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 function during zebrafish embryonic development. Expression of multiple endocardial specific markers is absent or greatly reduced in Etsrp knockout 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 cmlc2 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. 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 (Schönebeck 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 (Schönebeck 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 (Schönebeck 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 cmlic2 (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.

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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 Sugii, 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, flt1 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, flt1 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 endothelial and endocardial lineages, and die before E11.0 (Lee et al., 2008; Fardous 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(kdr:EGFP)str (Jin et al., 2005), Tg(fli1:EGFP)y1 (Lawn and Weinstein, 2002); Tg(cmlc2:GFP)apw1 (Huang et al., 2003); etsrpy11 (Pham et al., 2007), Tg(etsrp:EGFP)y1 (Proulx et al., 2010) and wild type (Ekkwille). 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(cmlc2:mCherry) founders were recovered with nearly identical expression patterns and levels. Tg(cmlc2:mCherry)y1 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. etsrp y1; y2 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 etsrp 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 etsrp y1 mutant phenotype and no other morphological abnormalities were observed. In the majority of the experiments, a single Foxc1a MO2 (sequence CGCCCTGCATGACTCTCCTCAACAC) 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 Etsrp morphant embryos.

**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, nfatc1 (Open Biosystems, catalog number EDR1052-91190630, Accession Number CN320837) and fli1 (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 fli1 and etsrp:GFP, immediately following in situ hybridization, embryos were washed in PBST, manually deyolked 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×PBS]. Embryos incubated in anti-GFP-Alexa488 (Invitrogen #A21101) 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 #A11078) 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 sphere to 30% epiboly stages by using capillary needles and adjusting balance 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*-XεX 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 1a* (ef1a). 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.

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**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*Y11–/– 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 track boundary (upper arrows), but is sparse and diffuse in *etsrp*Y11–/– 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*Y11–/– 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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Image capture and processing
Stained embryos were imaged by either whole mounting on glass slides in 2% methyleneblue or dehydrating in ethanol and whole mounting in araldite. Alternatively, flat mounting of stained or fixed transgenic embryos was carried out by either manually deyolking and mounting in 50% glycerol or dehydrating in ethanol and flat mounting in araldite. Images were captured with a Sony DSC-H9 digital camera mounted on a Zeiss SV8 microscope or on an AxioImager Z1 (Zeiss) compound microscope with Axioesc color camera or monochrome cameras (Zeiss). Images in different focal planes were combined using the Extended Focus module within Axiovision software (Zeiss). Image levels were adjusted using Adobe Photoshop CS2 to increase the contrast.

Cell counting
Etsrp-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 ImageJ for cell counting using pickpointer or counted in Imaris using the cell detection algorithm.

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 nfatc1 ETS site were labeled with [32P]-dCTP, using Klenow to fill in overhanging 5’ ends. Labeled probes were purified on a nondenaturing polyacrylamide-

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 etsrp Y11-/-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 kdr (Fig. 1A,C) and cdh5 (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 fibronectin 1-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 etsrp Y11-/- mutants at the 8- to 12-somite stages, as analyzed by in situ hybridization. (E-H) fn1-expressing endocardial precursors (arrowheads) migrate to the midline in wild-type sibling embryos, while they remain localized bilaterally in etsrp Y11-/- mutants at the 14- to 16-somite stages. (I-P) fn1 expression largely overlaps with etsrp-GFP expression in the presumptive endocardial precursor cells (white arrowheads) in uninjected control embryos and etsrp morphants. Maximum projection image of etsrp-GFP immunofluorescence (I,J) and fn1 in situ hybridization staining (K,L). Merged images of bright-field (BF) and GFP channels (M-P). There is slight expansion of fn1 and etsrp-GFP staining in etsrp 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.
in the loss vascular endothelial and endocardial staining for both
kdrl (Fig. 1B,D) and cdh5 (Fig. 1F,H). Similar absence or strong
reduction in endocardial kdrl expression was observed in both etsrp
morphants and etsrp\textsuperscript{y11} 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\textsuperscript{y11} embryos, nfatc1 endocardial expression is
completely missing at 24 hpf (Fig. 1K,L). By 48 hpf and 72 hpf,
etsrp\textsuperscript{y11} 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\textsuperscript{y11}; fli1:GFP and etsrp MO-injected
kdrl:GFP embryos at 30 hpf (Fig. 1Q-X). As expected, both lines
exhibited severe reduction in endothelial GFP expression in trunk
and tail region upon inhibition of etsrp function. Notably,
etsrp\textsuperscript{y11}; fli1:GFP transgenic embryos exhibited variable
penetration in endocardial reduction despite fairly uniform
endothelial defects, as determined by the absence of intersegmental
vessels. Seventy-nine percent of mutant embryos (22 out of 28) had
very few remaining GFP\textsuperscript{+} endocardial cells, forming only small
rudimentary tubes that failed to extend (Fig. 1T). The remaining
21% (six out of 28) of 30 hpf etsrp\textsuperscript{y11} mutants exhibited
significant but less severe reduction in length and width of the
endocardium (see Fig. S1 in the supplementary material).

