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An arterial-specific enhancer of the human *Endothelin-converting enzyme 1 (ECE1)* gene is synergistically activated by *Sox17*, *FoxC2*, and *Etv2*

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Abstract

Endothelin-converting enzyme-1 (Ece-1), a crucial component of the Endothelin signaling pathway, is required for embryonic development and is an important regulator of vascular tone, yet the transcriptional regulation of the *ECE1* gene has remained largely unknown. Here, we define the activity and regulation of an enhancer from the human *ECE1* locus *in vivo*. The enhancer identified here becomes active in endothelial progenitor cells shortly after their initial specification and is dependent on a conserved FOX:ETS motif, a composite binding site for Forkhead transcription factors and the Ets transcription factor *Etv2*, for activity *in vivo*. The *ECE1* FOX:ETS motif is bound and cooperatively activated by *FoxC2* and *Etv2*, but unlike other described FOX:ETS-dependent enhancers, *ECE1* enhancer activity becomes restricted to arterial endothelium and endocardium by embryonic day 9.5 in transgenic mouse embryos. The *ECE1* endothelial enhancer also contains an evolutionarily-conserved, consensus SOX binding site, which is required for activity in transgenic mouse embryos. Importantly, the *ECE1* SOX site is bound and activated by *Sox17*, a transcription factor involved in endothelial cell differentiation and an important regulator of arterial identity. Moreover, the *ECE1* enhancer is cooperatively activated by the combinatorial action of *FoxC2*, *Etv2*, and *Sox17*. Although *Sox17* is required for arterial identity, few direct transcriptional targets have been identified in endothelial cells. Thus, this work has important implications for our understanding of endothelial specification and arterial subspecification.

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Keywords

Etv2; FoxC2; Sox17; Ece-1; Endothelin; vasculogenesis; artery; vein; mouse; embryo

INTRODUCTION

Endothelin signaling is essential for embryonic development and is an important regulator of vascular tone postnatally (Barton and Yanagisawa, 2008; Kurihara et al., 1999). Endothelin signaling occurs via two G protein-coupled receptors, ET_A and ET_B, which are bound by Endothelin peptides and activate downstream intracellular signaling to control many diverse cellular outputs (Barton and Yanagisawa, 2008; Kedzierski and Yanagisawa, 2001). There are three distinct Endothelin ligands, ET-1, ET-2, and ET-3, which are encoded by three different genes and are initially produced as larger pro-proteins (Barton and Yanagisawa, 2008; Kedzierski and Yanagisawa, 2001). Preproendothelin proteins are cleaved by ubiquitously expressed Furin proteases to produce a precursor of Endothelin protein, referred to as Big Endothelin (Kido et al., 1997). Big Endothelins are subsequently processed into the mature 21 amino acid ligands in a regulated process by either of two highly specific proteases, Endothelin-converting enzyme (Ece)-1 or Ece-2 (Kedzierski and Yanagisawa, 2001). Inactivation of the *Ece1* gene in mice results in perinatal lethality and essentially phenocopies the genetic loss of both Endothelin receptors *in vivo* (Yanagisawa et al., 1998). In contrast, mice lacking Ece-2 protein appear grossly normal, suggesting that Ece-2 plays a relatively more minor role in the pathway and that the majority of Endothelin converting activity in the embryo is mediated by Ece-1 (Yanagisawa et al., 2000).

Consistent with its critical role in Endothelin signaling, Ece-1 is widely expressed during development and in adulthood (Yanagisawa et al., 2000). In adults, Ece-1 is expressed in various organ systems but is particularly prominent in the vascular endothelium (Korth et al., 1999). In the developing mouse embryo, *Ece1* is expressed in multiple tissues, including the cardiovascular system (Yanagisawa et al., 1998). Ece-1 function in the heart has been proposed to be important in the formation of the cardiac conduction system (Hall et al., 2004; Takebayashi-Suzuki et al., 2000). Four Ece-1 isoforms have been described (Schweizer et al., 1997; Valdenaire et al., 1999); the different *ECE1* transcripts are initiated from distinct transcriptional start sites, which results in slight variations in the N-termini of the different proteins produced (Funke-Kaiser et al., 2000; Li et al., 2012; Orzechowski et al., 1997; Valdenaire et al., 1999). However, how *ECE1* is controlled transcriptionally remains incompletely understood. Some analyses of proximal promoter elements in cell culture-based studies have been described (Funke-Kaiser et al., 2000; Li et al., 2012; Orzechowski et al., 1998; Orzechowski et al., 1997; Valdenaire et al., 1999), but the regulation of the *ECE1* gene *in vivo* has not been addressed previously.

The Sox family of high mobility group (HMG) transcription factors is important for development (Kamachi and Kondoh, 2013). In particular, members of the SoxF subfamily, Sox7, Sox17, and Sox18, are key regulators of endothelial cell development and differentiation (Francois et al., 2010). Sox7 is an important regulator of vascular development (Costa et al., 2012; Wat and Wat, 2014). Sox18 is a key regulator of venous

and lymphatic development and functions in a partially redundant manner with Sox7 (Francois et al., 2008; Tammela and Alitalo, 2010). Sox17, which is widely appreciated for its role in endoderm development (Hudson et al., 1997; Tam et al., 2003), is also a critical regulator of endothelial gene expression and arterial development (Choi et al., 2012; Corada et al., 2013; Sakamoto et al., 2007). Endothelial-specific deletion of *Sox17* in the mouse results in embryonic demise with defects in vascular remodeling and an apparent failure to form major arteries (Corada et al., 2013; Kim et al., 2007; Lee et al., 2014). Though it is clear that SoxF factors are key regulators of endothelial development, few direct endothelial targets have been identified to date.

