Genome-Wide Analysis of the Zebrafish ETS Family Identifies Three Genes Required for Hemangioblast Differentiation or Angiogenesis

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Abstract—ETS domain transcription factors have been linked to hematopoiesis, vasculogenesis, and angiogenesis. However, their biological functions and the mechanisms of action, remain incompletely understood. Here, we have performed a systematic analysis of zebrafish ETS domain genes and identified 31 in the genome. Detailed gene expression profiling revealed that 12 of them are expressed in blood and endothelial precursors during embryonic development. Combined with a phylogenetic tree assay, this suggests that some of the coexpressed genes may have redundant or additive functions in these cells. Loss-of-function analysis of 3 of them, erg, fli1, and etsrp, demonstrated that erg and fli1 act cooperatively and are required for angiogenesis possibly via direct regulation of an endothelial cell junction molecule, VE-cadherin, whereas etsrp is essential for primitive myeloid/endothelial progenitors (hemangioblasts) in zebrafish. Taken together, these results provide a global view of the ETS genes in the zebrafish genome during embryogenesis and provide new insights on the functions and biology of erg, fli1, and etsrp, which could be applicable to higher vertebrates, including mice and humans. (Circ Res. 2008;103:1147-1154.)

Key Words: zebrafish ■ gene duplication ■ ETS transcription factors ■ hemangioblast ■ angiogenesis

Zebrafish has been recognized as an excellent genetic and developmental biology model to study hematopoiesis and vessel development. Large numbers of genetic mutants and transgenic lines in both blood and endothelial lineages have become available. More importantly, developmental mechanisms, including the functions of many known important regulators involved in both blood and vessel development, are conserved between higher vertebrates and zebrafish.1 It has been proposed that hematopoietic and endothelial cells share a common progenitor, termed the hemangioblast.2 This idea was initially conceived from the observation that these 2 cell types develop in close proximity within the embryo.3 Coexpression of many important regulators of hematopoiesis and vasculogenesis in early embryos further supports the existence of a common progenitor for hematopoietic and endothelial cells. Furthermore, loss of function of several of these regulators, including the zebrafish cloche (clo) mutant, affects both cell types.4–7 Recent work by us and others subsequently showed that several important hematopoietic and endothelial regulators in mammals, such as Scl/Tal1 and Lmo2, also play similar roles in zebrafish.8–10

ETS domain genes encode a super family of transcription factors organized into 11 subfamilies based on domain structures, which are conserved from worm and fly to mammals.11 These transcription factors are involved in cell fate specification, proliferation, migration, and differentiation during embryonic development and adulthood and have been linked with diverse biological processes, from hematopoiesis, vasculogenesis, and angiogenesis to neurogenesis. Many important blood and endothelial regulators have well-conserved and functional ETS binding sites in their promoter and/or enhancer regions.12–15 For instance, the stem cell leukemia gene, Scl, a key regulator during hemangioblast formation and hematopoiesis, is regulated by a complex that consists of ETS transcription factors (Fli1 and Elf1) and other partners.12 Moreover, many ETS genes are coexpressed in hematopoietic and endothelial progenitor cells, which suggests they may have redundant roles or are synergistically required during hematopoiesis and vasculogenesis.11 Defects in human ETS counterpart genes, either by inappropriate expression or by fusions with other proteins, cause carcinogenesis, such as leukemia.16

To comprehensively explore the functional roles of ETS factors during hematopoiesis and vasculogenesis in zebrafish, we have used bioinformatics to systematically analyze the ETS family from the zebrafish genome (Table I in the online data supplement, available at http://circres.ahajournals.org). Interestingly, 12 of the 31 ETS genes identified showed expression in blood and endothelial precursors during embryonic development, which, together with phylogenetic analysis, suggested that some of them may have redundant or additive functions. Functional analysis of 3 of them, etsrp,
erg and fli1, which are strongly expressed in blood and endothelial precursors/hemangioblasts, demonstrated that they are required for hemangioblast differentiation and angiogenesis.

