Ets1-Related Protein Is a Key Regulator of Vasculogenesis in Zebrafish

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During embryonic development, multiple signaling pathways control specification, migration, and differentiation of the vascular endothelial cell precursors, angioblasts. No single gene responsible for the commitment of mesenchymal cells to the angioblast cell fate has been identified as yet. Here we report characterization and functional studies of Etsrp, a novel zebrafish ETS domain protein. etsrp embryonic expression is only restricted to vascular endothelial cells and their earliest precursors. Morpholino knockdown of Etsrp protein function resulted in the complete absence of circulation in zebrafish embryos. Angioblasts in etsrp–morpholino-injected embryos (morphants) failed to undergo migration and differentiation and did not coalesce into functional blood vessels. Expression of all vascular endothelial molecular markers tested was severely reduced in etsrp morphants, whereas hematopoietic markers were not affected. Overexpression of etsrp RNA caused multiple cell types to express vascular endothelial markers. etsrp RNA restored expression of vascular markers in cloche mutants, defective in hematopoietic and endothelial cell formation, arguing that etsrp functions downstream of cloche in angioblast formation. etsrp gene function was also required for endothelial marker induction by the vascular endothelial growth factor (vegf) and stem cell leukemia (scl/tal1). These results demonstrate that Etsrp is necessary and sufficient for the initiation of vasculogenesis.

Introduction

Vasculogenesis, or formation of vascular endothelial cells de novo, begins very early after the initiation of gastrulation in a vertebrate embryo. In a mammalian embryo, vasculogenesis starts with the formation of the blood islands in the yolk sac and angioblast precursors in the head mesenchyme [1]. In the zebrafish, the first angioblasts arise from the lateral plate mesoderm, migrate to the trunk midline between ten- and 15-somite stages in response to hedgehog signaling, and coalesce to form the primary axial vessels of the trunk, the dorsal aorta, and the cardinal vein [2–4]. During subsequent angiogenesis, the axial vessels sprout to form secondary vessels in the trunk region of a zebrafish embryo [5]. In the zebrafish, as well as the mammalian yolk sac, there is a close association between the primitive hematopoietic cells and the developing endothelium, suggestive of a common precursor, the hemangioblast [6]. Zebrafish cloche mutants lack nearly all blood and endothelial cells suggesting that the hemangioblast lineage has been affected [7,8]. A basic helix-loop-helix transcription factor Scl/tal1 is expressed in hematopoietic and endothelial cells in both mouse and zebrafish suggesting a possible role in specification of the hemangioblast [9–12]. Recent knockdown analysis has demonstrated its essential function in blood and dorsal aorta formation in zebrafish embryos [13,14].

Members of the Ets family of transcription factors play multiple roles during vasculogenesis, angiogenesis, and hematopoiesis [15]. They share a conserved DNA-binding domain of 85 amino acids which folds into a winged helix-turn-helix motif. Ets1, the founding member of the family, is expressed in the embryonic endothelial cells as well as putative hemangioblasts, lymphoid precursors, and myeloid hematopoietic cells [16–19]. The expression of Ets1 transcripts is associated in vivo with the activation of endothelial cells and the induction of angiogenesis. In vitro, Ets1 is expressed by proliferating and migrating endothelial cells but not after these cells have reached confluence [20,21]. In endothelial cells in vitro, Ets1 has been shown to regulate expression of the two vascular endothelial growth factor (Vegf) receptors Flt1 and Flk1 [22,23], the endothelial cell-specific adhesion protein VE-cadherin [24], the vascular-specific tyrosine kinases Tie1 and Tie2 [25], and numerous other target genes [15]. Ets1 antisense oligonucleotides inhibited angiogenesis in vitro [26,27]. However, no defects in vascular development have been found in the mouse embryos, nearly completely deficient in Ets1 function [28,29]. Therefore, the function of Ets1 in vascular development in vivo, if any, remains unclear.

