Genomes & Developmental Control

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; Medvinsky 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 targeting 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 micro-injection experiments. The radiation-induced spt^{b104} mutant line was obtained from C. Kimmel (Eugene, OR) and the spontaneous mutant clo^{m39} 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′-GCG GCG 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′-GC**A** GC**A** TTA CCT GTT A**C**T AGT **A**GC G-3′). Morpholinos were resuspended in Danieau's solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM Hepes, pH7.6). 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 morpholinos 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 anti-sense 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), βe3 globin (Brownlie et al., 2003), lmo2, c-myb, fli1, flk1 (Thompson et al., 1998), fli1b (Zhu et al., manuscript in preparation), efnb2 (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 whnb (Schorpp 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% $KOH/0.9\%$ H_2O_2 for 15 min to remove pigment, followed by $2\times$ rinses in PBST, and re-fixation in 4% PFA.

Reverse transcriptase-PCR

RNA was harvested from pools of 20 embryos (uninjected or morpholino injected) at the 20-somite stage using Trizol reagent according to the manufacturer's protocol (InVitrogen). First strand cDNA was prepared using Superscript II RT according to the manufacturer's protocol (InVitrogen). PCR primers to amplify scl sequence were: SCL seq10 (5′-TCC CAG AGA CCC GCT GAG CG-3′) and SCL seq3 (5′-CAG GAG GGT GTG TTG GGA TG-3′). Control primers for eF1α were: EF1ADO (5′-ATA CCA GCC TCA AAC TCA CC-3′) and EF1AUP (5′-ATC TAC AAA TGC GGT GGA AT-3′).

RNA microinjection

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 spt 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 anti-sense 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, confirming 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 drastically 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 evaluated 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 (E/I1.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 (Burns 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 *βe3-hemoglobin* gene is normally expressed in differentiating erythrocytes within the ICM as early as the 15-somite stage. Consistent with the loss of *gata1*, transcripts for *βe3-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.

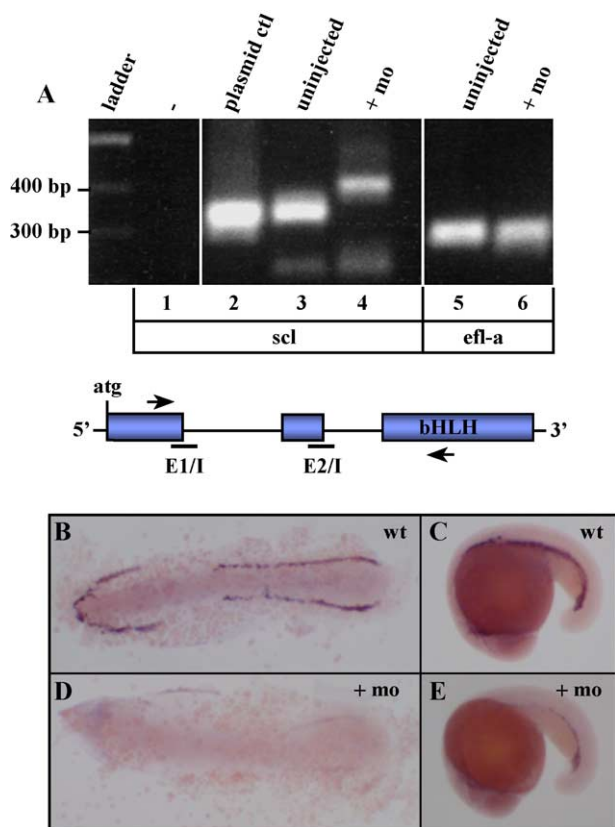The function of *scl* in anterior primitive myelopoiesis was evaluated by analyzing the expression of genes