Materials and Methods

Fish Strains and Embryos
Zebrafish embryos were obtained by natural spawning of adult AB strain zebrafish. Embryos were raised and maintained at 28.5°C in system water and staged as described.17 flk1:gfpiata1:dscrd transgenic line and etsrp11 mutant were kindly provided by Stefan Schulte-Merker (Utrecht, The Netherlands) and Brant Weinstein (NIH), respectively.

Bioinformatics
To search for ETS genes, the sequences of the human and mouse ETS domains were used to TBLASTN the zebrafish genome in NCBI (http://www.ncbi.nlm.nih.gov). The predicted zebrafish ETS genes and proteins were named based on the alignment of their coding regions to known entries in public databases. To find the conserved domain in the protein sequences, NCBI conserved domain database was used (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). To generate a phylogenetic tree, the full-length amino acid sequences of ETS proteins were aligned using the CLUSTALW program. Chromosome locations of ETS genes and their surrounding genes were obtained from the Ensembl zebrafish genome database as above.

Morpholinos
Antisense morpholinos (MOs) were obtained from GeneTools (Philomath, Ore). The MOs used in this work were erg atgMO (5′-AGTGACACTCAGTCTCCTGAGTA-3′), erg spliceMO (5′-AGACCGCGCTATCTGACCTCAGA-3′), and fli1 utrMO (5′-TTTCCGCAATTTCTCGAGGCCC-3′). etsrp atgMO was as reported previously.18 Stock solutions (1 mmol/L) in dH2O were prepared, and 2 to 10 ng amounts were injected into 1- to 2-cell stage embryos at the yolk/blastomere boundary. Both erg atgMO and spliceMO gave similar phenotype and coinjection of both MOs did not give a more severe phenotype than single injection.

Reverse Transcription–Polymerase Chain Reaction
RT-PCR analysis to determine efficacy of MO splicing inhibition was carried out with primers 5′-AGGGCGTGGTCGGTGGTGA-3′ (erg forward) and 5′-TCGGCTGGCGTCTGGAGG-GTAG-3′ (erg reverse). cDNA template was produced from mRNA isolated from 24 hpf wild-type (wt) and erg spliceMO-injected embryos. PCR products were analyzed on 1% agarose gel.

Whole-Mount In Situ Hybridization
Whole mount in situ hybridization was described as such.19 The following probes were used: fli1, scl, gata1, flk, pu.1,9,18 etsrp,18 pax2.1,20 erg, ets2, fev, elk4, gabpa, elf1, and elf2 were first reported in this work and their EST clones were obtained from ImaGenes (Berlin, Germany).

Photography
Images of live embryos were captured using a Nikon SMZ 1500 dissecting microscope (×1 HR Plan Apo objective; numeric aperture, 0.13) with a Nikon D5000 digital camera driven by Nikon Act-1 version 2.12 software (Nikon). Confocal images were acquired with a Zeiss LSM 510 META confocal laser microscope and 3D projections were generated using Zeiss LSM software (Carl Zeiss Inc).

Results

Identification and Phylogenetic Analysis of Zebrafish ETS Domain Genes
The presence of ETS binding sites in the regulatory elements of key hematopoietic and/or endothelial genes led us to systematically search the whole zebrafish genome (National Center for Biotechnology Information [NCBI]; Ensembl, zv7/Vega) for the potentially responsible ETS factors regulating hemangioblast development. Computational searching of sequence databases revealed 31 ETS domain-containing genes in the zebrafish genome sequence (supplemental Table I and data not shown), and their putative chromosomal locations were mapped according to the latest zebrafish genome mapping information (supplemental Table I).

Phylogenetic analysis with the complete open reading frames of the ETS transcription factors, identified 11 subfamilies within the ETS gene family (supplemental Table I and Figure 1), which is consistent with the classification of mammalian counterparts based on their structural composition and their similarities in the DNA-binding ETS domains. Besides the conserved ETS domain, a subset of ETS proteins has another evolutionarily conserved domain called the POINTED (PNT) domain at their N-terminal regions, which is required for protein–protein interactions. Notably, because of genome duplication, there are several genes that are closely related in this family (supplemental Table I). For example, there are 3 erl2-like genes, erl1, erl2, and erl3,
similar to erf itself, which are the orthologs of the mammalian erf gene. There are another 3 pairs of ETS genes, ie, elk4 and elk4l1, elf2 and elf2l1, spic and spicl1, which show high homology to a single mammalian gene.

