Interplay Between Etsrp/ER71, Scl And Alk8 Signaling

Controls Endothelial And Myeloid Cell Formation

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Short title: Etsrp regulates endothelial and myeloid lineages

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ABSTRACT

Vascular endothelial and myeloid cells have been proposed to originate from a common precursor cell, hemangioblast. Mechanism of endothelial and myeloid cell specification and differentiation is poorly understood. We have previously described the endothelial-specific zebrafish Ets1-related protein (Etsrp), which was both necessary and sufficient to initiate vasculogenesis in the zebrafish embryos. Here we identify human Etv2/ER71 and mouse ER71 proteins as functional orthologs of Etsrp. Overexpression of mouse ER71 and Etsrp caused strong expansion of hemangioblast and vascular endothelial lineages in a zebrafish embryo. In addition, we show that *etsrp* is also required for the formation of myeloid but not erythroid cells. In the absence of *etsrp* function, the number of granulocytes and macrophages is greatly reduced. *Etsrp* overexpression causes expansion of both myeloid and vascular endothelial lineages. Analysis of mosaic embryos indicates that *etsrp* functions cell-autonomously in inducing myeloid lineage. We further demonstrate that the choice of endothelial versus myeloid fate depends on combinatorial effect of *etsrp, scl* and *alk8* genes.
INTRODUCTION

Hematopoiesis, the differentiation of hematopoietic stem cells into various blood cell lineages, and vasculogenesis, the differentiation of endothelial cell progenitors into vascular endothelial cells, are closely related in many different organisms. Endothelial and hematopoietic cells emerge in the embryo in close proximity and time, suggesting the possibility of a common progenitor, the hemangioblast \(^1\). In the extraembryonic visceral yolk sac of a mouse embryo, vascular plexus and blood islands are closely associated with each other and share expression of multiple genes such as \textit{flk1, flt1, tie1, tie2} and others \(^2\). Differentiating embryonic stem cells contain blast colony-forming cells which can differentiate into both hematopoietic and endothelial cells in vitro \(^3\). In the zebrafish, which recently emerged as an excellent model system for the studies of vertebrate hematopoiesis and vasculogenesis / angiogenesis, both primitive hematopoietic and endothelial progenitors originate within lateral plate mesoderm, later forming an intermediate cell mass region, equivalent to the mouse blood islands \(^4,5\). The zebrafish \textit{cloche} mutants are deficient in both vascular endothelial and hematopoietic lineages \(^6\). Zebrafish vascular endothelial and hematopoietic cell progenitors also share expression of common markers such as a basic helix-loop-helix transcription factor \textit{scl/tal1} \(^7,8\) and an ETS domain transcription factor \textit{fli1} \(^9\). Overexpression of \textit{scl} can induce both hematopoietic and endothelial markers \(^7,8,10\) while knockdown of \textit{scl} results in defective hematopoietic and endothelial development \(^11,12\). Single cell-labeling in a zebrafish gastrula embryo demonstrated that individual cells can give rise to both erythroid and
endothelial cells\textsuperscript{13}, providing a definitive evidence for the existence of hemangioblast in vivo.

In the zebrafish, anterior lateral plate mesoderm gives rise to the progenitors of myeloid cells and head vessels while the posterior lateral plate mesoderm gives rise to the primitive erythroid cells, axial and intersegmental blood vessels\textsuperscript{14}. Two recent studies have demonstrated that the interplay between an early myeloid-specific transcription factor \textit{pu.1 / spi1} and an erythroid-specific \textit{gata1} determines the choice between myeloid and erythroid fates in the progenitor cells\textsuperscript{15,16}). \textit{Pu.1} is first expressed in both myeloid and erythroid cell progenitors, and later its erythroid-specific expression is repressed by \textit{gata1}, ensuring that \textit{pu.1} expression remains restricted to the myeloid cells within anterior lateral mesoderm. \textit{Scl} signaling is critical for myeloid development as \textit{scl}-deficient embryos lack \textit{pu.1}-expressing cells\textsuperscript{12}. BMP receptor Alk8 signaling functions in parallel to \textit{scl}, also providing an instructive input for myelopoiesis\textsuperscript{17}. However, the mechanism of transition from the putative hemangioblast to the myeloid and endothelial lineages is still unknown. Although a recent study demonstrated the existence of the common erythroid-endothelial progenitor cells\textsuperscript{13}, myeloid-endothelial progenitor cells have not been analyzed in this study. Therefore, the definitive evidence for the existence of a common myeloid-endothelial hemangioblast progenitor cells is still missing.

We have recently described a novel \textit{ets1-related protein, etsrp}\textsuperscript{18}. \textit{Etsrp} expression is localized to the presumptive vascular progenitor cells from the early somitogenesis stages. Knockdown of \textit{etsrp} results in the complete lack of circulation. Angioblasts in \textit{etsrp}-deficient embryos do not differentiate, fail to migrate towards midline and do not express other vascular markers. \textit{Scl} expression is strongly
downregulated in the anterior lateral mesoderm in etsrp-morpholino (MO) injected embryos (morphants) while its posterior erythroid-specific expression is not affected. Consistent with this, erythroid lineage is not affected in etsrp morphants. Overexpression of etsrp RNA is sufficient to induce expression of endothelial and hemangioblast markers, including scl, in many different regions of an embryo. This data show that etsrp is both necessary and sufficient for the initiation of vasculogenesis in a zebrafish embryo. Although this study clearly established etsrp as a critical regulator of zebrafish vasculogenesis, it was not clear whether etsrp function is conserved in other vertebrates, including mammals.

