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FoxH1 negatively modulates *flk1* gene expression and vascular formation in zebrafish

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Summary

Flk1 is the major receptor for VEGF on endothelial cells. During embryogenesis, *flk1* is required for both vasculogenesis and angiogenesis and abnormally elevated *flk1* expression is often associated with pathological conditions in adults. While the biological function of *flk1* has been studied extensively, very little is known about how the *flk1* gene is regulated at the transcriptional level. Our transgenic study led to the identification of a *flk1* endothelial enhancer positioned approximately 5 kb upstream of the *flk1* translation initiation site. Binding sites for FoxH1, scl, ets and gata factors are found in the zebrafish *flk1* endothelial enhancer, as well as in upstream sequences of mouse *flk1* and human *kdr* genes, suggesting that the regulatory machinery for *flk1/kdr* is conserved from fish to mammals. The roles of scl, ets and gata factors in hemangioblasts have been well defined, but the significance of FoxH1 in vessel formation has not been explored previously. Here we show that FoxH1 binds to the *flk1* endothelial enhancer *in vitro* and functions as a repressor for *flk1* transcription in cultured cells. Consistent with these findings, the expression level of *flk1* is elevated in embryos lacking both maternal and zygotic FoxH1. We further show that overexpression of FoxH1 has a negative effect on vascular formation that can be counteracted by the down-regulation of smad2 activity in zebrafish embryos. Taken together, our data provide the first evidence that *flk1* is a direct target of FoxH1 and that FoxH1 is involved in vessel formation in zebrafish.

Keywords

angiogenesis; blood vessel; FoxH1; flk1; zebrafish

Introduction

A functional vascular system is required for the proper development of vertebrate embryos and the survival of adults. A number of signaling pathways, including VEGF and TGF β , have been

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shown to be involved in vascular formation, but the VEGF pathway is the only one known to be involved in both vasculogenesis (the formation of vessels from hemangioblasts) and angiogenesis (the sprouting and remodeling of existing vessels) (for review, see (Roman and Weinstein, 2000)).

flk1 (also known as *KDR* and *VEGFR2*) is an endothelial-specific receptor of vascular endothelial growth factor (VEGF). During embryogenesis, *flk1* expression is first detected in hemangioblasts, the common precursor of the endothelial and blood lineages, and remains active in endothelial cells during vascular formation. Consistent with the importance of *flk1* in vessel formation, mouse embryos lacking *flk1* do not have cells from endothelial or hematopoietic lineages (Shalaby et al., 1995). In zebrafish, embryos lacking the activity of the VEGF receptor, *flk1/kdra*, display a partial loss of segmental arteries (Habeck et al., 2002;Covassin et al., 2006). This mild phenotype is likely to be the result of the compensatory effect of a second *flk1* homologue, *kdrb*. In fact, while *kdrb* morphants do not display apparent abnormalities in blood vessel formation, embryos lacking both *flk1/kdra* and *kdrb* show a complete loss of segmental arteries (Covassin et al., 2006).

The expression of *flk1* in endothelial cells is tightly regulated. During embryogenesis, *flk1* expression is down-regulated after the vasculature is established. In adults, significant *flk1* expression is only seen in tissues undergoing active angiogenesis, such as the mammary glands during pregnancy. Abnormal elevation of *flk1* expression is often associated with pathological conditions, such as neovascularization in tumorigenesis (for review, see (Robinson and Stringer, 2001)). The precise regulatory circuit that regulates the expression of *flk1* is not well understood. However, the limited information available from studies in the mouse model has revealed critical roles for *scl*, *ets* and *gata* factors in *flk1* regulation. Consensus binding sequences of these transcription factors are found in the mouse *flk1* enhancer and in the human *KDR* promoter (Patterson et al., 1995;Kappel et al., 2000) and mutations in these transcription factor binding sites abolish endothelial expression of the *flk1* gene in the mouse model (Kappel et al., 2000;Elvert et al., 2003). The regulatory relationship between *flk1* and *scl* has also been studied in the zebrafish. Overexpression of *scl* increases *flk1* expression in wild type embryos and can induce *flk1* expression in the *cloche* mutant, which lacks endothelial and blood cells (Liao et al., 1998;Liao et al., 2000), demonstrating that *scl* is an important transcription factor that regulates *flk1* gene expression. In addition, a recent study shows that overexpression of *Etsrp*, an endothelial-specific *Ets*-1 related protein, induces ectopic *flk1* expression in wild type and *clo* mutant embryos (Pham et al., 2006;Sumanas and Lin, 2006). These findings suggest that the regulatory mechanisms of *flk1* expression may be conserved among vertebrates.

FoxH1 (also known as *Fast1*) is a forkhead transcription factor that binds to *Smad2/3* and mediates *TGF β* signaling (for review, see (Attisano et al., 2001)). Gene expression analysis showed that FoxH1 is expressed primarily during early developmental stages. Consistent with this finding, overexpression of FoxH1 induces the expression of a broad range of genes downstream of *activin* and leads to abnormal axis formation in *Xenopus* (Watanabe and Whitman, 1999). Furthermore, mouse and zebrafish embryos carrying mutations in FoxH1 develop gastrulation defects (Pogoda et al., 2000;Sirotkin et al., 2000;Hoodless et al., 2001;Yamamoto et al., 2001). The roles for FoxH1 at later developmental stages or in adults are not as clear. However, the findings that FoxH1^{-/-} mouse embryos do not have the outflow tract or the right ventricle reveal a role of FoxH1 in the formation of the anterior heart field (von Both et al., 2004). Furthermore, the well-documented effects of *TGF β* signaling on the proliferation, differentiation, migration and even survival of endothelial cells (for review, see (Goumans and Mummery, 2000;Goumans et al., 2003)), suggest a role for FoxH1 in the regulation of endothelial cells.

