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ScienceDirect
Developmental Biology 303 (2007) 772–783
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Genomes & Developmental Control

Combinatorial function of ETS transcription factors in the developing vasculature

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Received for publication 12 September 2006; revised 17 October 2006; accepted 20 October 2006
Available online 25 October 2006

Abstract

Members of the ETS family of transcription factors are among the first genes expressed in the developing vasculature, but loss-of-function experiments for individual ETS factors in mice have not uncovered important early functional roles for these genes. However, multiple ETS factors are expressed in spatially and temporally overlapping patterns in the developing vasculature, suggesting possible functional overlap. We have taken a comprehensive approach to exploring the function of these factors during vascular development by employing the genetic and experimental tools available in the zebrafish to analyze four ETS family members expressed together in the zebrafish vasculature; *fli1*, *fli1b*, *ets1*, and *etsrp*. We isolated and characterized an ENU-induced mutant with defects in trunk angiogenesis and positionally cloned the defective gene from this mutant, *etsrp*. Using the *etsrp* morpholinos targeting each of the four genes, we show that the four ETS factors function combinatorially during vascular and hematopoietic development. Reduction of *etsrp* or any of the other genes alone results in either partial or no defects in endothelial differentiation, while combined reduction in the function of all four genes causes dramatic loss of endothelial cells. Our results demonstrate that combinatorial ETS factor function is essential for early endothelial specification and differentiation.

Keywords: Zebrafish; ETS transcription factors; Intersegmental vessels; Vascular development; Angiogenesis

Introduction

The ETS factors are a large family of transcriptional regulatory proteins involved in a wide variety of developmental and postnatal processes, in an equally diverse array of tissues. The first known ETS family member was the proto-oncogene Ets1, the cellular progenitor of v-ets, a viral oncogene found in the genome of the E26 acute leukemia retrovirus (Leprince et al., 1983; Nunn et al., 1983). More than 50 additional members of this family have now been identified, all of which share an 85 amino acid conserved DNA binding domain, the "ETS domain," a winged helix-turn-helix motif generally located in the C-terminal half of the protein. The ETS domain binds to DNA sequence consisting of a core GGAA/T motif. Most ETS family members are transcriptional activators except for a few (Erf, Net, Tel) that have been shown to have repressor activity. The activity of ETS transcription factors is regulated through their interaction with a large number of different structurally unrelated transcription factors such as AP1, MafB, and CBP (for a comprehensive list, see Lelievre et al., 2001; Verger and Duterque-Coquillaud, 2002).

Recent studies have suggested that a number of different ETS factors play important roles in hematopoietic and vascular development during early embryogenesis, although most *in vivo* loss-of-function data have not provided compelling evidence for essential roles for these genes in the specification or differentiation of these tissues (reviewed in Lelievre et al., 2001; Sato, 2001). During murine development, the Ets1 gene is initially expressed broadly in ventral

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0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.10.030

mesoderm, becoming progressively restricted to hemangiogenic mesoderm and then endothelium (Pardanaud and Dieterlen-Lievre, 1993; Queva et al., 1993). Antisense oligonucleotides against Ets1 inhibit angiogenesis in the chick chorioallantoic membrane (CAM) assay (Wernert et al., 1999), and dominant-negative Ets1 inhibits angiogenesis in endothelial cells *in vitro* and in implanted matrigel plugs (Nakano et al., 2000), all supporting the idea that this factor plays an important role in angiogenesis. However, Ets1 null mice are viable and fertile and have no detectable vascular defects (Barton et al., 1998; Bories et al., 1995; Muthusamy et al., 1995). In zebrafish, however, a recent study reported that morpholino knockdown of the novel ets1 related protein etsrp does cause defects in blood vessel formation (Sumanas and Lin, 2005).

Like Ets1, the Fli1 and closely related ERG (Ets-related) genes become progressively restricted to endothelial cells in the trunk during zebrafish and *Xenopus* development (Brown et al., 2000; Meyer et al., 1995). Overexpression of either Fli1 or ERG by microinjection into *Xenopus laevis* embryos causes ectopic endothelial differentiation, in addition to other defects (Baltzinger et al., 1999; Remy et al., 1996). However, transgenic mice overexpressing Fli1 in a variety of tissues under H-2Kk promoter control do not exhibit vascular abnormalities, although they do develop a lethal renal immunologic disease (Zhang et al., 1995). Furthermore, mice homozygous for a targeted disruption of Fli1 form a functional network of blood vessels, indicating that vasculogenesis and angiogenesis proceed normally, although they develop CNS hemorrhage (Hart et al., 2000; Spyropoulos et al., 2000). Mice with targeted disruption of the TEL repressor have normal vasculogenesis, with histologically normal dorsal aorta, intersomitic vessels and head veins at E9.5. However, they exhibit defective yolk sac angiogenic remodeling as well as intra-embryonic apoptosis of mesenchymal and neural cells (Wang et al., 1997). It has been suggested that Tel functions in the maintenance of the developing vascular network rather in the specification, differentiation, or proliferation of endothelial cells.