A different Ets family member Fli1 has been implicated in mouse megakaryopoiesis, hemostasis, and maintenance of vascular integrity [30,31]. Zebrafish fli1 homolog is expressed during early somitogenesis in the putative hemangioblast cells, while later being restricted to vascular endothelial cells [32]. Recently, an endothelial-cell-specific Ets1-related zebrafish protein Etsrp has been identified [33]. In the current study, we demonstrate a critical role of Etsrp during vasculogenesis. In the absence of Etsrp, angioblast precursors fail to migrate, differentiate, and coalesce into functional vessels. Furthermore, ectopic expression of etsrp is sufficient to induce...
endothelial markers in a variety of different cell types throughout the embryo. These results suggest that Etsrp functions as a critical regulatory gene, directing *etsrp*-expressing cells to adopt vascular endothelial cell fate during embryonic development.

**Results**

**Etsrp Encodes a Novel Ets1-Related Protein**

A novel Ets1-related protein Etsrp has been identified in a recent microarray study of the zebrafish *cloche* mutants [33]. We performed detailed analysis of the protein sequence and its developmental expression pattern. Etsrp-predicted protein sequence contains 366 amino acids and displays 29% identity (37% similarity) to the human Ets1 protein and 26% identity (34% similarity) to the human Ets2 (Figure 1A) (unpublished data). Although Etsrp and hEts1 proteins are 88% similar within the ETS domain region (Figure 1A), there is little similarity between the two proteins within the rest of the sequence, therefore Etsrp may not be a functional ortholog of the Ets1 protein. Supporting this idea, a recently published protein sequence of the zebrafish, Ets1 homolog, was found to be highly similar to the Ets1 subfamily throughout most of the protein sequence (Figure 1A) [34]. No other homology domains were found outside the ETS domain of Etsrp. We were unable to find an apparent Etsrp ortholog in other species. As evident from the homology tree, Figure 1.

**Figure 1.** Sequence Analysis of the Zebrafish Ets1-Related Protein Etsrp

(A) Shows alignment of Etsrp and its closest human and zebrafish homologs Ets1 proteins. Etsrp and hEts1 share 88% homology within the ETS DNA-binding domain (underlined in red), and a very limited homology within the rest of the sequence. Identical and similar amino acids are shaded in grey.

(B) The homology tree of Etsrp and its closest human, mouse, and zebrafish homologs. Length of horizontal branches is directly proportional to the evolutionary distance between the proteins. Zebrafish Ets1 and Ets2 protein sequences have been predicted using the available EST sequences TC282499 and TC270146 (http://www.tigr.org). GeneWorks 2.5 has been used to build the alignment and the homology tree. (C) Chromosomal location of the zebrafish etsrp, mouse, and human ets1 genes. In all cases, they are positioned next to a *fli1* homolog.

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Etsrp is the most closely related to Ets1 and Ets2 subfamilies (Figure 1B). We performed synteny analysis of human, mouse, and zebrafish ets1 or etsrp chromosomal regions. Human and mouse ets1 genes are positioned next to flt1 genes, which encode related ETS domain proteins (Figure 1C). ets1 and flt1 genes are transcribed in opposite directions and are likely to have originated from the same ancestral precursor via gene duplication. Zebrafish etsrp is also positioned in the opposite direction and next to a flt1-related gene fltlb [34]. This analysis suggests that etsrp is evolutionarily related to the mammalian ets1 genes. As the zebrafish genome has undergone an additional duplication [35], etsrp and ets1 may have diverged from each other and acquired separate functions. Alternatively, it is possible that ets1 duplicated prior to the divergence of teleosts and tetrapods, and etsrp was subsequently lost in the tetrapod lineage.

