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Zebrafish scl functions independently in hematopoietic and endothelial development

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Abstract

The SCL transcription factor is critically important for vertebrate hematopoiesis and angiogenesis, and has been postulated to induce hemangioblasts, bipotential precursors for blood and endothelial cells. To investigate the function of scl during zebrafish hematopoietic and endothelial development, we utilized site-directed, anti-sense morpholinos to inhibit scl mRNA. Knockdown of scl resulted in a loss of primitive and definitive hematopoietic cell lineages. However, the expression of early hematopoietic genes, gata2 and lmo2, was unaffected, suggesting that hematopoietic cells were present but unable to further differentiate. Using gene expression analysis and visualization of vessel formation in live animals harboring an lmo2 promoter-green fluorescent protein reporter transgene (Tg(lmo2:EGFP)), we show that angioblasts were specified normally in the absence of scl, but later defects in angiogenesis were evident. While scl was not required for angioblast specification, forced expression of exogenous scl caused an expansion of both hematopoietic and endothelial gene expression, and a loss of somitic tissue. In cloche and spadetail mutants, forced expression of scl resulted in an expansion of hematopoietic but not endothelial tissue. Surprisingly, in cloche, lmo2 was not induced in response to scl over-expression. Taken together, these findings support distinct roles for scl in hematopoietic and endothelial development, downstream of hemangioblast development.

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Introduction

Hematopoiesis is a dynamic process that occurs in successive stages and distinct anatomical locations during the vertebrate life span (reviewed in Galloway and Zon, 2003). In zebrafish, the first wave of hematopoiesis, termed primitive or embryonic, occurs in two distinct regions of the lateral mesoderm. In the posterior mesoderm, bilateral stripes of both erythroid and endothelial precursors migrate toward the midline of the embryo to form the intermediate cell mass (ICM), which is the equivalent of the extraembryonic mammalian yolk sac blood islands. The endothelial precursors form discrete axial vessels, and surround the inner mass of proliferating erythroblasts that enter circulation by approximately 28 h post-fertilization (hpf). A second site of primitive hematopoiesis occurs within the anterior lateral mesoderm, in close association with endothelial precursors, and gives rise to macrophages and granulocytes (Bennett et al., 2001; Herbomel et al., 1999; Lieschke et al., 2002). Lineage-specific genes, such as l-plastin and gata1, are restricted to the anterior myeloid or posterior erythroid populations, respectively (Bennett et al., 2001; Herbomel et al., 1999; Lieschke et al., 2002). In contrast to the transient primitive wave, definitive hematopoiesis generates long-term hematopoietic stem cells (HSCs) that continuously provide erythroid, myeloid, and lymphoid lineages throughout adulthood. Definitive hematopoiesis in the zebrafish is thought to

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initiate by 32 hpf, at which time runx-1 and c-myb expression occurs in presumptive definitive HSC along the ventral wall of the dorsal aorta (Burns et al., 2002; Kalev-Zylinska et al., 2002; Thompson et al., 1998). This site is analogous to the origin of definitive HSCs in the mammalian aorta-gonad-mesonephros (AGM) region (Jaffredo et al., 1998; Medvin-sky and Dzierzak, 1996; North et al., 2002). Zebrafish lymphoid precursors expressing the T-cell-specific genes, recombination activating gene-1 and -2 (rag1 and rag2) (Willett et al., 1999) are found in the thymus as early as 3 dpf. By 5 dpf, hematopoietic expression occurs in the kidney primordia, which will become the site of adult zebrafish hematopoiesis (Al-Adhami and Kunz, 1977; Zapata, 1979).

The scl gene encodes a basic helix–loop-helix transcription factor (Begley et al., 1990; Chen et al., 1990; Finger et al., 1989) and is a key regulatory molecule in hematopoietic development. Gene targetting studies and chimeric analyses in mice demonstrate that scl is essential for the generation of all primitive and definitive hematopoietic lineages (Porcher et al., 1996; Robb et al., 1995, 1996; Shivdasani et al., 1995). The scl gene is also expressed in endothelial cells (Hwang et al., 1993; Kallianpur et al., 1994), and the expression in both cell types has suggested a possible role for scl in specification of the hemangioblast, a bipotential precursor for both hematopoietic and endothelial cells. Historically, the existence of a hemangioblast was hypothesized due to the close association of both cell types during avian embryonic development (Murray, 1932; Sabin, 1920). More recently, hematopoietic and endothelial precursors were shown to develop in cell culture from a single blast colony forming cell (BL-CFC), thus representing an in vitro equivalent of the hemangioblast (Choi et al., 1998). Although scl expression occurs in endothelial cell types, Scl−/− mice have normal angioblast specification, and only display later defects in vascular remodeling (Robb et al., 1995; Shivdasani et al., 1995; Visvader et al., 1998). These findings are consistent with in vitro studies in which Scl−/− murine ES cells, following differentiation, are unable to generate hematopoietic precursors, but can generate adherent, flk1+ endothelial cells (Elefanty et al., 1997; Robertson et al., 2000). Based on these observations, it can be concluded that murine Scl is not required for hemangioblast specification.

Transcripts for zebrafish scl appear at approximately the 2- to 3-somite stage in erythroid and endothelial precursors in the posterior lateral mesoderm and in myeloid and endothelial precursors in the anterior lateral mesoderm (Gering et al., 1998; Liao et al., 1998). Analyses in vitro have demonstrated that SCL functions in a multimeric transcriptional complex with LMO2, a LIM-only protein (Wadman et al., 1997; Warren et al., 1994). Similar to scl, targeted deletion of lmo2 in mice demonstrated a requirement in primitive and definitive hematopoiesis and angiogenesis, but not angioblast specification (Yamada et al., 1998, 2000). Zebrafish scl and lmo2 are co-expressed spatiotemporally, and the scl−/− lmo2− precursor cells in the posterior lateral mesoderm differentiate to generate adjacent but mutually exclusive hematopoietic (scl−/− lmo2− gata1+) and endothelial (scl−/− lmo2− flk1+) populations (Davidson et al., 2003; Gering et al., 1998). The expression of scl persists in both cell types through approximately 28 hpf.

