Etv5a regulates the proliferation of ventral mesoderm cells and the formation of hemato-vascular derivatives

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Summary
Hematopoietic and vascular endothelial cells constitute the circulatory system and are both generated from the ventral mesoderm. However, the molecules and signaling pathways involved in ventral mesoderm formation and specification remain unclear. We found that zebrafish etv5a was expressed in the ventral mesoderm during gastrulation. Knockdown of Etv5a using morpholinos increased the proliferation of ventral mesoderm cells and caused defects in hematopoietic derivatives and in vascular formation. In contrast, the formation of other mesodermal derivatives, such as pronephros, somites and the gut wall, was not affected. Knockdown specificity was further confirmed by over-expression of an etv5a construct lacking its acidic domain. In conclusion, our data reveal that etv5a is essential for the inhibition of ventral mesoderm cell proliferation and for the formation of the hemato-vascular lineage.

Introduction
The circulatory system is one of the first functioning organ systems to develop in vertebrate embryos. The coordinated development of the heart, blood vessels and blood cells allows for the establishment of blood circulation, which is critical for early survival. During gastrulation, the mesoderm is induced and patterned across the dorsal-ventral axis; later, the ventral mesoderm cells proliferate and differentiate to form a transient progenitor cell termed the hemangioblast that gives rise to both endothelial and hematopoietic cell lineages (Amatruda and Zon, 1999; Lugus et al., 2005; Orkin and Zon, 2008; Patan, 2000; Xiong, 2008). Subsequently, during both
vasculogenesis and angiogenesis, the endothelial cells line the vasculature, while hematopoietic progenitors differentiate into distinct blood lineages through hematopoiesis. Studies in different vertebrate models showed that these developmental processes and the genetic program that controls them are generally conserved throughout vertebrate evolution (Orkin and Zon, 2008; Paik and Zon, 2010).

Several molecules play a vital role in the formation of the circulatory system. The GATA family of transcription factors is essential for both primitive and definitive hematopoiesis. Gata1 is required for the proper maturation of erythroid, mast, and megakaryocytic precursors and for the specification of eosinophils, whereas Gata2 is necessary for mast cell development and for the maintenance and expansion of hematopoietic stem cells (Fujiwara et al., 2004; Ohneda and Yamamoto, 2002). Gata3 is required for the development of T lymphocytes (Hosoya et al., 2009). Other critical factors in hematopoiesis are Rag1, which is essential for the differentiation of T and B lymphocytes (Petrie-Hanson et al., 2009), and Runx1, which regulates the formation of hematopoietic progenitors, myeloid cells and the vasculature (Jin et al., 2012; Kalev-Zylinska et al., 2002). On the other hand, the development of endothelial cells and vascular patterning is regulated by vascular endothelial growth factor (VEGF) receptors Flt1 and Flk1 (Hirashima, 2009; Orkin and Zon, 1997; Siekmann et al., 2008), whereas the basic helix-loop-helix transcription factor Scl is critical for both hematopoiesis and vasculogenesis (Gering et al., 1998). Although the molecules that regulate blood cell and blood vessel formation are well characterized, those involved in the specification of the hemangioblastic fate in ventral mesoderm remain much less well characterized.
Ets transcription factors contain a conserved winged helix-turn-helix ETS domain that is essential for DNA binding. Members of this family play multiple roles in cell proliferation, differentiation and migration, and many of them are critical for hematopoiesis and blood vessel development (Liu and Patient, 2008). For example, Ets1, Ets2 and Fli1 regulate both vaculogenesis and angiogenesis (Randi et al., 2009). Etsrp is required to drive haemangioblasts towards a vascular fate and is essential for the formation of myeloid cells (Ellett et al., 2009; Sumanas and Lin, 2006). Erg is essential for normal adult hematopoietic stem cell homeostasis as well as endothelial differentiation and vascular development (Kruse et al., 2009; Randi et al., 2009). Defects in many human ETS genes have been associated with cancer, such as leukemia (Sharrocks, 2001).