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 *ef1-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.
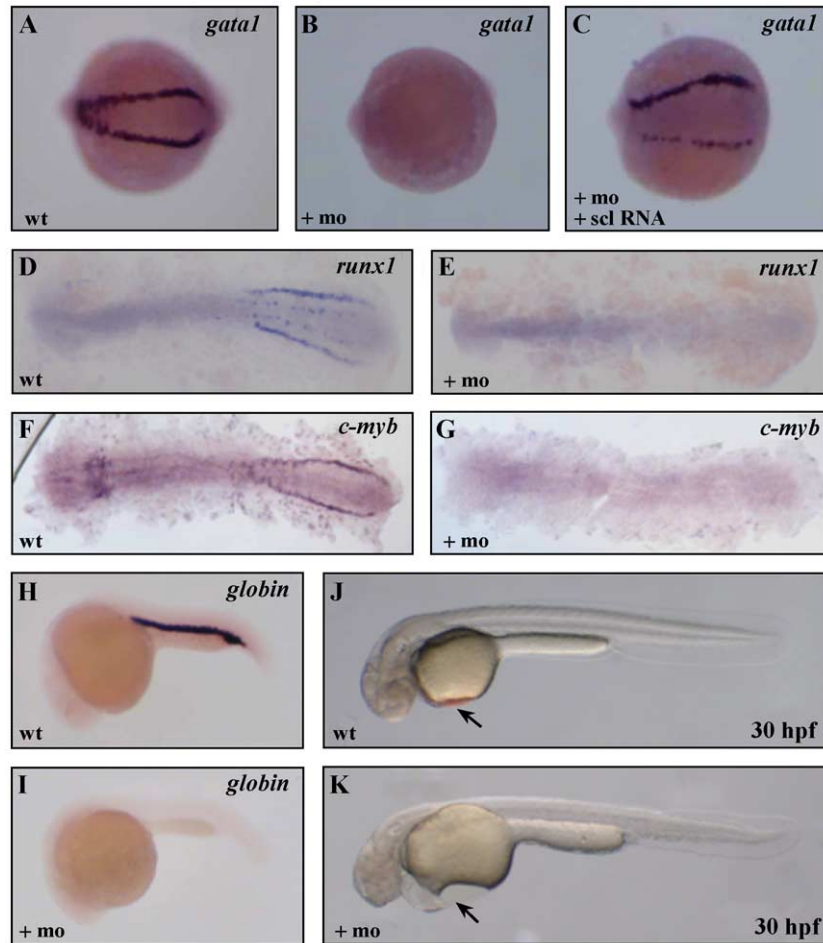
Fig. 2. Loss of embryonic erythroid development in *scl* morphants. (A, B) Loss of *gata1* expression as detected by ISH in the *scl* morphants at the 10-somite stage. (C) Co-injection of 300 pg of *scl* RNA rescued the *gata1* expression. Loss of transcripts for *runx1* (D, E) and *c-myb* (F, G) at the 10 somite stage, and *ße3-globin* transcripts at 24 hpf (H, I), in *scl* morphants. (J, K) Absence of circulating erythroid cells in the *scl* morphant embryos (arrow), in comparison to uninjected control embryos, as visualized under a dissecting microscope. Whole (A–C, H–I) or flat-mounted (D–G) embryos are shown 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-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-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-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.
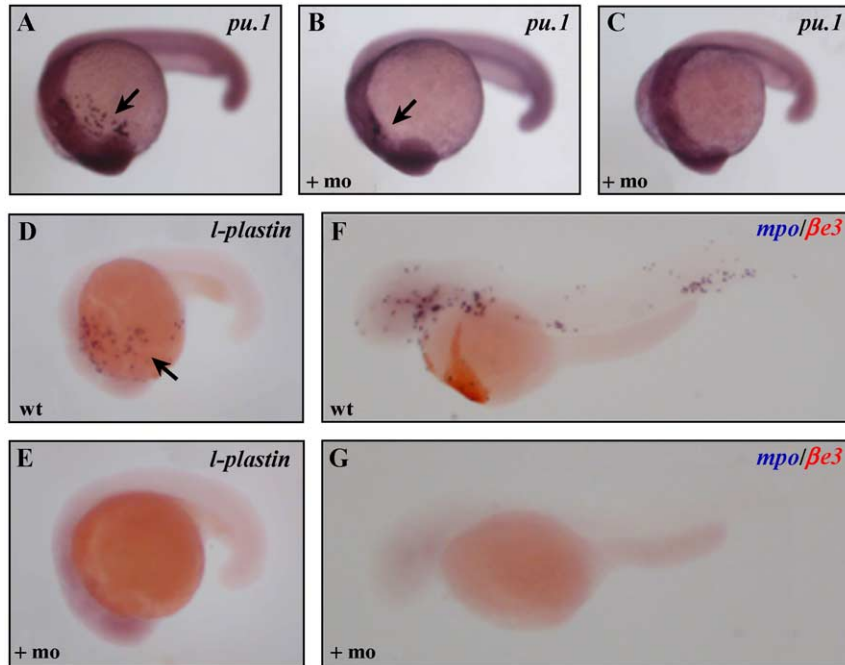
Fig. 3. Loss of myeloid development in *scl* morphants. Transcripts for *pu.1* were reduced (B) or absent (C) in the *scl* morphants, compared to uninjected controls (C), as indicated by the arrows. *l-plastin* (D, E), and *mpo* transcripts were not detected in *scl* morphants. As a control, the *mpo* ISH was performed as a double with *βe3-globin* (*βe3*) staining in red (F, G).
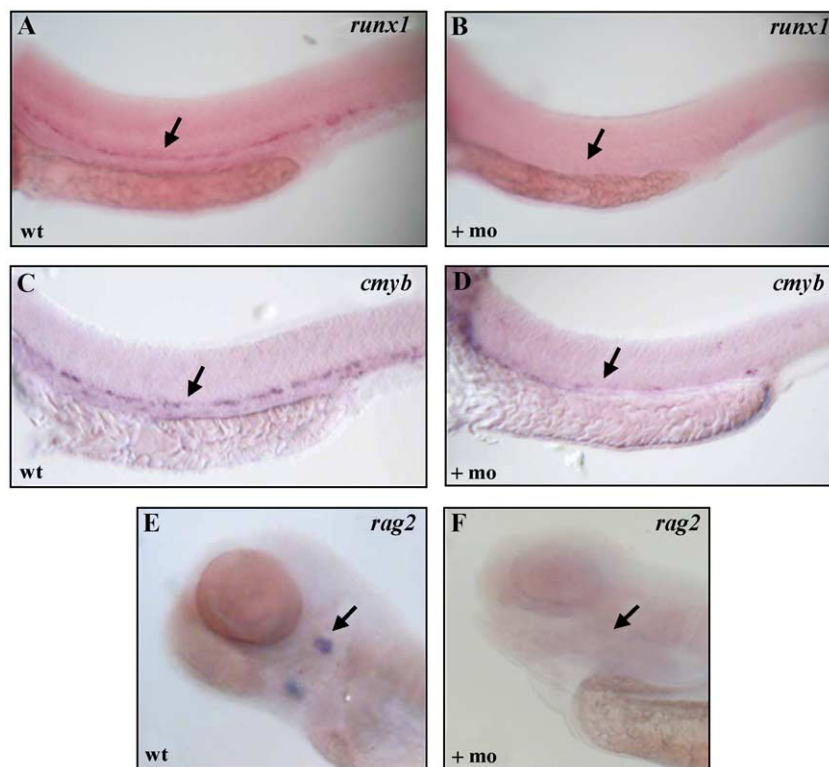