In the current study, we demonstrate by the phylogeny analysis that zebrafish etsrp is related to mouse ER71 and human etv2/ER71 genes. We show that mouse ER71 is functionally related to Etsrp by performing overexpression of both proteins in zebrafish embryos. We also further investigate etsrp function within the putative hemangioblast cells. We show that etsrp is both necessary and sufficient to initiate the myeloid cell formation, in addition to the endothelial lineage. We demonstrate that etsrp function is specific to the anterior but not the posterior hemangioblasts, thus providing the first distinction between the two pools. We also show that as the endothelial and myeloid lineages separate, etsrp is excluded from the myeloid lineage, and remains restricted to the endothelial cell precursors. Finally, we show that etsrp-expressing cells can give rise to both myeloid and endothelial lineages, supporting the existence of a common myeloid-endothelial cell progenitor, the hemangioblast.
MATERIALS AND METHODS

Microinjection of MOs. In all experiments, unless otherwise noted, 8-10 ng of etsrp-specific MO1 and MO2 mixture (4-5 ng each) was injected at the 1-2 cell stage 18. Typically, two independent experiments were performed, with at least 15-20 embryos per experiment analyzed. Penetration of observed phenotypes was greater than 90%, unless otherwise noted. Other morpholinos used: 1.2 ng alk8 MO: ACAACTCCTCAAGTGACTCTCAGCG (Open Biosystems) 19; scl MO: GCTCGGATTTCAGTTTTTCCATCAT (Open Biosystems) 18; 8 ng pu.l MO: GATATACTGATACTCCATTGGTGGT 15 (kind gift of J.P. Kanki); 7 ng gata1-ATG MO: CTGCAAGTGTAGTATTGAAGATGTC 16 (kindly donated by L. Zon).

Overexpression and epistasis experiments. 75-150 pg of etsrp mRNA was injected into the zebrafish embryos at the 1-8 cell stages 18. Approximately 100 pg of scl mRNA 8 and 10 pg of CA-alk8 mRNA 19 (kindly donated by M. Hammerschmidt) was used in the overexpression and epistasis experiments. Etsrp DNA microinjection construct was made by subcloning full-length etsrp cDNA into the Hind III site of pXeX 20. 25-75 pg of circular etsrp-XeX plasmid was injected into the blastomere at the 1-cell stage. hER71 coding sequence was cloned into pCMV-SPORT6 vector (Open Biosystems). 75-100 pg of hER71 DNA was injected into the flk1-GFP zebrafish embryos at one-cell stage. mER71 coding sequence has been subcloned into pcDNA3.1/V5-His vector (Invitrogen). 75 pg of mER71 DNA was injected in overexpression experiments. For hEts1 RNA injections, hEts1 ORF (kindly provided by P. Oettgen) has been subcloned into the SpeI site of pT3TS 21, subsequently linearized.
with BamHI and transcribed with T3 mMessage Machine kit (Ambion). For hEts1 DNA injections, 200 pg hEts1 cDNA in pSG5neo vector (Stratagene) (kindly provided by R. Forough) was injected.

**In situ hybridization.** In situ hybridization was performed as described. For the two color conventional in situ hybridization, the same protocol was followed except that embryos were incubated with two probes, etsrp labeled with fluorescein-UTP (Roche), and the other cDNA labeled with digoxygenin-UTP (Roche). Following the first color (blue) development, embryos were fixed in the 4% paraformaldehyde/PBS solution for 1 h, washed in PBT, incubated with anti-fluorescein antibody conjugated with alkaline phosphatase (Roche) overnight at 4 °C, washed in PBT, and developed using 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, Sigma) and iodonitrotetrazolium chloride (INT, Sigma) as the substrate for red color. To detect fluorescein-dextran and pu.1 localization, a fluorescent two-color in situ protocol was followed (J. Schoeneback, B. Keegan and D. Yelon, unpublished). Briefly, fixed embryos were hybridized with DIG-labeled pu.1 probe at 65 °C, washed in 0.2x SSC, blocked in 1x blocking reagent (Roche), incubated with 1:500 anti-DIG POD (Roche), washed in PBT, incubated with DNP-tyramide at 1:50 (Perkin Elmer), washed in PBT, blocked in 1x blocking reagent, incubated with anti-DNP POD at 1:500 (Perkin Elmer), washed in PBT, incubated with Cy3-tyramide at 1:25 (Perkin Elmer), sequentially washed in PBT, 1% H2O2, PBT, 0.1 M pH2.2 glycine, PBT, blocked in 1x blocking reagent, incubated with anti-FITC POD at 1:1000 (Roche), washed in PBT, incubated with FITC-tyramide (Perkin Elmer), washed in PBT and imaged as described below.
The following probes were used: *etsrp* \(^{23}\); *pu.l* \(^{24}\); *gata1* \(^{25}\); *flk1* \(^{26}\); *scl* \(^{8}\); *mpx* \(^{27}\) (made by subcloning *mpx* cDNA into the pCR4 vector, Invitrogen, kindly provided by J. Larson); *lcp1* \(^{28}\) (made by subcloning *lcp1* cDNA into the pCR4 vector, kindly provided by J. Larson).

**Transplantation.** Donor embryos (wt or *flk1*-GFP homozygous) were injected with a mixture of *etsrp* RNA (100 pg) and fluorescein isothiocyanate-dextran (2ng; Mw 2 MDa, Sigma) or tetramethyl rhodamine isothiocyanate-dextran (2ng; Mw 2MDa, Sigma) into the blastomere at the 1-cell stage. Recipient embryos (wt or *flk1*-GFP) were injected with 7.5-8 ng of *etsrp* MO1 and MO2 1:1 mixture at the 1-2 cell stage. Embryos were transferred to 1x Danieau solution and manually dechorionated prior to transplantation. 10-50 cells were transplanted at the sphere to 30% epiboly stages by using capillary needles and adjusting balance pressure of PLI-100 microinjector (Harvard Apparatus) to move cells up and down the needle. Embryos were fixed at the 8-10 somite stages and analyzed for *pu.l* expression and the presence for fluorescein. Two color conventional or fluorescent in situ hybridization protocol was followed to detect DIG-labeled *pu.l* and fluorescein (only *pu.l* probe was used).