**Etsrp Expression Pattern Analysis**

We analyzed the expression pattern of etsrp RNA in early embryos using in situ hybridization. No expression was observed prior to the one-somite stage (unpublished data). From the two-somite stage onward, etsrp RNA was localized to two stripes of cells within the lateral mesoderm (five-somite embryo, Figure 2A). Two distinct expression domains, in the anterior and posterior parts of an embryo, were apparent. The anterior stripes merged at the prechordal plate. The etsrp-expressing cells are likely endothelial cell precursors based on the expression of the gene at later stages. At the 15-somite stage, expression in two bilateral stripes of cells within the lateral mesoderm in the anterior and middle/posterior parts of an embryo, was apparent (Figure 2B–2D). In addition, a stripe of etsrp-expressing cells is apparent at the midline and extends through the middle and posterior parts of an embryo (arrows, C and D). etsrp is expressed in vascular endothelial cells of the axial, head, and intersomitic vessels. Note a group of etsrp-expressing cells bilaterally located in the intermediate mesoderm (arrowhead). Transverse section through the trunk region of a 30 hpf embryo. Arrowhead shows one of the etsrp-expressing cells located within the lateral/intermediate mesoderm. Expression of etsrp in the axial vessels is weak at this stage and not apparent in this section. nt, neural tube; n, notochord; y, yolk; (E) 36 hpf stage. etsrp is expressed in a subset of head vessels, the aortic arches (aa), the cardinal vein plexus (pl) region, posterior intersomitic vessels, and the dorsal longitudinal anastomotic vessel (dlav). Note a group of etsrp-expressing cells located in the endodermal region (arrowhead). (H) 52 hpf stage. etsrp expression is observed in a subset of head vessels, common cardinal vein, pectoral fin bud blood vessels (fb), cardinal vein plexus region, and weakly in posterior intersegmental vessels and the dorsal vessel.

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**Figure 2. Expression Pattern of etsrp as Analyzed by In Situ Hybridization**

Anterior is to the left except as noted. (A) five-somite stage. etsrp is expressed within lateral mesoderm in two distinct expression domains, in the anterior and posterior parts of an embryo. (B) 15-somite stage, lateral view. (C) dorsal view. (D) transverse section. etsrp is expressed in two bilateral stripes of presumptive angioblasts within the lateral mesoderm in the anterior and the trunk and posterior parts of an embryo (arrows, D). In addition, a stripe of etsrp-expressing cells is apparent at the midline and extends through the middle and posterior parts of an embryo (arrowhead, C and D). etsrp is expressed in vascular endothelial cells of the axial, head, and intersomitic vessels. Note a group of etsrp-expressing cells bilaterally located in the intermediate mesoderm (arrowhead). (F) Transverse section through the trunk region of a 30 hpf embryo. Arrowhead shows one of the etsrp-expressing cells located within the lateral/intermediate mesoderm. Expression of etsrp in the axial vessels is weak at this stage and not apparent in this section. nt, neural tube; n, notochord; y, yolk; (G) 36 hpf stage. etsrp is expressed in a subset of head vessels, the aortic arches (aa), the cardinal vein plexus (pl) region, posterior intersomitic vessels, and the dorsal longitudinal anastomotic vessel (dlav). Note a group of etsrp-expressing cells located in the endodermal region (arrowhead). (H) 52 hpf stage. etsrp expression is observed in a subset of head vessels, common cardinal vein, pectoral fin bud blood vessels (fb), cardinal vein plexus region, and weakly in posterior intersegmental vessels and the dorsal vessel.

Morpholino Knockdown of Etsrp Function Results in Loss of Circulation

We used antisense morpholino (MO) oligonucleotides [36,37] to knockdown the function of Etsrp. Etsrp–MO–injected embryos (morphants) showed no apparent circulation. While red blood cells were actively circulating in wild-type (wt) embryos at 34 hpf, in etsrp morphants they stayed at their formation site, the ICM region (Figure 3A and 3B). This observation was confirmed by the o-dianisidine staining of heme in the red blood cells (Figure 3A–3E). We performed microangiography analysis by injecting fluorescein-labeled high molecular weight dextran into the sinus venosus of the 2-day-old embryos. Lower doses of etsrp MOs caused partial loss of circulation in the intersegmental and axial vessels, particularly, in the posterior part of the embryo (Figure 3F and 3G). Head circulation was not affected at this dose (Figure 3G). Higher doses of etsrp MOs resulted in the complete loss of functional blood vessels (Figure 3H and 3I, Table 1). Other than the described defects, etsrp morphants appeared morphologically normal (Figure 3B). At 2–3 days-post-fertilization (dpf), etsrp morphants developed pericardial edema and eventually became necrotic and died (unpublished data). Injection of two different etsrp-specific
Figure 3. MO Knockdown of Etsrp Protein Function Disrupts Blood Vessel Formation in the Zebrafish Embryos