The Ets transcription factor Etv2 is a central regulator of endothelial cell specification (Lammerts van Bueren and Black, 2012; Meadows et al., 2011). Inactivation of the *Etv2* gene in mice results in loss of the endothelial and hematopoietic lineages (Ferdous et al., 2009; Lee et al., 2008). Similarly, morpholino knock down of the *Etv2* ortholog *etsrp* in zebrafish results in profound defects in vascular development (De Val et al., 2008; Sumanas and Lin, 2006). Co-expression of Etv2 and the Forkhead transcription factor FoxC2 results in dramatic cooperative activation of Etv2-dependent endothelial genes (De Val et al., 2008). Numerous endothelial genes may be direct transcriptional targets of Etv2 and FoxC2 via a composite DNA binding element, referred to as the FOX:ETS motif (De Val et al., 2008). Previously, we identified over 1500 evolutionarily-conserved FOX:ETS motifs associated with approximately 1200 endothelial genes in the human genome (De Val et al., 2008). Among the putative enhancers identified in that prior study was a small element from the human *ECE1* gene (De Val et al., 2008). Indeed, a preliminary test of the *ECE1* element in a transient transgenic mouse embryo assay found that it functioned as an endothelial-specific enhancer at E9.5 (De Val et al., 2008).

In the present study, we characterized the activity and regulation of the *ECE1* endothelial-specific enhancer in detail. We found that the *ECE1* enhancer is active in early endothelial progenitors and then rapidly restricted only to arterial endothelium and endocardium. We also found that enhancer activity was dependent on the previously predicted FOX:ETS motif, which we found was a *bona fide* element for cooperative activation by FoxC2 and Etv2. In addition, we show that the enhancer is dependent on an evolutionarily-conserved SOX site, which is bound and activated by the arterially-restricted Sox factor Sox17. Finally, we show that the Sox17, Etv2, and FoxC2 cooperatively activate the *ECE1* endothelial enhancer. Thus, these studies define the first *bona fide in vivo* enhancer of *ECE1* transcription, identify a direct arterial-specific target of Sox17, and establish cooperativity of Sox17 with other core endothelial transcription factors.

MATERIALS AND METHODS

Plasmids, cloning, and mutagenesis

FoxC2 and Etv2 expression vectors have been described previously (De Val et al., 2008). The mouse Sox17 cDNA was subcloned from the previously described plasmid pCIG-Sox17 (Sinner et al., 2007) into the pRK5 expression vector (Clontech) using the *XhoI* restriction site to create plasmid pRK5-Sox17. The reporter plasmid pTK-E-gal has been briefly described previously (Schachterle et al., 2012; Verzi et al., 2007) and contains a minimal

promoter from the Herpes Simple virus *thymidine kinase* gene, sufficient to activate expression in cell culture and competent to respond to neighboring enhancer sequences when inserted into the vector, upstream of a *lacZ* reporter gene. *ECE1*[623]-*lacZ* has also been briefly described previously: a fragment from the human *ECE1* locus was PCR amplified using the following primers: 5c-cataatcccggggcaaaaacacgcga-3c and 5c-tgactgaccgggccagacatcacc-3c, and the resulting 623 bp product was inserted into the transgenic reporter plasmid *hsp68-lacZ* vector after *Xma*I digest (De Val et al., 2008). For cell culture experiments, a shorter, 164 bp region of the *ECE1* enhancer encompassing the core enhancer was PCR amplified using the following primers:

5ccataatcccggggcaaaaacacgcga-3c and 5c-attgctgctggcagatctgtcct-3c. The resulting amplicon was digested with *Bgl*II and was then ligated into the *Bgl*II and *Bam*HI restriction sites in the pTK-E-gal reporter plasmid to create plasmid *ECE1*-TK-E-gal. Mutations were introduced into the *ECE1* enhancer sequence by PCR as described (Dodou et al., 2003) to create the following mutant sequences: mutant FOX:ETS motif (mFEM), 5c-tagctaagaggcaggaggcacaat-3c; mSOX, 5c-ggaagggaggcaggtagagaggaagt-3c. For the SOX site/FOX:ETS motif double mutant (mF/mS), the SOX site was mutated in the mFEM context using the same mutagenic primers.

Generation of transgenic mice

Transgene fragments were gel purified away from the parental plasmid backbone, prepared, and injected into oocytes by pronuclear injection as previously described (Dodou et al., 2003). The presence of a *lacZ* transgene in embryo yolk sacs or tail biopsies was determined by Southern blot or by PCR using the following primers: 5c-ctgtccaagagatgcttctg-3c and 5c-ctcagttggatgttcttgag-3c. All experiments using animals were reviewed and approved by the UCSF Institutional Animal Care and Use Committee and complied with all institutional and federal guidelines.

X-gal staining and immunofluorescence

X-gal staining for detection of β -galactosidase and counterstaining with Nuclear Fast Red were conducted as described previously (Anderson et al., 2004). For section immunofluorescence, embryos were sectioned, dewaxed, boiled in antigen retrieval solution (Biogenex), and blocked in PBS containing 10% sheep serum and 0.1% Triton X-100. The following primary antibodies were used at 1:100 dilutions in blocking serum: goat anti-ECE1 (R&D AF1784), goat anti-Nrp1 (R&D AF566), goat-anti Nrp2 (R&D AF2215), goat anti-connexin40 (Santa Cruz sc-20466), mouse anti-COUP-TFII (Perseus Proteomics PP-H7147-00), and chicken anti- β -galactosidase (Abcam ab9361). The following secondary antibodies were used: rabbit anti-goat 594 (Invitrogen A11080), rabbit anti-chicken 488 (Jackson ImmunoResearch 303-545-003), and rabbit anti-mouse 594 (Invitrogen A11062). Slides were mounted and photographed as described previously (Rojas et al., 2009).