**Image processing and analysis.** TRITC-labeled live *flk1*-GFP embryos or FITC-labeled *pu.l*-Cy3 hybridized embryos were imaged using a monochrome CCD camera (Hamamatsu C4742-95) on a compound microscope (Axioplan, Zeiss) equipped with rhodamine and GFP filter sets. To image stained embryos after in situ, they were either positioned in 1x PBS on the agarose-coated dishes, or they were manually deyolked and mounted in 50% glycerol (two-color in situ), or araldite (single-color). Images were taken using a color digital CCD camera (Axiocam, Zeiss) and a dissecting (Stemi SV11, Zeiss).
or a compound microscope (Axioskop 2, Zeiss) with 5x, 10x or 20x objective. Typically, frames in different focus planes were manually combined using Adobe Photoshop to yield the maximum clarity image. During image analysis of transplanted embryos, color levels, hue and saturation have been adjusted for the whole image. Color of the transplanted cells was analyzed using the Eyedropper tool (Photoshop), averaged from 7-10 samplings, and normalized for the intensity in red channel. The following color ratios were calculated for the cells 1-5 (Fig. 6H), channels red : green : blue. 1, 1 : 1.02±0.03 : 1.28±0.12; 2, 1 : 0.74±0.05 : 0.56±0.05; 3, 1 : 0.54±0.10 : 0.59±0.11; 4, 1 : 0.77±0.05 : 0.93±0.09; 5, 1 : 0.89±0.04 : 1.03±0.05. The ratios were used to select representative colors shown in the rectangulars in Fig. 6H.

RESULTS

mER71 and hEtv2/ER71 proteins are functional orthologs of zebrafish Etsrp.

The previous study suggested that zebrafish Etsrp may be evolutionary related to mammalian Ets1 proteins. However, the homology between Etsrp and Ets1 proteins is limited to the ETS DNA binding domain. Other domains such as Transactivation and Pointed domains, present in other Ets1 family members, are not recognizable within the Etsrp sequence. To identify a potential Etsrp ortholog in other vertebrates, we performed phylogenetic analysis of the sequences and protein structure of different Ets family members. ER71 proteins from different vertebrates, including medaka (Oryzias latipes), stickleback (Gasterosteus aculeatus), frog (Xenopus laevis), mouse and humans appeared
as potential orthologs of Etsrp (Fig. 1). Mouse ER71 was originally isolated as a testis-specific factor \(^1\(^{29,30}\), but its embryonic expression has not been previously investigated. Etsrp sequence is 57% and 61%, respectively, identical to its medaka and stickleback homologs, while it displays lower identity of 26% (37% similarity) to the human ER71 protein and 26% identity (36% similarity) to the mouse ER71 (Fig. 1A). Etsrp and hER71 proteins are 71% similar within the ETS domain region, while homology is much lower within the rest of the sequence. Interestingly, similar to Etsrp, other vertebrate ER71 proteins have no recognizable structural domains outside the ETS domain. Recent functional studies determined a potent transactivation domain present within the mouse ER71 N-terminal region \(^3\(^{30}\). Syntenic analysis shows that vertebrate etsrp/ER71 chromosomal regions are highly conserved (Fig. 1C).

To test if mammalian ER71 and zebrafish Etsrp proteins are functionally conserved, we injected DNA encoding mouse and human ER71 into early zebrafish embryos. Overexpression of both mER71 and hER71 resulted in the strong expansion of hemangioblast marker \(scl\) and vascular endothelial \(flk1\)-GFP expression, similar to the effect of etsrp expression (Fig. 1D-J; data not shown). On the other hand, overexpression of related human Ets1 DNA and RNA caused no apparent effect on early development and no increase in \(flk1\)-GFP expression (data not shown). These results argue that ER71 proteins, similar to Etsrp can initiate vasculogenesis in the zebrafish embryos, therefore Etsrp and ER71 but not Ets1 proteins are functional orthologs.

**Etsrp is necessary and sufficient for the formation of myeloid cells.** In the previous study, we showed that \(scl\) expression in the anterior lateral mesoderm is nearly completely missing in etsrp morphants \(^1\(^{18}\). \(Scl\) knockdown has been previously shown to
affect the myeloid lineage\textsuperscript{12}. Therefore, we investigated if myeloid cells were affected in etsrp morphants. \textit{L-plastin} (\textit{lcp1}) expressing macrophages\textsuperscript{28} and neutrophils\textsuperscript{31} and \textit{myeloid peroxidase} (\textit{mpx}) expressing heterophilic granulocytes\textsuperscript{27,32} were nearly completely absent in etsrp morphants (Fig. 2A-D). Expression of an early myeloid marker \textit{pu.1}\textsuperscript{24} was strongly downregulated in the anterior lateral plate mesoderm while the posterior erythroid-specific \textit{pu.1} expression was not significantly affected (Fig. 2E,F). These results show that etsrp function is required for the formation of myeloid lineage, in addition to its previously demonstrated requirement for endothelial lineage\textsuperscript{18}. Control 5-base mismatch morpholino caused no noticeable defects in embryonic development, including myeloid, hemangioblast and endothelial marker expression,\textsuperscript{18} confirming the specificity of the observed phenotypes (Suppl. Fig. 1; data not shown).

We have previously shown that overexpression of etsrp RNA was sufficient to induce strong ectopic \textit{scl} expression\textsuperscript{18}. \textit{Etsrp} RNA also induced ectopic \textit{pu.1} expression (Fig. 2G, H) and caused an increase in the number of \textit{lcp1}-expressing macrophages and neutrophils (Fig. 2I,J). Interestingly, while the total of 67\% of etsrp-overexpressing embryos displayed ectopic \textit{pu.1}, 60\% of the injected embryos had also reduced or completely absent endogenous \textit{pu.1} expression; 51\% displayed both effects at the same time (Table 1). \textit{Etsrp} RNA which lacked etsrp-MO binding sites induced ectopic \textit{pu.1} expression in etsrp morphants, demonstrating that ectopic \textit{pu.1} cells are not merely mislocalized endogenous \textit{pu.1} cells (Fig. 2K-N, Table 1). In the same experiment, etsrp RNA also caused ectopic expression of an endothelial marker \textit{flk1 (vegfr2)}\textsuperscript{26} in etsrp morphants, consistent with our previous results (data not shown; \textsuperscript{18}). \textit{Etsrp} overexpression can therefore induce cells to initiate myeloid or endothelial development.
Endogenous *pu.1* may become downregulated in *etsrp*-overexpressing embryos as those cells undertake endothelial fate.