Zebrafish (Danio rerio) has emerged as an excellent vertebrate model for the study of many aspects of the developmental process of the circulatory system, not only because of the transparency of the embryos, which facilitates in vivo imaging, but also because the embryos can survive for many days without circulating erythrocytes. In addition, most of the molecules involved in the development of the circulatory system are evolutionary conserved in this species (Belele et al., 2009). Here, we show that one Ets gene, etv5a, is expressed in the ventral mesoderm in zebrafish embryos. Knockdown of endogenous etv5a by antisense morpholinos resulted in increased proliferation of ventral mesoderm cells and defective hematopoietic cell and vascular endothelium cell formation without affecting other mesodermal derivatives. The effect of the morpholino knockdown was phenocopied by over-expression of a dominant-negative etv5a construct. Our data provide further insights into the roles of Etv5a in the formation of the ventral mesoderm and hemato-vascular cell lineages.
Results

*etv5a* is orthologous to mammalian *ETV5/Etv5*

Zebrafish *etv5a* (previously named *etv5*) and *etv5b* (*erm*) are both homologous to mammalian *ETV5/Etv5* and arose from a gene duplication in ray-finned fish (Roussigne and Blader, 2006). Sequence comparison showed zebrafish *Etv5a* displays more similarities with other vertebrate *ETV5* proteins than *Etv5b* (supplementary material Fig. S1A; also see (Roussigne and Blader, 2006) and (Liu and Patient, 2008)). A syntenic comparison of *etv5a* with mammalian homologues showed that gene loci flanking the zebrafish *etv5a* gene (chromosome 9) are highly conserved in human chromosome 3, mouse chromosome 16, and rat chromosome 11 (supplementary material Fig. S1B). Conversely, zebrafish *etv5b* is located in chromosome 6 and lacks significant synteny (supplementary material Fig. S1C).

These results substantiate the hypothesis that zebrafish *etv5a* is orthologous to mammalian *ETV5/Etv5*.

Expression of *etv5a* in the ventral mesoderm and its derivatives

*etv5a* expression was analyzed by whole mount in situ hybridization and quantitative real-time PCR of zebrafish embryos at developmental stages ranging from 1 cell-stage to 48 hours post fertilization (hpf). A strong and ubiquitous signal was detected from 1-cell to the oblong stage, which indicated that *etv5a* was expressed as a maternal mRNA (Fig. 1A-C). Ubiquitous expression was continued
through the whole embryo before gastrulation (Fig. 1D-E). During early gastrulation (75% epiboly), expression localized to the ventral mesoderm (arrowhead in Fig. 1F-G). At later stages, *etv5a* expression became confined to the lateral mesoderm as demonstrated by the presence of two longitudinal stripes at the 6-somite stage (Fig. 1H-J). A previous study showed that cells present in this region are multipotent progenitors that give rise to hematopoietic, endothelial and pronephric derivatives (Gering et al., 1998). *etv5a* expression was maintained in the posterior lateral mesoderm (Fig. 1K-N) during segmentation, but after 24 hpf it became restricted to the developing pronephric progenitors (Fig. 1O-T). In addition to mesodermal tissues, *etv5a* expression was detected in the developing nervous system, specifically in the brain and spinal cord, at the 6-somite stage, and was maintained until the latest stage analyzed (48 hpf, Fig. 1). The dynamic expression of *etv5a* suggests its importance in the developing central nervous system and mesodermal derivatives. In contrast, *etv5b* (*erm*) is expressed in the developing nervous system but not in the ventral mesoderm, lateral mesoderm or pronephric ducts (Munchberg et al., 1999; Raible and Brand, 2001). Mouse *Etv5* is also strongly expressed in the developing nervous system and in the intermediate and lateral plate mesoderm, but it is expressed late in the mesonephros (Chotteau-Lelievre et al., 1997; Chotteau-Lelievre et al., 2001). The results presented here reveal that the expression of *Etv5* and *etv5a* orthologues is evolutionarily conserved.

Defects in the circulatory system after Etv5a knockdown

To delineate the role of Etv5a during embryonic development, the morpholino (MO) knockdown approach was used to interfere with Etv5a expression. To block protein production, two 25 bp antisense morpholinos (MO1 and MO2) were
synthesized to target different regions located upstream of the translation start site of *etv5a* mRNA. The specificity of morpholinos was confirmed by rescue experiments in which the MOs were coinjected with cRNA for *etv5a*, as described for each experiment below. To confirm the efficacy of the morpholino knockdown approach, each of the two *etv5a* morpholinos was co-injected with the cRNA of a reporter construct that contained the *etv5a* MO1 and MO2 binding sequences upstream of an enhanced green fluorescent protein reporter (5′*etv5a*-EGFP). Effective knockdown, as revealed by the loss of EGFP protein, was observed upon co-injection with either of the two *etv5a* morpholinos, whereas no reduction in EGFP protein expression was observed upon co-injection of a control morpholino (supplementary material Fig. S2A).