Cell culture, transfection, and reporter assays

Cos-1 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. Transfections were performed with Fugene6 (Roche) according to manufacturers

instructions; transfection mixes contained 350ng of reporter construct and 450ng of total expression vectors. The total amount of transfected plasmid DNA was held constant in all cases. Cells were harvested 48 h after transfection, and β -galactosidase activity was determined using the Luminescent β -gal Detection Kit II (Clontech). Relative light units were normalized to total protein as measured by Bradford Assay (Bio-Rad).

Electrophoretic mobility shift assay (EMSA)

DNA binding assays were performed using modified 1 \times binding buffer (40mM KCl, 15mM HEPES pH7.9, 1mM EDTA, 0.5mM DTT, 50% glycerol) as described previously (Dodou et al., 2003). Recombinant proteins were made using the Promega TNT Coupled Reticulocyte System according to manufacturer's instructions. Full length FoxC2, Etv2, and Sox17 were expressed from their respective pRK5 vectors using SP6 polymerase. Control and mutated control probe sequences for FOX:ETS motif from the *Mef2c* gene and for the SOX site from the mouse *laminin 1a* gene have each been described previously (De Val et al., 2008; Niimi et al., 2004). *ECE1* probes were designed with guanine overhangs for labeling with Klenow and ³²P-dCTP. The sense strand sequences for the probes were: ECE1_FOX:ETS, 5'-gggagtctagctaaacaggaaggaggcacaatgagagga-3'; ECE1_mFEM, 5'-gggagtctagctaatgaggcaggaggcacaatgagagga-3'; ECE1_SOX, 5'-ggaaggaggcacaatgagaggaagt-3'; ECE1_mSOX, 5'-ggaaggaggcaggtagagaggaagt-3'.

RESULTS

ECE1 enhancer activity is restricted to arterial endothelium

Previously, we cloned a 623-bp fragment of human genomic sequence (*ECE1*[623]) located 384 bp upstream of one of the three transcriptional start sites for the *ECE1* gene (Fig. 1A; and De Val et al., 2008). This fragment contains the predicted FOX:ETS motif, and we previously established that this element functioned as an endothelial-specific transcriptional enhancer in transgenic mouse embryos at a single time point (De Val et al., 2008). To facilitate a more complete analysis of this regulatory element, we generated stable transgenic lines and analyzed enhancer activity in embryos from E7.5 to E18.5 (Fig. 2).

The *ECE1* endothelial enhancer first demonstrated activity *in vivo* beginning at E7.75 (Fig. 2A). At this stage, X-gal staining was apparent in cells within the lateral plate mesoderm (Fig. 2A), where vascular endothelial progenitors in the embryo proper originate (Patan, 2004). β -galactosidase activity was also evident more anteriorly in pre-cardiac mesoderm (Fig. 2A). By E8.5, X-gal staining was clearly visible in the paired dorsal aortae in the trunk and within the nascent endocardium in the heart (Fig. 2B). Following the onset of sprouting angiogenesis at approximately E9.0, *ECE1* enhancer activity could be readily observed throughout the vasculature, including in intersegmental vessels (Fig. 2C). Within the heart, reporter activity was restricted to the endocardium at E9.0 (Fig. 2C,D). At E9.5, activity of the *ECE1* enhancer was widely apparent in the vascular endothelium (Fig. 2E), but activity appeared to be restricting to arterial endothelium and endocardium, as X-gal staining was robust in arteries and very weak in developing veins (Fig. 2F). Enhancer activity remained robust in arterial endothelium throughout the remainder of mouse embryonic development.

At E12.5, β -galactosidase expression remained completely restricted to arterial endothelium and endocardium (Fig. 2G, H).

To examine the activity of the *ECE1* enhancer in more detail, we compared β -galactosidase expression from the *ECE1* reporter to the expression of endogenous Ece-1 protein in arteries and veins and to artery and vein-specific markers by co-immunofluorescence at E12.5 (Fig. 3). We found that endogenous Ece-1 was expressed throughout the endothelium in both arteries and veins (Fig. 3A). In contrast, β -galactosidase expression was detectable exclusively in arteries (Fig. 3A'). The overlap of β -galactosidase and endogenous Ece-1 only in arteries (Fig. 3A'') suggests that the *ECE1* enhancer described here controls only a subset of the endogenous expression pattern. Expression of the arterial markers Neuropilin-1 and Connexin40 (Buschmann et al., 2010; Herzog et al., 2001) overlapped completely with β -galactosidase expression (Fig. 3B,C), confirming the activity of the *ECE1* enhancer in arterial endothelium. Likewise, comparison of β -galactosidase expression with the expression of the venous markers Neuropilin-2 and COUP-TFII (Chong et al., 2011; Herzog et al., 2001; You et al., 2005) also confirmed the arterial restriction of *ECE1* enhancer activity since β -galactosidase expression did not overlap with expression of the venous markers (Fig. 3D,E). Taken together, the results presented in Figures 2 and 3 establish that *ECE1*[623] is an arterial-restricted enhancer *in vivo*.

FoxC2 and Etv2 regulate the *ECE1* arterial enhancer

Although the *ECE1* enhancer was originally identified based on the presence of a conserved FOX:ETS motif (De Val et al., 2008), the predicted FOX:ETS motif in the enhancer has not been previously validated as a *bona fide* binding site for FoxC2 and Etv2. Therefore, we performed electrophoretic mobility shift assays (EMSA) with recombinant FoxC2 and Etv2 proteins (Fig. 4A and B, respectively). Both factors efficiently bound to the *ECE1* FOX:ETS motif (Fig. 4A,B, lane 2 in each panel). Unlabeled oligonucleotide probes representing a control FOX:ETS motif from an endothelium-specific enhancer of the mouse *Mef2c* gene and the *ECE1* FOX:ETS motif itself efficiently competed for binding (Fig. 4A,B, lanes 3 and 5 in each panel). In contrast, mutant versions of each FOX:ETS motif failed to compete for binding even when present in ~100-fold excess (Fig. 4A,B, lanes 4 and 6 in each panel). Taken together, the results of the EMSA establish that the FOX:ETS motif in the *ECE1* enhancer is a *bona fide in vitro* binding element recognized by FoxC2 and Etv2.