*Etsrp* affects both hematopoiesis and vasculogenesis in the anterior but not the posterior region. We have previously shown that *etsrp* knockdown and overexpression did not significantly affect formation of erythroid cells, including erythroid-specific expression of *gata1*<sup>18</sup> (Suppl. Fig. 2). We investigated whether *etsrp* function is limited to the myeloid cells within anterior lateral plate mesoderm. In wild-type embryos, *pu.1* is expressed in both erythroid and myeloid progenitors by the 10 somite stage, and its erythroid expression disappears during later somitogenesis stages<sup>24</sup>. Knockdown of *gata1* has been reported to cause erythroid cells to undertake myeloid fate resulting in the persistence of posterior *pu.1* expression<sup>15,16</sup> (Fig. 3C). Co-injection of *etsrp* and *gata1* MOs resulted in the loss of *pu.1* expression in the anterior but not the posterior region (Fig. 3D), supporting *etsrp* requirement for the formation of anterior but not posterior myelo-erythroid progenitors. Knockdown of *pu.1* has been reported to result in the myeloid cells undertaking erythroid fate and expressing *gata1* in the anterior region<sup>15</sup> (Fig. 3G). These anterior *gata1*-expressing cells were absent in the *etsrp* and *pu.1* double morphants (Fig. 3H), consistent with *etsrp* affecting myelo-erythroid progenitors in the anterior region.

*Scl* functions downstream of *etsrp* in the myeloid but not endothelial lineage. *Scl* has been previously implicated in the myelopoiesis as well as vasculogenesis<sup>11,12</sup>. We studied the interaction of *etsrp* and *scl* in myeloid and vascular development. We have previously shown that *etsrp* overexpression induces strong ectopic *scl* expression<sup>18</sup>. *Scl* overexpression also enhanced *etsrp* expression, which was limited mostly to the somitic
mesoderm (Suppl. Fig. 3; Fig. 5G,I). Scl knockdown had no apparent effect on etsrp expression while etsrp morphants had strongly reduced scl expression in the anterior and trunk domains \(^{18}\). Addition of scl RNA restored the endogenous pattern of pu.1 expression in etsrp morphants (Fig. 4A-D; Suppl. Table 1). In the sibling embryos from the same experiment, scl RNA failed to rescue endothelial flk1 expression in etsrp morphants, although it was sufficient to expand flk1 expression in control embryos (Fig. 4E-H), consistent with our previously published data \(^{18}\). As reported previously, pu.1 expression was severely reduced or absent in scl morphants (Fig. 4I,J) \(^{11,12}\). Etsrp RNA failed to rescue pu.1 expression in scl morphants (Fig. 4K,L). In the sibling embryos from the same experiment, etsrp induced ectopic flk1-GFP expression in scl morphants, although to the smaller extent than etsrp overexpression alone (Fig. 4M-P). These experiments argue that scl functions downstream of etsrp in the myeloid induction. However, in the vascular induction etsrp is the critical factor, and scl may only modify etsrp expression or function but is not absolutely required. Overexpression of pu.1 mRNA was also sufficient to rescue lcp1 expression in etsrp morphants (data not shown).

A recent study demonstrated that the alk8 signaling provides a necessary input for myelopoiesis, apparently functioning in parallel to scl pathway \(^{17}\). We tested if etsrp RNA could induce ectopic pu.1 in alk8 morphants. As shown in Fig. 4Q-T, alk8 is required for etsrp RNA to induce ectopic pu.1 expression. Overexpression of constitutively active alk8 (CA-alk8) RNA caused mild ventralization of injected embryos, and the number of myeloid cells was not changed significantly. Co-injection of CA-alk8 and etsrp RNAs resulted in the dramatic expansion of myeloid lineage (Fig. 4U-
X). These results support the idea that etsrp and alk8 signaling are both necessary parallel inputs during myeloid formation.

**Etsrp and pu.1 expression is mutually exclusive.** To understand better how overexpression of endothelial-specific etsrp induces myeloid pu.1 expression, we investigated expression of etsrp, pu.1 and scl in greater detail by the two-color in situ hybridization. Etsrp expression first appears at the 1-2 somite stage\(^{18}\) and precedes pu.1 expression which is first apparent at the 6-somite stage\(^{24}\). Etsrp and pu.1-expressing cells are positioned immediately adjacent to each other, but their expression does not overlap at the 6-somite stage, soon after pu.1 expression first appears (Fig. 5A,B). In contrast, etsrp and scl expression overlap nearly perfectly at the 6-somite stage in the anterior region (Fig. 5C). In the posterior region, etsrp expression extends further into the trunk; scl and etsrp expression start to separate as scl expression becomes restricted to the erythroid cells (Fig. 5D).

Because etsrp and pu.1 are expressed in the neighboring but not the same cells, we tested the hypothesis that etsrp and pu.1 may negatively regulate each others expression. As we described earlier, knockdown of Etsrp translation resulted in the enhanced etsrp RNA expression, suggesting the presence of a negative autoregulatory loop\(^{18}\). Expansion of etsrp expression in etsrp morphants appeared to extend into the region where the myeloid cells would have normally formed, which no longer expressed pu.1 (Fig. 5E-H). As we described above, scl RNA could restore pu.1 expression in etsrp morphants (Fig. 4A-D). We analyzed pu.1 and etsrp expression in etsrp MO and scl RNA-injected embryos. Scl RNA and etsrp MO co-injected embryos displayed scattered pu.1 cells, which were embedded within the expanded domain of etsrp-expressing cells.
(Fig. 5J). Noticeably, expression of etsrp and pu.1 still did not overlap in these embryos, resulting in the salt-and-pepper pattern of intermingled pu.1 and etsrp-expressing cells. This experiment shows that etsrp expression expands into the myeloid domain in etsrp morphants. It also shows that etsrp and pu.1 expression is mutually exclusive even in the absence of Etsrp protein function.