Altogether, these results argue that etsrp function is required for
early endocardial differentiation.

**Fn1-expressing endocardial progenitors fail to
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 in the 8–16-
ARSEOM stages in wild-type and etsrp\textsuperscript{y11} 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
endothelial 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\textsuperscript{+} 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

![Fig. 3. Migration of etsrp:GFP-expressing cells is not affected
while myocardial markers are expanded in etsrp morphants.](image)

(A) etsrp:GFP expression in the midline population (white arrows) of
presumptive endocardial progenitors is not affected in Etsrp morphants (right
panels) at the 18- to 20-somite stages. Maximum intensity projection of fixed
Tg(etsrp:GFP) embryos, ventral flat-mounted view, anterior is upwards.

(B) At the 18- to 20-somite stage, the
relative numbers of etsrp:GFP-
putative cardiac progenitors that
migrate to the midline are similar
between uninjected controls and etsrp
morphants. Data are mean±s.e.m.

(C) The calculated average area (mm\textsuperscript{2})
of cmlc2 in situ hybridization staining
at the 20-somite stage shows a 58% increase in etsrp morphants compared
with uninjected wild-type controls
depicted in E. (D) hand2 expression
extends into the rostral ALPM (black
arrowheads) in etsrp morphants but is
absent from this region in the
uninjected controls at the 10-somite
stage. (E) cmlc2 expression at 20-
somite stage reveals radial expansion
of the cardiac plate in etsrp morphants
(right panels) compared with
uninjected wild-type embryos (left
panels). Anterodorsal whole-mount
view, anterior is upwards.
towards the midline in wild-type embryos (Fig. 2E,G). In etsrp mutants, fn1 expression remains bilateral, and fn1-expressing endocardial cells are absent from the midline, similar to the clo mutant phenotype (Fig. 2F,H).

To confirm the identity of fn1-expressing cells in wild-type and etsrp knockdown embryos, we used a etsrp:GFP reporter line to analyze fn1 and etsrp:GFP co-expression using a combination of in situ hybridization and immunostaining against GFP. At the 12- to 14-somite stages, etsrp and fn1 expression partially overlaps in wild-type embryos in the MOC region (Fig. 2I,K,M,O), where endocardial cells first emerge, as we have previously demonstrated by the time-lapse imaging of etsrp:GFP transgenic embryos (Proulx et al., 2010). We have previously shown that etsrp expression in the ALPM is expanded in etsrp morphants (Sumanas et al., 2008), which suggests that additional cells within the ALPM are recruited to express etsrp in an attempt to initiate vascular development. Similar to etsrp expansion, fn1 expression is also slightly expanded and closely overlaps with etsrp expression in etsrp morphants (Fig. 2J,L,N,P). These data suggest that fn1·etsrp+ double-positive cells include endocardial progenitors.

