Vascular endothelial and endocardial progenitors differentiate as cardiomyocytes in the absence of Etsrp/Etv2 function

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SUMMARY

Previous studies have suggested that embryonic vascular endothelial, endocardial and myocardial lineages originate from multipotential cardiovascular progenitors. However, their existence in vivo has been debated and molecular mechanisms that regulate specification of different cardiovascular lineages are poorly understood. An ETS domain transcription factor Etv2/Etsrp/ER71 has been recently established as a crucial regulator of vascular endothelial differentiation in zebrafish and mouse embryos. In this study, we show that etsrp-expressing vascular endothelial/endocardial progenitors differentiate as cardiomyocytes in the absence of Etsrp during zebrafish embryonic development. Expression of multiple endocardial specific markers is absent or greatly reduced in Etsrp knockdown or mutant embryos. We show that Etsrp regulates endocardial differentiation by directly inducing endocardial nfatc1 expression. In addition, Etsrp function is required to inhibit myocardial differentiation. In the absence of Etsrp function, etsrp-expressing endothelial and endocardial progenitors initiate myocardial marker hand2 and cmic2 expression. Furthermore, Foxc1a function and interaction between Foxc1a and Etsrp is required to initiate endocardial development, but is dispensable for the inhibition of myocardial differentiation. These results argue that Etsrp initiates endothelial and endocardial, and inhibits myocardial, differentiation by two distinct mechanisms. Our findings are important for the understanding of genetic pathways that control cardiovascular differentiation during normal vertebrate development and will also greatly contribute to the stem cell research aimed at regenerating heart tissues.

KEY WORDS: Endocardial, Endothelial, Multipotent progenitor, Myocardial, Zebrafish

INTRODUCTION

The heart is composed of diverse muscle and non-muscle cell lineages. Until recently, it has been believed that cardiac progenitor cells commit early during development to exclusively generate cardiomyocytes, whereas other heart lineages such as endocardial cells are specified independently. However, a growing body of evidence from multiple laboratories suggest that, with respect to lineage diversification, there may be a single stem/progenitor cell that can generate all major cell types during heart formation (Kattman et al., 2006; Moretti et al., 2006; Wu et al., 2006; Yang et al., 2008). However, signaling pathways that direct the formation of these cell lineages remain to be elucidated.

Although it is difficult to study cardiovascular lineage formation in mammals due to embryo inaccessibility, the zebrafish offers an advantageous in vivo system to dissect the mechanisms of cardiovascular lineage formation. Similar to mammalian embryos, zebrafish endocardial, myocardial and vascular endothelial cells form in close vicinity within the anterior lateral plate mesoderm (ALPM). Lineage-tracing experiments in avian and zebrafish embryos have shown that the spatial separation of endocardial and myocardial progenitors happens very early and can already be observed in early gastrula stage embryos (Lee et al., 1994; Keegan et al., 2004). However, cardiac progenitor cells retain flexibility to adopt a different cardiovascular fate and do not commit to their final cell fates until later somitogenesis stages. The zebrafish ALPM region rostral to the myocardium-forming region harbors latent myocardial developmental potential (Schoenebeck et al., 2007). This rostral ALPM region normally gives rise to head vessels, myeloid cells and endocardium. Expression of the transcription factor hand2 corresponds to the myocardium forming region, as demonstrated by fate-mapping studies (Schoenebeck et al., 2007). In cloche (clo) mutants, which are almost completely devoid of endothelial, endocardial and hematopoietic cells (Stainier et al., 1995; Liao et al., 1997; Liao et al., 1998), hand2 expression extends beyond its normal boundary, exhibiting strong expression throughout the rostral ALPM, resulting in a significant increase in cardiomyocytes (Schoenebeck et al., 2007). Similarly, double knockdown of etsrp and scl, two known regulators of vasculogenesis and hematopoiesis, respectively, resulted in a similar rostral expansion of myocardial hand2 expression. Conversely, combinatorial overexpression of scl and etsrp RNA resulted in the reduction of myocardial-specific hand2 and cmic2 (myl7 – Zebrafish Information Network) expression. Similar expansion of hematovascular and loss of myocardial development was recently observed upon inhibition of FGF signaling (Simoes et al., 2011). However, it is unclear whether the observed myocardial expansion in the absence of hematovascular development is due to the cell fate switch of hematovascular progenitors into myocardial progenitors. Furthermore, the molecular mechanism of the suppression of myocardial differentiation in the rostral ALPM region during normal development is not understood.
Although significant progress has been made towards elucidating the morphogenetic events and transcriptional control underpinning the patterning of myocardium, the early endocardial development remains still poorly understood (Lough and Sugi, 2000; Harris and Black, 2010). Similar to mammalian embryos, endocardial cells in zebrafish originate bilaterally within the anterior lateral plate mesoderm (ALPM). Vascular endothelial and endocardial cells share expression of multiple markers, including cdh5, fli1 and kdrl (Brown et al., 2000; Larson et al., 2004; Sumanas et al., 2005). Very few molecular markers are specific to endocardium; one of them is Nfatc1, which is expressed in the mouse endocardial, but not vascular endothelial, cells, indicating that the two endothelial subtypes are biochemically distinct (de la Pompa et al., 1998). Although Nfatc1 homologs have not been previously characterized in zebrafish, fibronectin 1 (fn1) expression is thought to label early endocardial but not vascular endothelial precursors (Trinh and Stainier, 2004). In vitro studies have demonstrated that endocardial lineage can develop from the same multipotent progenitor cells as myocardial and vascular endothelial lineages (Misfeldt et al., 2009). Endocardial precursors can be distinguished from other vascular endothelial cells as they migrate medially and posteriorly, and fuse at the midline between the 15- and 18-somite stages (Bussmann et al., 2007). Subsequently, they undergo a complex leftward movement to position the endocardial primordium at the left side of the embryo where they form the lining of the primitive heart tube. However, the signaling pathways that regulate specification, migration and differentiation of endocardial progenitors in vivo are largely unknown.