To further test whether the FOX:ETS motif in the *ECE1* arterial enhancer is a functional element for FoxC2 and Etv2, we tested the ability of FoxC2 and Etv2 to transactivate the core region of the *ECE1* enhancer (Fig. 1C). FoxC2 alone significantly activated the *ECE1*-TK-E-gal reporter (Fig. 4C, compare lanes 1 and 3; $p=0.0043$). This activation was dependent on an intact FOX:ETS motif since mutation completely abolished FoxC2-dependent transactivation (Fig. 4C, compare lanes 3 and 4). Similarly, Etv2 also significantly activated the *ECE1* reporter (Fig. 4C, compare lanes 1 and 5; $p=0.0100$) and this activation was also dependent on an intact FOX:ETS motif (Fig. 4C, compare lanes 5 and 6).

Importantly, and consistent with previous studies of other FoxC2/Etv2-dependent enhancers (De Val et al., 2008), co-transfection of FoxC2 and Etv2 expression constructs with the

ECE1-TK- β -gal reporter resulted in strong (>40-fold) activation of the *ECE1* enhancer compared to control (Fig. 4C, compare lanes 1 and 7; $p < 0.001$). Mutation of the FOX:ETS motif nearly abolished reporter activation (Fig. 4C, compare lanes 7 and 8). The cooperative activation of the reporter by both factors together represents a significant increase over the transactivation by either factor alone ($p < 0.001$ versus either FoxC2 or Etv2 alone). These data demonstrate that the *ECE1* endothelial enhancer is cooperatively regulated by FoxC2 and Etv2.

To determine if the *ECE1* FOX:ETS motif was necessary for reporter activity *in vivo*, we generated embryos with either wild type *ECE1*[623]-*lacZ* or a mutant version with a disrupted FOX:ETS motif and examined enhancer activity at E9.0 (Fig. 4D, Dc). As expected, X-gal staining demonstrated robust β -galactosidase activity in embryos harboring the wild transgene (Fig. 4D). In contrast, embryos transgenic for the mutant transgene (mFEM) exhibited no detectable X-gal staining (Fig. 4D'). These data demonstrate that the *ECE1* FOX:ETS is required for enhancer activity *in vivo*. Taken together, the results of Fig. 4 support the idea that the *ECE1* FOX:ETS motif is a *bona fide* binding element for FoxC2 and Etv2 and is required for enhancer activation *in vitro* and *in vivo*.

A SOX binding site is essential for *ECE1* enhancer activity

Comparison of *ECE1* enhancer sequences from human, mouse, and opossum in the core region of human:mouse conservation revealed that only three short stretches of sequence are perfectly conserved between the two placental mammals and the marsupial opossum (Fig. 1B). Two of those elements are the FOX:ETS motif and a neighboring ETS site. The only other perfectly conserved sequence in the core enhancer is a consensus SOX site (Fig. 1B; and Hosking et al., 1995; Niimi et al., 2004). This candidate site attracted our attention given the essential role of the SoxF subfamily in vascular endothelial development (Francois et al., 2010). In particular, Sox17 expression is restricted to arteries and has recently been described to be a key regulator of arterial development (Corada et al., 2013). Therefore, we reasoned that Sox17 might be a regulator of the *ECE1* arterial-restricted enhancer via the consensus SOX site.

To determine whether the SOX motif in the *ECE1* enhancer might function as a Sox17 binding site, we performed EMSA with recombinant Sox17 protein (Fig. 5A). Sox17 bound efficiently to the *ECE1* SOX motif (Fig. 5A, lane 2). Binding to the SOX site by Sox17 was specific since addition of excess unlabeled oligonucleotide probe from a control Sox17 site from the *laminin 1D* gene competed with the radiolabeled probe and prevented the shift from occurring (Fig. 5A, lane 3). Similarly, binding of Sox17 to the *ECE1* SOX site was abolished by addition of an unlabeled double-stranded oligonucleotide with the exact *ECE1* SOX site sequence (Fig. 5A, lane 5). Mutant versions of the control SOX site and the *ECE1* SOX site were unable to compete for binding even when added at ~100-fold excess (Fig. 5A, lanes 4 and 6, respectively). These results establish that the consensus SOX motif in the *ECE1* enhancer is *bona fide* binding site recognized by Sox17 *in vitro*.

We also tested the ability of Sox17 to transactivate the *ECE1* enhancer (Fig. 5B). Co-transfection of a Sox17 expression vector with the *ECE1*-TK- β -gal reporter resulted in a significant increase in reporter activity compared to the activation obtained with the parent

expression vector (Fig. 5B, compare lanes 1 and 3; ~13-fold activation, $p=0.0038$). Mutation of the SOX site in the enhancer significantly reduced activity, although some residual reporter activity remained (Fig. 5B, compare lanes 3 and 4; $p=0.0330$). The slight residual activity of the enhancer even with the mutant SOX site may be due to the existence of nonconsensus binding sites in the *ECE1*[164] core enhancer that allow for some activation of the reporter to occur, or it may be due to indirect activation by Sox17. Overall, however, the results presented in Fig. 5B demonstrate that Sox17 is sufficient for activation of the *ECE1* enhancer, and that this activation is mediated primarily by binding to the conserved SOX site in the core enhancer.