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 cmhc2 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; cmhc2:mCherry embryos at 30 hpf. In wild-type embryos, etsrp:GFP expression is restricted to the endocardium, whereas cmhc2: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 cmhc2:mCherry (16 out of 33 embryos analyzed contained etsrp+cmhc2+ cells; Fig. 4C). GFP/mCherry double-labeled cells were always in the myocardial layer in etsrp+cmlc2+ double-positive cells include endocardial progenitors.

Fig. 4. etsrp-expressing cells differentiate as cardiomyocytes in the absence of Etsrp function. (A,B) Rostrally expanded hand2 (purple) overlaps with etsrp (red) expression in Etsrp morphants (B) but not in wild-type embryos (A) at the 10-somite stage, as analyzed by two-color in situ hybridization. Red and purple arrows indicate the anterior-posterior span of etsrp and hand2 expression, respectively. Black arrows indicate the areas of overlapping expression. Ventral flat-mounted view, anterior is upwards. (C) At 30 hpf, etsrp:GFP and cmhc2:mCherry expression overlaps (yellow arrowheads) in the myocardial layer of Etsrp morphant hearts (three right columns show three different morphants) but not in control uninjected embryos (left column). Left lateral whole-mount views of fixed Tg(etsrp:GFP; cmhc2:mCherry) embryos, anterior is towards the left. Projections of only few selected slices are shown.
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 induce 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 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.** 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 [log2] scale. Data are means±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 is present in many endothelial specific enhancers and that binding of Etv2 and FoxC 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 (Fig. 6A). 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

![Fig. 6. Zebrafish Nfatc1 promoter contains a conserved ETS-binding site that interacts with Etv2 protein in vitro.](image)
co-injected, the endocardium is severely reduced (Fig. 8D). This is also true for endocardial expression of kdrl at the 20-somite stage (Fig. 8E-H) and of nfatc1 (Fig. 8I-L) at 30 hpf. By contrast, expression of myocardial markers hand2 and cmlc2 was not affected in etsrp, foxc1a and double etsrp/foxc1a morphants using the same MO doses (Fig. 8M-U). These results argue that etsrp and foxc1a interact during endocardial differentiation, whereas etsrp inhibits myocardial development in 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 nfatc1 expression. At the same time, Etsrp function is required to inhibit myocardial differentiation. In the absence of Etsrp function, etsrp-expressing endothelial/endoocardial 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/endoocardial and myocardial progenitors. During normal development, 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 etsrp is never expressed in myocardial progenitors and the two lineages have already separated by the one-somite stage, when etsrp expression is first initiated within ALPM. However, because endocardial progenitors can differentiate as myocardial cells in the absence of 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-endoocardial precursors cell-autonomously initiate myocardial differentiation in the absence of Etsrp function. It is possible that myocardial development is the ‘default’ fate within the ALPM in the absence of hematovascular development. However, our results show that in the absence of foxc1a function, endocardial differentiation is inhibited while myocardial differentiation is not affected. Furthermore, although co-injection of subphenotypic doses of etsrp MO and 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-endo-cardiac development by itself is not sufficient to initiate myocardial development and high inhibition levels of Etsrp function are necessary. Furthermore, these results suggest that etsrp represses myocardial development in 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 cmlc2 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
Fig. 9. A proposed model for specification of cardiovascular lineages within the zebrafish ALPM. Etsrp promotes vascular endothelial and endocardial differentiation by interacting with Foxc1a, whereas Etsrp function is required to inhibit myocardial differentiation in Foxc1a-independent manner. In the absence of Etsrp function, multipotent progenitors initiate myocardial differentiation.

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.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.064998/-/DC1