Previous studies have established that an ETS domain transcription factor, Etsrp/Etv2 functions on top of the transcriptional cascade that regulates vascular endothelial development in zebrafish (Sumanas and Lin, 2006; Pham et al., 2007). Morpholino knockdown of Etsrp function results in nearly complete loss of early vascular development as angioblasts fail to differentiate or migrate towards the midline. Etsrp overexpression alone is sufficient to induce precocious and ectopic expression of multiple vascular-specific markers, including kdrl, fli1 and cdh5 (Sumanas and Lin, 2006; Pham et al., 2007). Etsrp function is conserved during vertebrate development, with mouse Er71/Etv2 and human ETV2 proteins representing functional orthologs of Etsrp (Lee et al., 2008; Sumanas et al., 2008). Homozygous Etv2 knockout mouse embryos display the lack of blood islands, as well as endocardial and endothelial lineages, and die before E11.0 (Lee et al., 2008; Ferdous et al., 2009). It has been shown that multiple vascular endothelial specific genes share a conserved regulatory enhancer that cooperatively binds Etsrp/Etv2 and FoxC family of transcription factors (De Val et al., 2008). However, only limited analysis of cardiac defects has been performed in mouse Etv2 knockout embryos and its role in the formation of endocardial and myocardial lineages is poorly understood.

In this study, we have investigated the requirement of Etsrp for the development of endocardial and myocardial lineages in the zebrafish model system. We show that, in the absence of Etsrp function, early endocardial progenitors fail to differentiate, whereas myocardial progenitors expand into the rostral ALPM region. Furthermore, etsrp-expressing (etv2 – Zebrafish Information Network) endocardial progenitors initiate myocardial marker expression and differentiate as cardiomyocytes in the absence of Etsrp function. We further show that Foxc1a function is required for early endocardial differentiation but is dispensable for the inhibition of myocardial differentiation within the rostral ALPM. These results argue that Etsrp/Etv2 acts as a crucial switch in cardiovascular lineage differentiation, and it promotes endocardial and inhibits myocardial differentiation via two different mechanisms.

**MATERIALS AND METHODS**

**Zebrafish lines**

The following zebrafish lines were used for experiments: Tg(kdrl:EGFP)dp43 (Jin et al., 2005), Tg(fli1:EGFP)y1 (Lawson and Weinstein, 2002); Tg (cm122:GFP)aw41 (Huang et al., 2003); etsrp1T (Pham et al., 2007), Tg(etsrp:EGFP)y1 (Proulx et al., 2010) and wild type (Ekkwilli). A cmlc2:mCherry construct was generated by cloning a 900 bp fragment of the cmlc2 promoter (Huang et al., 2003) upstream of a promoter-less mCherry construct (Shaner et al., 2004). Five Tg(cm122:mCherry) founders were recovered with nearly identical expression patterns and levels. Tg(cmlc2:mCherry)aw7 exhibited the strongest expression and thus was employed for these studies.

Embryos were incubated at 28.5°C for analysis at 24 hpf and later stages, and at 23.5°C for analysis during somitogenesis stages. Embryos were staged as described previously (Kimmel et al., 1995). Embryos were treated with 1-pheny-2-thiourea (PTU) to inhibit pigment formation for stages 24 hpf and beyond. etv2$$^{–/–}$$ mutants were identified prior to 24 hpf by downregulation of fli1:GFP expression or at 24 hpf by the absence of intersomitic vessels and defective development of the axial vessels prior to in situ hybridization as previously reported (Pham et al., 2007).

**Microinjection of MOs**

For the majority of Etsrp knockdown experiments, 12.5 ng total of etsrp MO1 and MO2 1:1 mixture was injected at the 1- to 2-cell stage (Sumanas and Lin, 2006). To knock down etv2 function in Tg(etsrp:GFP)y1 line, 20 ng MO1 was used because MO2 is designed against the 5'UTR region and inhibits etsrp:GFP fluorescence. MO1 injection phenocopied the etv2$$^{–/–}$$ mutant phenotype and no other morphological abnormalities were observed. In the majority of the experiments, a single Foxc1a MO2 (sequence CGCCTGACTGCTCCTCCAAAAC) was injected at doses 1.5-3.0 ng per embryo, as reported previously (De Val et al., 2008). For 4 ng injections, a mixture of 4 ng of foxc1a MO2 and 2.5 ng p53 MO was injected to alleviate non-specific effects associated with high MO doses (Robu et al., 2000) and cmlc2 (Yelon et al., 1999) probes were synthesized as described. cmlc2 expression area was quantified by measuring width and length of the staining area using Adobe Photoshop CS2 in wild-type and FOXC1A founders were recovered with nearly identical expression patterns and levels. Tg(cm122:mCherry)aw7 exhibited the strongest expression and thus was employed for these studies.