In contrast to the mouse studies described above, previous functional analyses of scl in zebrafish have suggested a role in the specification of hemangioblasts. The zebrafish mutant, cloche (clo), displays a loss of hematopoietic and endothelial tissue (Stainier et al., 1995). Forced expression of scl in clo mutants rescues both primitive erythroid and vascular gene expression (Liao et al., 1998; Porcher et al., 1999), suggesting that scl functions downstream of clo in specification of hemangioblasts. In wild-type embryos, the expansion of posterior erythroid and endothelial gene expression in response to scl occurs at the expense of somitic mesoderm (Gering et al., 1998), and forced co-expression of scl and lmo2 results in enhanced induction of anterior endothelial gene expression at the expense of cardiac mesoderm (Gering et al., 2003). Thus, exogenous scl is able to reprogram mesodermal fate to blood and endothelial tissue.

To date, mutations in the scl locus have not been identified from large-scale mutagenesis screens in zebrafish, precluding loss-of-function analyses. To investigate the function of endogenous scl during zebrafish hematopoietic and endothelial development, we created an scl knockdown by utilizing site-directed, anti-sense morpholinos to inhibit proper mRNA splicing. The results described demonstrate that zebrafish scl is essential for the development of all primitive and definitive hematopoietic lineages, but not for the specification of angioblasts. When the scl morpholino was injected into animals harboring an lmo2 promoter-green fluorescent protein reporter transgene (Tg(lmo2:EGFP)), allowing for visualization of vessel formation by GFP fluorescence in live animals (Zhu et al., manuscript in preparation), later defects in angiogenesis were observed. The scl morphant phenotype is similar to that of Scl−/− mice, and highlights the evolutionary conservation of scl function. To further investigate the ability of exogenous scl to induce endothelial gene expression, we over-expressed scl in the zebrafish mutants, clo and spadetail (spt), a bloodless mutant that displays abnormal posterior mesoderm development (Griffin et al., 1998; Ho and Kane, 1990; Kimmel et al., 1989). In both clo and spt, a robust induction of hematopoietic tissue was observed, whereas the induction of endothelial tissue was attenuated. These results strongly indicate that although scl has the ability to induce both tissues, there are distinct requirements for each. Interestingly, in clo, lmo2 was not induced upon scl over-expression, indicating that scl can function independently of lmo2 when present in excess. Our findings demonstrate that scl is normally required downstream of hemangioblast formation for hematopoietic development and angiogenesis.
Materials and methods

Fish husbandry

Zebrafish were maintained as described (Westerfield, 1993), and staged as described (Kimmel et al., 1995). Either the Tü or WIK lines were used for all wild-type microinjection experiments. The radiation-induced spr mice mutant line was obtained from C. Kimmel (Eugene, OR) and the spontaneous mutant clo was obtained from M. Fishman (Charlestown, MA). The Tg(lmo2:EGFP) line has been described (Zhu et al., manuscript in preparation).

Anti-sense morpholino knock down

The zebrafish scl intronic sequence was determined by comparison of zebrafish scl cDNA sequence, fugu rubripes genomic scl sequence (Barton et al., 2001), and the Sanger Center zebrafish genome sequence database (http://www.ensembl.org/Danio_rerio/). Anti-sense morpholino oligos (Gene Tools, Corvalis, OR) were designed to target the exon/intron boundary sequences of either exon 1 or exon 2 as follows: SCL E1/I: (5'-CGC GGC TTA CCT GTT AAT AGT GGC G-3'); SCL E2/I: (5'-AAT GCT CTT ACC ATC GTT GAT TTC-3'). As a control for specificity, a 4 base pair mismatch was introduced into the SCL E1/I sequence as follows: SCL E1/I.X (5'-GCC GGG TTA CCT GTT AAT AGT GGC G-3'); SCL E2/I: (5'-AAT GCT CTT ACC ATC GTT GAT TTC-3'). As a control for specificity, a 4 base pair mismatch was introduced into the SCL E1/I sequence as follows: SCL E1/I/X (5'-GCC GGG TTA CCT GTT AAT AGT GGC G-3'); SCL E2/I: (5'-AAT GCT CTT ACC ATC GTT GAT TTC-3'). As a control for specificity, a 4 base pair mismatch was introduced into the SCL E1/I sequence as follows: SCL E1/I/X (5'-GCC GGG TTA CCT GTT AAT AGT GGC G-3'); SCL E2/I: (5'-AAT GCT CTT ACC ATC GTT GAT TTC-3').

Whole mount RNA in situ hybridization

Embryos were microinjected at the 1–4 cell stage with 1 nl of morpholino, alone or in combination, at a final concentration of 0.5 mM. Phenol Red was co-injected as a tracer. The phenotypes observed for SCL E1/I alone, SCL E2/I alone, or both morphinos together were indistinguishable, as assessed by circulating blood cells in live animals or in situ hybridization (ISH) for blood cell markers. The results shown in all figures are for co-injection of both SCL E2/I and SCL E1/I.