To further understand how the *flk1* gene is regulated at the transcriptional level in zebrafish, we systematically studied the regulatory elements of *flk1/kdra* (hereafter referred to as *flk1*). We identified a ~6.4kb genomic sequence upstream of the translation initiation site of zebrafish *flk1* that drives GFP expression in the zebrafish vasculature, resembling the endogenous *flk1* expression pattern. Deletion analysis revealed that a ~800 bp DNA fragment positioned approximately 4.3kb upstream of the *flk1* translation initiation site is sufficient to drive *flk1* expression in endothelial cells. Consensus sequences of transcription factor binding sites including scl, ets and gata factors are present in this 800bp minimal *flk1* enhancer suggesting conserved regulatory machinery from fish to mammals. In addition, we provide evidence that FoxH1 is part of the regulatory machinery for *flk1* gene expression in zebrafish. We found multiple FoxH1 binding sites in the upstream regulatory regions of zebrafish, mouse and human *flk1* genes. By gel shift assay, we show that FoxH1 binds to the *flk1* regulatory element. We further show that FoxH1 can repress the activity of the *flk1* minimal enhancer in HEK293T cells. Consistent with this finding, the expression level of *flk1* is moderately elevated in zebrafish embryos lacking both maternal and zygotic FoxH1 and overexpression of FoxH1 disrupts the formation of zebrafish vasculature. Moreover, we showed that *flk1* gene expression is up-regulated in *smad2* morphants and that such changes in *flk1* expression levels could be counteracted by overexpression of FoxH1, indicating that the negative effects of FoxH1 on *flk1* gene expression is *smad2* dependent. These data suggest that FoxH1 functions as a negative modulator of *flk1* gene expression and indicate interplay between TGF β and VEGF signaling pathways in embryonic vascular formation.

Materials and Methods

Zebrafish husbandry and the generation of *TG(flk1:GFP)^{la116}* transgenic lines

The AB strain was used for this study. Adult fish and embryos were maintained as previously described (Westerfield, 2000).

A ~6.4 kb genomic fragment upstream of the zebrafish *flk1* gene (from -6410 to -1 upstream of *flk1* translational initiation site) was cloned into pKS-GM2r vector 5' to green fluorescent protein (GFP) (kind gift from S. Lin) to create the *flk(-6.4)-GFP* construct. Approximately 50pg of linearized *flk(-6.4)-GFP* DNA was microinjected into zebrafish embryos at the 1-cell stage. The injected embryos were raised to adulthood and screened for founder fish with germline integration. Images of GFP expression patterns of *TG(flk1:GFP)^{la116}* embryos were acquired using a Zeiss SV-11 epifluorescence microscope or an Olympus IX70 confocal microscope equipped with a 10X objective.

Deletion constructs of *flk(-6.4)-GFP*

Serial deletion constructs of *flk(-6.4)-GFP* were generated using the ERASE-A-BASE system (Promega). Sequencing analysis showed that *flk(-5.7)-GFP*, *flk(-5.0)-GFP*, *flk(-4.3)-GFP*, *flk(-3.5)-GFP* and *flk(-1.5)-GFP* have sequences upstream of *flk1* translational initiation site starting from -5680, -5016, -4353, -3565 and -1503, respectively. DNA fragments corresponding to sequences from -5045 to -3543, -4353 to -3543, and -5045 to -4326 were amplified by PCR using the following primers: -5045F, 5'-CCGCGGTTGTATGAAGTTTCTGTGTGAC-3'; -4353F, 5'-CCGCGGTCACCTTCTGCTAGTAAAACC-3'; -4326R, 5'-GCGGCCGCGAATGAGGTTTTAACTAGCAGA-3'; -3543R, 5'-GCGGCCGCAATCCAAAGTAATTGATCCCTG-3'. These DNA fragments were then cloned into *flk(-1.5)-GFP* to create *flk(-5.0,-3.5/-1.5)-GFP*, *flk(-5.0,-4.3/-1.5)-GFP* and *flk(-4.3,-3.5/-1.5)-GFP*.

Approximately 50pg of each of these constructs were microinjected into zebrafish embryos at the 1-cell stage. The injected embryos were screened for transient expression of GFP using a Zeiss SV-11 epifluorescence microscope after one day of development.

mRNA and morpholino injection

mRNA encoding FoxH1 (kind gift from D. Meyer) (Pogoda et al., 2000), FAST-Eng and FAST-VP16 (kind gifts from M. Whittman) (Watanabe and Whitman, 1999) were synthesized using mMACHINE (Ambion) and injected into *TG(flkl:GFP)^{la116}* embryos at the 1- to 4-cell stage.

An morpholino modified oligonucleotide specifically targeting the translation initiation site of *smad2*, *smad2MO*, was synthesized (GAGTGAAAGGCAAGATGGAGGACAT) (Genetools). 0.8 ng of *smad2* morpholino were injected into *TG(flkl:GFP)^{la116}* embryos at the 1- to 4-cell stage.

The vasculature of the FoxH1 mRNA or *smad2MO* injected embryos were analyzed at between the 18-somite stage and 24 hpf (hour post fertilization). Embryos were photographed using a Zeiss SV-11 epifluorescence microscope and AxioCam digital camera and the intensity of GFP signals was quantified by the NIH ImageJ program. The *p* value was calculated by the student's t-Test.

GST fusion proteins

The zebrafish FoxH1 cDNA (from D. Meyer) was cloned into the *Sma*I site of pGEX-2TK (GE Healthcare) to create the pGEX-2TK-FoxH1 construct. pGEX-2TK-FoxH1 and pGEX-2TK were transformed into BL21 (Stratagene). The FoxH1-GST and GST proteins were purified using Bulk GST Purification Module (GE Healthcare). Protein concentrations were determined using the DC Protein Assay (Bio-Rad).