Embryos injected with MO1 or MO2 were analyzed at 24 hpf, 2 days post-fertilization (dpf), 3 dpf and 4 dpf for morphological defects. The injection of 2 ng of MO1 or 10 ng of MO2 resulted in identical phenotypes and therefore only embryos injected with 2 ng of MO1 are shown (for both of the morpholinos produced same phenotypes see supplementary material Fig. S3). Although *etv5a* knockdown did not result in significant morphological abnormalities (supplementary material Fig. S2B), it caused a reduced heart beat rate (control = 75.28 ± 5.82 beats/min versus *etv5a* morphants = 32.43 ± 4.53 beats/min at 24 hpf) and a lower blood cell count at all stages analyzed. Concomitant injection of morpholinos with *etv5a* cRNA rescued these phenotypes, indicating that the morpholino-induced defect was due to loss of Etv5a function.

Defective hemato-vascular derivatives in Etv5a knockdown embryos
The reduced number of blood cells observed in etv5a morphants suggested a hematopoietic defect; thus, we examined the formation of erythroid cells by assessing the expression of gata1, a marker of erythroid precursors (Detrich et al., 1995), by in situ hybridization and quantitative real-time PCR. The results showed that gata1 expression was down-regulated in etv5a knockdown embryos (Fig. 2). We also found that injection of etv5a morpholinos down-regulated markers for granulocytes (mpo) (Bennett et al., 2001) and lymphoid cells (rag1) (Willett et al., 1997) (Fig. 2). These results suggested that the common progenitor for these hematopoietic derivatives was affected by loss of Etv5a. Further analysis of gata2, which marks hematopoietic stem cells at the 10 somite stage (Detrich et al., 1995; Yamauchi et al., 2006), demonstrated that its expression was decreased in etv5a knockdown embryos (Fig. 2), indicating that Etv5a is required for the formation of hematopoietic stem cells.

We next analyzed the formation of blood vessels by using Tg(fli1:eGFP) zebrafish embryos in which vascular endothelial cells are labeled by green fluorescence protein (Bennett et al., 2001). In etv5a knockdown embryos, the main artery and vein initially formed at 24 hpf; however, the GFP fluorescence was significantly weaker than in the controls, as shown by direct observation and quantification of GFP by Western blot analysis (Fig. 3). The defect in blood vessel formation became more severe as angiogenesis progressed, as evidenced by the weaker GFP signals in the intersomitic and sub-intestinal venous vessels, the formation of which is used as an indicator of proper angiogenesis (Isogai et al., 2001) (Fig. 3A).

During development, blood and endothelial cells develop from a common
progenitor, the hemangioblast (Gering et al., 1998). Since our results showed defects in both blood cells and vessels, we examined the expression of *scl*, which marks hemangioblasts in lateral plate mesoderm (Gering et al., 1998). During normal development, transcripts for *scl* were seen in two pairs of lateral stripes flanking the mesoderm. By contrast, *scl* expression was down-regulated in Etv5a knockdown embryos (Fig. 2A,B). Taken together, these results show that knockdown of Etv5a is sufficient to inhibit the formation of hemato-vascular progenitors, which results in defective hematopoiesis and vasculogenesis.

Previous studies have shown that morpholinos can cause off-target apoptosis mediated by p53 activation (Robu et al., 2007). To rule out this possibility, all of the *etv5a* MOs were co-injected with a *tp53* MO. The results showed no significant differences between the phenotypes of embryos co-injected with *etv5a* and *tp53* MOs or those injected with *etv5a* MO alone (Fig. 2). Since the phenotypes caused by *etv5a* morpholino injection were also rescued by concomitant injection of *etv5a* cRNA as noted above, the phenotypes of the *etv5a* morphants were not the result of p53 activation but the result of specific inhibition of Etv5a function.