Whole mount RNA in situ hybridization

Digoxigenin (DIG)- or fluorescein (FLU)-labeled antisense RNAs were transcribed from linearized templates using RNA Labeling Kit (Sp6/T7) according to manufacturer’s protocol (Roche). Antisense riboprobes to scl (Liao et al., 1998), gata1, gata2, (Detrich et al., 1995), b23 globin (Brownlie et al., 2003), lmo2, c-myb, flil, flkl (Thompson et al., 1998), flilb (Zhu et al., manuscript in preparation), enib2 (Lawson et al., 2002), runx1 (Kalev-Zylinska et al., 2002), draculin, l-plastin (Herbomel et al., 1999), rag2 (Willett et al., 1999), pu.1 (Lieschke et al., 2002), mpo (Bennett et al., 2001), hhex (Ho et al., 1999), and who (Schopp et al., 2002), have been described previously. Whole mount RNA ISH were performed as previously described (Ransom et al., 1996). DIG- or FLU-labeled riboprobes were detected using alkaline phosphatase conjugated anti-DIG or anti-FLU antibodies (Roche), followed by detection of alkaline phosphatase activity in orange or blue using NBT/INT or NBT/BCIP substrate (Sigma), respectively. For double ISH, the first color reaction was inactivated by 2 × 15 min incubation in 100 mM Glycine, pH 2.2, following fixation in 4% PFA. After inactivation, embryos were incubated in the second antibody and processed as for single ISH. When necessary, embryos raised to time points beyond 24 hpf were incubated in 0.8% KOH/0.9% H2O2 for 15 min to remove pigment, followed by 2× rinses in PBST, and re-fixation in 4% PFA.

Reverse transcriptase-PCR

Full length scl RNA was transcribed from linearized pCS2+-SCLA2.1 (Liao et al., 1998; GenBank accession no. AF045432) using mMessage mMachine according to manufacturer’s protocol (Ambion). For morpholino rescue experiments, 300 pg of scl RNA was injected separately, immediately following the morpholino injection. Embryos for microinjection of clo and spr mutants were obtained from heterozygous incrosses, and 100 pg of scl mRNA was injected between the 1- and 4-cell stages. Embryos were fixed in 4% PFA for whole mount in situ hybridization. Mutant spt embryos were identified morphologically. Rescued clo embryos were genotyped using SSR marker z1496 (Liao et al., 2000).

Results

Loss of scl results in loss of all hematopoietic lineages

The role of scl in hematopoietic and endothelial development was investigated by creating a knockdown using antisense morpholinos targeted to the splice donor site of either exon 1 or exon 2 of the zebrafish scl gene. Splice-site-directed morpholinos inhibit pre-mRNA splicing events (Draper et al., 2001), resulting in a loss of functional mRNA. To visualize the aberrant scl message, reverse transcriptase-PCR was performed on uninjected and mor-
pholino injected embryos with primer sites flanking the
splice junctions of exons one and two (Fig. 1A). The
expected product of 325 bp was detected in uninjected,
control embryos, but not in scl morpholino-injected
embryos. Instead, aberrant products were observed, con-
firming the inhibition of proper pre-mRNA splicing in
the presence of the morpholinos. The expression of scl in
the scl morphant was further assessed by whole mount RNA
in situ hybridization. Detection of transcript levels was
dramatically reduced at the 10-somite stage, and the reduction
persisted, shown here at the 20-somite stage (Figs. 1B–D).
The initial reduction in scl transcript is likely due to
nonsense mediated decay of the aberrant message, a process
known to occur in eukaryotes following transcription of
some nonsense alleles (reviewed in Mendell and Dietz,
2001; Wilkinson and Shyu, 2002). The presence of aberrant
transcripts as detected via non-quantitative RT-PCR and the
loss of scl transcripts as detected by whole mount RNA in
situ hybridization confirmed the effective knockdown of scl
via morpholino injection.

To investigate the role of scl in primitive erythroid
development, erythroid-specific gene expression was eval-
uated in the scl morphants. Transcripts for gata1, one of the
earliest known erythroid-specific genes, were undetectable
at the 10-somite stage in scl morphant embryos (n = 96/120;
80%) (Figs. 2A, B). To confirm that the loss of gata1 was
specific to the loss of scl, the scl morpholino was co-
injected with scl RNA. The exogenous RNA is unaffected
by splice-site directed morpholinos and should therefore
rescue the gata1 expression. Expression of gata1 was
rescued in 100% of the co-injected embryos (n = 48). Of
these embryos, most had an asymmetric expression pattern
(n = 34/48; 71%), as shown in Fig. 2C, and the remaining
appeared wild type (n = 14/48; 29%). As a further control
for specificity, a 4-bp mismatch morpholino (E1/I.X) was
injected at the same or a two-fold higher dose as that of the
scl morpholino. All of the resulting embryos displayed
normal gata1 expression at the 10-somite stage at either
dose (n = 20/20 or n = 31/31, respectively; data not shown).
Therefore, the phenotype observed in the scl morpholino
injected embryos was specific to the loss of scl.

The genes runx1 and c-myb are expressed in primitive
hematopoietic cells within the bilateral stripes of the
posterior mesoderm (Buns et al., 2002; Thompson et al.,
1998). Morpholino knockdown of runx1 has suggested a
role in primitive hematopoiesis (Kalev-Zylinska et al.,
2002). In the scl morphant at approximately the 12 somite
stage, transcripts for runx1 were not detected (n = 32/43;
75%), nor were transcripts for c-myb (n = 28/40; 70%)
(Figs. 2D–G), further demonstrating a defect at the earliest
stages of hematopoietic development. Later, the β3-
hemoglobin gene is normally expressed in differentiating
erthrocytes within the ICM as early as the 15-somite stage.
Consistent with the loss of gata1, transcripts for β3-
hemoglobin were undetectable (n = 59/61; 97%) in
morphant embryos (Figs. 2H, I). By the onset of circulation
at 28 hpf, qualitative assessment of circulating red blood
cells visualized under a dissecting microscope revealed an
absence of erythrocytes in the scl morphants (n = 93/97;
96%), in contrast to uninjected control animals (Figs. 2J, K).
This result was confirmed by the absence of hemoglobin as
detected by o-dianisidine staining (data not shown).
Therefore, the loss of scl inhibited the earliest stages of embryonic
erythroid development. In addition to the anemia, a mild
pericardial edema was evident from 28 hpf onward.
Otherwise, the overall morphology of the scl morpholino-
injected embryos was grossly normal at this time. By 2 days
post-fertilization (dpf), scl morphants display severe edema,
necrosis, and ultimately die between 6 and 7 dpf.