(A,B) Morphological analysis of live etsrp morphants at 34 hpf. (A) Uninjected control embryo. (B) 5 ng of etsrp MO1-injected embryo. Notice that red blood cells are scattered throughout the circulatory system in the control uninjected embryo while they accumulate at their formation site within the intermediate cell mass (arrow, B) in the etsrp morphant. (C–E) o-dianisidine staining of heme in the red blood cells of uninjected control (C), 5 ng of etsrp MO1-injected (D) and 5 ng of etsrp MO2-injected (E) embryos at 34 hpf. While many circulating blood cells are apparent within the common cardinal vein before entering the heart in the control embryo (arrow, C), they stay at their formation site within the ICM region in etsrp morphants (arrows). (F–I) Microangiography analysis of the circulatory system by injecting fluorescein-labeled dextran into the sinus venosus of etsrp morphants at 55 hpf. (F) Control uninjected. (G) 1 ng of etsrp MO1-injected. (H) 2.5 ng of etsrp MO1-injected. (I) 5 ng of etsrp MO1-injected embryos. Note that the embryo in (G) has lost circulation in the posterior vessels, the embryo in (H) has lost circulation in most vessels, and the embryo in (I) has no circulation at all. (J–M) Analysis of blood vessels in live flk1-GFP transgenic embryos at 26 hpf. (J) Control uninjected. (K) 1 ng of etsrp MO1-injected. (L) 2.5 ng of etsrp MO1-injected. (M) 15 ng of etsrp MO1-MO2 (1:1) mix-injected embryos. Note the gaps in formation of intersegmental vessels in (K) (arrowhead), the missing (arrowhead) and abnormally branched, (arrow) intersegmental vessels in (L), and the nearly completely eliminated flk1 expression from axial vessels (arrow) in (M).

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Table 1. Dose Dependence of Etsrp MO Phenotype

| Dose Injected | Normal Circulation, % of Embryos | No or Defective Intersegmental Circulation, % of Embryos | No Intersegmental and No or Defective Axial, % of Embryos | Complete Loss of Circulation, % of Embryos |
|---------------|----------------------------------|--------------------------------------------------------|--------------------------------------------------------|------------------------------------------|
| 1–1.5 ng MO1  | 62 ± 17                          | 27 ± 16                                                 | 7 ± 3                                                  | 1 ± 1                                    |
| 2.5 ng MO1    | 19 ± 6                           | 34 ± 9                                                  | 42 ± 8                                                 | 1 ± 1                                    |
| 5–6 ng MO1    | 9 ± 3                            | 35 ± 9                                                  | 35 ± 10                                                | 22 ± 3                                   |
| 1 ng MO2      | 4 ± 1                            | 7 ± 4                                                   | 63 ± 11                                                | 27 ± 5                                   |
| 2.5 ng MO2    | 6 ± 3                            | 4 ± 1                                                   | 47 ± 4                                                 | 40 ± 11                                  |
| 5–6 ng MO2    | 4 ± 1                            | 5 ± 5                                                   | 18 ± 1                                                 | 71 ± 11                                  |

Two different etsrp-specific translation-blocking MOs cause the same phenotype, resulting in defective intersegmental and axial circulation at lower doses and complete loss of circulation at higher doses. Live embryos were scored at 2 dpf by visual observation.