We next asked if the SOX site was required for *ECE1* enhancer activity *in vivo*. To test this, we generated transgenic mouse embryos harboring wild type *ECE1*[623]-*lacZ* and a mutant version with a disrupted SOX site (Fig. 5C,Cc). As described above, embryos transgenic for the wild type *ECE1*[623]-*lacZ* construct showed strong X-gal staining in the vascular endothelium at E9.5 (Fig. 5C). In contrast, we did not observe any staining in embryos carrying the mutant version of the reporter construct (Fig. 5Cc). Thus, the activity of the *ECE1* enhancer *in vivo* is dependent on the SOX binding site.

Sox17, FoxC2, and Etv2 synergistically activate the *ECE1* enhancer

The proximity of the FOX:ETS and SOX binding sites in the core enhancer suggested the possibility that FoxC2, Etv2, and Sox17 might function collaboratively to activate the *ECE1* enhancer. To test this possibility, we examined the combined effects of these three transcription factors on *ECE1* enhancer transactivation (Fig. 6A). As expected, FoxC2 and Etv2 exhibited strong cooperativity on the *ECE1* enhancer (Fig. 6A, lane 5). Importantly, co-expression of Sox17 with either FoxC2 or Etv2 also resulted in synergistic activation of the reporter (Fig. 6A, lanes 6 and 7). Co-expression of all three factors resulted in even more robust activation of the *ECE1* reporter (>160-fold) than the activation observed with any combination of two factors (Fig. 6A, lane 8). Taken together, these observations suggest that Sox17 can function cooperatively with Etv2 and FoxC2 separately or combinatorially to activate endothelial-specific regulatory elements.

We next tested whether the FOX:ETS and SOX sites in the *ECE1* enhancer were required for cooperative activation by FoxC2, Etv2, and Sox17 (Fig. 6B). Mutation of either the FOX:ETS motif or the SOX site dramatically reduced, but did not completely eliminate, transactivation of the reporter by the three factors (Fig. 6B, lanes 2 and 3). In contrast, simultaneous disruption of the FOX:ETS motif and the SOX site completely abolished all reporter activity (Fig. 6B, lane 4). These data establish that maximal synergy by FoxC2, Etv2, and Sox17 requires the presence of functional binding sites for each factor.

DISCUSSION

Modular regulation of *ECE1* transcription

Ece-1 functions as an important regulatory node in Endothelin signaling by controlling the production of the mature Endothelin peptide (Turner and Murphy, 1996). In spite of its importance in embryonic development and in the control of vascular tone postnatally, the

transcriptional regulation of *ECE1* has not been extensively investigated. In the present study, we conducted the first analysis of *ECE1* transcriptional regulation using an *in vivo* approach. Compared to endogenous *Ece1*, which is expressed broadly in the developing mouse embryo, including throughout the vasculature (Yanagisawa et al., 1998; and data not shown), the *ECE1* enhancer described here only marks a subset of the endogenous gene expression pattern. This suggests that additional enhancers regulate *ECE1* in other parts of its expression domain. Modular regulation by multiple, distinct enhancers is a recurrent mechanism for many developmental genes with complex expression patterns, such as *ECE1*, and is thought to help achieve precise temporal and spatial control of expression (Spitz and Furlong, 2012).

Restriction of *ECE1* enhancer activity to arterial endothelium and endocardium

The *ECE1* enhancer described here rapidly restricts to arterial endothelium. However, at early times, activity appears to be present more broadly in the endothelium, including in veins or possibly in their progenitors. This may be due to reporter expression in the endothelial progenitor population and the perdurance of β -galactosidase in veins once they have formed. Alternatively, it is possible that the enhancer remains active in presumptive veins until the venous character of the vessel has been fully established. This may suggest that a population of early endothelial cells may be neither arterial nor venous and that the *ECE1* enhancer is active in these early cells, in addition to being active subsequently in arterial endothelium.

The three SoxF family members, Sox7, Sox17, and Sox18 are each expressed in endothelial cells, including in the arterial endothelium (Francois et al., 2010; Young et al., 2006), but only Sox17 is thought to restrict exclusively to arterial endothelium within the vasculature (Corada et al., 2013; Liao et al., 2009). Consistent with this notion, deletion of *Sox17* specifically in vascular endothelium results in arteriovenous malformations (Corada et al., 2013). Furthermore, an arterial enhancer of *Dll4* contains a SOX binding site that is crucial for activity in arterial endothelium (Sacilotto et al., 2013). Our findings are consistent with a role for Sox17 as a key regulator of arterial fate and arterial gene expression; the evidence presented here suggests that Sox17 is a *bona fide* regulator of the *ECE1* arterial enhancer. However, we can't rule out that other SoxF factors or other transcription factors acting via the SOX consensus element may be involved in *ECE1* enhancer activity and contribute to its arterial restriction during embryogenesis.

Notch signaling in the vascular endothelium is also restricted to arteries and is crucial for arterial identity (Lawson et al., 2001; Roca and Adams, 2007; Zhong et al., 2001). However, it is currently unknown how many arterial genes are direct Notch signaling targets. Recently, a binding site for RBPJ, a downstream effector of Notch signaling, has been identified in the *Dll4* arterial enhancer (Sacilotto et al., 2013; Wythe et al., 2013), suggesting that *Dll4* is a direct Notch target. In contrast, the *ECE1* arterial-restricted enhancer described here does not contain any discernible RBPJ binding sites (Fig. 1B), suggesting that arterial restriction of this enhancer may occur independent of direct Notch activation. This finding supports the idea that regulatory pathways upstream or parallel to Notch may also be sufficient for artery-restricted expression and is consistent with some recent studies that suggest that Sox17

functions in arterial identity upstream or possibly independent of Notch signaling (Clarke et al., 2013; Corada et al., 2013). Conversely, other recent studies suggest that Notch functions upstream of Sox17 (Lee et al., 2014). Thus, it will be important for future studies to resolve the relationship between Notch signaling and Sox17 and their respective roles in arterial identity.