The function of scl has suggested a role in anterior primitive myelopoiesis
was evaluated by analyzing the expression of genes

![Fig. 1. Efficient knockdown of scl by morpholino injection. (A) Reverse-
transcriptase (RT)-PCR analysis of uninjected and scl morphant embryos.
An aberrant PCR product was observed in the scl morphant (+mo, lane 4)
compared to the 325 bp product observed in uninjected control embryos
(uninjected, lane 3). An approximate 325 bp product was observed with a
pcMV-scl plasmid control (plasmid ctl, lane 2), and no PCR product was
observed in the absence of template (--, lane 1). The relative locations of
E1/I and E2/I are indicated as black bars in the schematic below, and the
relative locations of primers used for RT-PCR are indicated as arrows. As a
control, primers designed to amplify efi-alpha yielded the same sized
product from uninjected or scl morphant embryos (lanes 5, 6). B–E Whole
mount RNA in situ hybridization (ISH) for scl in uninjected (B, C) or scl
morphant embryos (D, E). In B and D, the yolk was removed and the 10-
somite embryos were flat mounted on glass slides for imaging. In C and E,
lateral views of the whole embryo at the 20-somite stage are shown. All
embryos are oriented with anterior to the left.

encoding the early myeloid-specific transcription factor, \( pu.1 \), the granulocyte-specific gene, \( myeloperoxidase (mpo) \), and the macrophage-specific gene, \( l\text{-plastin} \), in the \( scl \) morpholino injected embryos. Expression of \( pu.1 \) was absent (\( n = 30/42; 71\% \)) or strongly reduced (\( n = 9/42; 21\% \)) in the \( scl \) morphants (Figs. 3A–C). In embryos with a reduced amount of \( pu.1 \) expression, these cells remained in the dorsal midline rather than migrating laterally over the yolk sac as in uninjected embryos. Expression of \( l\text{-plastin} \) at the 20-somite stage was strongly reduced (\( n = 12/35; 34\% \)) or absent (\( n = 23/35; 66\% \)) in morpholino-injected embryos (Figs. 3D, E), and similar to results for \( pu.1 \), in embryos that had a reduced amount of staining, the \( l\text{-plastin}^+ \) cells remained in the midline (data not shown). Later, \( mpo \) expression at 40 hpf was also strongly reduced (\( n = 4/25; 16\% \)) or absent (\( n = 21/25; 84\% \)) in the \( scl \) morphant embryos (Figs. 3F, G). In embryos with reduced staining, the positive cells were located in the posterior ventral tail region (data not shown). Thus, loss of \( scl \) affected anterior hematopoiesis, resulting in a loss of differentiated primitive macrophage and monocyte lineages.

Because the \( scl \) morphant survives to approximately 7 dpf, and definitive hematopoietic gene expression is presumed to occur as early as 32 hpf, it was possible to assess the effects of \( scl \) knockdown on definitive hematopoiesis. In the \( scl \) morphant, both \( runx1 \) (\( n = 18/26; 69\% \)) and \( c-myb \) (\( n = 15/17; 88\% \)) expression was undetected at 36 hpf, suggesting a failure to form AGM-derived HSCs (Figs. 4A–D). Consistent with a loss of definitive HSCs, \( rag2 \) expression in the thymus was undetectable (\( n = 40/40; 100\% \)) in morpholino-injected embryos (Figs. 4E, F). Failure to express \( rag2 \) was unlikely to be the result of abnormal thymic development, since expression of the \( whnb \) gene, which is expressed in thymic epithelium (Schorpp et al., 2002), was unaffected (data not shown). Thus, the knockdown of \( scl \) resulted in a loss of primitive and definitive hematopoietic lineages, demonstrating a key regulatory role for \( scl \) in hematopoietic development.
Fig. 3. Loss of myeloid development in scl morphants. Transcripts for *pu.1* were reduced (B) or absent (C) in the *scl* morphants, compared to uninjected controls (C), as indicated by the arrows. *l-plastin* (D, E), and *mpo* transcripts were not detected in *scl* morphants. As a control, the *mpo* ISH was performed as a double with β3-globin (β3) staining in red (F, G).

Fig. 4. Loss of definitive hematopoietic gene expression in *scl* morphants. Transcripts for *runx1* (A, B), *cmyb* (C, D), and *rag2* (E, F) were absent in *scl* morphants. In A–D, arrow indicated the region of expression along the ventral wall of the dorsal aorta. In E and F, arrow indicates expression in the thymic rudiment, ventral views.
Loss of scl causes a defect in angiogenesis following normal angioblast specification

If scl is required for the specification of hemangioblasts, the loss of scl should result in a loss of endothelial cells. The genes, fli1, encoding an ets family transcription factor (Brown et al., 2000), hhex, encoding a homeobox transcription factor (Ho et al., 1999; Liao et al., 2000), and flk1 (Liao et al., 1997), encoding a receptor tyrosine kinase, are expressed in endothelial precursors as early as the 10-somite stage, and by 24 hpf, these cells form the axial and intersomitic vessels. In scl morpholino-injected embryos at 26 hpf, fli1 and hhex were expressed in the trunk region (Figs. 5A–D), indicating normal angioblast specification. However, the angioblasts remain diffuse and the axial and intersomitic vessels did not form [(n = 22/24; 92%) and (n = 20/23; 87%), respectively], in comparison to uninjected control embryos. The same results were observed for analysis of flk1 expression (data not shown). Consistent with the loss of vessel formation, the expression of efnb2, a marker of the terminally differentiated dorsal aorta, was undetectable (n = 16/22; 73%) in the scl morphants (Figs. 5E, F).