Fig. 4. Loss of definitive hematopoietic gene expression in *scl* morphants. Transcripts for *runx1* (A, B), *cmyb* (C, D), and *rag2* (E, F) were absent in *scl* morphants. In A–D, arrow indicated the region of expression along the ventral wall of the dorsal aorta. In E and F, arrow indicates expression 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.
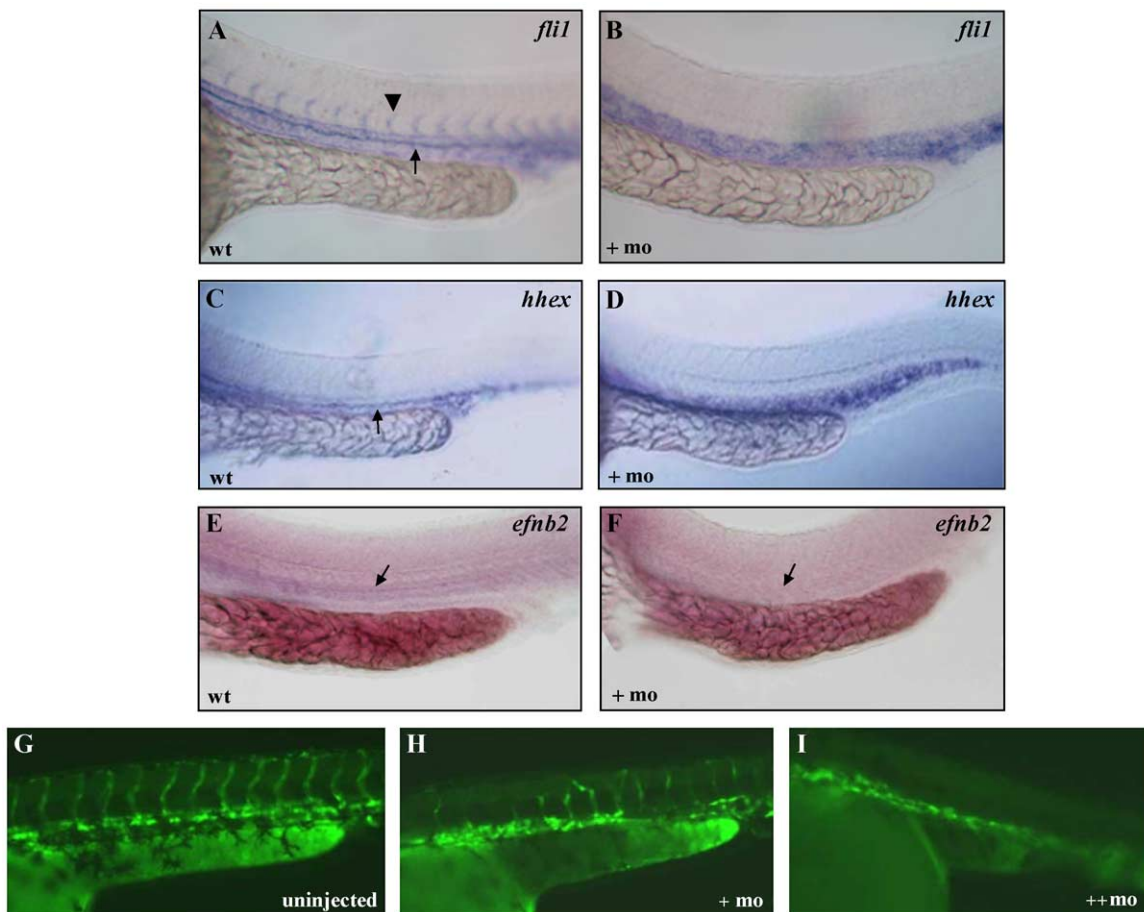