We tested if pu.1 itself could possibly downregulate etsrp expression in the myeloid precursors. However, pu.1 RNA overexpression and MO-knockdown did not significantly affect etsrp expression (data not shown).

**Etsrp functions cell-autonomously in myeloid and endothelial lineages.** As we showed so far, etsrp overexpression results in the induction of both vascular and myeloid markers. We hypothesized that etsrp induces common myeloid-endothelial precursor cells, hemangioblasts, which then differentiate into myeloid and/or endothelial cells. To test whether etsrp functions cell-autonomously in myeloid and endothelial induction, we traced the lineage of etsrp-expressing cells by performing cell transplantation experiments. To test etsrp function in endothelial induction, flk1-GFP donor embryos were injected with the mixture of tetramethylrhodamine isothiocyanate (TRITC)-conjugated dextran and etsrp RNA. Cells from donor embryos were transplanted into etsrp morphants at the beginning of epiboly, which were imaged for the presence of TRITC and flk1-GFP expression at the 8-10 somite stages (Fig. 6A). In two independent experiments, 24 out of 61 successfully transplanted embryos displayed flk1-GFP expressing cells. In every single case, all flk1-GFP expressing cells contained also TRITC label, arguing that they originated from etsrp-expressing cells (Fig. 6B-D).
To test whether \textit{etsrp} functions cell-autonomously in inducing myeloid lineage, donor embryos were injected with a mixture of fluorescein-labeled dextran and \textit{etsrp} RNA; cells from the donor embryos were transplanted into \textit{etsrp} morphants at the beginning of epiboly, which were assayed for \textit{pu.1} expression and fluorescein presence at the 8-10 somite stages. Overall, among 127 successfully transplanted embryos, 40 ectopically located \textit{pu.1}-expressing cells were detected. Although most \textit{etsrp} morphant recipient embryos had no endogenous \textit{pu.1} expression in the anterior region and only posterior erythroid-specific \textit{pu.1} stripes were present, occasional embryos displayed a few \textit{pu.1}-expressing cells in the anterior lateral mesoderm. These cells, positioned in the location of endogenous \textit{pu.1} expression, were not counted in this experiment. Among the 40 ectopic \textit{pu.1} cells in the transplanted embryos, 39 displayed also the presence of fluorescein and one could not be reliably determined, as assayed by fluorescent or conventional two color in situ hybridization (Fig. 6E-H). These data argue that the \textit{pu.1}-expressing myeloid progenitor cells originate from \textit{etsrp}-expressing cells, which downregulate \textit{etsrp} expression as they undertake the myeloid fate.

**DISCUSSION**

In this study, we show that Etsrp function is evolutionary conserved and identify mammalian ER71 proteins as functional orthologs of Etsrp. Both phylogeny and chromosomal synteny analysis argues that Etsrp and ER71 proteins are homologous to each other. In addition, mammalian ER71 and zebrafish Etsrp but not mammalian Ets1
proteins result in a similar phenotype when overexpressed in zebrafish embryos. Similarly, overexpression of mouse ER71 in embryonic stem cells causes induction of multiple hemangioblast and vascular endothelial markers (Lee et al., submitted). Mouse ER71 expression domains include yolk blood islands and major vessels (Lee at al., submitted), supporting the idea that Etsrp is related to ER71 protein family. Thus it appears that ER71/Etsrp proteins are critical to induce vascular endothelial cell fate in undifferentiated progenitor cells in different vertebrates.

In addition, we demonstrate that *etsrp* is not only a critical factor that initiates vasculogenesis, but it also participates in the induction of myelopoiesis. In the absence of *etsrp*, myeloid cells were nearly completely absent, while overexpression of *etsrp* resulted in the formation of ectopic myeloid cells. We show that *etsrp* regulates myeloid development by functioning through transcription factor *scl*, a known regulator of myeloid as well as erythroid and vascular development. We also show that *etsrp*-expressing cells give rise to both vascular endothelial and myeloid cells, with *etsrp* becoming localized to the endothelial cells and excluded from myeloid progenitors. This data suggest the following two possibilities. First, Etsrp functions within the common myeloid-endothelial progenitor, hemangioblast cells in the anterior part of an embryo (Fig. 7A). Alternatively, there may be two separate pools of Etsrp-expressing cells giving rise to endothelial and myeloid lineages (Fig. 7B). Although at present we cannot distinguish between the two models, we favor the first model, based on the recent study demonstrating the existence of posterior hemangioblast. Thus we propose a model where *etsrp* is necessary and sufficient for the upregulation of *scl* in hemangioblasts, positioned within the anterior lateral plate mesoderm (Fig. 7). Etsrp function within
hemangioblasts is necessary for their further differentiation into both endothelial and myeloid lineages. As hemangioblasts divide, giving rise to angioblasts and myeloid precursors, etsrp becomes localized to the endothelial progenitor cells and excluded from the myeloid precursors. Scl appears to support etsrp function within angioblasts, either by upregulating etsrp transcription or / and functioning together with etsrp to upregulate expression of its target genes such as flk1.

It is currently not clear how etsrp expression is excluded from the myeloid cells. All the experimental data support the idea that etsrp and pu.1 expression is mutually exclusive. This mutually exclusive expression pattern is maintained even in the absence of Etsrp protein, as seen in the etsrp morphants injected with scl RNA. It is possible that pu.1 directly or indirectly represses etsrp transcription in the myeloid cells, although our data do not support this model as etsrp expression does not change significantly upon pu.1 downregulation or overexpression. Alternatively, a different signaling pathway may be involved in maintaining this relationship. Interestingly, etsrp itself appears to repress pu.1 expression as etsrp RNA-overexpressing embryos often have the endogenous pu.1 downregulated or even totally absent. This may help to ensure that pu.1 is never expressed in the endothelial cells.