Electrophoretic Mobility Shift Assays

Oligonucleotides corresponding to *flkl* upstream sequences from -3870 to -3844 were synthesized. Sequences of the upper strands are as follows: flk-FoxH1, 5'-CCGAATATTGTATTTCGAGAAATATC-3', and flk-FoxH1-mt, 5'-CCGAATATTcTGTATTTCGAGAAATATC-3' (the FoxH1 binding sites are underlined and the lowercase letter represents the substituted nucleotide). Oligonucleotides were annealed in 20mM Tris pH8.0, 1mM EDTA pH8.0 and 50mM NaCl.

3μg of purified FoxH1-GST and GST proteins were incubated with ³²P end-labeled probes for 20min at room temperature in binding buffer containing 4% glycerol, 1mM MgCl₂, 0.5mM EDTA, 0.5mM DTT, 50mM NaCl, 10mM Tris-HCl (pH 7.5) and 0.05mg/ml poly(dI-dC). Protein-DNA complexes were separated on a 6% nondenaturing polyacrylamide gel (37.5:1 acrylamide/bis-acrylamide) in 0.5X TBE. Unlabeled flk-FoxH1 or flk-FoxH1-mt were used as competitors at 50-, 100-, or 200-fold molar excess of labeled probes.

Luciferase Assay

FoxH1 cDNA was subcloned into pSG5 (Stratagene) to create pSG5-FoxH1 expression vector and upstream sequences of *flkl* from -532 to -371 containing predicted TATA box and transcription starting site were amplified by PCR using flkP-F (5'-ACTGAGCGGCCGCTAGCCTGAATAAGTAGATAGC-3') and flkP-R (5'-CGGGATCCTACCTCTGACTTTTCTACTGG-3'). The amplified DNA fragment was then cloned into a luciferase reporter vector, pGL3-basic (Promega), to create the pGL3-flkP construct. Upstream sequences of *flkl* from -5045 to -3543 was amplified by PCR using the

–5045F and –3543R primers, and cloned into pGL3-flkP to create pGL3-flk(–5.0, –3.5)P. The Gs in all three FoxH1 consensus binding sequences were substituted with Cs using QuikChange II Site-Directed Mutagenesis Kit (Stratagene) to create the pGL3-flk(–5.0, –3.5)P-mt construct. Primers used for site-directed mutagenesis are as follows: mt1-F; 5'-ATAATAATAAAGAGGCGTATTTATACATCTGTGCCAGG-3', mt1-R; 5'-CCTGGCACAGATGTATAAATACGCCTCTTTATTATTAT-3', mt2-F; 5'-GCTAGGCCGAATATTCTGTATTCGAGAAATATC-3', mt2-R; 5'-GATATTTCTCGAATACAGAATATTCGGCCTAGC-3', mt3-F; 5'-CATTAATACCAATGAAGATCTGTAATCTAAAATTAT-3' and mt3-R; 5'-ATAATTTTAGATTACAGATCTTCATTGGTATTTAATG-3'.

HEK293T cells were plated on 24-well plates at a density of 50,000 cells per well and transfected using Lipofectamine™ and Plus™ reagents (Invitrogen) with 0, 100 or 500 ng of pSG5-FoxH1, 10ng of luciferase reporter plasmid (pGL3-flk(–5.0, –3.5)P or pGL3-flk(–5.0, –3.5)P-mt), 2ng of pRL-CMV and various amounts of pSG5 plasmid to keep the total amount of DNA at 512ng for each transfection. Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega) and normalized with Renilla Luciferase activity for transfection efficiency.

Quantitative RT-PCR—Total RNA was extracted from the un-injected control and 100pg FoxH1 mRNA injected embryos at the 18-somite stage using RNAwiz (Ambion) and the first strand cDNA was synthesized using the SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen). The relative *flk1* expression level was determined by quantitative PCR using DNA Engine Opticon (MJ Research). β -actin was used as the internal control for normalization. Primers used: β -actin forward primer, 5'-CTGTCTTCCCATCCATCGTGGGTC-3'; β -actin reverse primer, 5'-CTCCATATCATCCCAGTTGGTGACA-3'. *flk1* forward primer, 5'-GAGAACGGAACCAACAAGATCCACGAG-3'; *flk1* reverse primer, 5'-CCCTCCAGCAGAAGTACTCCTTAC-3'.

Results and Discussion

Identification of an endothelial-specific regulatory element of the *flk1* gene

As the first step toward understanding the regulation of *flk1* at the transcriptional level, we isolated a ~6.4kb DNA fragment upstream of the translation initiation site of the zebrafish *flk1* gene by long-range PCR. We created a reporter construct, flk(–6.4)-GFP, using this 6.4kb *flk1* upstream DNA fragment to drive GFP expression.

We generated a transgenic zebrafish line $TG(flkl:GFP)^{la116}$ that carries a germline-integrated flk(–6.4)-GFP transgene and found that the GFP expression pattern in $TG(flkl:GFP)^{la116}$ resembled the endogenous *flk1* expression pattern. GFP signals can be observed in $TG(flkl:GFP)^{la116}$ embryos as early as the 6-somite stage. At the 8-somite stage, GFP signals are detected in three patches of cells in $TG(flkl:GFP)^{la116}$ embryos (Fig 1B,D), similar to the endogenous *flk1* expression pattern (Fig.1A,C). The similarities between the endogenous *flk1* expression pattern and GFP expression pattern in $TG(flkl:GFP)^{la116}$ embryos continue as development proceeds. GFP expression is detected in the dorsal aorta, cardinal vein, intersomitic vessels and endothelial cells in the brain by 24 hours post fertilization (hpf), and in paired dorsal longitudinal anastomotic vessels (DLAV) by 48 hpf (Fig. 1E-L). By three days of development, endothelial cells in the pharyngeal arches can be clearly detected by GFP expression in $TG(flkl:GFP)^{la116}$ embryos (Fig. 1M). This pattern is distinct from that observed in $TG(fli:EGFP)^{y1}$ embryos where both the aortic arches and the mesenchyme of the forming jaw are GFP positive (Lawson and Weinstein, 2002). Furthermore, the GFP expression patterns in $TG(flkl:GFP)^{la116}$ embryos are consistent with the other two independent transgenic lines

using the same 6.4kb regulatory element (Cross et al., 2003;Beis et al., 2005;Jin et al., 2005), indicating that the ~6.4kb *flk1* upstream regulatory element is sufficient to drive gene expression in endothelial cells and that this endothelial expression pattern is not an artifact of the integration site of the *flk*(-6.4)-GFP transgene.