**In situ hybridization**

In situ hybridization was performed as previously described (Jovett, 1999). A two-color in situ hybridization protocol was used as described (Sumanas et al., 2008). To synthesize DIG-labeled probes, nef1 (Open Biosystems, catalog number EDR1052-918306, Accession Number CN320837) and fn1 (Open Biosystems, catalog number EDR1052-96834665) cDNA clones, both in pExpress1 vector, were digested with EcoRI and transcribed with T7 RNA polymerase (Promega, fli1:kdrl (Thompson et al., 1998), cdh5 (Sumanas et al., 2005); hand2 (Yelon et al., 2000) and cmlc2 (Yelon et al., 1999) probes were synthesized as described. cmlc2 expression area was quantified by measuring width and length of the staining area using Adobe Photoshop CS2 in wild-type and Etsrp morphant embryos.

**Immunoﬂuorescent detection of etsrp:GFP**

To perform double staining of fn1 and etsrp:GFP, immediately following in situ hybridization, embryos were washed in PBST, manually deyolkel and blocked for 1 hour at room temperature in saponin blocking solution (SBS) [0.2% (w/v) saponin, 2 mg/ml BSA, 10% sheep serum (v/v), 1×PBST]. Embryos incubated in anti-GFP-Alexa488 (Invitrogen #A21480) at 4 μg/ml diluted in SBS overnight at 4°C. Embryos were washed three times for 10 minutes per wash with PBS/0.2% saponin and incubated with goat anti-rabbit-Alexa488 (Invitrogen #A11076) at 4 μg/ml diluted in SBS for 2 hours at room temperature. After washing with PBS/0.2% saponin, PBS and 30% glycerol/PBS, embryos were ventrally flat mounted in Vectashield (Vector Labs H-1000).
Cell transplantation

Donor cmlc2-GFP embryos were injected with a mixture of etsrp DNA (55 pg) and tetramethyl rhodamine isothiocyanate (TRITC)-dextran (2 ng; Mw 2 MDa; Sigma-Aldrich) into the blastomere at the one-cell stage. Embryos were manually dechorionated prior to transplantation. Fifty to 100 cells were transplanted into recipient cmlc2-GFP-uninjected embryos at the 30% epiboly stage with capillary needles and adjusting pressure of PLI-100 microinjector (Harvard Apparatus, Holliston, MA) to move cells up and down the needle.

Overexpression and real-time RT-PCR analysis

Tg(kdrl:GFP) embryos were injected at the one- to two-cell stage with 55 pg of circular etsrp-XeX or human Ets1 plasmid DNA (Sumanas et al., 2008). Batches of 20 injected and control uninjected embryos were frozen on dry ice at the 10- and 20-somite stages. Total RNA was purified using the RNAquous-4PCR kit (Ambion). cDNA was synthesized using SuperScript III Reverse Transcriptase and oligo-dT primer (Invitrogen). Real-time PCR was performed using Chromo4 thermal cycler (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad). The following PCR profile was used: 95°C for 5 minutes; 95°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute, detection at 82°C for 10 seconds; steps 2-5 repeated 44 times. Relative cDNA amounts for most myocardial markers and nfatc1 were calculated using the iCycler software (Bio-Rad) and normalized to the expression of elongation factor 1α (ef1α). As PCR amplification of cmlc2 resulted in minor amounts of nonspecific products, the relative amount of specific cmlc2 PCR product was calculated using ImageJ software (NIH) from the intensity values of an image of an ethidium bromide-stained agarose gel. Primer sequences are shown in Table S1 in the supplementary material.

Fig. 1. etsrp function is required for endocardium formation. (A-H) Morpholino knockdown of etsrp results in the loss of early endocardial precursors (arrows) that migrate to the midline, as analyzed by in situ hybridization for endothelial/endocardial markers kdrl (A-D) and cdh5 (E-H) at the 19-somite (A,B,E,F) and 24-somite (C,D,G,H) stages. (I-L) etsrp<sup<y11/-</sup> mutants lack kdrl (I,J) and nfatc1 (K,L) expression within the endocardial tube (arrows) at 24 hpf. Normal kdrl expression within the endocardium is outlined in yellow (I). nfatc1 expression in olfactory placodes is not affected (white arrowheads). (M-P) At 48 hpf (M,N) and 72 hpf (O,P) stages, nfatc1 expression in wild-type sibling embryos (M,O) is concentrated at the atrial/ventricular boundary (lower arrows) and the ventricular/outflow tract boundary (upper arrows), but is sparse and diffuse in etsrp<sup<y11/-</sup> mutants (N,P). (A-L) Ventral flat-mount view, anterior is upwards; (M-P) Ventral whole-mount view. (Q-X) Tg(fli1:GFP) (Q-T) and Tg(kdrl:GFP) (U-X) lines reveal loss of endocardial GFP in etsrp<sup<y11/-</sup> mutants (R,T) and etsrp morphants (V,X) at 30 hpf (insets in Q,R,U,V are shown a higher magnification in S,T,W,X, respectively). As expected, loss of Etsrp function results in the absence of intersegmental vessels (white arrowheads) and downregulation of kdrl:GFP and fli1:GFP in the axial vessels (yellow arrowheads). Lateral whole-mount view, anterior is towards the left. Arrows indicate endocardial tube.
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**RESULTS**

