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The role of the ETS factor *erg* in zebrafish vasculogenesis

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ABSTRACT

Erg, a member of the ETS family of transcription factors, has been implicated by previous studies in endothelial and haematopoietic development. Deregulation of the human *ERG* locus is associated with acute myeloid leukaemia, prostate cancer and Ewing's sarcoma. To better understand the role of Erg during early development, we utilised the zebrafish as a model amenable to descriptive and functional studies *in vivo*. Zebrafish have a single *erg* gene that is expressed in mesoderm and its vascular derivatives during angioblast migration, vasculogenesis and early angiogenesis. Mutant and morphant expression analyses placed *erg* in a genetic pathway downstream of *cloche*, *tal1/scl* and *etsrp* during early angioblast migration. Furthermore, a combination of gain-of-function and loss-of-function studies suggested a redundant yet specific role for *erg* in both angioblast specification/proliferation and early angiogenesis, and a synergistic interaction with the critical ETS factor *etsrp*.

1. Introduction

During zebrafish embryonic development, vasculogenesis begins at the 6-somite stage with the specification of angioblasts from a putative haemangioblast subset in the lateral plate mesoderm (Liao et al., 1998). Between the 10- and 15-somite stages, angioblasts migrate to the midline of the embryo, where they coalesce to form the dorsal aorta and cardinal vein, the first functional embryonic blood vessels. At the 26-somite stage, angiogenesis begins, with secondary vessels sprouting from these primary vessels along the trunk of the embryo (reviewed in Childs et al., 2002).

Many of the transcriptional regulators directing zebrafish mesodermal specification, vasculogenesis and angiogenesis belong to the ETS family of transcription factors. This large family contains 26 members that share a common DNA-binding "ETS domain", and is delineated into 9 subfamilies according to conservation of various other domains (for review see Oikawa and Yamada, 2003).

During mesodermal specification, the ETS factor *spi1/pu.1* drives myeloid specification in the anterior mesoderm (Rhodes et al., 2005), while *etsrp* is required to drive haemangioblasts towards vascular fate (Sumanas and Lin, 2006). Expression of the closely-related ETS factors *fli1*, *fli1b* and *ets1* overlaps that of *etsrp* during the specification and migration of angioblasts, and functional studies indicate redundant roles for these factors during vasculogenesis in zebrafish (Pham et al., 2007).

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The human ets related gene (*ERG*) is an ETS family member 96% homologous to FLI1 (Maroulakou and Bowe, 2000) first identified in 1987 (Reddy et al., 1987). ERG is a transcription factor containing four functional domains, each responsible for sequence specific DNA-binding, transcriptional activation, and repression of transcriptional activation (Reddy and Rao, 1991; Siddique et al., 1993). Human *ERG* is also an important proto-oncogene with roles in a range of human malignancies, including Ewing's sarcoma, acute myeloid leukaemia, and more than half of all prostate cancers (Ichikawa et al., 1994; Sorensen et al., 1994; Tomlins et al., 2005). Malignancies are linked to chromosomal translocations fusing *ERG* to *EWS*, *TLS/FUS* or *TMPRSS2*, producing either transcriptionally volatile fusion proteins or *ERG* overexpression. High *ERG* expression levels are also an independent correlate of poor prognosis in acute myeloid leukaemia (Marcucci et al., 2007).

Deletion studies of ERG *in vitro* have identified two distinct domains that take part in formation of homodimers, heterodimers and ternary complexes, both with itself and other ETS proteins (Carrere et al., 1998). *In vitro* studies in mammalian cells have demonstrated ERG to be important in a number of developmental processes. These include early haematopoiesis; during which ERG is expressed highly in early myeloid cells (Murakami et al., 1993), and endothelial cell differentiation (McLaughlin et al., 2001). In the mouse, *Erg* is expressed in mesodermal tissues including precartilaginous, urogenital and endothelial cells, positioning it for functions in cell migration and differentiation, as well as for establishment of endothelial fate in mesenchymal cells (Vlaeminck-Guillem et al., 2000). Transient expression of *Erg* has also been described during murine T cell lineage specification (Anderson et al., 1999). A missense mutation of a conserved amino acid in the DNA-binding ETS domain of ERG, resulting in a putative loss-of-function allele, has recently been shown to result in haematopoietic stem cell (HSC) deficiencies in mice (Loughran et al., 2008). Homozygosity of this mutation results in loss of definitive erythropoiesis and death by E13.5. Mice heterozygous for this mutation have lower numbers of lineage-negative Sca-1+c-kit+ (LSK) cells, which represent the long-term repopulating HSC compartment. Furthermore, remaining LSK cells appear functionally deficient when competitively transplanted with wild-type LSK cells into lethally irradiated mice. These studies suggest a role for ERG in either the specification or function of haematopoietic progenitor cells during development and/or steady-state production and survival of haematopoietic precursors during adult life.