The later vascular defect was further demonstrated using a transgenic line in which the lmo2 promoter directs GFP expression in both hematopoietic and endothelial cells (Zhu et al., manuscript in preparation), providing a means to visually assess vessel formation in live embryos. In the Tg(lmo2:EGFP) scl morphants, the axial vessels are disorganized and very few intersomitic vessels were formed, in contrast to uninjected, lmo2-GFP control embryos (Figs. 5G, H). At a higher morpholino concentration, the intersomitic vessels were completely absent, and GFP+ cells aggregated along the midline (Fig. 5I). The presence of GFP expression in the Tg(lmo2:EGFP) scl morphants further demonstrates that expression from the lmo2 promoter was unaffected by the scl knockdown. These results confirm the altered pattern of endothelial gene expression described above, and further illustrate the disorganization of the vasculature. Thus, scl is not required for angioblast specification, but is necessary at a later stage for proper angiogenesis.

Fig. 5. Angiogenesis is disrupted in scl morphants. Transcripts for fli1 (A, B), hhex (C, D), and efnb2 (E, F) in uninjected and scl morphant embryos. In A, the arrowhead indicates the expression in intersomitic vessels, and in A, C, E–F, the arrow indicates expression in the dorsal aorta. G) Uninjected Tg(lmo2:EGFP) embryos, showing fluorescence in the axial and intersomitic vessels and blood cells. In the presence of increasing amounts scl morpholino (H, I), increased disruption of angiogenesis occurred. All images are lateral views of trunk region, anterior to the left.
Hematopoiesis is blocked at an early stage in scl morphants

The absence of hematopoietic differentiation in the scl morphant could be the result of a failure to specify HSCs from mesoderm or a block in differentiation following HSC specification. To further investigate these possibilities, the expression of gata2 and lmo2, both of which are normally co-expressed with scl as early as the 2–3 somite stage, was evaluated. At the 12 somite stage, transcripts for both gata2 (n = 39/39; 100%) and lmo2 (n = 35/35; 100%) were expressed normally in the scl morphant embryos (Figs. 6A–D), and their expression persisted until at least the 20-somite stage (data not shown). Previous studies in vitro have shown that gata2 and lmo2 are expressed in Scl−− murine ES cells (Elefanty et al., 1997). Our results extend these findings by demonstrating that, based on anatomical location, the gata2+lmo2+ cells appear to represent early hematopoietic cells blocked in further differentiation due to the loss of scl.

To further evaluate the stage at which the scl knockdown was inhibiting hematopoietic development, the expression of draculin(dra) in the scl morphant was evaluated. The dra gene encodes a novel zinc finger protein initially expressed in early ventral mesoderm and later restricted to hematopoietic tissue in both the anterior and posterior mesoderm (Herbomel et al., 1999). The function of dra remains unclear. In scl morpholino-injected embryos, dra expression at the 1-somite stage was normal (data not shown), indicating proper development of ventral mesoderm. By the 10-somite stage, when dra expression is specific to the hematopoietic tissue, its expression was either severely decreased (n = 29/55; 53%) (Figs. 6E, F) or absent (n = 24/55; 44%) in the scl morphants. By the 15-somite stage, dra expression was not detected in the posterior nor anterior hematopoietic mesoderm (data not shown). Therefore, the temporal loss of dra expression provides further support that scl is required for the maintenance or differentiation of early hematopoietic precursors.

Previous results have demonstrated that forced expression of scl results in expansion of hematopoietic and endothelial tissue at the expense of other mesodermal derivatives, such as somitic and pronephric tissue (Gering et al., 1998, 2003). It was therefore possible that cross-antagonism between factors involved in tissue specification could allow the permissive expansion of non-hematopoietic mesodermal tissues in the scl morphant. Transcripts for gata2 and lmo2 are present in endothelial cells, and to confirm that the gata2+lmo2+ cells were not angioblasts, the expression of fli1b was evaluated. The fli1b gene is one of the paralogs of the ets-domain transcription factor fli1, and is specifically expressed in endothelial cells (Zhu et al., manuscript in preparation). The expression pattern of fli1b appeared normal (n = 28/28; 100%) in scl morphant embryos (Figs. 6G, H). Double ISH with lmo2 and fli1b further illustrated that endothelial fate was not expanded in the scl morphant, relative to wild type (Figs. 6I, J). Similarly, the expression pattern of pax2.1, a pronephric duct marker, appeared normal in the scl morphant (data not shown).
shown). Therefore, endothelial and kidney fates were not expanded, and cells with an early hematopoietic identity were specified in the absence of scl.

**Forced expression of scl in wild type, clo and spt reveals separable effects on hematopoietic and vascular tissue**

To further investigate the differences between the loss-of-function phenotype described here and the previously reported gain-of-function effects (Gering et al., 1998, 2003; Liao et al., 1998), we injected scl RNA into wild-type and mutant embryos. In wild-type embryos, forced expression of scl resulted in an increase in both gata1 and flk1 expression (Figs. 7A, B) and a concomitant loss of myoD⁺ somitic tissue (Figs. 7C, D), consistent with previously reported results. Using double RNA ISH, we further demonstrated that the expansion of gata1⁺ and flk1⁺ tissue was spatially distinct and non-overlapping; the gata1 expresion domain expanded laterally, while the flk1 expression domain expanded medially. This suggested that scl may function independently within the two tissue domains to achieve induction.