Developmental Expression Patterns of Twelve Hematopoietic or Endothelial ETS Genes

To systemically characterize expression of these ETS genes in wt embryos, we performed a detailed expression pattern analysis at various developmental stages (Figure 2, supplemental Figure I, and data not shown). We obtained cDNA clones for 27 of the 31 ETS genes (the other 4 clones are duplicate genes, ie, erf2l, elk4l1, elf2, and spicl1, which should give similar expression patterns because of high similarity of the sequences) (supplemental Table I) and subjected zebrafish embryos to in situ hybridization at 1-somite (1s), 6s, 10s, and 26-hpf stages when hemangiblasts and erythroid/myeloid and endothelial lineages are clearly identifiable (Figure 2, supplemental Figure I, and data not shown). Compared to the known hemangiblast marker, scl (supplemental Figure I, A through D), we found that 12 ETS genes are expressed in the right place and early enough to be involved in hemangiblast formation (Figure 2 and supplemental Figure I). These are ets1, ets2, etsrp, fli1, fli1b, erg, fev, gabpa, elf1, elf2, pu.1, and elk4. Importantly, among them, ets2, erg, fev, gabpa, elf1, elf2, and elk4 are reported for the first time in this study. Of note, etsrp is the earliest ETS gene strongly expressed in the anterior lateral plate mesoderm (ALM) (Figure 2A), which will give rise to myeloid and endothelial cells in zebrafish. In the posterior lateral plate mesoderm (PLM), fli1 is the earliest and strongest expressed at 1s among these 12 genes (Figure 2E). erg is also expressed in the PLM at the 1s stage (Figure 2M). Later on, all of them except pu.1 are expressed in endothelial cells at 26 hpf (Figure 2 and supplemental Figure I), suggesting that they might play roles in angiogenesis.

Expression Analysis in clo Mutants Confirms That Twelve ETS Genes Are Specifically Expressed in Hematopoietic or Endothelial Cells

To confirm the expression of the 12 ETS genes in hematopoietic and endothelial cells, we tested whether their expression was affected in clo mutants, which lack both blood and endothelial cells. So far, clo mutants exhibit the earliest hematopoietic and endothelial lineage defects in zebrafish.7 As shown in Figure 3 and supplemental Figure II, endothelial expression of 11 ETS genes, ie, ets1, ets2, etsrp, fli1, fli1b, erg, fev, gabpa, elf1, elf2, and elk4, and myeloid expression of pu.1 were reduced or absent in clo embryos at 26 hpf, suggesting that they are indeed expressed in hematopoietic or

Figure 2. etsrp, fli1, fli1b, and erg are expressed in hematopoietic and/or endothelial cells during zebrafish embryogenesis. etsrp (A through D), fli1 (E through H), fli1b (I through L), and erg (M through P). Flat-mount view: A through C, E through G, I through K, and M through O; lateral view, D, H, L, and P. Anterior to the left in all images.

Figure 3. Expression of etsrp, fli1, fli1b, and erg was reduced or lost in clo mutant embryos at 26 hpf. A, etsrp. B, fli1. C, erg. D, etsrp. Note that endothelial-specific expression was reduced or absent in the clo embryos (arrows). Anterior to the left and dorsal to the top.

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endothelial cells. Of note, neural crest expression of fli1, ets1, gabpa, elf1, and elf2 were relatively normal in clo embryos (Figure 3B and supplemental Figure II, A, D, E, and F), compared to wt siblings.

etsrp Is Required for Anterior Hemangioblast (Endothelial/Myeloid Progenitor) Differentiation