Transcription factor interactions in the endothelial cell gene regulatory network

Etv2 is essential for endothelial progenitor cell specification and is thought to be a central upstream regulator of the gene regulatory network that establishes the endothelial cell phenotype (De Val and Black, 2009; Lammerts van Bueren and Black, 2012). Previous work has shown that Etv2 activity is dramatically enhanced by cooperative interactions with other transcription factors (De Val et al., 2008; Shi et al., 2014). We show here that the *ECE1* enhancer, like other previously described endothelial enhancers (De Val et al., 2008), is cooperatively activated by FoxC2 and Etv2 through its conserved FOX:ETS motif. Importantly, we found that Sox17 also functions cooperatively with Etv2 to activate the *ECE1* enhancer and that transactivation by all three factors, FoxC2, Etv2, and Sox17, results in additional synergistic activation.

After the initial specification of the endothelial lineage, Etv2 expression is rapidly extinguished (Ferdous et al., 2009; Kataoka et al., 2011; Lee et al., 2008), whereas Sox17 becomes restricted to the arterial compartment within the vasculature (Corada et al., 2013; Liao et al., 2009). FoxC2 is expressed throughout the early vascular endothelium and its expression persists in the vascular endothelium (Seo et al., 2006). Our observation that Sox17 cooperates with FoxC2 without a requirement for Etv2 (Fig. 6A) is consistent with the possibility that the combined activities of FoxC2 and Sox17 may account for the continued activation of the *ECE1* enhancer in arteries, following initial enhancer activation by Etv2, possibly in combination with Forkhead and SoxF factors. It is interesting to speculate that this may represent a general model for early endothelial gene activation in which Etv2 and its cofactors are involved in the initiation of gene expression at early times in the endothelial gene regulatory network. Subsequently, as Etv2 expression is rapidly switched off, other transcription factors function combinatorially to maintain and restrict endothelial gene expression to specific vascular compartments. It will be important to decipher these transcription factor relationships in order to understand the endothelial cell gene regulatory network more completely.

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Highlights

- The human *ECE1* locus contains an arterial-restricted enhancer
- The *ECE1* arterial enhancer is dependent on a conserved FOX:ETS motif
- The *ECE1* arterial enhancer is dependent on a conserved SOX binding site
- Etv2, FoxC2, and Sox17 directly binding and activate the *ECE1* arterial enhancer
- Spx17, Etv2, and FoxC2 cooperatively activate the *ECE1* arterial enhancer

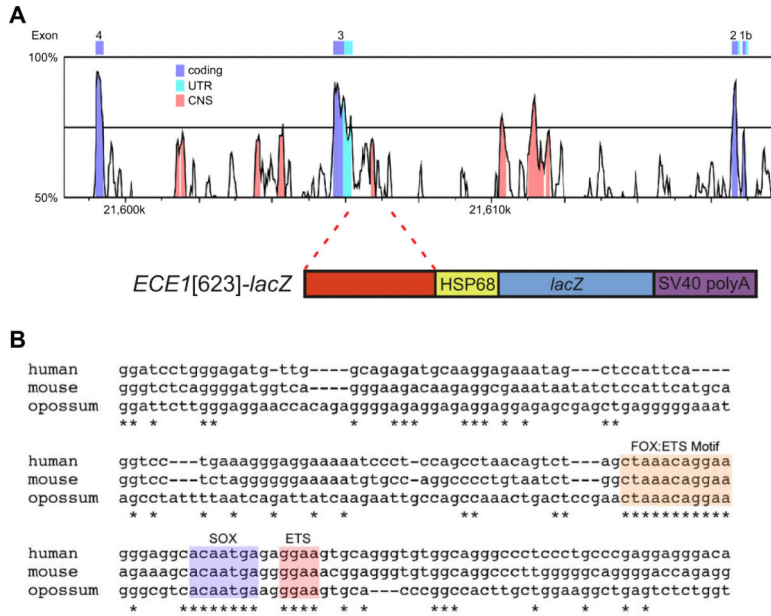


FIG. 1. A conserved, endothelial-specific enhancer in the *ECE1* locus. (A) VISTA plot comparing human and mouse sequence within the *ECE1* locus (upper schematic). Human sequence from hg19 (Build GRCh37.p13, Chr1: 21606465-21606628) is used as the reference sequence. CNS, conserved, non-coding sequence; UTR, untranslated exon sequence. A 623-bp fragment encompassing the CNS containing the FOX:ETS motif was cloned upstream of the *hsp68* minimal promoter for expression of E-galactosidase (lower panel). (B) ClustalW multiple sequence alignment of the core 164-bp *ECE1* enhancer region from human, mouse, and opossum. Conserved nucleotides are indicated with asterisks. The conserved FOX:ETS motif, a second conserved ETS site, and the conserved SOX site are indicated.