The spt mutant was utilized to further address the role of somitic mesoderm in the expansion of hematopoietic and endothelial tissue described above. The spt mutant has a severe loss of trunk somitic mesoderm and a loss of posterior hematopoietic cells, while initial specification of angioblasts appears relatively normal (Griffin et al., 1998; Griffin et al., 1998; Kimmel et al., 1989; Rohde et al., 2004; Thompson et al., 1998; Weinberg et al., 1996). If the induction of either hematopoietic or endothelial tissues in response to forced expression of scl occurs at the expense of somitic mesoderm, the induction might be attenuated in spt mutants.

In spt⁻/⁻ embryos, scl RNA injection resulted in an efficient induction of gata1 expression, when compared to uninjected spt⁻/⁻ embryos, which have no detectable gata1 expression (Figs. 7E, F). In contrast, an expansion of flk1 expression in the posterior lateral mesoderm was not observed, relative to uninjected spt⁻/⁻ controls (arrows, Figs. 7G, H). A slight increase in the anterior flk1 expression and an altered pattern in the posterior tail bud were observed. This altered flk1 expression pattern in the tail bud was not observed in scl RNA injected wild-type embryos. Thus, an absence of somitic tissue correlated with an inability of scl to induce endothelial, but not hematopoietic tissue, in the posterior mesoderm.

The clo mutant has a loss of hematopoietic and endothelial tissue, but normal somitic mesoderm (Stainier et al., 1995). Upon forced expression of scl in clo mutants, an expansion of hematopoietic and endothelial tissue and a concomitant loss of somitic tissue might be expected, similar to results in wild-type embryos. In the clo⁻/⁻ embryos, scl RNA injection resulted in an efficient expansion of gata1 expression (Figs. 8C, D), comparable to that observed in wild-type embryos (Figs. 8A, B). In contrast, only a very modest induction of flk1 expression

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Fig. 7. Forced expression of scl RNA in wild-type and spadetail mutants. (A, B) Double ISH for flk1 (blue) or gata1 (red) in uninjected (A) or scl RNA injected (B) embryos. (C, D) myoD transcripts normally present in adaxial cells (black arrow) and somites (blue arrow) are not detected in the scl RNA injected embryos. (E–H) The expression of gata1 (E, F) or flk1 (G, H) in uninjected (E, G) or scl RNA injected (F, H) spt⁻/⁻ embryos. Brackets indicate anterior head mesoderm; arrow indicates flk1 expression in posterior mesoderm; arrowhead indicates tail bud. All embryos are at the 12-somite stage.
was observed in the posterior mesoderm (Figs. 8G, H), particularly evident when compared to the induction in wild-type embryos (Figs. 8E, F). In the anterior mesoderm of injected clo<sup>−/−</sup> embryos, an efficient induction of flk1 expression was observed. In addition, a less dramatic loss in somitic tissue upon scl RNA injection was observed in clo<sup>−/−</sup>-injected embryos (Figs. 8A–D).

To further investigate the observed differences in endothelial versus hematopoietic induction in clo, expression of gata2 and lmo2 was evaluated following scl RNA injection. In wild-type embryos, a strong induction of gata2 was observed following scl RNA injection (Figs. 8I, J). In clo<sup>−/−</sup>-embryos, gata2 expression is normally very low, and only just detectable in the most posterior mesoderm. Upon scl over expression, gata2 is induced (Figs. 8K, L), although to a lesser degree than observed in wild-type embryos. A decrease in myoD expression is also visible, as shown (Fig. 8L). In wild-type embryos, a robust expansion of lmo2 is observed following scl RNA injection (Figs. 8M, N). This effect was also observed earlier, at the 5-somite stage (data not shown) and later, at the 20-somite stage (Fig. 8Q, R). Surprisingly, in clo<sup>−/−</sup>-injected embryos, lmo2 was not induced at any of the stages observed (Figs. 8O, P, S, T, and data not shown), despite the robust induction of gata1. Thus, in clo, scl is capable of inducing hematopoietic tissue independently of endothelial tissue, and in the absence of lmo2.

Because previous rescue experiments place scl downstream of clo in hematopoietic and endothelial development (Liao et al., 1998), it is of interest to compare early gene expression in the scl morphant to that of clo. In the scl morphant, both lmo2 and gata2 expression was comparable to that of wild type, as described above (Figs. 6A–D). In clo at the 10 somite stage, the expression of gata2 was dramatically reduced, with only a few cells in the most posterior bilateral mesoderm showing expression (Fig. 8K). The expression of lmo2 at the 10-somite stage in clo was greatly reduced and only present in the most lateral of the posterior stripes (Fig. 8O). Double ISH with the pronephric marker, pax2.1 indicates that this represents pronephric tissue (our unpublished results). Together, these results extend previous findings and demonstrate that the loss of lmo2 and gata2 during early embryogenesis in clo is not due to the loss of scl.

The results are summarized in Fig. 9. The induction or expansion of gata1 expression in response to scl RNA did not require a loss of somitic tissue, since the observed effects occurred in spt<sup>−/−</sup> embryos, which lack trunk somitic mesoderm. In contrast, the expansion of endothelial tissue was attenuated in spt<sup>−/−</sup> and clo<sup>−/−</sup> embryos. In clo<sup>−/−</sup>, the lack of posterior expansion of endothelial tissue correlated with an inability to induce lmo2. Interestingly, despite the lack of lmo2, a robust induction of gata1 expression occurred in response to exogenous scl. These
Discussion

Our results demonstrate that zebrafish \textit{scl} is necessary for primitive and definitive hematopoietic development and for angiogenesis. Angioblasts were specified normally in the \textit{scl} morphants, demonstrating that hemangioblast formation was unaffected. Our analyses in \textit{clo} and \textit{spt} indicate that the ability of \textit{scl} to expand both hematopoietic and endothelial tissue is separable, and further, demonstrate that \textit{scl} is capable of inducing hematopoietic gene expression independent of \textit{lmo2}.