In addition, we found that while the spatial GFP expression of *TG(flkl:GFP)^{la116}* resembles the endogenous *flkl* expression pattern revealed by the in situ hybridization, the temporal regulation is different. In zebrafish, the expression of *flkl* in endothelial cells is greatly reduced after 3 days of development (Fouquet et al., 1997;Liao et al., 1997). However, the endothelial GFP signal remains strong in *TG(flkl:GFP)^{la116}* throughout embryogenesis and persists into adulthood (Fig. 1N-P), indicating that the regulatory sequences responsible for down-regulating *flkl* is not present or is not intact in the ~6.4kb *flkl* upstream DNA fragment.

Identification of the minimal *flkl* endothelial enhancer

To identify critical cis-regulatory elements for *flkl* expression in endothelial cells within the ~6.4kb *flkl* regulatory element, we made a series of deletion variants of *flk*(-6.4)-GFP. The *flk*(-5.7)-GFP, *flk*(-5.0)-GFP, *flk*(-4.3)-GFP, *flk*(-3.5)-GFP and *flk*(-1.5)-GFP deletion variants have the GFP reporter gene under the control of the ~5.7kb, 5.0kb, 4.3kb, 3.5kb, and 1.5kb DNA fragments upstream from the *flkl* translation initiation site, respectively. We injected these deletion constructs into zebrafish embryos at the 1-cell stage to evaluate the ability of these constructs to drive GFP expression in the zebrafish embryonic vasculature. We found that of the 196 embryos injected with *flk*(-6.4)-GFP, 158 (80.6%) had strong GFP expression in endothelial cells (Fig.2A,B). Similar results were observed in embryos injected with *flk*(-5.7)-GFP, *flk*(-5.0)-GFP and *flk*(-4.3)-GFP (52.0%, n=125, 52.4%, n= 145 and 51%, n=200, respectively) (Fig.2A, C, D). However, none of the embryos injected with the *flk*(-3.5)-GFP or *flk*(-1.5)-GFP constructs had GFP expression in endothelial cells (n=181 and 130, respectively) (Fig.2A). These data suggest that critical regulatory elements for *flkl* endothelial expression are located at least 3.5 kb upstream of the *flkl* translational initiation site.

Since *flk*(-4.3)-GFP was capable of driving GFP expression in endothelial cells but *flk*(-3.5)-GFP could not, we focused our analysis on the region of -4353 to -3543 upstream of *flkl*. We did not detect any GFP expression in embryos injected with the *flk*(-4.3,-3.5)-GFP construct, indicating that this fragment alone is not sufficient to drive gene expression *in vivo* (Fig.2A). One possible cause is that this construct lacks a functional promoter. The TATA box and the predicted transcriptional initiation site of *flkl* are located within the 1.5kb fragment immediately upstream of the *flkl* translation initiation site. Therefore, we created three additional constructs with various lengths of *flkl* 5' sequences from -5kb to -3.5kb fused to *flk*(-1.5)-GFP, *flk*(-5.0,-3.5/-1.5)-GFP, *flk*(-4.3,-3.5/-1.5)-GFP and *flk*(-5.0,-4.3/-1.5)-GFP. We injected these constructs into wild type zebrafish embryos at the 1-cell stage and found that while no GFP signals were detected in embryos injected with *flk*(-1.5)-GFP or *flk*(-5.0,-4.3/-1.5)-GFP (n=130 and n=176, respectively), transient GFP expression in endothelial cells was driven by *flk*(-5.0,-3.5/-1.5)-GFP and *flk*(-4.3,-3.5/-1.5)-GFP constructs (68.8%, n= 218 and 53.9%, n=293, respectively) (Fig.2A, E-G). These findings indicate that the DNA fragment of -4353 to -3543 has the information required to drive *flkl* expression in endothelial cells.

We established stable transgenic lines carrying germline-integrated *flk*(-5.0,-3.5/-1.5)-GFP or *flk*(-4.3,-3.5/-1.5)-GFP transgenes to analyze their GFP expression patterns in detail. We found that endothelial cells are marked by GFP expression in embryos carrying *flk*(-5.0,-3.5/-1.5)-GFP or *flk*(-4.3,-3.5/-1.5)-GFP transgenes, consistent with the findings obtained from transient expression analysis that sequences from -4353 to -3543 have all critical information

to drive gene expression in endothelial cells. However, when analyzed closely, we found that while the GFP expression pattern of $flk(-5.0,-3.5/-1.5)$ -GFP transgenic embryos is identical to that of $TG(flkl:GFP)^{la116}$ embryos, $flk(-4.3,-3.5/-1.5)$ -GFP transgenic embryos have GFP expression in the brain, the eyes and the neural tube in addition to endothelial cells (Fig.3). These observations, together with our finding that $flk(-5.0,-4.3/-1.5)$ -GFP does not drive GFP expression in zebrafish embryos (Fig.2A,E), indicate that while the DNA fragment from -4353 to -3543 is sufficient to drive *flkl* expression in endothelial cells, sequences from -5045 to -4325 are required to restrict *flkl* gene expression to the endothelial cells.