**Increased proliferation of ventral mesoderm cells after Etv5a knockdown**

In zebrafish embryos, hemangioblasts originate from the lateral plate mesoderm, and the lateral plate mesoderm is derived from the ventral mesoderm (Bockamp et al., 2009; Davidson and Zon, 2004). We next examined the expression of *eve1*, which is specifically restricted to the ventral mesoderm during gastrulation (Seebald and Szeto, 2011). Intriguingly, the expression of *eve1* was significantly up-regulated in *etv5a* morphants (Fig. 4A,B). The increase in the expression of *eve1* was not a
result of embryo ventralization since head and notochord structures, which are the
first structures affected in ventralized embryos, were morphologically normal
(Neave et al., 1997) (supplementary material Fig. S2). The observed increase in eve1
could, however, result from increased cell proliferation, reduced cell death, or
inhibition of differentiation of ventral mesoderm cells. We first examined cell
proliferation by phosphohistone H3 antibody and counterstained with eve1, which
revealed increased proliferation of ventral mesoderm cells in etv5a morphants (Fig.
4C-F). We next examined for apoptosis by examining for the presence of activated
caspase-3 using immunohistochemistry. No significant differences were observed
between etv5a morphants and controls at 75% epiboly (Fig. 5). Intriguingly,
significantly increased apoptotic cells were detected 2 h later at the tail bud stage
and this abnormally increased apoptosis ceased 1 h later (6-somite stage), suggesting
that the apoptosis was triggered by deregulation of proliferation. These results
demonstrate that knockdown of Etv5a increases the proliferation of ventral
mesoderm cells.

In addition to hemato-vascular progenitors, the ventral mesoderm also gives
rise to somites, pronephric cells and the gut wall. To examine whether knockdown
of etv5a affects other ventral mesoderm derivatives, the expression of myoD (a
marker for early somitic mesoderm) (Weinberg et al., 1996), pax2a (a marker of
pronephric ducts) (Majumdar et al., 2000) and foxf1 (a marker of the gut wall)
(Madison et al., 2009) were analyzed. The result showed that the expression of these
markers was not affected by Etv5a knockdown (supplementary material Fig. S4),
suggesting that Etv5a specifically regulates the formation of the hemato-vascular
lineage. Taken together, these findings demonstrate that the proliferation of ventral
mesoderm cells and the differentiation of hemato-vascular progenitors were
dependent on the proper expression of Etv5a.

Abrogation of the acidic domain in etv5a causes a dominant-negative effect

Widespread expression of Etv5a by injection of etv5a cRNA did not show any significant phenotype compared to the controls (Fig. 6B,C). To gain insight into the structural requirements for Etv5a function, we created two deletion variants: one lacking the region containing the ETS DNA binding domain up to the carboxy-terminus of Etv5a (etv5a^ΔETS), and the other one lacking the amino terminus end, including the acidic domain (etv5a^Δacidic) (Fig. 6A). Injection of etv5a^ΔETS cRNA did not result in any significant change in the phenotype compared to controls (Fig. 6B,C). However, injection of etv5a^Δacidic resulted in reduced levels of all hematopoietic derivatives including hematopoietic stem cells and hemangioblasts (Fig. 6B,C) and increased the expression of the ventral mesoderm marker eve1 (Fig. 6B,C). The phenotypes caused by etv5a^Δacidic injection were identical to those observed in Etv5a knockdown embryos. These results indicate that although deletion of the amino terminus end, including the acidic domain (etv5a^Δacidic), abolishes Etv5a transactivation, the remaining ETS motif may compete with endogenous Etv5a for the DNA binding site, thereby causing a dominant-negative effect. By contrast, deletion of the ETS domain (etv5a^ΔETS) would produce a non-functional protein, incapable of competing with endogenous Etv5a. These results confirm the specificity of the phenotypes conferred by etv5a morpholino injection.

Discussion

In this study, we used etv5a morpholinos and a dominant-negative etv5a variant
to show that interference with *etv5a* expression in zebrafish embryos results in an increase in the proliferation of ventral mesoderm cells. In addition, the formation of hemato-vascular derivatives was defective whereas other derivatives developed normally. These abnormally proliferated hemangioblastic ventral mesoderm cells later underwent apoptosis during a very short time interval and consequently caused defective hematopoietic cells and vessels, however, cell differentiation seemed unaffected since those cells that escape apoptosis differentiate normally according to the residue expression of hematopoietic and vascular markers. Taken together, our results suggest that *Etv5a* specifically regulates the proliferation of ventral mesoderm cells with hemato-vascular potential.