The absence of multi-lineage hematopoiesis observed in the \textit{scl} morphant is likely due to a requirement in HSCs or early progenitor cells, rather than a requirement for \textit{scl} in each of the differentiated lineages, by analogy to recent studies of conditional \textit{scl} knockout in mice (Mikkola et al., 2003). These studies demonstrated that following specification of HSCs, \textit{Scl} is required for proper differentiation of the erythroid and megakaryocyte lineages, but not lymphoid or myeloid lineages. Furthermore, in zebrafish, anterior myelopoiesis occurs independently of posterior ICM erythropoiesis, as \textit{spt} and \textit{kugelig (kgg)} mutants lack ICM hematopoiesis but have normal anterior myeloid development (Davidson et al., 2003; Lieschke et al., 2002). In both \textit{spt} and \textit{kgg} mutants, \textit{scl} expression is absent in the posterior hematopoietic region but normal in the anterior hematopoietic region, correlating with the hematopoietic phenotypes. Our results demonstrate that, although posterior erythroid and anterior myeloid development can occur independently, both require \textit{scl}, presumably in HSCs or early progenitor cells.

Transcripts for \textit{gata2} and \textit{lmo2} were unaffected in the \textit{scl} morphant, despite the loss of molecular markers for more differentiated hematopoietic lineages. Gene expression profiling of in vitro differentiated \textit{Scl}−/− murine ES cells have also shown \textit{gata2} and \textit{lmo2} expression comparable to that of differentiated wild-type ES cells (Elefanty et al., 1997). Both \textit{gata2} and \textit{lmo2} are also expressed in early endothelial cells. In the \textit{scl} morphant, embryonic patterning was normal as assessed by both morphology and tissue-specific gene expression. The ability to observe normal \textit{gata2} and \textit{lmo2} expression in the embryo proper suggests that mesodermal cells have committed to a hematopoietic fate, but in the absence of \textit{scl}, are blocked in further differentiation. The temporal loss of \textit{dra} expression in the \textit{scl} morphant further indicates a block in differentiation or maintenance. The fate of the \textit{gata2+ lmo2+} presumptive hematopoietic precursors is unclear. An increase in apoptosis as detected by acridine orange staining was not observed through to the 20-somite stage (data not shown), but it is possible that these cells undergo apoptosis at a later stage of development.

Fig. 9. Summary of \textit{scl} gain- and loss-of-function phenotypes in wild type, \textit{spt}, and \textit{clo}. (A) In the \textit{scl} morphant, loss of \textit{scl} results in a loss of hematopoietic tissue, while endothelial and somitic tissues are unaffected. (B) In wild-type embryos, forced expression of \textit{scl} RNA results in mutually exclusive expansion of hematopoietic (red) and endothelial tissue (green) at the expense of somitic tissue (yellow). (C) \textit{spt}−/− embryos lack hematopoietic and trunk somitic tissue. Upon forced expression of \textit{scl} RNA, an expansion of hematopoietic but not endothelial tissue was observed. (D) \textit{clo}−/− embryos lack both hematopoietic and endothelial tissue but have normal somitic tissue. Upon forced expression of \textit{scl} RNA, an efficient expansion of hematopoietic tissue, but only a modest expansion of endothelial tissue, occurred. Somites are slightly less affected in injected \textit{clo}−/− embryos than in injected wild-type embryos.
development. The results demonstrate that the initial induction and patterning of hematopoietic tissue in the lateral mesoderm occurs normally in the absence of *scl*, but a later defect occurs within the HSCs or progenitor cells as assessed by a loss of more differentiated molecular markers.

The loss of definitive HSCs in the *scl* morphant was concluded from the loss of *runx1* and *cmyb* expression at 36 hpf in the AGM region. The defect in vascular remodeling is evident at this time, and the lack of AGM HSCs could be a secondary effect due to the loss of a correctly organized dorsal aorta. In mice, chimeric analyses showed that *Scl<sup>−/−</sup>* cells do not contribute to definitive hematopoietic lineages, demonstrating a cell autonomous role for *scl* in making definitive HSC (Porcher *et al.*, 1996; Robb *et al.*, 1996). By analogy, these observations suggest that the loss of AGM HSCs was the result of the absence of *scl*. However, because of the role of *scl* in vascular remodeling, a further evaluation of the requirement for *scl* in definitive HSC formation awaits the advent of conditional gene targeting techniques in the zebrafish.

The specification of angioblasts occurred normally in the *scl* morphant. Because forced expression of *scl* is capable of partially rescuing both hematopoietic and endothelial gene expression in *clo* (Liao *et al.*, 1998), it has been hypothesized that *scl* is required for hemangioblast specification. Based on the normal expression of endothelial genes in the *scl* morphant, the loss of endothelial gene expression in *clo* is independent of *scl*. The loss of *lmo2* and *gata2* in *clo* also appears to be independent of the loss of *scl*, as the *scl* morphant displayed normal *lmo2* and *gata2* expression. It has previously been demonstrated that forced expression of *scl* can induce *lmo2* and *gata2* expression (Gering *et al.*, 2003). Our results demonstrate that although *scl* is sufficient to induce expression of *gata2*, *lmo2*, and endothelial genes, as discussed further below, their expression does not require *scl*.