**Etsrp function is required for endocardial differentiation**

To determine whether Etsrp function is required for endocardial development, we analyzed Etsrp morpholino (MO) knockdown embryos (morphants) and eetsrp<sup>y11</sup>-null mutant embryos for endothelial and endocardial marker expression by in situ hybridization. Although endocardial precursors and vascular endothelial cells share expression of multiple genes, endocardial precursors can be distinguished from vascular endothelial cells after the 14-somite stage as they migrate to the midline to form the endocardial plate and the heart tube. At the 19- and 24-somite stages, uninjected embryos express fn1<sup>+/+</sup> (Fig. 1A,C) and <i>cdh5</i> (Fig. 1E,G) in vascular endothelial cells, located bilaterally, and in the endocardial cells at the midline. MO knockdown of Etsrp resulted in the failure of fn1<sup>-/-</sup>-expressing endocardial precursors to migrate towards the midline. (A-D) Fibronectin 1 (fn1) expression (arrowheads) in the presumptive endocardial precursors is slightly expanded rostrally in <i>eetsrp<sup>y11</sup></i>-null mutants at the 8- to 12-somite stages, as analyzed by in situ hybridization. (E-H) fn1<sup>+</sup>-expressing endocardial precursors (arrowheads) migrate to the midline in wild-type sibling embryos, while they remain localized bilaterally in <i>eetsrp<sup>y11</sup></i>-null mutants at the 14- to 16-somite stages. (I-P) fn1<sup>+</sup> expression largely overlaps with eetsrp:GFP expression in the presumptive endocardial precursor cells (white arrowheads) in uninjected control embryos and eetsrp morphants. Maximum projection image of eetsrp:GFP immunofluorescence (I,J) and fn1<sup>+</sup> in situ hybridization staining (K,L). Merged images of bright-field (BF) and GFP channels (M-P). There is slight expansion of fn1<sup>+</sup> and eetsrp:GFP staining in eetsrp morphants. O,P are higher magnification views of the insets in M,N, respectively. (A-P) Ventral views of flat-mounted embryos, anterior is upwards.

**Electrophoretic mobility shift assay (EMSA)**

DNA-binding reactions were performed as described previously (Dodou et al., 2003). Briefly, double-stranded oligonucleotides corresponding to the Mef2c-F10 ETS site (De Val et al., 2008) or the zebrafish <i>nfatc1</i> ETS site (Dodou et al., 2003). Briefly, double-stranded oligonucleotides corresponding to the Me2C-F10 ETS site used for EMSA is 5’-GGCAAACGCTTT-ACACAACAGGAAC-3’. The sense strand sequence of the mutant <i>nfatc1</i> ETS site is 5’-GGCAAACGCTTTACACATCTAGAAAC-3’. Mouse Etv2 protein was synthesized using the TNT Coupled Transcription-Translation System (Promega). Plasmid pCS2-Etv2, used for in vitro synthesis of Etv2, has been described previously (De Val et al., 2008).

**Cell counting**

<i>Etsrp</i>:GFP-expressing cells in ventrally mounted flattened embryos were counted using Bitplane Imaris, Autoquant and ImageJ software packages. Briefly, acquired z-stack images were cropped using the advanced cropping feature in Autoquant software to select those cells that had migrated to the midline. Cropped images were attenuation corrected and 3D deconvolved to remove out of focus fluorescence. Deconvolved z-stacks were exported as tiff files and imported into ImageJ or Imaris for cell counting. Tiff stacks were then maximum intensity projected using Imaris and exported to Adobe Photoshop CS2 to increase the contrast.
in the loss vascular endothelial and endocardial staining for both kdr (Fig. 1B,D) and cdh5 (Fig. 1F,H). Similar absence or strong reduction in endocardial kdr expression was observed in both etsrp morphants and etsrp<sup>y11</sup> mutant embryos at 24 hpf, when the heart has formed a linear heart tube (Fig. 1I,J; data not shown).

We recently isolated the zebrafish nfatc1 homolog, which is expressed specifically in endocardial but not vascular endothelial cells starting from 21 hpf and can be used as a marker for endocardial differentiation (K. S. Wong, K. Proulx, S. Palencia-Desai, V. Kohli, W. Hunter, J. D. Uhl and S. Sumanas, unpublished). In etsrp<sup>y11</sup> embryos, nfatc1 endocardial expression is completely missing at 24 hpf (Fig. 1K,L). By 48 hpf and 72 hpf, etsrp<sup>y11</sup> embryos exhibit partial recovery of nfatc1 expression, which remains reduced and diffuse throughout the endocardium (Fig. 1M-P).