It is also not known how cells make a choice between the endothelial and myeloid fates. Our results support the idea that continued etsrp expression drives cells towards endothelial fate. In contrast, combination of etsrp and alk8 signaling combined with the subsequent etsrp downregulation is sufficient to direct multiple cells towards myeloid fate. It is possible that additional as yet undiscovered pathways participate in the cross-talk between endothelial and myeloid cells and influence this fate decision.
Until now, it has been generally accepted that the anterior and posterior hemangioblasts are equivalent in their potential. For example, the myeloid-forming anterior hemangioblasts can give rise to erythroid cells in the absence of *pu.1*, and the erythroid-forming posterior hemangioblasts can give rise to myeloid cells in the absence of *gata1*. *Etsrp* is the first known gene to specifically affect anterior hemangioblasts, while in the posterior region it functions in the angioblasts but does not affect hematopoietic cells. The most likely explanation is that in the anterior region *etsrp* is expressed within the hemangioblast cells while the posterior *etsrp* expression is limited to the angioblasts. It is possible that related Ets-domain proteins such as Ets1 may play similar role in the posterior hemangioblasts; it has been demonstrated that *etsrp, ets1, fli1* and *fli1b* genes can function somewhat redundantly. Interestingly, *etsrp* overexpression resulted in the expansion of hemangioblasts which differentiate into endothelial and myeloid, but not erythroid cells. Possible explanations are that *etsrp* induces hemangioblasts in both regions but they behave differently in the posterior region, or *etsrp* induces hemangioblasts in the anterior but not the posterior region. *Scl* has been previously shown to be necessary for erythroid, myeloid, and to certain extent, endothelial development. *Scl* overexpression also induces erythroid, myeloid and endothelial lineages. *Etsrp* induces strong ectopic *scl* expression while *scl* can feedback and induce *etsrp* expression as well, although this induction is limited to the portions of lateral, somitic and intermediate mesoderm. Apparently, *etsrp*-induced *scl*-expressing cells cannot differentiate into erythroid lineage, either because *etsrp* presence directs them towards endothelial fate or additional genes upregulated by *etsrp* ensure
endothelial fate of these cells, even after etsrp expression is lost or downregulated (which happens in a subset of etsrp-expressing cells).

Transplantation experiments show that etsrp-expressing cells can give rise to both endothelial and myeloid lineages. A relatively low number of transplanted cells (less than 1%) displayed pu.1 expression. It is possible that not all etsrp expressing cells have the ability to differentiate into both lineages. There may be a certain bias in the transplantation experiments, e.g. the transplanted cells do not end up in the myeloid-competent region often enough. We noticed that the ectopic pu.1-expressing cells in the overexpression and the transplantation experiments were often located in the ventral and almost never in the dorsal side of an embryo, while flk1-expressing cells were located mostly at the dorsal side, possibly reflecting different myeloid and endothelial competences within distinct regions of an embryo. Not all etsrp-expressing cells differentiated into endothelial cells at the time of analysis, which may reflect also different competence to undertake endothelial fate, or simply a delay between the etsrp expression and flk1 induction. Although our experiments show that etsrp-expressing cells can give rise to both lineages, thus supporting the hemangioblast idea, additional experiments are needed to convincingly demonstrate the existence of a common endothelial-myeloid progenitor.
ACKNOWLEDGMENTS

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AUTHORSHIP

Contribution: S. Sumanas designed and performed experiments, analyzed data and wrote the paper; G.G. and Y.Z. performed experiments and analyzed data; C. P. and K. C. contributed reagents; S.L. designed experiments and analyzed data.

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Table 1. Effect of *etsrp* overexpression and knockdown on *pu.1* expression at the 8-10 somite stages. *Etsrp* RNA causes ectopic *pu.1* expression in control embryos and *etsrp* morphants and also results in the reduction of endogenous *etsrp* expression. Results are percentage averages from two independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>Normal or slightly distorted <em>pu.1</em> pattern</th>
<th>Normal endogenous + ectopic <em>pu.1</em> expression</th>
<th>Reduced or absent <em>pu.1</em> expression</th>
<th>Reduced or absent endogenous + ectopic <em>pu.1</em> expression</th>
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<tbody>
<tr>
<td>Control <em>(n&gt;50)</em></td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td><em>etsrp</em> MO <em>(n=40)</em></td>
<td>5 ± 5</td>
<td>0</td>
<td>95 ± 5</td>
<td>0</td>
</tr>
<tr>
<td><em>etsrp</em> RNA <em>(n=57)</em></td>
<td>24 ± 11</td>
<td>16 ± 1</td>
<td>9 ± 9</td>
<td>51 ± 19</td>
</tr>
<tr>
<td><em>etsrp</em> MO + RNA <em>(n=56)</em></td>
<td>1 ± 1</td>
<td>0</td>
<td>27 ± 1</td>
<td>72 ± 2</td>
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FIGURE LEGENDS