Conserved regulatory control of *flkl*

To further investigate regulatory mechanisms of *flkl* gene expression, we searched for transcription factor binding sites in *flkl* upstream sequences from -5045 to -3543. In the region that is critical for restricting *flkl* expression to endothelial cells (-5043 to -4326), we noted an Ets and a GATA factor consensus binding site but did not identify binding sites for other transcription factors with known function in endothelial gene expression, including FoxH1 (see below) (Fig.4B). However, multiple consensus binding sites of transcription repressors, including five engrailed, three pbx and two brn-3 binding sites, were found in this region. Interestingly, all these genes are expressed in the brain and the eyes (DeCarvalho et al., 2004; Erickson et al., 2006), raising a possibility that they are part of the machinery restricting *flkl* expression to endothelial cells. Further studies on molecular mechanisms by which -5045 to -4326 influences *flkl* gene expression, including the regulatory effects of engrailed, pbx and brn-3 on *flkl*, should provide more insights into *flkl* gene regulation.

The *flkl* upstream segment from -4353 to -3543 is critical for driving GFP expression in endothelial cells. In this region, we found multiple binding sites of scl, Ets and GATA factors. Interestingly, these transcription binding sites are also present in the mouse *flkl* minimal enhancer and human *flkl/KDR* promoter (Patterson et al., 1995; Kappel et al., 2000) (Fig.4), suggesting that the regulatory machinery of *flkl* gene expression is conserved from fish to mammals.

In addition to scl, ets and GATA binding sites, there are three FoxH1/Fast1 binding sites in the *flkl* minimal regulatory element (-4353 to -3543) (Fig.4). While the role of FoxH1 in endothelial cells has not been investigated, the effects of TGF β signaling on the proliferation, differentiation, migration and permeability of endothelial cells have been well documented in animal models and in cultured cells (for review, see (Goumans and Mummery, 2000; Goumans et al., 2002), raising a possibility that *flkl* is a target gene of FoxH1-mediated TGF β signaling. We therefore investigated whether FoxH1 binding sites are also present in the regulatory regions of mouse and human *flkl/KDR* genes. As illustrated in Fig.4B, multiple FoxH1 binding sites are present within a 5kb region upstream of the human and mouse *flkl* genes, supporting the notion that FoxH1 is part of *flkl* regulatory machinery that is conserved from fish to mammals.

FoxH1 binds to the *flkl* minimal endothelial enhancer *in vitro*

To explore the possibility that FoxH1 regulates *flkl* gene expression directly, we tested whether FoxH1 could bind to the consensus FoxH1 binding sites in the *flkl* minimal regulatory element. We performed a gel shift assay using a synthetic oligonucleotide, flk-FoxH1, corresponding to *flkl* upstream sequences from -3870 to -3844, consisting of one FoxH1 binding site, as a probe. As shown in Fig.5A, FoxH1 binds to flk-FoxH1 *in vitro* and this binding is competed away by unlabeled probes in a dosage-dependent manner (Fig.5A). It was previously shown that a G to C substitution in the FoxH1 binding site consensus sequence abolishes its interaction with FoxH1 (Chen et al., 1996). We found that flk-FoxH1-mt, synthetic oligonucleotides

harboring the same G to C mutation in the consensus sequences of the FoxH1 binding site did not compete out FoxH1 interaction with the labeled probes (Fig.5A), indicating that the binding of FoxH1 to the *flk1* minimal endothelial enhancer is sequence-specific.

FoxH1 suppresses *flk1* gene expression in HEK293T cells

Previous studies show that FoxH1 can function as an activator as well as a repressor (Labbe et al., 1998; Watanabe and Whitman, 1999; Osada et al., 2000; Saijoh et al., 2000; Kofron et al., 2004; Chen et al., 2005). We therefore explored the regulatory relationship between FoxH1 and *flk1* in HEK293T cells. pGL3-*flk*(-5.0,-3.5)P is a construct that uses the *flk1* upstream sequences from -5045 to -3543 and a 162bp *flk1* basal promoter to drive luciferase expression. Co-transfecting pSG5-FoxH1 with pGL3-*flk*(-5.0,-3.5)P represses the production of luciferase in a dosage-dependent manner ($p < 0.05$) (Fig.5B), demonstrating that FoxH1 functions as a repressor for *flk1*. To investigate if the repressive activity of FoxH1 is DNA-binding dependent, we created the pGL3-*flk*(-5.0,-3.5)P-mt construct where the Gs in all three FoxH1 binding sites on the minimal *flk1* regulatory element were substituted with Cs (Chen et al., 1996). HEK293T cells transfected with pGL3-*flk*(-5.0,-3.5)P-mt have similar luciferase activity as those cells transfected with pGL3-*flk*(-5.0,-3.5)P, indicating that loss of FoxH1 binding does not affect the activity of *flk1* minimal enhancer. Furthermore, no significant difference in luciferase activity was observed when FoxH1 expression plasmid was cotransfected with pGL3-*flk*(-5.0,-3.5)P-mt (Fig.5B), suggesting that the repressive effect of FoxH1 on *flk1* expression is DNA-binding dependent.

FoxH1 negatively regulates zebrafish vascular formation

We further explored the regulatory effect of FoxH1 on the formation of the embryonic vascular system in zebrafish. Our data show that FoxH1 represses the transcription activity of the *flk1* minimal endothelial enhancer in cultured cells. If the same regulatory relationships were relevant during embryonic vascular formation, one would expect *flk1* expression levels to be increased in zebrafish embryos lacking FoxH1 function. The zebrafish *schmalspur* (*sur*) locus encodes FoxH1/Fast1 (Pogoda et al., 2000; Sirotkin et al., 2000). We analyzed *flk1* expression levels in the maternal-zygotic *sur* homozygotes (MZ_{sur}) derived from the *sur*^{ty68b} allele which carries a point mutation in the conserved FKH domain (Pogoda et al., 2000). As shown in Fig. 6A, the *flk1* expression levels are indeed moderately elevated in MZ_{sur} embryos, supporting the notion that FoxH1 is a repressor of *flk1*. We further analyzed the forced expression effects of FoxH1 on vascular formation *in vivo*. Data collected from three independent experiments showed that 44 out of 73 *TG(flk1::GFP)*^{la116} embryos injected 50pg of FoxH1 mRNA (60%) and 265 out of 282 *TG(flk1::GFP)*^{la116} embryos injected with 100pg of FoxH1 mRNA (94%) have missing patches of endothelial cells (Fig.6B), suggesting that FoxH1 negatively regulates vascular formation in zebrafish and that this negative effect of FoxH1 on zebrafish vascular formation is dose-dependent. Furthermore, we measured relative *flk1* gene expression level in control and FoxH1 mRNA injected embryos using quantitative RT-PCR. From two independent experiments performed in triplicate, we consistently observed that the levels of *flk1* transcripts in those embryos injected with 100 pg of FoxH1 mRNA were 48% of the levels in un-injected embryos (standard deviation = 4.2%, $p < 0.001$), supporting the notion that FoxH1 represses *flk1* gene expression.