Molecules and regulatory mechanisms involve in the development of mesoderm cells and their progeny have attracted increasing attention due to their potential applications in regenerative medicine and stem cell-based therapies for human diseases. The mechanisms involved in hematopoiesis are well-characterized; however, only a small number of factors involved in ventral mesoderm and hemato-vascular specification have been identified. Only a few mutant lines showing defects in mesoderm and mesodermal derivatives are available in zebrafish. Moreover, the defects in these cell lines are not restricted to the hemato-vascular lineage but affect many other mesodermal derivatives as well (Davidson et al., 2003; Gering et al., 1998; Griffin et al., 1998; Thompson et al., 1998). Our results showing that *Etv5a* specifically regulates the formation of the hemato-vascular lineage without affecting other mesodermal derivatives, suggests that *etv5a* is one of the earliest players in the generation of the hemato-vascular progenitors from the ventral mesoderm. In contrast, the *cloche* (*clo*) mutant displays severe deficiencies in the development of both endothelial and all hematopoietic cells, without affecting pronephros or somite
formation, indicating that cloche regulates mesoderm cell specification and that the cloche mutation affects specifically hemangioblast formation (Stainier et al., 1995).

The phenotype caused by Etv5a deficiency is similar to that of the cloche mutant, suggesting a possible interaction between Etv5a and Cloche. However, whether Etv5a and Cloche interact and work synergetically to regulate ventral mesoderm proliferation requires further confirmation.

Several molecules belonging to the BMP, FGF and Wnt signaling pathways have been implicated as early inducers and patterning factors of the ventral mesoderm; however, how they direct the ventral mesoderm into different cell fates is still unclear. Here, we showed that Etv5a is a novel regulator of ventral mesodermal proliferation and specification. To our knowledge, Etv5a is the first transcription factor shown to be essential for the inhibition of proliferation of ventral mesoderm cells and for hemato-vascular specification. However, the upstream regulator for Etv5a in ventral mesoderm proliferation still remains to be identified. A previous study in mouse embryos demonstrated that BMP signaling positively regulates the expression of another Ets transcription factor, Er71/etsrp, in mesoderm cells, which contribute to hematopoietic and endothelial, skeletal, and smooth muscle cell lineages (Lee et al., 2008). A study in zebrafish also showed that BMP signaling was required for the expression of flil (also a member of Ets family) in hemangioblasts of the lateral mesoderm (Liu et al., 2008). We also found inhibition of BMP signaling upregulated etv5a expression (data not shown). Previous studies in zebrafish showed that etv5a transcription was down-regulated by inhibition of the FGF signaling pathway at 24 hpf (Mao et al., 2009; Roussigne and Blader, 2006), in contrast, we found inhibition of FGF/MEK signaling did not affect the expression of etv5a at 75% epiboly (data not shown). Therefore, how etv5a responds to BMP and FGF signaling and whether this
regulation is essential for ventral mesodermal proliferation remains to be examined.

ETV5/ERM regulates cell proliferation in several tumor cell lines and during spermatogenesis and has been suggested to act as a proto-oncogene (Chen et al., 2005; Oh et al., 2012). Our results demonstrate that Etv5a is essential for the proliferation of mesoderm cells and suggest that Etv5a is a positive regulator of hemato-vascular lineage development. However, embryos over-expressing the full etv5a cRNA had a normal phenotype. A possible explanation for this finding is that ETV5/ERM protein requires phosphorylation for its activity (Janknecht et al., 1996). Therefore, it will be interesting to determine whether activated-Etv5a is sufficient to induce abnormal hematopoiesis and vasculogenesis. In line with this idea, ETV5 is over-expressed in lymphoid leukemia and lymphoma (Charfi et al., 2011; Korz et al., 2002). This observation also reinforces the positive correlation between ETV5 expression and hematopoiesis. Whether the embryonic role of Etv5 found in this study is a feature of leukemias and other tumors warrants further investigation.

Materials and Methods

Ethics Statement

All experiments were performed in strict accordance to standard guidelines for zebrafish work and approved by the Institutional Animal Care and Use Committee of Chang Gung University (IACUC approval number: CGU08-86 and CGU11-118).

Fish Maintenance and Mutants
Tü (wild type) zebrafish embryos were purchased from the Zebrafish International Resource Center (ZIRC, Oregon, USA) and were raised, maintained and paired under standard conditions. Tg(fli1:eGFP) zebrafish was obtained from Taiwan Zebrafish Core Facility at ZeTH with the permission from Zebrafish International Resource Center (ZIRC). The embryos were staged according to the number of somites, hours post fertilization and days post fertilization (Kimmel et al., 1995).