The early and specific expression of ETS factors in vascular tissues and their mesodermal progenitors has led to speculation that these factors might be important in the establishment of this lineage and the differentiation of angioblasts and endothelial cells, but as noted above *in vivo* evidence for this has been difficult to obtain from loss-of-function experiments. It is likely that a significant difficulty in probing the functional roles of ETS factors in the vasculature is the extensive overlap between these factors in this tissue, making it difficult to perform effective loss-of-ETS-function experiments. We have taken a comprehensive approach to exploring ETS factor function in the vasculature by using the zebrafish to simultaneously reduce the levels of multiple ETS family members. We identified four different vascular ETS factors in the zebrafish and characterized a genetic mutant in one of these genes, *etsrp*. We show that the four genes function cooperatively in the differentiation and maintenance of endothelium.

Materials and methods

Zebrafish methods

Zebrafish (*Danio rerio*) embryos were obtained and raised and fish maintained as described (Kimmel et al., 1995; Westerfield, 1995). The *Tg* (*fli1:EGFP*)^{y1} transgenic zebrafish line was described previously (Lawson and Weinstein, 2002). An ENU F3 genetic screen was performed using this line to isolate the *etsrp*^{y11} mutant (Lawson et al., unpublished results). Embryos imaged post-1.5 dpf were treated with 1-phenyl-2-thiourea (PTU) to inhibit pigment formation (Westerfield, 1995).

Meiotic and physical mapping of the y11 mutation

Meiotic and physical mapping was performed essentially as described previously (Roman et al., 2002) using an EK *Tg*(*fli1:egfp*)^{y1}; *y11*/TL polymorphic mapping cross. Bulked segregant analysis was performed using a 192-marker panel of CA repeat markers (the list of markers in this panel is available upon request). Oligonucleotide sequences for the markers noted in Fig. 2 are available at http://zebrafish.mgh.harvard.edu/zebrafish/ssrQuery.aspx. BAC clones were identified by screening the CHORI 211 Zebrafish BAC library filters (Children's Hospital Oakland Research Institute) using ^{32}P labeled oligonucleotide probes. PAC clones were identified from DNA pools by PCR as described by the supplier (Chris Amemiya Lab). BAC and PAC DNAs were prepared using Nucleobond columns (Clontech). BAC end sequences were obtained from http://trace.ensembl.org/perl/ssahaview?server=danio_rerio. PAC end sequences were determined by sequencing. Additional genomic sequences were obtained through SSAHA2 searches of trace data from the Sanger Institute (http://www.ensembl.org/Danio_rerio/blastview). PCR primers designed against non-repetitive regions of PAC and BAC ends and genomic sequences were used to establish physical contigs and look for polymorphisms for use in meiotic mapping. To identify polymorphisms, PCR products amplified from genomic DNA from an individual wild-type, heterozygous, and mutant embryos (genotyped based on flanking markers) were sequenced. SNPs were assayed as RFLPs, when possible, or were converted to RFLPs using derived cleaved amplified polymorphic sequence (dCAPS) analysis using a dCAPS Finder program (Neff et al., 2002), http://helix.wustl.edu/dcaps/dcaps.html.

Cloning of full-length zebrafish etsrp, fli1b, and ets1 cDNAs

etsrp

Sequences encompassing the 5′ UTR to 3′ UTR of *etsrp* were obtained using available zebrafish EST (GenBank #s AI877585, AL915831, AI 793509, AI793542) and genomic trace sequences. 5′ RACE was performed from 24 hpf cDNA to obtain the complete 5′ UTR sequence. The full-length cDNA of etsrp was obtained by high fidelity PCR on 24 hpf cDNA using PfuUltra High-Fidelity DNA polymerase (Stratagene) and the primers 5′-CTTTAAGATATG-GAAATGTACCAATCGG-3′ and 5′-CCAATCCTTCGATTCCTCCTCTA-3′. A single product was obtained, cloned into pCRII-TOPO (Invitrogen), and verified by sequencing.

fli1b

The 3′ end of *fli1b* was obtained using available zebrafish EST sequence (GenBank # CA472045). 5′ RACE was performed from 24 hpf cDNA to obtain the complete 5′ UTR and coding sequence. The full-length coding sequence of zebrafish *fli1b* was obtained by high fidelity PCR as above with primers 5′-CAGAAATCTGCAATGGACT-3′ and 5′-GTGACTGTTTTAA-TAATAAGTGTTC-3′. A single product was obtained, cloned into pCRII-TOPO (Invitrogen), and verified by sequencing.

ets1

The full-length coding sequence of zebrafish *ets* (GenBank # BC092935) was amplified by high fidelity PCR as above using primers 5′-ACAGACTCTG-TACGTTTGAATGCGT-3′ and 5′-GTCCAGACTTTACTCGTCCGTGTC-3′. A single product was obtained, cloned into pCRII-TOPO (Invitrogen), and verified by sequencing.