Fig. 5. Angiogenesis is disrupted in *scl* morphants. Transcripts for *fli1* (A, B), *hhex* (C, D), and *efnb2* (E, F) in uninjected and *scl* morphant embryos. In A, the arrowhead indicates the expression in intersomitic vessels, and in A, C, E–F, the arrow indicates expression in the dorsal aorta. 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
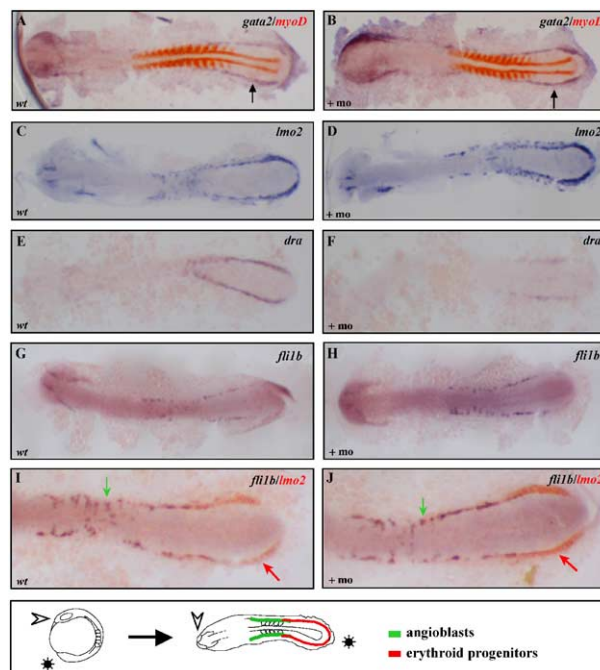