Gene expression profiling showed that fli1, erg, and etsrp are strongly expressed in the putative hemangioblast population in 10s stage embryos and later on are restricted to endothelial cells (Figure 2). We reasoned that they might be involved in angiogenesis and in some leukemia cases in mammalian as well as in angiogenesis. erg has previously been implicated in angiogenesis and in some leukemia cases in mammalian cell lines.21 Because no knockout mice for erg has previously been implicated in angiogenesis and in some leukemia cases in mammalian cell lines.21 Because no knockout mice for erg had been reported (but see Discussion), we decided to use the zebrafish as a model to study the function of erg in vivo. To knockdown erg expression in zebrafish, we designed 2 antisense MOs (Figure 4A). The erg atgMO was designed to target the erg ATG and inhibit translation. The erg spliceMO was directed against the boundary of exon 4 and intron 4, spanning part of the PNT domain, to skip exon 4 and generate a truncated protein containing only a small portion of the PNT domain and, because of a frameshift, no downstream ETS DNA binding domain (Figure 4A). To test MO efficiency, RT-PCR analysis of erg spliceMO-injected embryos was carried out, revealing the formation of aberrantly spliced RNAs (Figure 4B, lower arrow in spliceMO-injected embryos), instead of the wt product, which was virtually abolished (Figure 4B, upper arrow in control embryos). Thus, endogenous erg activity was substantially knocked down with the splice MO. spliceMO-injected embryos developed normally without any obvious morphological defects, apart from a hemorrhage in the head area between 2.5 and 3 days postfertilization (see below). The atgMO gave a similar hemorrhage phenotype (data not shown), indicating that the hemorrhage is a specific phenotype caused by erg knockdown.

To determine the consequences of loss-of-function of fli1, we designed an antisense MO against the 5’ untranscribed region immediately upstream of the fli1 translation initiation codon. This fli1 utrMO completely abolished the green fluorescent protein (GFP) expression in fli1:gfp embryos, suggesting that this antisense MO knockdown strategy was efficient (Figure 4D). The fli1 morphants also developed a hemorrhage in the head area around 2.5 days postfertilization (supplemental Figure IV).

etsrp has been demonstrated to be involved in endothelial differentiation but is dispensable for erythroid development in zebrafish embryos.18 However, its role in the earliest precursor, the hemangioblast, has not yet been tested. Knockdown experiments showed that whereas fli1MO and ergMO had no effects on the early hemangioblast program in both ALM and PLM, etsrpMO caused significant reduction of scl and an absence of pl1 and flk1 expression in the ALM, which gives rise to myeloid and endothelial lineages in wt embryos (supplemental Figure III). In contrast, the erythroid marker gatal was not affected in the PLM of all 3 morphants (supplemental Figure III, U through X).

To confirm the etsrpMO phenotype, we used the available etsrp mutant, y11, in zebrafish.22 The predicted Etsrp polypeptides synthesized in the homozygous mutant contain only a quarter of the wt Etsrp protein and do not have the well-conserved ETS DNA binding domain essential for the function of ETS genes. Therefore, it is very likely to be a null allele, which should phenocopy the loss-of-function phenotype induced by etsrp atgMO injection. Whole-mount in situ hybridization shows that the hemangioblast marker, scl, was significantly reduced or absent in the ALM of y11 embryos (Figure 5A). The myeloid marker pl1 was completely absent, and the endothelial marker flk1 was also absent in both the ALM and the PLM (Figure 5B, 5C, and 5E). In the PLM, scl expression was downregulated in y11 embryos (Figure 5D), suggesting that etsrp is also involved in posterior hemangioblast differentiation into the endothelial lineage. The erythroid marker gatal was not affected at either the 10s stage in the PLM (Figure 5F) or at 24 hpf in the intermediate cell mass (data not shown), confirming that etsrp is not required for erythroid differentiation.18
Knockdown of *erg* and *fli1* Causes Angiogenesis Defects and Downregulation of VE-Cadherin

To further characterize the *erg* morphant phenotype, we injected *erg*MOs into *flk1:gfp-gata1:dsRed* transgenic embryos to visualize the whole vasculature and blood circulation. The hemorrhage phenotype was reproduced in ~40% of injected embryos. Confocal microscopy clearly showed that the hemorrhage occurred in the head vasculature from 60 hpf onward in *erg* spliceMO-injected embryos (Figure 6A through 6F, arrows). In addition, we also found that intersomitic vessel (ISV) patterning was slightly affected (Figure 6H). In the controls, ISVs were arranged in very regular chevron shapes (Figure 6G). However, in the *erg* morphants, ISVs often did not follow this chevron shape and grew either straight or reversely (Figure 6H).