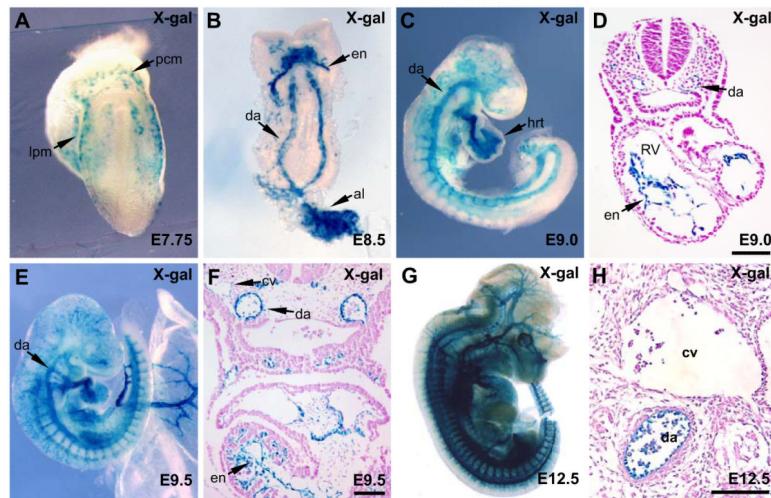
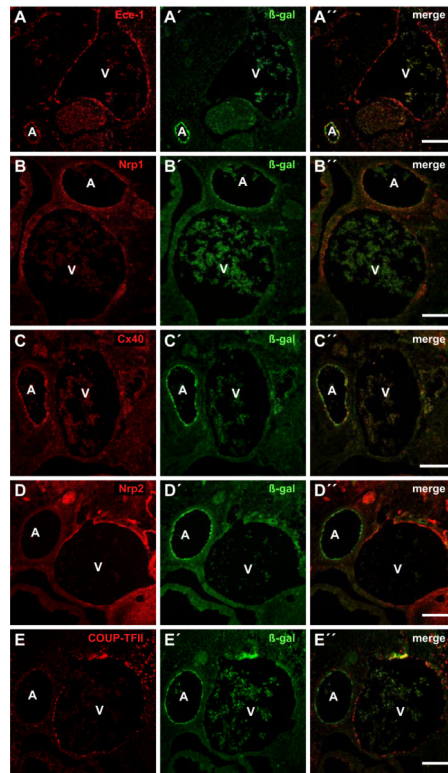


FIG. 2. The *ECE1* enhancer is endothelial specific and restricts to arterial endothelium. *ECE1[623]-lacZ* transgenic embryos were collected at E7.75 (A), E8.5 (B), E9.0 (C,D), E9.5 (E,F) and E12.5 (G,H) and were X-gal stained and then analyzed by whole mount (A,B,C,E,G) or were sectioned and counterstained (D,F,H) to analyze *ECE1* enhancer activity. al, allantois; cv, cardinal vein; da, dorsal aorta; en, endocardium; hrt, heart; lpm, lateral plate mesoderm; pcm, precardiac mesoderm; RV, right ventricle. Scale bars, 100 μm.

**FIG. 3.**

ECE1 enhancer activity is restricted to arterial endothelium at E12.5. Immunostaining of transverse sections through the trunk region of *ECE1[623]-lacZ* transgenic embryos collected at E12.5. Endogenous *Ece-1* protein expression (A) is present in arteries and veins; β -galactosidase protein expression from the *ECE1[623]-lacZ* transgene (*A_c*) is restricted to arteries. β -galactosidase protein expression (*B_c*, *C_c*) overlaps with expression of the arterial markers *Nrp1* (B) and *Cx40* (C). β -galactosidase expression (*D'*, *E'*) is found only in arteries and is mutually exclusive from the expression of the venous markers *Nrp2* (D) and *COUP-TFII* (E). Respective merged images for each of the markers are shown in *A''-E''*. A, artery, V, vein. Scale bars, 100 μ m.

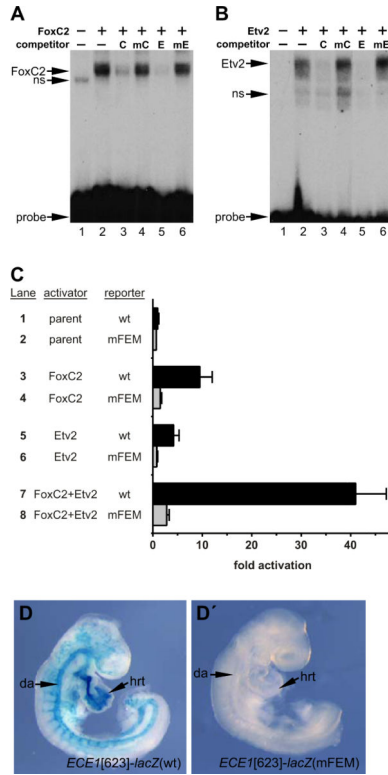


FIG. 4.

The *ECE1* enhancer contains a *bona fide* FOX:ETS motif. (A,B) EMSA using a radiolabeled double-stranded oligonucleotide probe encompassing the *ECE1* FOX:ETS motif.

Recombinant FoxC2 (A) and recombinant Etv2 (B) each bound the site (lane 2). Addition of excess unlabeled control FOX:ETS motif probe [C] or the *ECE1* FOX:ETS motif probe [E] abolished binding of FoxC2 and Etv2 (lanes 3, 5). Competitor oligonucleotides in which the FOX:ETS motifs were mutated [mC, mE] did not compete for binding, even when added at ~100-fold excess (lanes 4, 6). Unprogrammed reticulocyte lysate is shown in lane 1 in each panel. Non-specific [ns] bands and free probe are denoted. (C) Cos-1 cells were co-transfected with pRK5 expression vector (lanes 1,2), pRK5-FoxC2 (lanes 3,4), pRK5-Etv2 (lanes 5,6), or pRK5-FoxC2 and pRK5-Etv2 (lanes 7,8) and the wild type [wt] *ECE1*-TK- β -gal reporter (lanes 1,3,5,7) or a version of the reporter with a mutated FOX:ETS motif [mFEM] (lanes 2,4,6,8) and assayed for β -galactosidase activity. FoxC2 and Etv2 each activated the reporter (lanes 3,5). Cooperative activation was observed when FoxC2 and Etv2 were co-expressed (lane 7). In all cases, activation was dependent on an intact FOX:ETS motif. Data are expressed as the mean fold activation plus SEM for 3 independent transfections and analyses. (D, D') Wild type [wt] *ECE1*[623]-*lacZ* (D) or *ECE1*[623]-*lacZ* with a mutated FOX:ETS motif [mFEM] (D') transgenic embryos were collected at E9.0 and were X-gal stained to detect β -galactosidase activity. The wild type enhancer was active throughout the arterial vasculature (7/9 independent transgenic lines; 2 lines showed little or no expression). Mutation of the FOX:ETS motif site completely abolished all activity in 3/3 independent transgenic lines examined. Representative transgenic embryos are shown. da, dorsal aorta; hrt, heart.