Fig. 6. Analysis of early gene expression in the *scl* morphants. Expression of *gata2* (A, B), *lmo2* (C, D), *dra* (E, F) and *fli1b* (I, J) at the 10- to 12-somite stage in *scl* morphants and uninjected controls. As an internal control, the *gata2* ISH was performed as a double with *myoD*, a marker of the somitic tissue, detected in red (A, B). (I, J) Double ISH using probes for *lmo2* (red arrow) and *fli1b* (green arrow) in uninjected and *scl* morphant embryo, showing higher magnification of posterior mesoderm. All embryos were flat mounted and shown with anterior to the left. In D, a schematic illustrates the orientation of embryos and regions of erythroid progenitors and angioblasts.

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* expression 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;

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
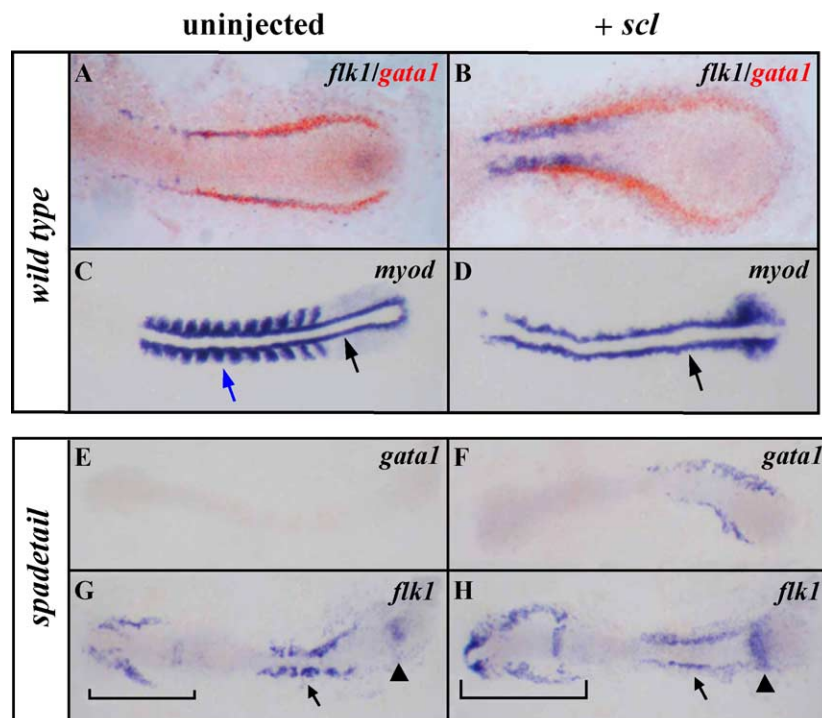

