain in the posterior ICM. The posterior ICM contributes to form the VVR of the posterior tail. More anteriorly, *SCL* expression is detected in dorsal bilateral stripes at 24 hpf. These *SCL*-positive cells migrate toward the trunk midline as development progresses. Preliminary studies suggest that these cells colonize the AGM (aorta-gonad-mesonephros) region, a site of definitive hematopoiesis in vertebrates (M.A. Thompson and L. Zon, unpubl.). At 4 dpf, *SCL* and *c-myb* are coexpressed at high levels in the VVR. In mice, *c-myb* is associated with definitive hematopoiesis (Mucenski et al. 1991). We propose that the VVR represents a larval site of definitive hematopoiesis in zebrafish. AGM formation (at 36 hpf) precedes that of the VVR (4 dpf), and we believe both regions form niches for definitive blood. The relative contribution of VVR and AGM to definitive blood remains to be elucidated by detailed transplantation and cell lineage studies.

Our data also support the notion of angioblast heterogeneity. We find *SCL* expressed in cranial angioblasts and in the ICM but not in precardiac angioblasts that contribute to the endocardium. The ICM has been implicated to contain angioblast potential (Pardanaud et al. 1996), and may be involved in forming the dorsal aorta and axial vein of the trunk and tail. The lack of *SCL* transcripts in the precardiac angioblasts suggests a molecular difference in the developmental programs that specify different vascular structures.

Our experiments suggest an instructive role for *SCL* in specifying both hematopoietic and vascular progenitors during zebrafish embryogenesis. Recent evidence from *Xenopus* animal cap assays suggests that *SCL* is sufficient to specify the hematopoietic mesoderm (P. Mead and L. Zon, unpubl.). The dual role of *SCL* acting in hematopoiesis and yolk sac vitelline vessel angiogenesis has also been implicated by mouse chimera and transgenic studies (Visvader et al. 1998). These lines of evi-

SCL specifies hematopoiesis and vasculogenesis

Figure 4. Forced expression of *SCL* rescues both the hematopoietic and vascular defects in *clo*. Microinjection rescue of *clo*^{-/-} embryos with *cmv-SCLa2.1*. Embryos are shown in lateral views in A–C, dorsal views in D, and lateral close-ups of posterior tail in E. (A) *SCL* in situ hybridizations of injected embryos at 24 and 48 hpf; (B) *GATA-1* in situ hybridization; (C) *o*-dianisidine staining; (D) *flk-1* and (E) *tie-1* in situ hybridization. Wild-type *clo* (wt), mutant *CLO*^{-/-} (mut), and rescued *clo*^{-/-} (rsc) are shown, all staged at 2 dpf. (See text for details.)

dence corroborate that *SCL* has an important dual function during vertebrate embryogenesis in blood and vascular development. This adds *SCL* to a growing list of bHLH transcription factors that have key roles in organogenesis, such as *NeuroD* (Naya 1997) and *MyoD* (for

review, see Olson 1990), in pancreas and muscle formation, respectively.

Materials and methods

Zebrafish strains and maintenance

Zebrafish were maintained as described (Westerfield 1993), and staged as described (Kimmel et al. 1995). Embryos raised to time points beyond 24 hpf were treated with 0.003% phenylthiourea (PTU) to prevent melanization (Sigma). The spontaneous *clo* allele, *clo*^{m39} (Stainier et al. 1995), was obtained from Mark Fishman, MGH (Charlestown). The *clo*^{m39} allele was outcrossed to the standard wild-type strain (AB) for two generations. Heterozygotes carrying AB *clo*^{m39} allele were crossed to a wild-type strain in the isogenic DAR genetic background to generate a AB/DAR mapping strain. Embryos used for in situ and microinjection experiments were collected from pairwise matings between identified *clo*^{m39} heterozygotes. Haploids used for linkage analysis were generated essentially as described (Westerfield 1993).

Isolation of zebrafish SCL cDNA

Full-length zebrafish *SCL* cDNA was isolated by low stringency hybridization using the bHLH domain of mouse and *Xenopus SCL* as the labeled probe. The *SCL* bHLH fragments were generated by PCR with the following primers: murine *SCL* (5'-TTCTTTGGGAACCGGATG, 3'-CTCCTCCTGGTCATTGAG), *Xenopus SCL* (5'-TTTG-TTGACCCAGACACC, and 3'-ATCGAGAAG-TTTGGCAAG). An adult zebrafish kidney cDNA library (provided by J. Rast, Children's Hospital, St. Petersburg, FL) was screened, and one full-length clone (*SCLa2.1*) was sequenced (DNA Sequencing Core Facility, Children's Hospital, Boston) and used in the experiments described herein (GenBank accession no. AF045432). Sequence analysis using BLAST, BESTFIT, and PILEUP programs was done with the UWGCG software package. Peptide sequence alignment was done with Gene Inspector (Textco).

In situ hybridization, o-dianisidine staining, and histological analysis
In situ hybridization and riboprobe synthesis were performed as described (Schulte-Merker et al. 1992), with modifications: Proteinase K digestion was extended to 2 min in 20 µg/ml, and hybridization steps were carried out at 65°C. The *GATA-1* (Detrich et al. 1995), *flk-1*, and *tie-1* (M.A. Thompson and L. Zon, pers. comm.) probes were prepared as described previously. *o*-Dianisidine staining was done as described (Detrich et al. 1995). Embryos for histological sections were treated with acetone and embedded in epon-araldite (Polysciences, Inc.) plastic resin, for histological sections. Sections of 6 µm were cut on LKB microtome and counterstained with 0.5% eosin, as described (Hyatt et al. 1996).

Mapping of SCL and polymorphism analysis in clo

A SSLP was amplified with the following primers [forward, GG-GATTGAGCAGCCCTATC; reverse primer, GCAGGGCTAAAGT-TGGGGT(T/G)]. The 190-bp (AB) or 188-bp (DAR) product was resolved on 8% denaturing urea-polyacrylamide gel. This SSLP was used to type *clo*^{m39} and *clo*^{m39} haploid individuals, and 96 individuals of a diploid AB/DAR map panel (J. Postlethwait).

cDNA expression constructs and microinjection

The *SCLa2.1* full-length cDNA defined by *Bam*HI and *Xho*I sites was subcloned into *Bam*HI and *Xho*I sites of the pCS2⁺ vector, containing the CMV promoter [D. Turner (Fred Hutchinson Cancer Research Center, Seattle, WA) and R. Rupp (Friedrich-Miescher Laboratorium, Tübingen, Germany)]. The injection construct (*cmv-SCLa2.1*) plasmid DNA was diluted to 100 ng/µl in sterile ddH₂O. A control plasmid (*cmv-GFP*) was constructed with the green fluorescent protein (B. Seed, MGH, Boston), gene inserted into *Clal* and *Xba*I sites of the pCS2⁺ vector, and diluted to 125 ng/µl. Microinjection was performed essentially as described (Westerfield 1993), utilizing Nikon picoinjector and Narishige micromanipulator.

Figure 5. Proposed model of *SCL* function during hematopoiesis and vasculogenesis.

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SCL/Tal-1 transcription factor acts downstream of *cloche* to specify hematopoietic and vascular progenitors in zebrafish

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