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Vascular Endothelial Markers Are Lost in Etsrp Morphants

We performed analysis of different molecular markers in *etsrp* morphants using high doses of *etsrp* MOs. Kinase insert domain receptor (keh, fli1) [40], caderin 5 (cdh5, VE-cadherin) [41], adenovomedulin receptor (admR) [42], dual specificity phosphatase 5 (dusp5) [42], and C1qR-like (crl) [42] genes are expressed in all vascular endothelial cells while fms-related tyrosine kinase 4 (flt4) [40] is restricted to the venous vessels in early zebrafish embryos. Expression of all of these markers in axial and intersegmental vessels was nearly completely abolished in *etsrp* morphants at 24 hpf (Figure 4A–4L). Head vessel expression, however, was reduced but still apparent even at high MO doses (Figure 4A–4D) (unpublished data). We were unable to use even higher MO doses to attempt to completely eliminate Etsrp function due to mild toxic effects observed at doses above 15 ng (unpublished data).

Expression of *friend leukemia integration 1* (*fli1*) [40] was downregulated but still apparent in *etsrp* morphants (Figure 4M and 4N). Interestingly, expression of *etsrp* itself was upregulated in *etsrp* morphants, suggesting the presence of a negative autoregulatory loop (Figure 4O and 4P). However, the number of *etsrp*-expressing angioblasts was reduced in *etsrp* morphants. These angioblasts remained scattered within the lateral mesoderm failing to coalesce into functional blood vessels (Figure 4P). Formation of hematopoietic precursors appeared unaffected as evident from *scl* and *gata1* expression (Figure 4Q–4T).

We analyzed formation of hematopoietic and vascular progenitors during early somitogenesis stages. A transcription factor *scl* is expressed in both hematopoietic and vascular endothelial cell precursors [12]. Expression of *scl* in *etsrp* morphants was reduced in the anterior domain and absent from the trunk domain while its posterior expression was not affected at the six- and ten-somite stages (Figure 5A–5D). *fli1* is expressed in the putative hemangioblast cells while later being restricted to vascular endothelial cells [32]. Anterior expression of *fli1* was absent in *etsrp* morphants while its tail expression was not affected at the six-somite stage (Figure 5E and 5G). At the ten-somite stage, *fli1* expression in *etsrp* morphants was absent from the anterior and trunk domains (Figure 5F and 5H). As described earlier, angioblasts migrate from the lateral mesoderm towards the axial and intersegmental vessels while *scl* expression remains absent in the trunk domain (Figure 5I–5L). As described earlier, angioblasts migrate from the lateral mesoderm towards the axial and intersegmental vessels while *scl* expression remains absent in the trunk domain (Figure 5I–5L).
midline during somitogenesis \[2,3\]. etsrp-expressing angioblasts were present within the lateral mesoderm but failed to migrate towards the midline in etsrp morphants (Figure 5I–5L). Also, no scl- or fli1-expressing migrating angioblasts were observed in etsrp morphants (unpublished data). etsrp expression level within the angioblasts was strongly increased in etsrp morphants suggesting the presence of negative autoregulation. All of the etsrp-expression domains, including the trunk domain, were present in etsrp morphants (Figure 5I–5L) (unpublished data).

Etsrp is Sufficient for the Vascular Endothelial Marker Induction

We analyzed if etsrp mRNA was sufficient for induction of the vascular endothelial markers. Injection of synthetic etsrp mRNA into zebrafish embryos at the 1–16 cell stage resulted in strong ectopic induction of vascular endothelial markers flk1, scl, fli1, and cdh5 during somitogenesis (Figure 6A–6F) (unpublished data). Large patches of intense ectopic flk1 expression were observed within different germ layers including dorsal, lateral, and ventral mesoderm, endoderm and neuroectoderm (Figure 6E and 6F) (unpublished data). No induction of hematopoietic marker gata1 was observed (Figure 6G and 6H). These results indicate that etsrp is sufficient to induce vascular endothelial gene expression in a variety of cell types. Furthermore, etsrp specifically induces vascular markers without affecting closely related hematopoiesis.