To further confirm that FoxH1 does indeed have a negative effect on vascular formation, we injected FAST-Eng and FAST-VP16 mRNA into *TG(flk1::GFP)*^{la116} embryos at the 1-cell stage. FAST-Eng and FAST-VP16 encode proteins that have the wild type FoxH1 forkhead DNA-binding domain fused to the transcriptional repressor domain of Engrailed (FAST-Eng) and the viral transcriptional activator domain of VP16 (FAST-VP16), respectively (Watanabe and Whitman, 1999). Embryos injected with 10pg of FAST-VP16 mRNA died before vascular

formation. We, therefore, focused our analysis on lower levels of FAST-VP16 injection. None of the 130 embryos that received 4pg of FAST-VP16 had notable vascular defects (Fig.6D). On the other hand, embryos injected with FAST-Eng mRNA displayed a phenotype resembling that observed in FoxH1 mRNA injected embryos and this effect was dosage-dependent. While 43 out of 55 *TG(flk1::GFP)^{la116}* embryos injected with 10pg of mRNA encoding FAST-Eng had missing patches of endothelial cells, all 36 *TG(flk1::GFP)^{la116}* embryos injected with 25pg of FAST-Eng mRNA had missing patches of endothelial cells (Fig.6C). These data demonstrate that FoxH1 functions as a repressor and has a negative effect on zebrafish vascular development.

Studies in *Xenopus* have demonstrated both smad2-dependent and smad2-independent effects of FoxH1 in early embryogenesis (Kofron et al., 2004). We therefore evaluated whether the negative effects of FoxH1 on *flk1* gene expression are smad2-dependent. If indeed the negative effects of FoxH1 on *flk1* expression and zebrafish vascular formation were smad2-dependent, one would expect that knocking down smad2 activity could counteract the effects of FoxH1 overexpression. We found that while *TG(flk1::GFP)^{la116}* embryos injected with 0.8 ng of Smad2MO developed normally, the GFP signals in endothelial cells were brighter than their un-injected siblings (86%, n=112) and that co-injecting 50pg of FoxH1 mRNA with 0.8ng of smad2MO could suppress the smad2MO induced elevation of GFP signals (100%, n=102) (Fig.6E,F). We then quantified the GFP intensity at selected regions of interest in the brain and in the trunk using the NIH ImageJ software. As shown in Fig.6E and F, while there was no significant differences in the GFP signals between wild type embryos and embryos co-injected with smad2MO and FoxH1 mRNA (25 embryos analyzed from each group, $p>0.05$), the GFP signals were increased by 2.84-fold in head blood vessels and 1.78-fold in the dorsal aorta and cardinal vein of smad2 morphants when compared to wild type embryos (n=25 for each group, $p<0.001$). These data support the notion that the effects of FoxH1 on the developing zebrafish vasculature are smad2-dependent and indicate that TGF β signaling plays a role in *flk1* gene expression.

The role of FoxH1 in early embryogenesis has been studied extensively, but its relevance in vascular development has not been explored. In this report, we provide evidence that FoxH1 binds directly to the *flk1* endothelial enhancer and modulates *flk1* expression both *in vivo* and in cultured cells. We also show that FoxH1 has a negative effect on blood vessel formation in developing zebrafish embryos, offering the first evidence of the role of FoxH1 in patterning embryonic vasculature. It is interesting to note that TGF β has both stimulating and inhibitory effects on endothelial cells (Pepper, 1997;Piek et al., 1999). Recent studies indicate that cellular effects of TGF β signaling on endothelial cells are determined by the selection of downstream pathways (Goumans et al., 2002). In this model, TGF β signaling via Alk1/smad1,5,8 pathway induces cell proliferation, differentiation and survival, whereas the Alk5/smad2,3 pathway which involves FoxH1, inhibits endothelial cell proliferation and differentiation, and induces cell death (for review, see (Goumans et al., 2003). Our finding that FoxH1 negatively regulates *flk1* gene expression in a moderate and smad2-dependent manner is consistent with the negative effect of Alk5-mediated TGF β signaling on endothelial cells and raises the possibility that the VEGF/*flk1* pathway is a direct target of TGF β signaling. Detailed investigation of the regulation of the VEGF pathway by TGF β /FoxH1 will deepen our understanding of the interplay of these pathways in endothelial cells.