Similar reduction or absence of GFP-expressing endocardial cells was observed in etsrp<sup>y11</sup>; fli1:GFP and etsrp MO-injected kdr:GFP embryos at 30 hpf (Fig. 1Q-X). As expected, both lines has formed a linear heart tube (Fig. 1I,J; data not shown). etsrpy11–/–/– embryos at 30 hpf (six out of 28) of 30 hpf exhibited very few remaining GFP+ endocardial cells, forming only small vessels. Seventy-nine percent of mutant embryos (22 out of 28) had endothelial defects, as determined by the absence of intersegmental penetration in endocardial reduction despite fairly uniform morphants and reduction in endocardial (Fig. 1B,D) and etsrp expression was observed in both etsrp and etsrp<sup>y11</sup> mutants which are deficient in hematopoietic and vascular endothelial/endocardial lineages, fn1 midline expression, which presumably corresponds to the endocardial progenitors, is absent (Trinh and Stainier, 2004). To gain further insight into how etsrp may affect endocardial development, we analyzed the expression of fn1 at the 8- to 16-somite stages in wild-type and etsrp<sup>y11</sup> mutant, as well as in MO knockdown embryos. Although fn1 exhibits a complex expression pattern and is expressed in multiple cell types, the major group of anterior fn1-expressing cells at the 8- to 16-somite stages can be found in the ALPM, in the region corresponding to the midbrain organizing center (MOC) (Proulx et al., 2010), which gives rise to the majority of the cranial vessels, as well as to the myeloid and endocardial lineages (Fig. 2A,C). This fn1 expression domain partially overlaps with etsrp expression (Fig. 2I,K,M,O) and is likely to include endocardial progenitors. In y11<sup>−−</sup> mutant and etsrp morphant embryos at the 8- to 14-somite stages, fn1 expression in the ALPM is mostly normal except for a slight expansion, mostly apparent at the 12-somite stage (Fig. 2A-D,I-P). At the 14- to 16-somite stages, the bilateral groups of fn1-expressing cells migrate towards the midline in the absence of Etsrp function.

Fibronectin is thought to be one of the earliest markers for endocardial progenitors. In cloche mutants which are deficient in hematopoietic and vascular endothelial/endocardial lineages, fn1 midline expression, which presumably corresponds to the endocardial progenitors, is absent (Trinh and Stainier, 2004). To gain further insight into how etsrp may affect endocardial development, we analyzed the expression of fn1 at the 8- to 16-somite stages in wild-type and etsrp<sup>y11</sup> mutant, as well as in MO knockdown embryos. Although fn1 exhibits a complex expression pattern and is expressed in multiple cell types, the major group of anterior fn1-expressing cells at the 8- to 16-somite stages can be found in the ALPM, in the region corresponding to the midbrain organizing center (MOC) (Proulx et al., 2010), which gives rise to the majority of the cranial vessels, as well as to the myeloid and endocardial lineages (Fig. 2A,C). This fn1 expression domain partially overlaps with etsrp expression (Fig. 2I,K,M,O) and is likely to include endocardial progenitors. In y11<sup>−−</sup> mutant and etsrp morphant embryos at the 8- to 14-somite stages, fn1 expression in the ALPM is mostly normal except for a slight expansion, mostly apparent at the 12-somite stage (Fig. 2A-D,I-P). At the 14- to 16-somite stages, the bilateral groups of fn1-expressing cells migrate towards the midline in the absence of Etsrp function.

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Myocardium is expanded in the absence of Etsrp function

In contrast to the absence of endocardial marker expression at the midline in *etsrp* morphants, our previous studies have argued that the migration of presumptive *etsrp*:GFP-positive endocardial progenitors to the midline is not affected in *etsrp* morphants (Proulx et al., 2010). Indeed, in *etsrp* morphants a population of *etsrp*:GFP cells is present at the midline in the region that normally corresponds to the endocardial precursors (Fig. 3A). Furthermore, the number of *etsrp*:GFP progenitor cells present at the midline is similar in wild-type embryos and *etsrp* morphants (Fig. 3B). Because these cells do not express endocardial markers, we hypothesized that at least some of them may have switched their fates and no longer represent endocardial progenitors.

Earlier studies have shown that inhibition of hematovascular development results in the rostral expansion of myocardial marker expression (Schoenebeck et al., 2007). Thus, expanded *hand2* expression has been observed in the *clo* mutant embryos and upon simultaneous inhibition of *etsrp* and *scl* function. Because our earlier studies showed that *etsrp* function is required for *scl* expression in the ALPM (Sumanas et al., 2008) we hypothesized that inhibition of *etsrp* function alone should be sufficient for the expansion of myocardial markers. Indeed, knockdown of Etsrp results in the rostral expansion of *hand2* expression (Fig. 3D). Furthermore, myocardial *cmlc2* expression is significantly expanded in Etsrp morphants (Fig. 3C,E).