We tested if etsrp was sufficient for vascular induction in clo–/– mutant embryos. etsrp mRNA was injected into the progeny from clo+/– carriers, and the embryos were analyzed for flk1 expression at the ten- to 12-somite stages. As expected, 25% (23 out of 92) of the uninjected progeny from clo+/– carriers showed no flk1 expression. Among etsrp mRNA injected embryos (n = 110), 29% displayed normal flk1 expression pattern (with minor distortions in some embryos), 45% showed both endogenous and ectopic flk1 expression, 19% showed only ectopic flk1 expression, and 6% showed no detectable flk1 expression (Figure 6I–6L). The last two groups apparently represent clo–/– homozygous mutants. These results show that the induction of flk1 by etsrp is independent of clo function, suggesting that etsrp functions downstream of clo. etsrp mRNA did not fully restore the endogenous pattern of flk1 expression in clo–/– mutants most likely because it was expressed ubiquitously and not localized to vascular progenitors.

Etsrp Function Is Required for flk1 Induction by Vegf and Scl

We analyzed the epistatic relationship between vegf and etsrp. vegf overexpression has been reported to induce strong expression of vascular markers such as flk1 \[43\] (Figure 7A...
and 7B). Co-injection of vegf mRNA together with etsrp MO resulted in the loss of flk1 expression, similar to etsrp MO phenotype, indicating that etsrp function is required for flk1 induction by vegf signaling (Figure 7C and 7D). Downregulation of vegf expression resulted in the loss of flk1 expression in the intersegmental vessels (Figure 7E and 7F). etsrp expression in dorsal aorta was also not apparent while cardinal vein seemed expanded. This is consistent with the previous report of vegf involvement in regulating arterial fate in zebrafish [44]. Expression of etsrp was not affected in vegf morphants during mid-somitogenesis stages (unpublished data).

Scl has been implicated in the hematopoietic and vascular development in zebrafish [13,14]. Downregulation of Scl function using two different MOs had no significant effect on the etsrp expression in angioblasts while the hematopoietic expression of gata1 was completely eliminated (Figure 7G–7J). Overexpression of scl mRNA has been reported to cause induction of vascular markers [45]. We tested if etsrp MO would block this induction by co-injecting it together with scl mRNA. The embryos were analyzed for flk1 expression at the 15-somite stage (Figure 7K–7N). As expected, no or only extremely weak flk1 expression was observed in etsrp-MO injected embryos (Figure 7L). Overexpression of scl mRNA caused strong induction of flk1 (Figure 7M). Embryos, co-injected with scl mRNA and etsrp MO showed no or very weak flk1 expression (Figure 7N). These results demonstrate that etsrp function is required for flk1 induction by scl.

**Discussion**

In this study, we report characterization and functional analysis of a novel zebrafish Ets1-related protein, Etsrp. Knockdown of Etsrp resulted in the complete absence of functional blood vessels and downregulation of all endothelial-specific markers analyzed. In zebrafish, hedgehog signaling is necessary for migration of angioblasts from the lateral mesoderm toward the midline, where they subsequently differentiate within the ICM region [4]. Such migration is not apparent in the anterior region which gives rise to the anterior vessels including head vessels. Interestingly, vasculogenesis defects in etsrp morphants were more pronounced in the posterior region of the embryo which was particularly evident at lower downregulation levels. At high MO doses, endothelial cells were nearly completely absent from axial and intersegmental vessels. However, head expression of endothelial markers was not completely eliminated. It is possible that the remaining amount of Etsrp protein activity is sufficient for the head vasculogenesis. Upregulation of etsrp RNA was particularly strong in the head region in etsrp morphants, which may partially compensate for the MO-mediated translation inhibition. Alternatively, etsrp function may be redundant in the vasculogenesis of head vessels.