Fig. 7. Forced expression of *scl* RNA in wild-type and *spadetail* mutants. (A, B) Double ISH for *flk1* (blue) or *gata1* (red) in uninjected (A) or *scl* RNA injected (B) embryos. (C, D) *myoD* transcripts normally present in adaxial cells (black arrow) and somites (blue arrow) are not detected in 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 $clo^{-/-}$ 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 $clo^{-/-}$-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 $clo^{-/-}$ 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* over expression (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 $clo^{-/-}$-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 $spt^{-/-}$ embryos, which lack trunk somitic mesoderm. In contrast, the expansion of endothelial tissue was attenuated in $spt^{-/-}$ and $clo^{-/-}$ embryos. In $clo^{-/-}$, 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
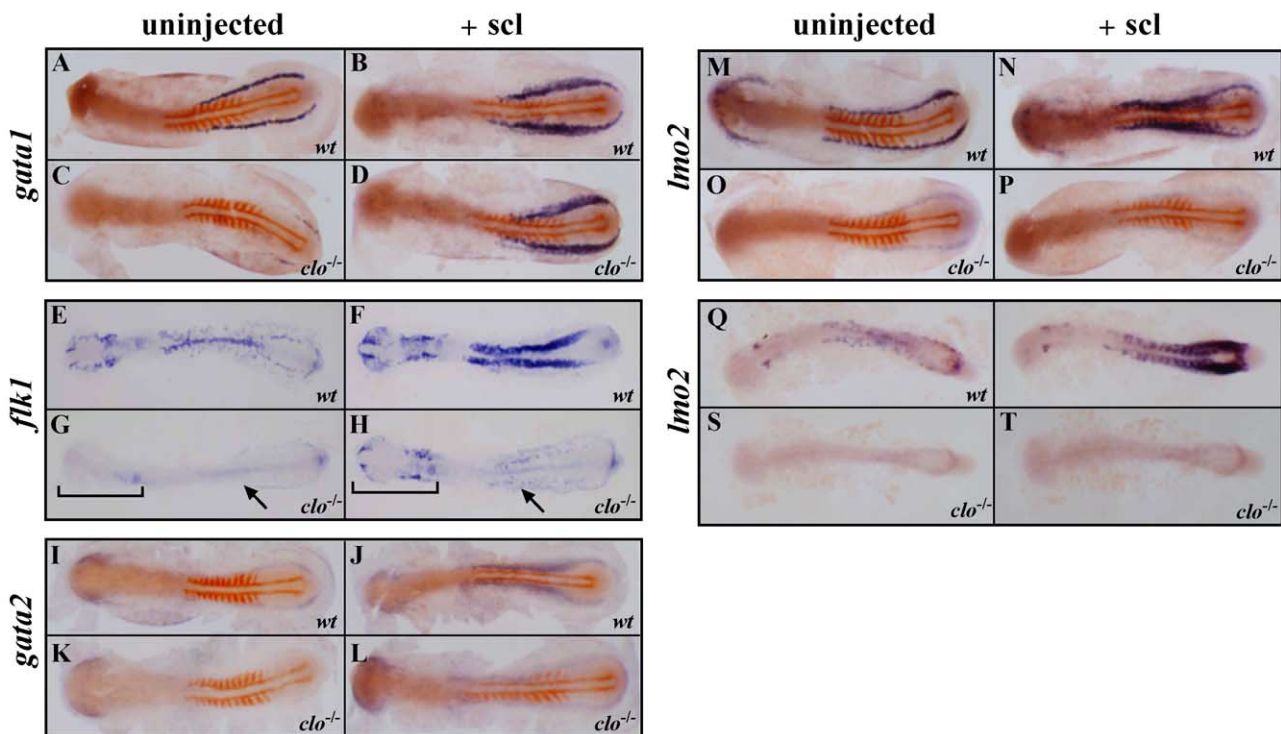

Fig. 8. Analysis of hematopoietic and endothelial expansion in *clo* in response to forced expression of *scl*. Double ISH (A–D, I–P) using *myoD* in red and *gata1*, *gata2*, or *lmo2* in blue, or single ISH using *flk1* (E–H) or *lmo2* (Q–T), in wild-type and $clo^{-/-}$ embryos injected with *scl* RNA (+scl) or uninjected controls, as indicated. In G, H, brackets indicate anterior endothelial cells, and arrows indicate posterior lateral mesoderm. In (I and J) arrowhead indicates expression of *flk1* in the tail bud. Embryos are between the 10- and 12-somite stage (A–P) or the 20 somite stage (Q–T).

results, suggest separable and independent effects on hematopoietic and endothelial development in response to forced expression of *scl*.

Discussion

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

The absence of multi-lineage hematopoiesis observed in the *scl* morphant is likely due to a requirement in HSCs or early progenitor cells, rather than a requirement for *scl* in each of the differentiated lineages, by analogy to recent studies of conditional *scl* knockdown in mice (Mikkola et al., 2003). These studies demonstrated that following specification of HSCs, *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 *spt* and *kugelig* (*kgg*) mutants lack ICM hematopoiesis but have normal anterior myeloid

development (Davidson et al., 2003; Lieschke et al., 2002). In both *spt* and *kgg* mutants, *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 *scl*, presumably in HSCs or early progenitor cells.