Etsrp-expressing cells develop as cardiomyocytes in the absence of Etsrp function

These data demonstrate that *etsrp* morphants display simultaneous loss of endothelial and endocardial, and expansion of myocardial, lineages. Although the expansion of myocardial lineage has been previously observed in the absence of hematovascular development (Schoenebeck et al., 2007), the origin of these ectopic myocardial progenitors has not been established. We hypothesized that, in wild-type embryos, *etsrp* inhibits myocardial development in endothelial and endocardial progenitors, which develop as cardiomyocytes in the absence of *etsrp* function. To determine whether *etsrp*-expressing cells may initiate myocardial marker expression in the absence of *etsrp* function, we performed two-color in situ hybridization for *etsrp* and *hand2* expression at the 10-somite stage in wild-type and *etsrp* morphant embryos. In wild-type embryos, *etsrp* is expressed bilaterally along the ALPM just anterior to *hand2* expression (Fig. 4A) with no overlap. In Etsrp morphants, *hand2* expression extends rostrally where it overlaps with *etsrp* expression (Fig. 4B). This argues that *etsrp*-expressing cells initiate myocardial development in the absence of *etsrp* function.

To confirm whether some cardiomyocytes in *etsrp* morphants may be derived from *etsrp*-positive cells, we analyzed GFP and mCherry colocalization in double transgenic *etsrp*:GFP; *cmlc2*:mCherry embryos at 30 hpf. In wild-type embryos, *etsrp*:GFP expression is restricted to the endocardium, whereas *cmlc2*:mCherry expression is restricted to the myocardial layer and no overlap between the two transgenes is observed (0 out of 7 embryos analyzed, Fig. 4C). In *etsrp* morphants, *etsrp*:GFP expression can be observed in both endocardial and myocardial layers, where it overlaps with *cmlc2*:mCherry (16 out of 33 embryos analyzed contained *etsrp*:mCherry and *cmlc2*:mCherry cells; Fig. 4C). GFP/mCherry double-labeled cells were always in the myocardial
layer, and randomly found throughout both the atrium and ventricle. Endocardial etsrp:GFP cells appear more sparse in etsrp morphants, consistent with the formation of reduced endocardium after 24 hpf. These data argue that in the absence of etsrp function, etsrp-expressing cells contribute to both endocardium and myocardium.

**Etsrp overexpression results in the inhibition of endogenous myocardial markers and ectopic induction of both endocardial and myocardial marker expression**

To determine whether etsrp overexpression is sufficient to inhibit myocardial and endocardial marker expression, we analyzed myocardial hand2, cmlc2 and endocardial nfatc1 expression in etsrp-overexpressing embryos by in situ hybridization and quantitative RT-PCR. As expected, microinjection of one-cell stage etsrp-overexpressing embryos by in situ hybridization and etsrp expression. In some cases, the entire left or right side of cmlc2 expression within the cardiac plate was missing (Fig. 5A-D). In addition, embryos often displayed fusion defects of myocardial precursors. The phenotype was variable, probably owing to the highly mosaic nature of DNA overexpression. Unexpectedly, multiple etsrp-overexpressing embryos also displayed ectopic patches of cmlc2-expressing cells (Fig. 5A-D; see Table S2 in the supplementary material). Similarly, both patches of missing hand2 expression and domains of ectopic hand2-expressing cells were present in etsrp-overexpressing embryos (Fig. 5E-L). As analyzed by qPCR, expression levels of myocardial hand2, cmlc2, mef2a, mef2ca, tbx20 and endocardial nfatc1 were significantly upregulated in etsrp-overexpressing embryos (Fig. 5M; see Table S3 in the supplementary material). However, nkx2.5 and vmhc expression was not significantly affected (Fig. 5M; see Table S3 in the supplementary material). Overexpression of related human ETS1 caused no significant induction in hand2, cmlc2 or nfatc1 expression (see Table S4 in the supplementary material), demonstrating that the phenotype is specific to Etsrp overexpression.

Because the induction of ectopic myocardial marker expression by Etsrp was unexpected, we investigated this phenotype further. To determine whether Etsrp functions cell-autonomously to initiate myocardial development, cell transplantation was performed from Etsrp- and TRITC-dextran-injected cmlc2:GFP transgenic embryos into recipient uninjected cmlc2:GFP embryos. The embryos were subsequently analyzed at 24 hpf for the localization of cmlc2:GFP and TRITC fluorescence. The majority of ectopically located cmlc2:GFP cells did not display TRITC fluorescence, which argues that they did not originate from Etsrp-expressing cells (see Fig. S2A-C in the supplementary material). As analyzed by two-color in situ hybridization, ectopic cmlc2-positive cells frequently did not overlap with etsrp expression in Etsrp DNA-injected embryos (see Fig. S2D-I in the supplementary material). These results argue that Etsrp overexpression results in non-cell-autonomous induction of at least partial myocardial differentiation. However, because Etsrp during normal development is not expressed in myocardial progenitors and its function is not required for their differentiation, this phenotype is probably an artifact of Etsrp overexpression at high levels in different cell types where it is not normally expressed.