Sequence Comparisons and Phylogeny

Amino acid sequences were aligned and displayed using the Vector NTI (Invitrogen). Phylogenetic tree calculation was performed with ClustalX (Thompson et al., 1997). The GenBank accession numbers of the compared proteins are as follows: human ETV5/ERM (NM_004454.2); rat ETV5/ERM (NM_001107082.1); mouse ETV5/ERM (NM_023794.2); chicken ETV5 (XM_422651.2); zebrafish Etv5a (NM_001126461.1), Etv5b (NM_131205.1).

Constructs Generation

The open reading frame of zebrafish etv5a was PCR amplified with high fidelity Pfu polymerase (Fermentas) and primers (5’-GAATTCGCCACCATGGACGGATTTTATGACC-3’ and 5’-GGAATTCCTCAGTACACGTAACCATCAGGG-3’) which were designed according to the GenBank sequence (accession number: NM_001126461.1). etv5aΔETS was created with primers (5’- GGAATTCATGGACGGATTTTATGACCAGCAAG -3’ and 5’-
GCTCTAGAGAGATCGCGCCGCTGATATG -3’), whereas etv5\textsuperscript{a\textsubscript{acidic}} was made
by primers (5’- GGAATTCATGTCGAGAGCTTGATGTTTCATG -3’ and 5’-
GCTCTAGAGTCAGTACACGTAACCATCAGG -3’). etv5\textsuperscript{a} MO1 and MO2

binding sequences were inserted upstream of an enhanced green fluorescent protein
reporter in the pCS2 vector to create 5’rgs4-EGFP construct to evaluate the
specificity and efficiency of morpholinos.

RNA and Morpholino Injection

Capped RNA encoding the full coding sequence of Etv5a, etv5\textsuperscript{a\textsubscript{ETS}} and etv5\textsuperscript{a\textsubscript{acidic}}
were prepared as described previously (Chung et al., 2011). Antisense morpholino
oligonucleotides were purchased from Gene Tools, LLC (Oregon, USA). Two
morpholinos against etv5\textsuperscript{a} were used: MO1
(TCACCTGGGTCTTCAAAGAGGCTCC) that overlaps the ATG start codon (-26
to -2), and MO2 (GATCTTCGCTTTAAAAGCGATAGCTG) that corresponds to -59
to -35 to the translation start site. Blast analysis revealed homology of less than 20
bp identity for MO1 or MO2 to other genomic sequences, none of which
corresponded to 5’ UTR or exon-intron splicing site of predicted or characterized
genes, suggesting that MO1 and MO2 will act specifically on etv5\textsuperscript{a}. A control
morpholino designed to a random sequence of nucleotides not found in the zebrafish
genome (5’-CCTCTTACCTCAGTTACAATTTATA-3’; Gene Tools) and a
morpholino with 5 bases mismatch to MO1 (5’-
TC\textsuperscript{a}GCTG\textsuperscript{a}GTC\textsuperscript{a}TT\textsuperscript{a}A\textsuperscript{a}A\textsuperscript{a}G\textsuperscript{a}C\textsuperscript{a}C -3’; mismatched bases are indicated by
small letters) was injected in an equal amount of MO1 as a control experiment. All
injections were performed at the one to two-cell stage and cRNAs or morpholinos
were introduced into blastomeres.
Histological Analysis

Digoxigenin-UTP labeled riboprobes were synthesized according to manufacturer’s instructions (Roche), and in situ hybridizations were performed as described previously (Cheng et al., 2012). The color reaction was carried out using NBT/BCIP substrate (Roche). For immunohistochemistry, the embryos were blocked in 5% goat serum and incubated with rabbit phospho-histone H3 antibody or rabbit monoclonal anti-active caspase-3 (1:200, Abcam). Goat anti-mouse IgG HRP or goat anti-rabbit IgG HRP (Roche) was used to detect the primary antibodies and DAB was used as a substrate for secondary antibody-conjugated HRP (Amresco). Embryos were mounted with Vectashield mounting medium (Vector Laboratories, Inc.).