**Figure 1.** Etsrp is a functional ortholog of the mammalian ER71 subfamily.

(A) Alignment of zebrafish Etsrp, medaka, stickleback, mouse and human ER71 amino acid sequences. Identical and similar amino acids are labeled in red and blue, respectively. Etsrp and hER71 share 71% homology within the ETS DNA-binding domain (underlined in grey). GenBank accession numbers used for the analysis are: human ER71 (O00321), mouse ER71 (NP_031985) and zebrafish ETSRP (AAY89037). Medaka ER71 (ENSORLP00000019929) and stickleback ER71 (ENSGACP00000016315) are Ensembl predictions. (B) Phylogenetic analysis of zebrafish Etsrp and its closest human, mouse, frog and fish homologs. The phylogenetic tree is built using the Neighbor Joining method. Length of horizontal branches is proportional to the evolutionary distance between the protein molecules. GenBank accession numbers used for the analysis are: human ER71 (O00321), mouse ER71 (NP_031985), zebrafish ETSRP (AAY89037), human ETS1 (NP_005229), mouse ETS1 (NP_035938), frog ETS1 (NP_001081621), zebrafish ETS1a (NP_001017558), human ETS2 (NP_005230), mouse ETS2 (NP_035939), frog ETS2 (NP_001081007), zebrafish ETS2 (NP_00101874), human FLI1 (NP_002008), mouse FLI1 (NP_032052), zebrafish FLI1a (NP_571423) and zebrafish FLI1b (NP_001008780). Medaka ER71 (ENSORLP00000019929), stickleback ER71 (ENSGACP00000016315), fugu ETS1 (SINFRUP000000163510) and medaka ETS1 (ENSORLP00000016939) are Ensembl predictions. NTI Vector (Invitrogen) has been used to build the alignment and the phylogenetic tree. (C) Chromosomal location of the zebrafish etsrp, medaka, stickleback, mouse and human ER71 genes. Numbers along side the chromosomal regions of interest correspond to the actual physical distances (Mb). (D-J) Mouse ER71 and zebrafish Etsrp overexpression causes ectopic expression of hemangioblast marker scl and vascular endothelial marker flk1. flk1-GFP transgenic embryos were injected with 75pg of either Etsrp or mER71 DNA at the one cell stage and analyzed at 8-10 somite stages. Relative to uninjected embryos (D,G), both Etsrp (E) and mER71 (F,H) result in the ectopic induction of scl when examined by in situ hybridization. Flk1 expression was also
induced by ER71 injections as revealed by ectopic GFP expression (J) relative to un.injected controls (I). Panels G-J are lateral views with anterior to the left.

**Figure 2.** Etsrp is necessary and sufficient for the formation of myeloid cells.

(A–F) Knockdown of Etsrp results in the nearly complete absence of myeloid cells as analyzed by in situ hybridization. Anterior is to the left. (A, C, E) Control uninjected embryos; (B, D, F) Etsrp morphants, injected with 12-15 ng of etsrp MO1 and etsrp MO2 1:1 mixture. (A, B) L-plastin (lcp1) expression at 24 hpf. Note that lcp1-expressing macrophages are nearly completely absent in etsrp morphants (B). (C, D) mpx expression at 24 hpf. Note that mpx-expressing neutrophils are nearly completely absent in etsrp morphants (D). (E, F) pu.1 expression at the 16-somite stage. Embryos have been flat-mounted with the yolk removed. Note that the anterior myeloid-specific pu.1 expression is severely reduced in (F) while posterior erythroid-specific expression is not significantly affected.

(G–J) Etsrp RNA overexpression induces ectopic myeloid cell formation. (G, I) Control uninjected embryos. (H, J) etsrp RNA-overexpressing embryos. (G, H) pu.1 expression at the 16-somite stage, anterior view (G), ventro-lateral view (H). Note the strong expansion of pu.1-expressing cells, some of which are located ectopically (H, arrows). (I, J) lcp1 expression at 24 hpf. Note the increase in the number of lcp1-expressing macrophages in (J).

(K–N) Etsrp RNA with missing MO-binding sites can restore pu.1 expression in etsrp morphants. Embryos are at the 8-somite stage; (K,L) anterior view; (M,N) anterior-ventral view. (K) Control uninjected embryo; (L) 10 ng etsrp MO2-injected embryo; (M)
100 pg etsrp RNA-injected embryo; (N) Embryo co-injected with 10 ng etsrp MO2 and 100 pg etsrp RNA.

**Figure 3.** Etsrp affects both hematopoiesis and vasculogenesis in the anterior but not the posterior region.

(A–D) Posterior ectopic pu.1 expression is independent of etsrp function as analyzed by in situ hybridization at 22 hpf. (A) Control uninjected embryo; (B) embryo injected with etsrp MOs; (C) gata1 MO-injected embryo; (D) gata1 MO and etsrp MO co-injected embryo. Note that ectopic pu.1 expressed in the erythroid cells in gata1 morphants (C) is unaffected in the double gata1 / etsrp morphants (D). Anterior myeloid-specific pu.1 expression (arrowheads, A, C) is missing in etsrp morphants (B, D).

(E–H) Anterior ectopic gata1 expression is dependent on etsrp function as analyzed by the in situ hybridization at 20-21 hpf. (E) Control uninjected embryo; (F) embryo injected with etsrp MOs; (G) pu.1 MO-injected embryo; (H) etsrp and pu.1 MOs co-injected embryo. Note that the ectopic myeloid-specific anterior gata1 expression in pu.1 morphants (arrowhead, G) is absent in double etsrp / pu.1 morphants (H). Posterior erythroid gata1 expression is not affected in etsrp morphants.

**Figure 4.** Analysis of interaction between etsrp and scl and alk8 signaling pathways.

(A–H) scl RNA can rescue myeloid but not vascular cell formation in etsrp morphants as evident from pu.1 and flk1 expression analysis at the 10-somite stage. (A-D) pu.1 expression, anterior view; (E-H) flk1 expression, dorsal view, anterior to the left. (A, E) Control uninjected embryos; (B, F) embryos injected with etsrp MOs; (C, G) scl RNA-
injected embryos; (D, H) Embryos co-injected with etsrp MOs and scl RNA. Note that the myeloid-specific pu.1 expression is restored in etsrp MO and scl RNA co-injected embryos (D). Also note that the vascular specific flk1 expression is absent in etsrp MO and scl RNA co-injected embryos in the same experiment (H).

(I–P) etsrp RNA can rescue vascular but not myeloid cell formation in scl morphants as evident from pu.1 and flk1 expression analysis at the 8-somite stage. (I-L) pu.1 expression as analyzed by in situ hybridization; anterior view; (M-P) GFP fluorescence in flk1-GFP transgenic embryos, dorsal view, anterior is to the left. (I, M) Control uninjected embryo; (J, N) scl MO-injected embryo; (K, O) etsrp RNA-injected embryo; (L, P) scl MO and etsrp RNA co-injected embryo. Note that etsrp RNA fails to rescue myeloid-specific pu.1 expression in scl morphants (L). Etsrp RNA can restore vascular flk1-GFP expression in the same experiment (P). Flk1-GFP fluorescence in wild-type embryos and scl morphants (M,N) is much weaker and not apparent under the same exposure.