*Fli1* and *Erg* belong to the same subfamily in the ETS family (supplemental Table I and Figure 1), and interestingly *fli1* and *erg* MOs gave similar hemorrhage phenotypes in the head at around 60 to 72 hpf (supplemental Figure IV, A through C). Moreover, *fli1* knockout mice also showed a hemorrhage phenotype in the head.23,24 These data suggest that *erg* and *fli1* are involved in establishing and/or maintain-

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**Figure 5.** *etsrp* is essential for anterior hemangioblast formation in zebrafish embryos. ALM expression of *scl* was nearly absent in the *etsrp* mutant (A, 6/6). *pu.1* expression in ALM was completely lost in *etsrp* mutants (B, 6/6). *flk1* expression in ALM was absent in *etsrp* mutants (C, 5/5). PLM expression of *scl* was reduced in *etsrp* homozygous mutants (D, 5/5), whereas *flk1* expression in PLM was completely lost in *etsrp* mutants (E, 6/6). *gata1* expression in PLM was normal in *etsrp* mutants (F, 5/5). Dorsal view and anterior to the top; wt and *y11* embryos were at 6s (A through F).

**Figure 6.** *erg* and *fli1* are additively required for angiogenesis. A through I, Confocal microscopy at 72 hpf showed that hemorrhage occurred specifically in the head vasculature by using *flk1*:GFP/*gata1*:dsRed (A through F, arrows) and slightly disorganized ISVs (H, red arrows), compared to control embryos (A through C and G). Double knockdown by *erg*MO and *fli1*MO gave more severely disorganized ISV patterning (K, 22/32, arrows). The dorsal longitudinal anastomotic vessel is indicated by dotted boxes. Lateral views with anterior toward left (A through I).
ing vessel integrity. Because the 2 genes have similar expression patterns in mice and also in zebrafish, these 2 ETS transcription factors could either be equally or redundantly required for angiogenesis and vessel remodeling after the endothelial cells have been specified (vasculogenesis). However, double knockdown of both fli1 and erg gave a more severe phenotype than the individual knockdowns, namely a more severe ISV patterning defect and higher penetrance of the hemorrhage (71% versus 41%, Figure 6I versus 6H and data not shown). In the double knockdown, ISVs were more disorganized and they did not grow properly toward the dorsal side of the embryo (Figure 6I, arrows). In addition, the dorsal longitudinal anastomotic vessel did not develop properly in the double knockdown morphants (Figure 6I, dotted box), which contrasts with the individual knockdowns (Figure 6H, dotted box). Thus, these data demonstrate that erg and fli1 are additively required for vessel integrity and angiogenic remodeling.

VE-cadherin, a key regulator of endothelial intercellular junctions, has recently been shown, by chromatin immunoprecipitation and functional assays, to be a direct target of erg in human umbilical vein endothelial cells. We, therefore, looked at VE-cadherin mRNA expression in the erg, fli1, and double morphants. At 24 hpf, VE-cadherin was equally expressed in both controls and the morphants (data not shown). However, there was a reduction of VE-cadherin expression in both the trunk and the head at 50 hpf in the morphants (supplemental Figure IV, D through G, arrows), just before the hemorrhage occurred, suggesting that down-regulation of VE-cadherin by erg and fli1 knockdown might be responsible for the hemorrhage and ISV patterning defects observed in the morphants.

Discussion
To initiate a comprehensive evaluation of ETS gene function in hematopoiesis and endothelial cells, we have systematically characterized the ETS family in an organism ideally suited to functional testing, the zebrafish. Bioinformatic analysis identified a total of 31 complete ETS genes in the zebrafish genome. Gene expression profiling showed that 12 of them are expressed in blood and/or endothelial lineages during embryogenesis. Functional analysis of erg and fli1 demonstrated that they are required for angiogenesis, possibly via direct regulation of VE-cadherin, a key regulator of endothelial intercellular junctions. Combined use of antisense knockdown and a genetic mutant clearly demonstrated that etsrp plays an early role during hemangioblast development to drive both myeloid and endothelial differentiation.