A defect in angiogenesis was observed in the *scl* morphant, despite normal specification of angioblasts, and the *Tg(lmo2:EGFP)* line allowed real time visualization of this defect. The vascular defect in the *scl* morphant is likely to be cell autonomous, as *scl* is expressed in vascular cells until 26 hpf. A cell non-autonomous defect from loss of a hematopoietic derived signal is unlikely, as mutants that lack circulating erythrocytes, such as *bloodless* and *vlad tepes*, develop normal vasculature (Liao *et al.*, 2002; Lyons *et al.*, 2002). The vascular phenotype in the *scl* morphant is similar to that reported for the *flk1* mutant (Habeeb *et al.*, 2000), the VEGF-A morphant (Nasevicius *et al.*, 2000), and the *phospholipase C gamma-1* (*plcg1*) mutant (Lawson *et al.*, 2003). The VEGF molecule signals through *flk1*, and *plcg1* is thought to function as effector of this signaling pathway in vessel formation. In addition to its role in vascular development, VEGF signaling has been reported to stimulate hematopoiesis and *scl* expression (Li et al., 2001; Martin *et al.*, 2004). Further studies are needed to address the role of *scl* in vascular remodeling and its relationship to the VEGF signaling pathway in this process.

Forced expression of *scl* in wild-type embryos resulted in the expansion of mutually exclusive populations of both hematopoietic and endothelial cells at the expense of somitic mesoderm. One model to explain this observation is that of an expansion of a bipotential hemangioblast population, followed by migration and differentiation to distinct fates in response to spatially restricted environmental signals. An alternative model is that of a functionally independent expansion of both tissues in response to forced expression of *scl*. By utilizing the *spt* and *clo* mutants, we provide evidence to support the latter possibility. Under normal conditions, angioblasts are specified in *spt*, as evidenced by *flk1* expression (Rohde *et al.*, 2004; Thompson *et al.*, 1998). Upon forced expression of *scl* in *spt*, the posterior *flk1<sup>+</sup>* endothelial tissue was not expanded, while *gata1<sup>−/−</sup>* hematopoietic tissue was induced. Thus, the expansion of endothelial tissue in response to forced expression of *scl* required trunk somitic tissue, or another *spt* dependent factor, whereas expansion of hematopoietic tissue did not.

Under normal conditions, *gata1* and *flk1* are only very weakly expressed in *clo* mutants. Upon forced expression of *scl* in *clo* mutants, *gata1* expression was robustly expanded, whereas only a very slight induction of *flk1* expression within the posterior mesoderm was observed. Previously, *scl* was reported to rescue *flk1* expression at 24 hpf in *clo* (Liao *et al.*, 1998). The number of *flk1<sup>+</sup>* cells present at the 10-somite stage following *scl* RNA injection may proliferate to generate the phenotype observed at 24 hpf, or alternatively, there may be a second wave of angioblast specification that is more efficiently rescued by forced expression of *scl*. In the rescued *clo* embryos, the somitic tissue was slightly less affected by over-expression of *scl* than it was in wild-type embryos, possibly reflecting the less efficient induction of endothelial tissue. Taken together, the analyses in the mutant backgrounds supports a model where *scl* is capable of inducing both hematopoietic and endothelial tissue via functionally independent mechanisms, and only the latter is attenuated in a *clo*- and *spt*-dependent manner.

Interestingly, *lmo2* was not induced in *clo* mutants in response to *scl*. This was unexpected, as murine *lmo2*, similar to *scl*, is essential for primitive and definitive hematopoiesis (Warren *et al.*, 1994; Yamada *et al.*, 1998). It is likely that endogenous *lmo2* is normally required for zebrafish hematopoiesis. However, under conditions where *scl* is present in vast excess, the requirement for such a cofactor could be overcome. Alternatively, it is possible that there is a compensatory upregulation of another LIM domain protein, such as *lmo1* or *lmo4*, which can then interact with *scl* in place of *lmo2*. In addition, the observed antagonism between mesodermal fates could be due to an ability of *scl* to squelch non-specific transcription factors or an ability to interact with other bHLH factors required for
tissue differentiation, such as myoD (reviewed in (Rudnicki and Jaenisch, 1995) and interfere with their function. It has recently been shown that SCL is capable of transforming mouse T cells in the absence of LMO2 by interfering with the function of the bHLH proteins, E47 and HEB (O’Neil et al., 2004). The ability of scl to both induce blood and alter the fate of other mesodermal derivatives could be the result of the combined effects of such mechanisms. Further investigation of zebrafish scl and lmo proteins will distinguish between these possibilities.

The inability of scl to induce flk1 expression in the posterior mesoderm of clo mutants correlates with the inability to induce lmo2, and is an intriguing possible explanation for this effect. However, in contrast to the posterior mesoderm, a rescue of flk1 expression was observed in the anterior mesoderm, despite the lack of lmo2 in this region. Previous analyses of forced co-expression of scl and lmo2 in wild-type embryos demonstrated that an expansion of anterior endothelial tissue correlated with a loss of anterior cardiac mesoderm (Gering et al., 2003). With scl over-expression alone, we did not observe a loss of the cardiac marker, nks2.5 (data not shown). Additionally, spt−/− embryos do express lmo2 in the posterior endothelial mesoderm (Rohde et al., 2004; Thompson et al., 1998), and is presumably not the reason for the inability of scl to induce flk1 in these embryos.

To date, large-scale mutagenesis screens in zebrafish have failed to identify mutations in the scl locus. The reason for this is unclear, since the scl morphant survives beyond the time at which previous genetic screens for hematopoietic mutants have been performed (Ransom et al., 1996; Weinstein et al., 1996; Dooley, K, Davidson, AJ, Schmid, B, Tü 200 Screen Consortium, and Zon, L.I., unpublished results). In the absence of a genetic mutant, we utilized morpholino knockdown technology and have demonstrated that zebrafish scl is important for hematopoietic development and angiogenesis, highlighting the evolutionary conservation of scl function. Together with over-expression analyses in zebrafish mutants, these results argue against a role for scl in the specification of hemangioblasts. To date, little is known about the targets of scl (Cohen-Kaminsky et al., 1998; Xu et al., 2003), either in hematopoietic or later endothelial development. The efficient knockdown of scl by morpholino injection provides a valuable tool for further analysis of in vivo targets of scl in both hematopoietic development and vascular remodeling.

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