Quantitative Analysis

For quantitative real time PCR (qPCR), embryos were homogenized in TRIzol reagent (Invitrogen) and total RNA was extracted using a standard method. cDNA was synthesized from total RNA with random hexamer priming using RevertAid First Strand cDNA Synthesis Kit (Fermentas). qPCR was performed on an ABI StepOne™ Real-Time PCR System (Applied Biosystems) with SYBR green fluorescent label (Fermentas). Primers for eve1 (F:

5’-CCCTGGTTAGGTGCTTCCA-3’;

R: 5’-GGGTTGTAGGCCTGCTAGCT-3’), scl (F:

5’-CGCAGACCTGCACCTTATGA-3’; R:

5’-AGGGTGTGTTGGGATGAGCTT-3’), gata2 (F:

5’-AAGCACGGCTCCAGTTTCCT-3’; R: 5’-TCCTTTTCGTCCATTCTTGCA-3’),
\textit{gata1} (F: 5'-ACACAGTCCAGTTCGCCAAGT-3'; R: 5'-TGGAGAGGTTTTTGGGAAA-3'), \textit{mpo} (F: 5'-TCTTTTTGCTGCTGATTTC-3'; R: 5'-ATTCCGCTTGTGCAGAT-3') and \textit{rag1} (F: 5'-CACTAAGCTCATCCCCACTGAAG-3'; R: 5'-CCCAAAGCATGGGTGTACCT-3') were used. Gene expression levels were normalized to \textit{gapdh} and assessed using the comparative CT (40 cycles) according to the manufacturer's instructions (Applied Biosystems).

For Western blot analysis, embryos were homogenized in SDS lysis buffer. 60 \(\mu\)g were loaded on 12\% SDS polyacrylamide gel and transferred to a PVDF membrane and detected with anti-GFP monoclonal antibody (1:1000, Invitrogen). After washes, membranes were incubated with goat anti-Mouse HRP-conjugated secondary Ab (Chemicon) and developed with ECL (Millipore). Band intensities were quantified using Multi Gaugre analysis software.

Statistical analysis was performed by student’s t-test using Microsoft Excel\textregistered\ 2007. The significance level was set at \(P < 0.05\). All Reaction was performed in triplicate for each sample.

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Figure Legends

Fig. 1. etv5a expression in the developing zebrafish embryo. etv5a expression was analyzed by in situ hybridization (A-T) and quantitative real-time PCR (U). Embryo stages are shown at the bottom right corner of each panel. Lateral view, dorsal to the right (E-F, H, K, M, O, Q); dorsal view (I, J, L, N, P, R, T). G is a coronal section of the ventral etv5a-stained region, showing that etv5a is expressed in the mesoderm (arrowhead in G). (J) a double in situ hybridization of etv5a (purple) and scl (red) showing that etv5a is expressed in the in the posterior lateral mesoderm. etv5a expression first appears in the ventral mesoderm at 75% epiboly (arrowhead in F) and later becomes restricted to the lateral mesoderm (arrows in I, J, L and N). The transcripts were detected in the pronephric ducts at 24 hpf (O and P) and were retained until the latest stage analyzed (48 hpf) (O-T). pd: pronephric ducts.

Fig. 2. Decreased levels of hemato-vascular progenitors and derivatives in Etv5a knockdown embryos. (A) In situ hybridization showing down-regulation of markers for hemangioblasts and hematopoietic lineages in etv5a morpholino-injected embryos. The phenotypes observed were rescued by co-injection with etv5a cRNA but not a tp53 morpholino. The insert panels are enlargements of the stained areas. The fraction of embryos exhibiting these phenotypes are shown at the top right corner and embryo stages are shown at the bottom right corner of each panel. scl, hemangioblast; gata2, hematopoietic stem cell; gata1, erythroid progenitor; mpo, granulocytes; rag1, lymphocyte. (B) The result shown in A was quantitatively confirmed by qPCR analysis. ***; p < 0.001.
Fig. 3. Knockdown of Etv5a results in defective blood vessel formation. (A) Tg
(fli1:eGFP) embryos showing defected green fluorescent blood vessels as a result of
etv5a morpholino injection. The sub-intestinal venous vessels (middle panels) and
intersomitic vessels (bottom panels) are indicated by arrows and arrowheads,
respectively. (B) Western blot analysis confirming that the levels of fli1 driven
eGFP expression in etv5a morphants were down-regulated compared to the levels in
the control. * p < 0.05.