Among the vascular-specific genes analyzed, expression of only fli1 and scl was not globally downregulated in the early embryos. The early fli1 expression, which overlaps with the hematopoietic factor gata2 expression, is unaffected in the clo mutants and scl morphants and has been suggested to mark...
the common blood and vascular precursors, hemangioblasts [13,32]. fli1 is later expressed specifically in the vascular endothelial cell precursors, and this expression was reduced but not completely eliminated in etsrp morphants. fli1 expression may not be directly regulated by etsrp, therefore undifferentiated angioblasts retain some of fli1 expression in etsrp morphants. scl is expressed in both hematopoietic and vascular progenitors throughout most of the early development. Although we did not see global downregulation of scl expression in etsrp morphants, it is likely that scl expression is lost in angioblasts but retained in hematopoietic precursors. Interestingly, expression of both fli1 and scl genes was lost from the anterior and trunk domains of the lateral mesoderm. Anterior domain contains angioblast and myeloid progenitors, which appear to be absent from the etsrp morphants. The difference between the trunk and posterior lateral mesoderm has not been previously analyzed in great detail. Possibly, the trunk region contains mostly angioblasts, while the tail region contains both angioblasts and hematopoietic precursors.

Overexpression of etsrp induced strong ectopic expression of vascular markers in multiple cell types, including even tissues that normally commit to a very different fate such as neuroectoderm. Furthermore, overexpression of etsrp specifically initiated vascular development without affecting related hematopoiesis, which argues that the observed effects are not caused by an early ventralization of the whole embryo. These results indicate that etsrp is sufficient to initiate vasculogenesis. As a contrast, overexpression of other regulators of vasculogenesis such as vegf and scl induced expression of vascular markers only within the lateral or somitic mesoderm [14,43,45].

Figure 7. etsrp Is Required for vegf and scl Signaling

(A–D) Etsrp is required for Vegf signaling as assayed for flk1 expression at 26 hpf. (A) Control uninjected embryo, (B) vegf RNA-injected embryo, (C) 7.5 ng of etsrp MO2-injected embryo, (D) vegf RNA- and etsrp MO2-co-injected embryo. Note that vegf RNA induces strong flk1 expression in (B) while vegf RNA and etsrp MO co-injection results in loss of flk1 expression in (D), similar to the etsrp morphant phenotype in (C). (E,F) Etsrp expression analysis in Vegf morphants at 26 hpf. (E) Control uninjected embryo; (F) 10.5 ng of vegf MO-injected embryo. Note that vegf morphants have lost etsrp expression in the intersegmental vessels (arrowhead). (G–I) Scl knockdown affects gata1 but not etsrp expression at the 15-somite stage. Dorsal view, anterior is to the left. (G,I) Control uninjected embryo; (H,J) 10 ng scl UTR-MO-injected embryo; (G,H) gata1 expression; (I,J) etsrp expression. (K–N) Etsrp is required for scl signaling in clo mutants as analyzed for flk1 expression at the 15-somite stage. (K) Control uninjected embryo; (L) 7.5 ng etsrp MO2-injected embryo; (M) scl RNA-injected embryo; (N) scl RNA- and etsrp MO2-co-injected embryo. Note that scl RNA causes ectopic flk1 expression in (M) which is lost upon knockdown of Etsrp in (N).

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Overexpression of vegf and scl did not have an effect on vascular development in the absence of etsrp function, which suggests that etsrp is an essential mediator of vegf and scl signaling, at least in the vascular induction. Loss of vegf or scl did not affect early expression of etsrp within angioblasts. Overexpression of etsrp caused strong scl induction, indicating that etsrp plays an important role in controlling scl expression, at least within angioblasts. This is also supported by the loss of scl expression in the anterior and trunk regions in etsrp morphants. Our data suggest that etsrp is necessary for scl expression within angioblasts, and both genes are then required for induction of multiple vascular endothelial genes.

The current study shows for the first time that a single gene can be necessary and sufficient for initiating vascular development in vertebrates. Our results demonstrate that Etsrp acts as a very early regulator of vasculogenesis. These findings will greatly advance our understanding of vascular development and the general mechanisms of cell fate specification.