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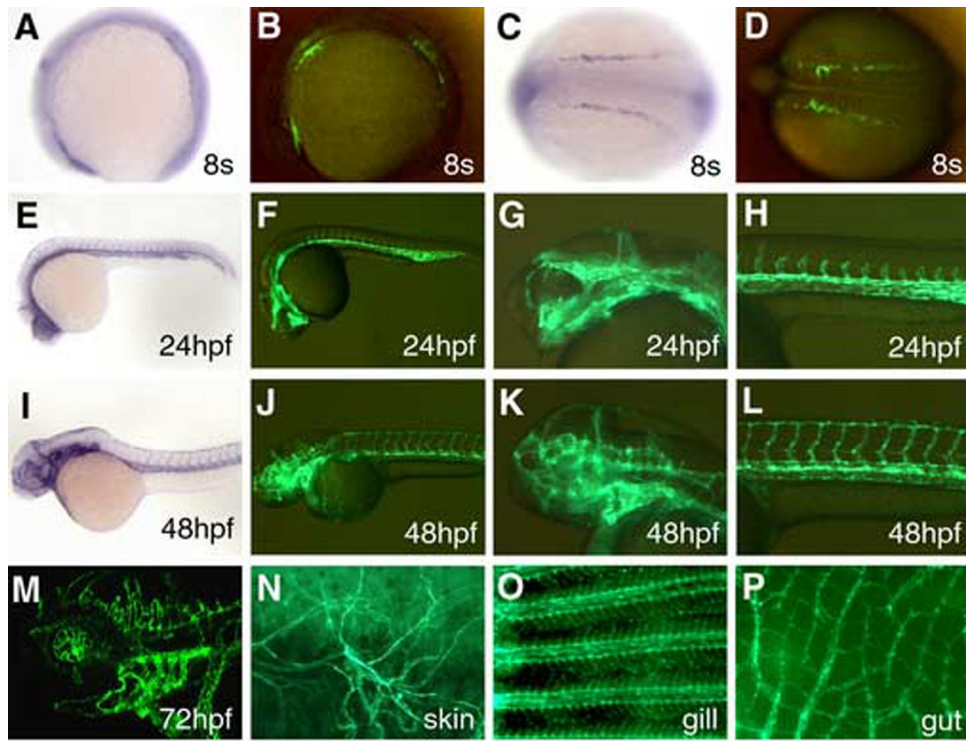


Fig1.
GFP expression in $TG(flkl:GFP)^{la116}$ embryos. (A-D) GFP signals driven by the $flk(6.4)$ -GFP transgene can be detected in $TG(flkl:GFP)^{la116}$ embryos as early as the 8-somite stage. $flkl$ expression is detected in three patches of cells by in situ hybridization (A). A similar pattern is seen in $TG(flkl:GFP)^{la116}$ embryos (B). Lateral views are shown in panels A and B and the dorsal views are shown in panels C and D. Anterior to the left. (E-H) GFP expression pattern of $TG(flkl:GFP)^{la116}$ embryos at 24hpf (F) resembles the $flkl$ pattern detected by in situ hybridization (E). Higher magnification images of the head and trunk are shown in G and H, respectively. (I-L) GFP expression pattern of $TG(flkl:GFP)^{la116}$ embryos at 48hpf (J) resembles the $flkl$ pattern detected by in situ hybridization (I). Higher magnification images of the head and trunk are shown in K and L, respectively. (M) Confocal image of GFP expression in endothelial cells in the brain and brachial arches in 3-day-old $TG(flkl:GFP)^{la116}$ embryos. (N-P) GFP expression remains active in adult $TG(flkl:GFP)^{la116}$ fish. Images show GFP signals in endothelial cells on the skin (N), in the gill (O) and gut (P).