(Q–T) etsrp RNA fails to rescue pu.1 expression in alk8 morphants. pu.1 expression analyzed at the 10-somite stage, anterior view, except for (S) which is anterior-ventral. (Q) Control uninjected embryo; (R) alk8 MO-injected embryo; (S) etsrp RNA-injected embryo; (T) alk8 MO and etsrp RNA co-injected embryo.

(U–X) etsrp and constitutively active CA-alk8 RNA synergize in inducing pu.1 expression at the 14-somite stage. Anterior-ventral views except for (X) which is ventrolateral view, anterior is to the top. (U) Control uninjected embryo; (V) CA-alk8 RNA-injected embryo; (W) etsrp RNA-injected embryo; (X) CA-alk8 RNA and etsrp RNA co-injected embryo.
Figure 5. Analysis of etsrp, pu.1 and scl expression by the two-color in situ hybridization. Flat-mounted embryos, anterior is to the left.

(A, B) Etsrp (red) and pu.1 (blue) expression in the anterior region of a flat-mounted embryo at the 6-somite stage. (B) is higher magnification of (A). Note that pu.1-expressing cells lie immediately adjacent to etsrp-expressing cells but expression of the two markers does not overlap.

(C, D) Etsrp (blue) and scl (red) expression in the anterior (C) and posterior (D) regions of a flat-mounted embryo at the 6-somite stage. Note that the two markers completely overlap in (C) while in (D) the trunk region contains only etsrp-expressing cells (arrowheads); scl expression partially overlaps with etsrp in the tail region where scl is restricted to erythroid cells during later stages (arrow and the right arrowhead).

(E, F) Etsrp RNA expression expands into the myeloid region in etsrp morphants injected with Etsrp translation blocking MOs. Etsrp expression in control uninjected embryos (E) and etsrp morphants (F) at the 9-somite stage. Note the more intense and expanded etsrp expression in (F).

(G–J) scl RNA restores pu.1 expression in etsrp morphants with pu.1 and etsrp-expressing cells intermingled. Two color in situ hybridization analysis for pu.1 (blue) and etsrp (red) expression at the 9-10 somite stage. Only the anterior part of an embryo is shown. (G) Control uninjected embryo; (H) etsrp MO-injected embryo; (I) scl RNA-injected embryo; (J) etsrp MOs and scl RNA co-injected embryo. Etsrp staining is very weak in the control embryos because of the short staining time, which was the same for all experimental batches. Note that etsrp morphants in (H) have absent pu.1 expression
and strongly upregulated and expanded etsrp expression. scl RNA-injected embryos (I) display upregulated etsrp expression. pu.1-expressing cells are intermingled with etsrp-expressing cells in (J) but they do not overlap.

**Figure 6.** Etsrp-expressing precursor cells give rise to both vascular endothelial and myeloid lineages. (A) Diagram of transplantation experiment. Donor embryos were injected at the 1 cell with etsrp RNA and TRITC (B-D) or FITC (E-H)-dextran, while recipient embryos were injected with 7.5ng etsrp MO1/MO2 mixture. Cells were transplanted at the beginning of epiboly. (B-D) flk1-GFP expressing cells are a subset of etsrp RNA/TRITC-labeled cells. Embryo is at the 8-somite stage, lateral view, anterior to the left. (B) TRITC-filter image. Only transplanted cells are visible. (C) GFP-filter image. Only flk1-GFP expressing cells are visible. (D) Overlay of the TRITC, GFP and transmitted light DIC images. Cells where GFP and TRITC fluorescence overlaps, are in yellow (arrows point to some of these cells). Note that every GFP-expressing cell has also TRITC fluorescence. (E–H) pu.1-expressing cells originate from etsrp-expressing cells, transplanted from etsrp RNA-overexpressing embryos into etsrp morphants. Etsrp RNA was coinjected with fluorescein-labeled dextran; pu.1 expression and fluorescein presence analyzed by two color fluorescent (E-G) or conventional (H) in situ hybridization at the 8-10-somite stages. (E-G) Anterior-lateral view of the same embryo, dorsal is up. (E) FITC-filter image. Only transplanted cells are visible. (F) pu.1 expression as detected by tyramide-Cy3 amplification, visualized through the rhodamine channel filter. Note the ectopically
located pu.1-expressing cells (arrows) and three remaining endogenous pu.1-expressing cells that are located bilaterally within the anterior lateral mesoderm (arrowheads). (G) Overlay of FITC, Cy3 and transmitted light images. Note that all three ectopic pu.1-expressing cells contain FITC label (arrows) while the endogenous pu.1 cells do not (arrowheads). (H) A posterior region from an embryo containing multiple pu.1 and fluorescein-positive cells. Embryo has been flat-mounted to show dorsal, lateral and ventral tissues. 1, Endogenous pu.1-expressing cells in the posterior lateral mesoderm; 2, fluorescein-labeled transplanted cells, 3-5, double pu.1 and fluorescein-positive cells. Average color for each cell group is shown in the boxes below (see Materials and Methods).

**Figure 7.** Proposed models for etsrp function within the anterior lateral mesoderm. (A) Etsrp induces scl in the hemangioblast cells, which give rise to both vascular endothelial and myeloid precursors. As hemangioblasts divide, etsrp expression becomes restricted to endothelial cells where it is both necessary and sufficient for vasculogenesis. (B) Alternatively, there are two separate pools of etsrp-expressing endothelial and myeloid precursors. As the cells initiate myeloid marker expression, they downregulate etsrp expression.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Interplay between Etsrp/ER71, scl and alk8 signaling controls endothelial and myeloid cell formation

Saulius Sumanas, Gustavo Gomez, Yan Zhao, Changwon Park, Kyunghee Choi and Shuo Lin