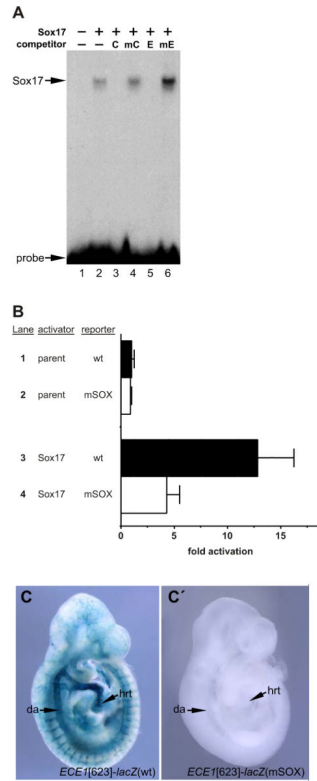


FIG. 5.

The *ECE1* enhancer contains a *bona fide* SOX site. (A) EMSA using a radiolabeled double stranded oligonucleotide probe corresponding to the *ECE1* SOX site. Recombinant Sox17 bound to the probe (lane 2). Addition of excess unlabeled control SOX site [C] or the *ECE1* SOX site [E] abolished binding (lanes 3,5). Unlabeled mutant versions of the SOX sites [mC, mE] did not compete for Sox17 binding at ~100-fold excess (lanes 4,6). Unprogrammed reticulocyte lysate is shown in lane 1. Free probe is denoted. (B) Cos-1 cells were co-transfected with pRK5 expression vector (lanes 1,2) or with pRK5-Sox17 (lanes 3,4) and the wild type [wt] *ECE1*-TK- β -gal reporter (lanes 1,3) or a version of the reporter with a mutated SOX site [mSOX] (lanes 2,4) and assayed for β -galactosidase activity. Sox17 strongly activated the reporter (lane 3) and this activation was largely dependent on an intact SOX site (lane 4). Data are expressed as the mean fold activation plus SEM for 3 independent transfections and analyses. (C, C') Wild type [wt] *ECE1*[623]-*lacZ* (C) or *ECE1*[623]-*lacZ* with a mutated SOX site [mSOX] (Cc) transgenic embryos were collected at E9.5 and were X-gal stained to detect E-galactosidase activity. The wild type enhancer was active throughout the arterial vasculature (7/9 independent transgenic lines; 2 lines showed little or no expression). Mutation of the SOX site completely abolished all activity in all 3/3 independent transgenic lines examined. Representative transgenic embryos are shown. da, dorsal aorta; hrt, heart.

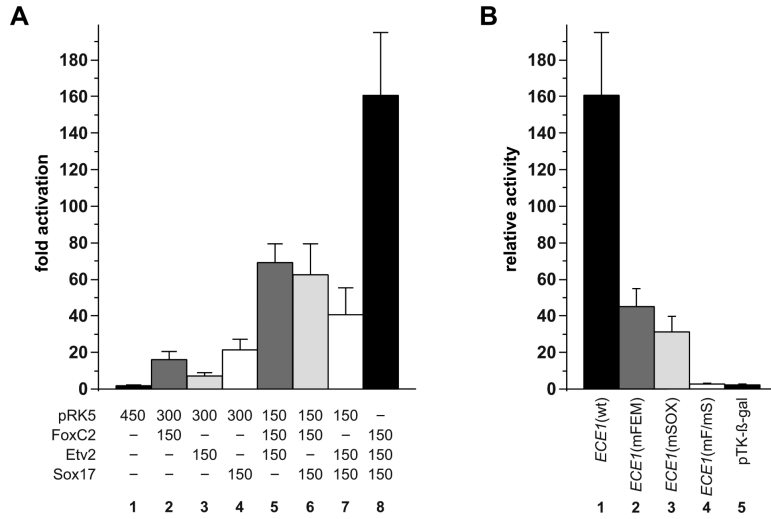


FIG. 6. Synergistic activation of the *ECE1* enhancer by combinatorial expression of Sox17, FoxC2, and Etv2 requires binding to the cognate binding site for each factor. (A) Cos-1 cells were transfected with the wild type *ECE1*-TK-β-gal reporter plasmid and various combinations of pRK5 expression plasmids for FoxC2, Etv2, and Sox17 and were assayed for E-galactosidase activity. The amount (ng) of each transfected expression plasmid is indicated. Data are expressed as the mean fold activation of reporter co-transfected with parental expression plasmid only plus SEM for 3 independent transfections and analyses. (B) Cos-1 cells were transfected with expression plasmids for FoxC2, Etv2, and Sox17 (150 ng each plasmid) and various *ECE1*-TK-β-gal reporter plasmids, including the wild type (wt, lane 1), mutated FOX:ETS motif (mFEM, lane 2), mutated SOX site (mSOX, lane 3), both FOX:ETS motif and SOX sites mutated (mF/mS, lane 4), and the parental pTK-E-gal reporter lacking *ECE1* elements (lane 5). Maximal activation by the three transactivators was only observed when both *cis*-acting elements were intact (lane 1). Data are expressed as the mean relative β-galactosidase activity plus SEM for 3 independent transfections and analyses.