References


Figure S2

Transplantation

cmlc2:GFP

TRITC

etsrp DNA injection

etsrp / cmlc2

cmlc2 only

Figure S2
Figure S3
<table>
<thead>
<tr>
<th>Marker</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>ef1α</td>
<td>TCACCCCTGGGAGTGAAACAGC</td>
<td>ACTTGCAGGGCAGATGGACAG</td>
</tr>
<tr>
<td>nfatc1</td>
<td>GTGACGGAGGACAGTGGTT</td>
<td>AGAGCAGGCAGTGGACT</td>
</tr>
<tr>
<td>cmic2</td>
<td>GAGGCTGCTAATGACCCAAGC</td>
<td>AGTCAAGGAATTTAAGGGCC</td>
</tr>
<tr>
<td>hand2</td>
<td>TACCATGGCACCTTGTACA</td>
<td>CTTCTCTTTTGGGGTCTG</td>
</tr>
<tr>
<td>mef2ca</td>
<td>ATCTGTCCTCTCTGTCTGCG</td>
<td>CTGCCGCTGATAGCTGCC</td>
</tr>
<tr>
<td>mef2a</td>
<td>GGCAGCTCCTAGCTCGCTGG</td>
<td>GCTTGACTGATGGGCTCTCC</td>
</tr>
<tr>
<td>tbx20</td>
<td>TTTTCTGAGCCCTGCGACC</td>
<td>CAGGTAAAGGTTGGATCTGCC</td>
</tr>
<tr>
<td>nkx2.5</td>
<td>GCTTTTAGGCAAAGAATCTCC</td>
<td>GATCTCACCTGTTGAGAGG</td>
</tr>
<tr>
<td>vmhc</td>
<td>ATGAGGAAGGTCAAGCGCTGC</td>
<td>ACTTGTTTCTTCACTTGCC</td>
</tr>
<tr>
<td>Phenotype</td>
<td>Uninjected (n)</td>
<td>Etsrp DNA (n)</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------------</td>
<td>---------------</td>
</tr>
<tr>
<td>hand2: fused or reduced</td>
<td>0% (n=6)</td>
<td>50% (n=14)</td>
</tr>
<tr>
<td>hand2: ectopic expression</td>
<td>0% (n=6)</td>
<td>79% (n=14)</td>
</tr>
<tr>
<td>cmic2: fused or reduced</td>
<td>4% (n=28)</td>
<td>49% (n=35)</td>
</tr>
<tr>
<td>cmic2: ectopic expression</td>
<td>0% (n=28)</td>
<td>49% (n=35)</td>
</tr>
</tbody>
</table>

Etsrp DNA overexpression results in two opposite phenotypes: reduction of endogenous hand2 and cmic2 expression, and induction of ectopic hand2 and cmic2 expression. In some cases, reduced bilateral expression domains were fused. n represents the total number of embryos scored.
Table S3. Induction of endocardial *nfatc1* and selected myocardial markers in *etsrp* DNA-injected embryos as analyzed by real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ratio of Etsrp DNA to uninjected controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>nfatc1, 20 somites</td>
<td>1270±131</td>
</tr>
<tr>
<td>hand2, 10 somites</td>
<td>9.2±1.7</td>
</tr>
<tr>
<td>cmic2, 20 somites</td>
<td>3.5±0.36</td>
</tr>
<tr>
<td>mef2a, 20 somites</td>
<td>1.4±0.24</td>
</tr>
<tr>
<td>mef2ca, 20 somites</td>
<td>2.0±0.95</td>
</tr>
<tr>
<td>tbx20, 20 somites</td>
<td>1.35±0.03</td>
</tr>
<tr>
<td>nkx2.5, 20 somites</td>
<td>0.91±0.2</td>
</tr>
<tr>
<td>vmhc, 20 somites</td>
<td>0.91±0.04</td>
</tr>
</tbody>
</table>

Averaged ratio of expression in *etsrp* DNA-injected embryos versus uninjected controls is shown. Expression values were normalized to EF1α expression level. All experiments were repeated at least three times; error is shown.
Table S4. Overexpression of human ETS1 DNA fails to induce endocardial *nfatc1* and myocardial *hand2* or *cmlc2* expression

<table>
<thead>
<tr>
<th></th>
<th>Ratio human ETS1 DNA to uninjected controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>nfatc1</em>, 20 somites</td>
<td>1.09±0.01</td>
</tr>
<tr>
<td><em>hand2</em>, 10 somites</td>
<td>1.04±0.01</td>
</tr>
<tr>
<td><em>cmlc2</em>, 20 somites</td>
<td>0.90±0.002</td>
</tr>
</tbody>
</table>