The ETS Family and Genome Duplication
Our phylogenetic tree analysis indicates that the zebrafish genome contains 3 pairs of duplicated ETS genes, ie, elk4 and elk4l1, elf2 and elf2l1, spic and spic1, and 1, erf; that has 3 diverged copies (supplemental Table I and Figure 1). The zebrafish-specific ETS gene duplicates may result from zebrafish-specific gene duplication or mammalian-specific gene loss, or both. Several lines of evidence suggest that whole-genome duplication and subsequent massive loss of duplicated genes occurred in the teleost. Syntenic analysis further confirmed the likelihood of a genome-wide duplication. For example, the etsl-fili pair on chromosome 18 has been duplicated and their duplicates, etsrp and fli1b, are located on chromosome 16. In the most extreme case, erf has 3 additional duplicates. These duplicated ETS genes, together with their surrounding genes, often form duplicated chromosome segments located on either the same chromosome or a different chromosome, further suggesting genome duplication occurred. Of note, their close neighboring genes also have putative mammalian orthologs located near the human counterpart genes, highlighting highly conserved syntenies between zebrafish and mammals such as human and mouse. Taken together, our data and published data strongly support the view that the zebrafish-specific ETS genes arose from genome-wide duplication.

A Role for Etsrp in Hemangioblast Formation
The list of genes involved in both blood and endothelial lineages is expanding, although the existence of a bipotential progenitor, the hemangioblast, is still contentious. Scl, Lmo2 and Gata2 have all been demonstrated to be critical for the hemangioblast program. Using binding and transactivation assays, Pimanda et al have implicated Fli1 in the control of scl and gata2 expression; however, no perturbations were performed to support such a role in hemangioblasts. We show here that, although erg and fli1 are expressed in the hemangioblast population, loss of their functions in zebrafish has no effect on hemangioblast development, suggesting that they are either not required or possibly redundantly required. We have recently found that fli1 is indeed essential for blood and endothelial development in Xenopus embryos and that gain-of-function of fli1 can induce many hemangioblast markers in zebrafish, consistent with a role for this family at the top of gene regulatory hierarchy. However, even a quadruple knockdown of erg, fli1, fli1b, and fev (belonging to the same subfamily) did not give a hemangioblast phenotype (supplemental Figure V), indicating that if redundancy is the explanation, it involves ETS factors outside this subfamily in zebrafish.

Etsrp, an ETS domain protein discovered in zebrafish, has been shown to be necessary and sufficient for the initiation of vasculogenesis. Here, we have clearly demonstrated, by using antisense MO knockdown and a genetic homozygous mutant, that Etsrp is absolutely required for anterior hemangioblast formation in zebrafish. This requirement could be cell-autonomous, because etsrp is coexpressed with scl and fkl1 in the same cell population, the anterior lateral plate mesoderm, during embryogenesis. As etsrp is dispensable for erythroid differentiation, it suggests that the anterior hemangioblast, only giving rise to myeloid and endothelial lineages, and the posterior hemangioblast population, giving rise to erythroid and endothelial lineages, are differentially programmed. It is highly possible that both cell-intrinsic differences and local environments account for the differential regulation of these 2 hemangioblast populations. Our studies have identified etsrp as a critical regulator required for generation of the hemangioblasts that differentiate into myeloid and endothelial lineages. Consistent with our finding, mice carrying a mutation in Er71, the homolog of etsrp in
mammals, have very recently been shown to lack both hematopoietic and endothelial lineages.34

Sumanas et al recently reported the dependence of myeloid gene expression on etsrp in the anterior hemangioblast population in zebrafish embryos.35 They showed, by etsrpMO knockdown that myeloid markers, such as lcp1, mpx, and pu.1, were reduced and that overexpression of etsrp could upregulate lcp1 and pu.1 expression. However, anterior expression of endothelial markers, such as flk1, was not analyzed. Together with our data, we can conclude that etsrp lies at the top of the genetic hierarchy programming both blood and endothelium in the anterior hemangioblast population. A recent study by Xiong et al36 showing that etsrp can partially rescue both hematopoietic and endothelial defects in cloche embryos is consistent with our finding here.