![Fig. 5. Etsrp overexpression results in the loss of endogenous myocardial markers and the induction of ectopic myocardium.](image-url)

**Fig. 5. Etsrp overexpression results in the loss of endogenous myocardial markers and the induction of ectopic myocardium.** cmlc2 (A-D) and hand2 (E-L) expression analysis in uninjected (A,E,I) and etsrp DNA-injected (B-D,F-H,J-L) embryos. (A-D) Etsrp-overexpressing embryos exhibit fusion defects, missing cells (B-D, white arrows) and ectopic cmlc2 expression at the 20-somite stage (B,D, black arrows). (E-L) At the 10-somite stage, Etsrp overexpression results in both disruption of endogenous hand2 expression (F,G, white arrows) and induction of ectopic expression (black arrows, G,H,J,L). (A-H) Anterodorsal whole-mount view, anterior is upwards; (I-K) mid-dorsal view, anterior is upwards; (L) posterior view, dorsal is upwards. (M) Normalized ratio of myocardial marker expression in etsrp DNA-injected embryos versus wild-type embryos, as analyzed by qPCR. y-axis is shown in [log]2 scale. Data are mean±s.e.m.
Etv2 directly binds to Fox:Ets consensus sequence within the nfatc1 promoter

Our results show that Etsrp function is both necessary and sufficient for early endocardial nfatc1 expression. We then investigated whether Etsrp may directly regulate nfatc1 expression. Recent evidence has demonstrated that a consensus Fox:Ets-binding motif bound by both Forkhead and Etsrp/Etv2 transcription factors synergistically induces endothelial specific gene expression (De Val et al., 2008).

By analyzing nfatc1 promoter regions of zebrafish and puffer fish Tetraodon nigroviridis, we identified a conserved region that contained consensus Ets and Fox:Ets-binding sites (De Val et al., 2008). As analyzed by EMSA assay, mouse Etv2 protein bound efficiently to the control and zebrafish nfatc1 Ets sites (Fig. 6B, lanes 2,8).

Binding to both sites was specifically competed by an excess of the wild-type, unlabeled control probe (Fig. 6B, lanes 3,9) but not by an equivalent amount of a mutant version of the control probe, which did not compete for binding to either probe (Fig. 6B, lanes 4,10). Unlabeled zebrafish nfatc1 ETS probe also efficiently competed for binding to the control ETS site (lane 5) and to itself (lane 11). A mutant version of the nfatc1 ETS probe in which the ETS consensus was disrupted did not compete for binding to either probe (Fig. 6B, lanes 6,12). These results argue that Etsrp/Etv2 directly binds to the nfatc1 promoter.

Foxc1a is required and interacts with Etsrp in initiating endocardial differentiation but not inhibiting myocardial development

Previous studies have shown that foxc1a is required for zebrafish vascular development (De Val et al., 2008). To test whether foxc1a was required for the initiation of endocardial and inhibition of myocardial development, foxc1a morphants were analyzed for myocardial hand2 and cmlc2, endocardial nfatc1, and kdrl:GFP expression. Knockdown of foxc1a using 1.5-4.0 ng foxc1a MO had no significant effect on myocardial hand2 or cmlc2 expression (Fig. 7A-H). A foxc1a MO injection (4 ng) resulted in apparent defects in somitogenesis (see Fig. S3 in the supplementary material), as reported previously (Topczewska et al., 2001). Doses of foxc1a MO above 4 ng resulted in high toxicity; therefore, we were not able to use higher MO doses for marker analysis. However, at the same doses, endocardial nfatc1 and endocardial/endothelial kdrl expression were strongly reduced in foxc1a morphants (Fig. 7I-L; data not shown). In morphants injected with 1.5 ng of foxc1a MO, nfatc1 expression is reduced, outlining a shorter thinner endocardium with fewer cells (Fig. 7J). Knockdown using 3 and 4 ng of foxc1a MO results in a severe reduction where most embryos have either no nfatc1 staining at all or very few cells located in the ventricle region (Fig. 7K-M). Similarly, kdrl:GFP reporter embryos injected with foxc1a MO display strongly reduced endocardium (see Fig. S4 in the supplementary material). Similar results were also observed using a cocktail of two previously published foxc1a-specific MOs (see Fig. S5 in the supplementary material) (Topczewska et al., 2001). These results argue that foxc1a function is required for the endocardial development but is dispensable for the inhibition of myocardial formation.

As FoxC and Etsrp/Etv2 have been shown to act synergistically to promote endothelial gene transcription (De Val et al., 2008), we wanted to determine whether foxc1a and etsrp act synergistically in endocardial development. To test this interaction, we injected subphenotypic doses of etsrp MO and foxc1a MO individually or together. The endocardial tube formation is not significantly affected in kdrl:GFP transgenic embryos injected with a low dose of etsrp MO or a low dose of foxc1a MO (Fig. 8A-C). When both MOs are
co-injected, the endocardium is severely reduced (Fig. 8D). This is also true for endocardial expression of \textit{kdrl} at the 20-somite stage (Fig. 8E-H) and of \textit{nfatc1} (Fig. 8I-L) at 30 hpf. By contrast, expression of myocardial markers \textit{hand2} and \textit{cmlc2} was not affected in \textit{etsrp}, \textit{foxc1a} and double \textit{etsrp}/\textit{foxc1a} morphants using the same MO doses (Fig. 8M-U). These results argue that \textit{etsrp} and \textit{foxc1a} interact during endocardial differentiation, whereas \textit{etsrp} inhibits myocardial development in \textit{foxc1a}-independent manner.