**Materials and Methods**

**Microinjection of MOs.** Two etsrp-specific MOs (MO1, TTGGTA CAATTCCATATCGAAGG and MO2, CACTGACTCTATTI CACTATATC; Gene Tools, Inc.) were used to inhibit the function of Etsrp protein. For the dose response curve, 1–6 nl of 1 mg/ml solution in the Danieau buffer supplemented with 15 mM Tris-Cl (pH 7.5) was injected into one- to two-cell-stage embryos [30]. For phenotypic and marker analysis, 3–6 nl of 2.5 mg/ml MO2 solution was injected. A five-base MO2 mismatch (CAGTGGAGCTTAATCAG TATAAC) was used as a control MO. A previously described Vegf-A-1 MO (kindly donated by S.C. Ekker) was used to inhibit Vegf function [46]. ScI translation-blocking UTR-MO (GCTCGGATTT CATTTCCATATCTTAAAGT and MO2, CACTGAGTCCTTATTT TATAAC) was used as a control MO. A previously described Vegf-A-1 MO (kindly donated by S.C. Ekker) was used to inhibit Vegf function [46].

**BAC modification and microinjection.** CHORI-211 BAC library from Children’s Hospital Oakland Research Institute, Oakland, California, United States was used to identify etsrp-containing–BAC of approximately 250 kilobases. Replacement of Etsrp coding sequence with GFP was essentially performed as described [38]. Purified BAC DNA (100–150 pg) was injected into blastomere at the one-cell-stage.

**RNA overexpression and epistasis experiments.** Etsrp overexpression constructs were generated by subcloning the open reading frame of etsrp into the SpeI site of pT3TS [47]. To synthesize mRNA, Etsrp-GFP BAC was linearized with XbaI and transcribed using T3 mMessage mMachine Kit (Ambion, Austin, Texas, United States). At the two- to 16-cell stage for overexpression studies, 75–15 pg of etsrp mRNA was injected into zebrafish embryos. For epistasis studies, approximately 50 pg of VEGF121 and VEGF165 mRNA 1:1 mixture was injected [43]. Approximately 300 pg of scl mRNA was injected for epistasis studies [12].

**In situ hybridization.** In situ hybridization was performed as described [48]. The following probes were used: etsrp [42], fkl [40], fkl [40], fl [40], fkl [40], scl [12], gataI [19], adma [14], cd5 [42], crl [42].

**Analysis of etsrp knockdown phenotype.** O-diamide heme staining was performed as described [19]. Microangiography was performed as described [46].

**Zebrafish strains.** Majority of etsrp knockdown analysis was performed in the wild-type zebrafish from Scientific Hatchingers (Huntington Beach, California, United States). As confirmed by sequencing, no polymorphisms in the etsrp MO-binding sites were detected in this wild-type strain. In addition, fkl-GFP transgenic zebrafish line was used [39]. chl line was used in etsrp overexpression experiments [7].

**Supporting Information**

**Figure S1.** Etsrp MO Blocks GFP Expression in the Embryos Injected with etsrp–GFP-Modified BAC Construct

Mosaic panels have been generated by taking individual images of randomly chosen embryos from each batch. Embryos are at the 90% epiboly stages. (A) Following microinjection, etsrp-GFP BAC is transiently expressed in a mosaic pattern. Note that the transient BAC expression commonly does not recapitulate the endogenous expression pattern. (B) Co-injection of etsrp-GFP BAC and 10 ng of etsrp MO2 completely eliminated GFP fluorescence. (C) Co-injection of etsrp-GFP BAC and 10 ng of 5-base MO2 mismatch had no effect on GFP fluorescence.

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**Competing interests.** The authors have declared that no competing interests exist.

**Author contributions.** SL and SS conceived and designed the experiments. SS performed the experiments. SL and SS analyzed the data. SS wrote the paper.

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