**Erg, Fli1, and Angiogenesis**

Although erg is dispensable for the hemangioblast program, its specific expression in endothelial cells at later stages of development suggests that it might be involved in angiogenesis. In addition, fli1 knockout mice showed a central nervous system hemorrhage phenotype, suggesting that it is not redundant in angiogenesis.23,24 In contrast, the very recently reported Erg<sup>atd</sup> mutation did not cause a vasculature phenotype in mice.37 A possible explanation is that this Mld2 mutation, which caused a substitution in the ETS DNA-binding domain but did not affect DNA binding affinity, might not therefore be a null allele. Many ETS genes have been implicated in angiogenesis.11 Pham et al have demonstrated a combinatorial function for ETS transcription factors, fli1, fli1b, ets1, and etsrp, in the developing vasculature in the zebrafish.32 The additive interaction of erg and fli1 discovered in this study supports such combinatorial functions among ETS factors in vessel development.

Downregulation of VE-cadherin, a key molecule involved in cell–cell junctions and cell survival, by erg and fli1 knockdown could explain why a hemorrhage occurred in both morphants. Targeted null mutation of VE-cadherin impairs the organization of vascular-like structures in embryoid bodies.39 More importantly, electrophoretic mobility shift assays and chromatin immunoprecipitation analyses showed that Erg binds to the VE-cadherin promoter.25,40 The strongest expression of VE-cadherin and erg/fli1 in the head vasculature indicates that they might be particularly essential for the vessel integrity and stabilities in that region. However, we have not been able to detect any discernible defects in the head vasculature, even using confocal microscopy imaging of the flk1:GFP/gata1:dsRed double transgenic line, although the hemorrhage always occurs in the head. The hemorrhage phenotype in the erg morphants is very similar to the hemorrhaged mutants recently identified in zebrafish, such as bubblehead/pkip and redhead/pak2a.31,42 It was suggested that βPix-Pak2a signaling pathways regulate cerebral vascular stability, possibly via the p38 mitogen-activated protein kinase pathway.41 Nevertheless, it would be intriguing to know whether expression of VE-cadherin was affected in these mutants, as in the erg and fli1 morphants described here. As a first test for effects on FGF or VEGF signaling, 2 pathways known to be important in angiogenesis, we asked whether activated forms of Erg or Fli1 could induce expression of the ligands (supplemental Figure VI, D through I). However, we found that vegf expression was unaffected and, even though fgf8 expression was upregulated, it was in the tail bud and not the vasculature. In contrast, both activated ETS factors, upregulated flk1 expression (supplemental Figure VI, A through C), raising the possibility that they might sensitize endothelial cells to VEGF signaling.

In conclusion, by using systematic bioinformatic analysis and gene expression profiling, we have comprehensively characterized the whole ETS gene family in zebrafish and identified 12 of the 31 ETS genes as expressing in hematopoietic or endothelial tissues. Functional assays have identified 3 genes, etsrp, erg, and fli1, that are specifically required for hemangioblast development and angiogenesis. Our studies provide new insights into how tissue-specific differentiation and maintenance is achieved by the ETS domain transcription factors during hematopoiesis, vasculogenesis, and angiogenesis.

**Acknowledgments**

We thank Aldo Ciau-Uitz for helpful comments.

**Sources of Funding**

This work was funded by the Medical Research Council (United Kingdom).

**Disclosures**

None.

**References**


Supplemental Table 1. Zebrafish ETS domain genes

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Supplemental Figure Legends

Figure S1. *ets1, ets2, fev, gabpa, elf1, elf2, pu.1, elk4* are expressed in haematopoietic and/or endothelial cells during zebrafish embryogenesis. *Scl*, the known hemangioblast marker in zebrafish was used for comparison (A-D). *ets1* (E-H), *ets2* (I-L), *fev* (M,N), *gabpa* (O,P), *elf1* (Q,R), *elf2* (S,T), *pu.1* (U,V) and *elk4* (W). Of note, *elk4* is not expressed at 6s stage. Anterior to the left in all panels.