Transient functional studies of ERG include knock-down of *ERG* in human umbilical vein endothelial cells (HUVECs) with GeneBloc antisense oligonucleotides/siRNA (McLaughlin et al., 2001; Huang et al., 2005; Birdsey et al., 2008) and overexpression studies in *Xenopus* (Baltzinger et al., 1999), both of which support a role for ERG in endothelial differentiation. (McLaughlin et al., 2001) described decreased expression of known regulators of angiogenesis, factors important in cellular remodelling and angiogenesis, *von Willebrand factor* (an adhesive glycoprotein for coagulation of platelets and a proposed factor involved in tumour angiogenesis) (Girma et al., 1987; Zanetta et al., 2000) and *RhoA*, a small GTPase involved in cellular adhesion pathways (Nobes et al., 1995). Furthermore, GenBloc treatment of HUVECs impaired the formation of tubular structures *in vitro*. ERG mediates angiogenesis in part by transcriptional regulation of VE-cadherin, overexpression of which rescues apoptosis in ERG-deficient HUVECs (Birdsey et al., 2008). In *Xenopus*, overexpression of *XIerg* by injection of mRNA led to ectopic endothelial cell accumulation and perturbations of the cardiovascular system (Baltzinger et al., 1999).

erg was first described in zebrafish as a vascularly-expressed gene by (Weber et al., 2005) based on differential expression in a morpholino microarray analysis, but this report did not present temporospatial expression patterns or any functional studies.

In this study, we detail the spatiotemporal expression pattern of *erg* during angioblast specification, vasculogenesis and early angiogenesis. From functional studies, we present data suggesting a redundant yet synergistic role for *erg* during blood vessel formation and its capacity to drive angioblast specification and/or proliferation in the lateral plate mesoderm during early somitogenesis.

2. Results

2.1. *erg is highly conserved between vertebrate lineages*

To identify the zebrafish *erg* locus, we utilised the BLAST tool at the Ensembl database to search the current zebrafish genome assembly (Zv7). The *erg* locus is located on chromosome 10 and encodes a predicted 2.8 kb mRNA translating into a 427 amino acid protein with two major functional domains: an N-terminal "pointed domain" implicated in protein–protein interactions; and an C-terminal ETS DNA-binding domain common to all ETS family members. Only one copy of the *erg* locus was identified in the zebrafish genome; unlike the *fli1* locus (Zhu et al., 2005), the *erg* gene has not undergone duplication.

To investigate the degree of conservation between zebrafish Erg and various vertebrate homologues and paralogues, amino acid sequences were compared both over the full-length of the protein and more specifically over the highly conserved ETS DNA-binding domain (Fig. 1). Zebrafish Erg is highly conserved between vertebrate lineages, sharing more than 60% identity with its murine and human homologues over the entire protein sequence, and more than 97% identity within the ETS domain.

2.2. *erg is expressed during vasculogenesis*

The basic spatiotemporal expression of zebrafish *erg* is included in an online database of high-throughput whole-mount *in situ* hybridisation (WISH) expression patterns and available through ZFIN (Thisse and Thisse, 2004) . Our studies expand on these observations with more detailed timecourse and co-expression analyses by WISH and RT-PCR (Fig. 2, Supplementary Fig. 1). *erg* expression was first observed at the 6-somite stage of development (12 hpf) in anterior and posterior domains of the lateral plate mesoderm. A two-colour WISH of *tal1* and *erg* at the 10-somite stage (14 hpf) confirmed that *erg* expression co-localised with the subset of *tal1* labelled mesodermal haemangioblasts destined for vascular development, angioblasts (Fig. 2B). At the 15-somite stage (16.5 hpf), *erg* was expressed in cells positioned at the midline of the embryo,