**DISCUSSION**

In this study, we show that a key regulator of vascular endothelial differentiation, Etsrp/Etv2, is also a crucial factor in endocardial-endothelial-myocardial lineage decisions. Our data show that Etsrp is required for endocardial differentiation by directly regulating \textit{nfatc1} expression. At the same time, Etsrp function is required to inhibit myocardial differentiation. In the absence of Etsrp function, \textit{etsrp}-expressing endothelial/endocardial progenitors differentiate as cardiomyocytes. Furthermore, Foxc1a function and Foxc1a/Etsrp interaction is required to initiate endocardial development but is dispensable for the inhibition of myocardial differentiation. This suggests that Etsrp initiates endothelial and endocardial and inhibits myocardial differentiation by two distinct mechanisms (Fig. 9).

It is currently not known whether myocardial, endocardial and endothelial lineages are derived from the same progenitor cells in zebrafish embryos. Earlier fate-mapping studies have argued that myocardial and endocardial lineages come from different spatial regions and are already separated during early gastrulation stages (Lee et al., 1994; Keegan et al., 2004). Our results support early separation of early endothelial/endocardial and myocardial progenitors. During normal development, \textit{etsrp}:GFP expression is observed only in endothelial and endocardial but not myocardial progenitors (Proulx et al., 2010). Because GFP has a long half-life and its fluorescence can be observed for at least 24 hpf, even after its transcription has terminated, this argues that \textit{etsrp} is never expressed in myocardial progenitors and the two lineages have already separated by the one-somite stage, when \textit{etsrp} expression is first initiated within ALPM. However, because endocardial progenitors can differentiate as myocardial cells in the absence of \textit{etsrp} function, this argues that endocardial cells retain developmental plasticity until much later stages and their fates can be reprogrammed.

Although previous studies have demonstrated myocardial expansion within the ALPM in the absence of hematovascular development (Schoenebeck et al., 2007), the origin of ectopic cardiomyocytes was not known. Our studies argue that endothelial-endocardial precursors cell-autonomously initiate myocardial differentiation in the absence of Etsrp function. It is possible that myocardial differentiation is the ‘default’ fate within the ALPM in the absence of hematovascular development. However, our results show that in the absence of \textit{foxc1a} function, endocardial differentiation is inhibited while myocardial differentiation is not affected. Furthermore, although co-injection of subphenotypic doses of \textit{etsrp} MO and \textit{foxc1a} MOs results in the absence of both endocardial and vascular endothelial marker expression within the ALPM, no increase in myocardial marker expression is observed. These results argue that inhibition of endothelial-endocardial development by itself is not sufficient to initiate myocardial development and high inhibition levels of Etsrp function are necessary. Furthermore, these results suggest that \textit{etsrp} represses myocardial development in \textit{foxc1a}-independent manner, perhaps by recruiting transcriptional repressors.
Previous studies have suggested that fn1 expression within ALPM corresponds to early endocardial progenitors (Trinh and Stainier, 2004), which is consistent with our results. Early fn1 expression only partially overlaps with etsrp expression, suggesting that not all fn1+ expressing cells within ALPM are endocardial progenitors. In contrast to other endocardial markers, fn1 expression in Etsrp mutants or morphants remains localized bilaterally, whereas a pool of etsrp:GFP-expressing cells migrate to the midline. These results argue that etsrp function is required for the midline migration of fn1+ endocardial progenitors. Furthermore, it suggests that etsrp:GFP cells present at the midline in etsrp morphants include myocardial progenitors, and etsrp function is not required for their migration. In support of this hypothesis, at least some etsrp:GFP cells co-express cmic2 and thus differentiate as cardiomyocytes in etsrp morphants.

Etsrp overexpression has been known to result in the precocious and ectopic induction of multiple endothelial-specific genes. As our results show, it also results in strong precocious induction of endocardial nfatc1 expression, as analyzed at the 20-somite stage. This argues that Etsrp is sufficient to induce both endothelial and endocardial differentiation. Based on EMSA analysis, Etsrp binds directly to the evolutionarily conserved nfatc1 enhancer, which argues that Etsrp directly regulates nfatc1 transcription. Unexpectedly, etsrp overexpression resulted in both inhibition of endogenous and induction of ectopic myocardial marker
expression. As demonstrated by cell transplantation analysis, ectopic myocardial marker induction is non-cell-autonomous. It is likely that overexpression of Etsrp results in an induction of a secreted signaling molecule that can promote myocardial development. However, because during normal development Etsrp is not expressed in myocardial progenitors and is not required for cardiomyogenesis, this Etsrp overexpression phenotype may be an artifact caused by high doses of Etsrp protein present in many different cell types. In summary, this study establishes Etsrp as a crucial regulator of early cardiovascular development, and argues that Etsrp promotes endothelial and endocardial development and represses myocardial differentiation by two independent mechanisms. Mouse Etv2 mutants also display endothelial and endocardial defects (Lee et al., 2008; Ferdous et al., 2009), which suggests that the Etsrp/Etv2 mechanism of function is evolutionarily conserved. These results are important for our understanding of normal cardiovascular development in vertebrates and will greatly contribute to the stem cell research aimed at regenerating heart tissues, eventually leading to new strategies in treating heart disorders.

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Competing interests statement
The authors declare no competing financial interests.

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