Figure S2. Expression of *ets1, ets2, fev, gabpa, elf1, elf2, pu.1, elk4* was reduced or lost in *cloche* mutant embryos at 26 hpf. (A) *ets1*, (B) *ets2*, (C) *fev*, (D) *gabpa*, (E) *elf1*, (F) *elf2*, (G) *pu.1* and (H) *elk4*. Note that endothelial or blood specific expression was reduced or absent in the *cloche* embryos (arrows). Anterior to the left and dorsal to the top.

Figure S3. *etsrp* but not *fli1* and *erg* is essential for anterior hemangioblast formation in zebrafish embryos. ALM expression of *scl* was not affected in *fli1*- (B, 10/10) and *erg*- morphants (C, 10/10), but was significantly reduced in *etsrp*-morphants (D, 12/12). *pu.1* expression in ALM was not affected in *fli1*- (F, 10/10) and *erg*- morphants (G, 10/10), but was completely lost in *etsrp* morphants (H, 10/10). 

*flk1* expression in ALM was only slightly affected in *fli1*- (J, 6/10) and *erg*- morphants (K, 4/9), but was absent in *etsrp* morphants (L, 10/10). PLM expression of *scl* was not affected in *erg*- (N, 11/12) and *fli1*- (O, 10/10) morphants, but was reduced in *etsrp*- morphants (P, 10/10), whereas *flk1* expression in PLM was slight affected in *erg*- (R, 4/10) and *fli1*- morphants (S, 6/11), but completely lost in *etsrp* morphants (T, 10/10). *gata1* expression in PLM was normal in *erg*- (V, 9/9), *fli1*- (W,
10/11) and etsrp- (X, 10/10) morphants. Dorsal view and anterior to the top; controls and MO-injected embryos were at 10s (A-X).

**Figure S4. Knockdown of **erg** and **fli1** causes downregulation of **ve-cadherin.**

(A,B, C) Live embryo at 3 dpf showed a hemorrhage in the head of erg morphants (B, arrows, 24/58) and of the fli1 morphants (C, arrow, 8/24), with otherwise perfectly normal overall morphology. Downregulation of ve-cadherin was seen in erg- (E, arrows, 10/13), fli1- (F, arrows, 14/14) and the double morphants (G, arrows, 23/24) at 50 hpf. Lateral views with anterior towards left (A-G).

**Figure S5. Hemangioblast gene expression is unaffected in fev or quadruple (erg, fli1, fli1b and fev) knockdowns.** scl expression was normal in the fev morphant (B, 10/10) and the quadruple knockdown (C, 9/10) at 10s. Dorsal views, anterior to the top.

**Figure S6. (A-I) Activated **erg** (B, 10/10) and **fli1** (C, 10/12) upregulate **flk1** expression, and also **fgf8** (E, 10/12; F, 13/14) but not **vegf** (H, 10/10; I, 9/10).**

Embryos are at 24 hours postfertilization (hpf), anterior to the left. CA-Erg and CA-Fli1 were constructed by subcloning the full length inserts into pBUT2VP16 vector made in the lab. Fli1 and Erg coding sequences were fused at their C-terminus with the viral constitutively active transactivation domain, VP16. The constructs were verified by sequencing. Capped mRNAs (50-100 ng/embryo) for injection were synthesized using the mMessage mMachine kit according to the manufacturer’s protocol (Ambion, Austin, USA).
Supplemental References

Liu_FigS1
Liu_FigS3

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MO erg MO + fli1 MO

E G
ve-cadherin

ve-cadherin

ve-cadherin

ve-cadherin
Liu_FigS5

A  WT  B  fevMO  C  4MOs

scl
Liu_Fig S6

control  CA-Erg  CA-Fli1

A  B  C
flk1

D  E  F
fgf8

G  H  I
vegf
Genome-Wide Analysis of the Zebrafish ETS Family Identifies Three Genes Required for Hemangioblast Differentiation or Angiogenesis
Feng Liu and Roger Patient

Circ Res. 2008;103:1147-1154; originally published online October 2, 2008;
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