Transcripts for *gata2* and *lmo2* were unaffected in the *scl* morphant, despite the loss of molecular markers for more differentiated hematopoietic lineages. Gene expression profiling of in vitro differentiated $Scl^{-/-}$ murine ES cells have also shown *gata2* and *lmo2* expression comparable to that of differentiated wild-type ES cells (Elefanty et al., 1997). Both *gata2* and *lmo2* are also expressed in early endothelial cells. In the *scl* morphant, embryonic patterning was normal as assessed by both morphology and tissue-specific gene expression. The ability to observe normal *gata2* and *lmo2* expression in the embryo proper suggests that mesodermal cells have committed to a hematopoietic fate, but in the absence of *scl*, are blocked in further differentiation. The temporal loss of *dra* expression in the *scl* morphant further indicates a block in differentiation or maintenance. The fate of the $gata2^+ lmo2^+$ 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
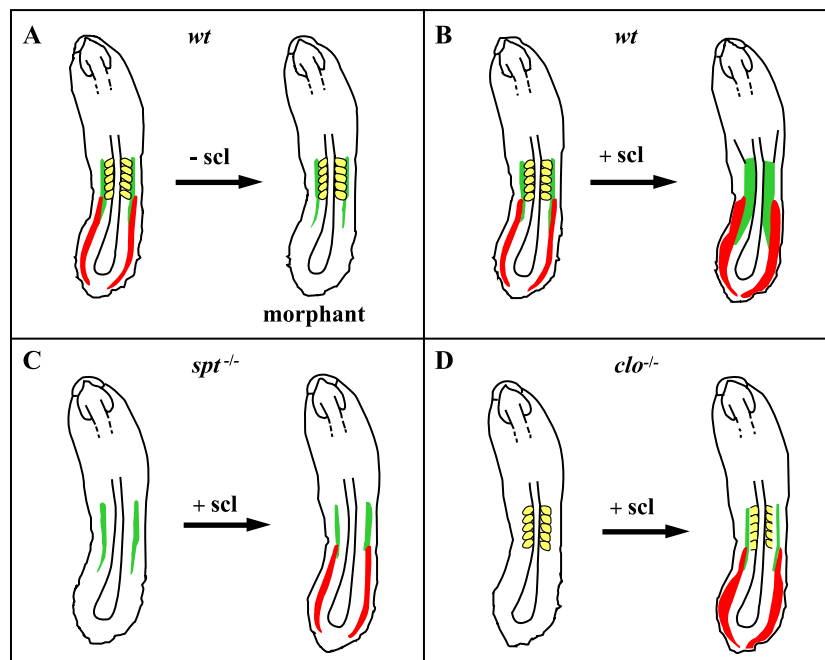

Fig. 9. Summary of *scl* gain- and loss-of-function phenotypes in wild type, *spt*, and *clo*. (A) In the *scl* morphant, loss of *scl* results in a loss of hematopoietic tissue, while endothelial and somitic tissues are unaffected. (B) In wild-type embryos, forced expression of *scl* RNA results in mutually exclusive expansion of hematopoietic (red) and endothelial tissue (green) at the expense of somitic tissue (yellow). (C) $spt^{-/-}$ embryos lack hematopoietic and trunk somitic tissue. Upon forced expression of *scl* RNA, an expansion of hematopoietic but not endothelial tissue was observed. (D) $clo^{-/-}$ embryos lack both hematopoietic and endothelial tissue but have normal somitic tissue. Upon forced expression of *scl* RNA, an efficient expansion of hematopoietic tissue, but only a modest expansion of endothelial tissue, occurred. Somites are slightly less affected in injected $clo^{-/-}$ 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 $Scl^{-/-}$ 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 (Habeck 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 (Liang 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 $flk1^+$ endothelial tissue was not expanded, while $gata1^+$ 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 $flk1^+$ 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 co-factor 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, *nkx2.5* (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, LI, unpublished results). In the absence of a genetic mutant, we utilized morpholino knockdown technology and have demonstrated that zebrafish *scl* is important for hemato-poietic 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 know 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.

Acknowledgments

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