Fig. 4. Increased proliferation of ventral mesoderm cells after Etv5a knockdown.
(A) In situ hybridization showing up-regulation of eve1 in etv5a
morpholino-injected embryos. This phenotype was rescued by co-injection with
etv5a cRNA but not a tp53 morpholino, as confirmed by qPCR analysis (B). (C)
etv5a morphants showed an increase in cell proliferation. Proliferating cells were
detected using an anti-phospho-histone H3 antibody (brown) and were
double-labeled with eve1 (purple). (D) The proportions of phospho-histone H3- and
eve1-positive cells among the total eve1-positive cells were quantified. (E), Ventral
view of flat-mounted embryos. Right panels are enlargements of areas marked in
dashed squares on the left. etv5a-expressing cells are pseudocolored in fluorescent
red and counterstained with phospho-histone H3 (fluorescent green) and DAPI
(fluorescent blue) to locate the cell nuclei. Cells double labeled with etv5a and
phospho-histone H3 are indicated by white arrowheads. (F) Quantification of
proliferating etv5a-positive cells in E. **, p < 0.01; ***, p < 0.001.

Fig. 5. Etv5a knockdown causes a temporary increased apoptosis of ventral
mesoderm cells.
(A) The control and Etv5a knockdown embryos were labeled by in situ
hybridization of *eve1* (left panels) and anti-activated caspase-3 antibody (fluorescent red in the right panels). In the right panels, *eve1*-expressing cells are pseudocolored in fluorescent green to favor a better presentation of the counterstaining. Note that significantly increased cell apoptosis can only be detected at tailbud stage. (B) Quantification of apoptotic *eve1*-positive cells in A. **, p < 0.01.

**Fig. 6.** Resemblance between the phenotypes of *etv5a*<sup>Acidic</sup> injected embryos and those of *etv5a* morphants. (A) Schematic illustration of *etv5a*<sup>ETS</sup> and *etv5a*<sup>Acidic</sup> deletion constructs. (B) Injection of *etv5a*<sup>Acidic</sup> was sufficient to down-regulate markers for hemangioblasts and hematopoietic derivatives, and to increase the expression of *eve1*. In contrast, over-expression of the fully coding sequence of *etv5a* or the *etv5a*<sup>ETS</sup> deletion mutant did not cause a significant phenotype. These results were confirmed by qPCR (C). ***, p < 0.001.
Figure 1

A 1 cell  B 32 cell  C oblong  D 50%

E shield  F 75%

H 6 ss  I 6 ss  J 6 ss

K 10 ss  L 10 ss  M 14 ss  N 14 ss

D pd  P 24 hpf  Q 24 hpf  R 36 hpf  S 48 hpf

etv5a  1-cell  32-cell  oblong  50%  shield  75%  6 ss  10 ss  14 ss  24 hpf  48 hpf
Figure 3

A

control  
1 dpf  
n = 97  
91%

etv5a MO1  
1 dpf  
n = 88  
84%

etv5a MO1 + etv5a cRNA  
1 dpf  
n = 89  
71%

1 dpf  
n = 66  
86%

3 dpf  
n = 72  
80%

3 dpf  
n = 61  
78%

3 dpf  
n = 57  
89%

3 dpf  
n = 96  
76%

3 dpf  
n = 73  
72%

B

relative gene expression

GFP  
actin

control  
1.2  
* 
estv5a MO  
1.0

estv5a MO + etv5a cRNA  
0.8
Figure 4

A

B

C

D

E

F

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Figure 5

A

control  
75% epiboly  
tailbud  
6ss  

etv5a MO  
75% epiboly  
tailbud  
6ss  

B

caspase 3+ & eve1+ / eve1+  

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<th>tailbud</th>
<th>6ss</th>
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** denotes a significant difference.
Figure 6

A

etv5a

etv5a^Δacidic

etv5a^ΔETS

B

<table>
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<th>etv5a^Δacidic</th>
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<td>14 ss</td>
<td>14 ss</td>
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<tr>
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C

- **gata1**
  - control
  - etv5a cRNA
  - etv5a^ΔETS
  - etv5a^Δacidic

- **gata2**
  - control
  - etv5a cRNA
  - etv5a^ΔETS
  - etv5a^Δacidic

- **mpo**
  - control
  - etv5a cRNA
  - etv5a^ΔETS
  - etv5a^Δacidic

- **scl**
  - control
  - etv5a cRNA
  - etv5a^ΔETS
  - etv5a^Δacidic

- **rag1**
  - control
  - etv5a cRNA
  - etv5a^ΔETS
  - etv5a^Δacidic

- **eve1**
  - control
  - etv5a cRNA
  - etv5a^ΔETS
  - etv5a^Δacidic