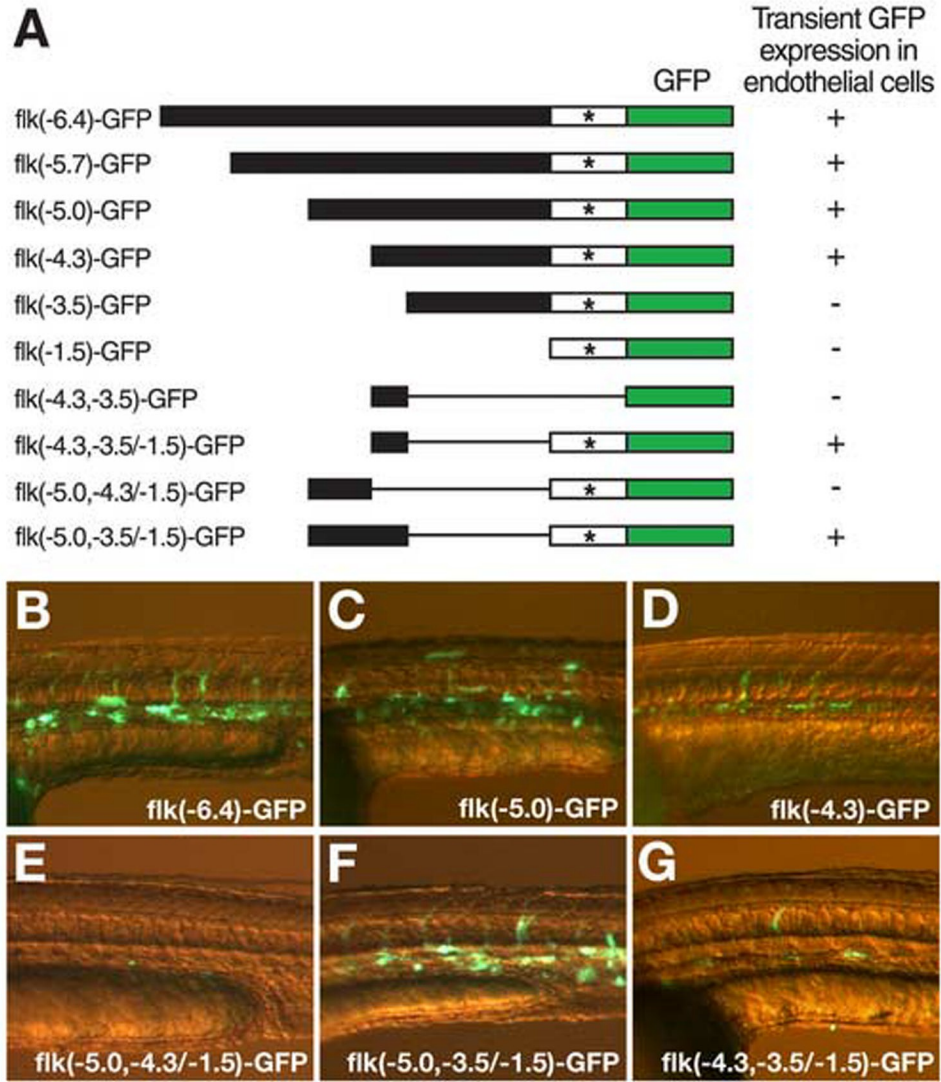


Fig2.
Deletion analysis of the *flk1* regulatory region identifies a highly conserved element that is necessary for endothelial expression. (A) Schematic diagram of the deletion constructs of *flk1*-GFP reporter. Linearized DNA of each construct was injected in wild type zebrafish embryos at the 1-cell stage. GFP expression in injected embryos was analyzed after 1 day of development. The transient endothelial expression directed by each construct is summarized by a plus (endothelial expression) or a minus (no detectable endothelial expression) to the right of the line representing each construct. * marks the transcription initiation site of *flk1*. (B-G) Transient GFP expression in endothelial cells of 1-day-old embryos injected with *flk*(-6.4)-GFP (B), *flk*(-5.0)-GFP (C), *flk*(-4.3)-GFP (D), *flk*(-5.0,-4.3/-1.5)-GFP (E), *flk*(-5.0,-3.5/-1.5)-GFP (F) or *flk*(-4.3,-3.5/-1.5)-GFP (G).

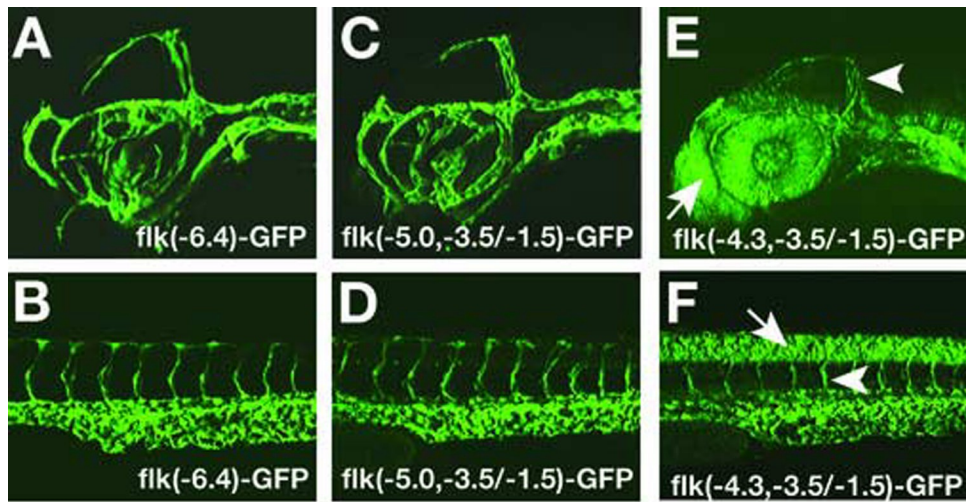


Fig3.
Transgenic analysis of the *flk1* regulatory region identifies a 1.5kb minimal endothelial specific enhancer. (A-B) GFP expression patterns in the brain (A) and trunk (B) of 2-day-old *TG(flkl:GFP)^{la116}* embryos. (C-D) GFP expression patterns of 2-dayold embryos carrying germ line integrated *flk(-5.0,-3.5/1.5)-GFP* transgene resembles the patterns observed in *TG(flkl:GFP)^{la116}*. (E-F) Embryos carrying germ line integrated *flk(-4.3,-3.5/1.5)-GFP* have GFP expression in endothelial cells (arrowhead) as well as neural tissues. Arrows point to GFP positive cells in forebrain in E and to GFP positive cells in neural tube in F.

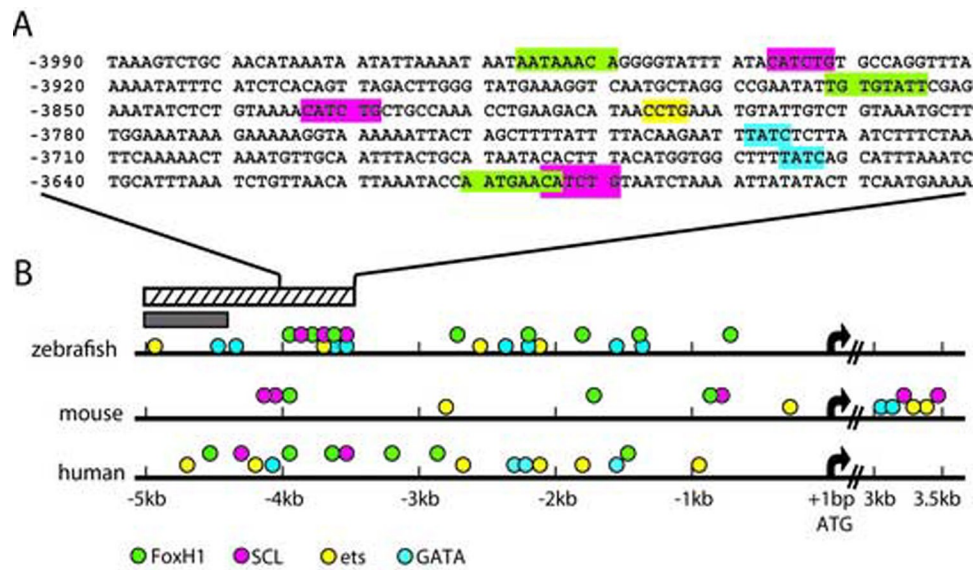


Fig4. Cross-species comparison of *flk1* 5'-flanking sequences. (A) Sequence of *flk1* enhancer from -3990 to -3571. (B) Schematic diagrams of 5'-flanking regions of zebrafish, mouse, and human *flk1* genes. Consensus sequences of FoxH1, scl, ets and GATA factor binding sites are highlighted in green, red, yellow and blue, respectively in (A) and represented with dots with the same color scheme in (B). Translation initiation sites are marked by arrows. The hatched bar indicates the zebrafish *flk1* upstream sequence from -5.0kb to -3.5kb and the grey bar indicates the region from -5.0kb to -4.3kb.

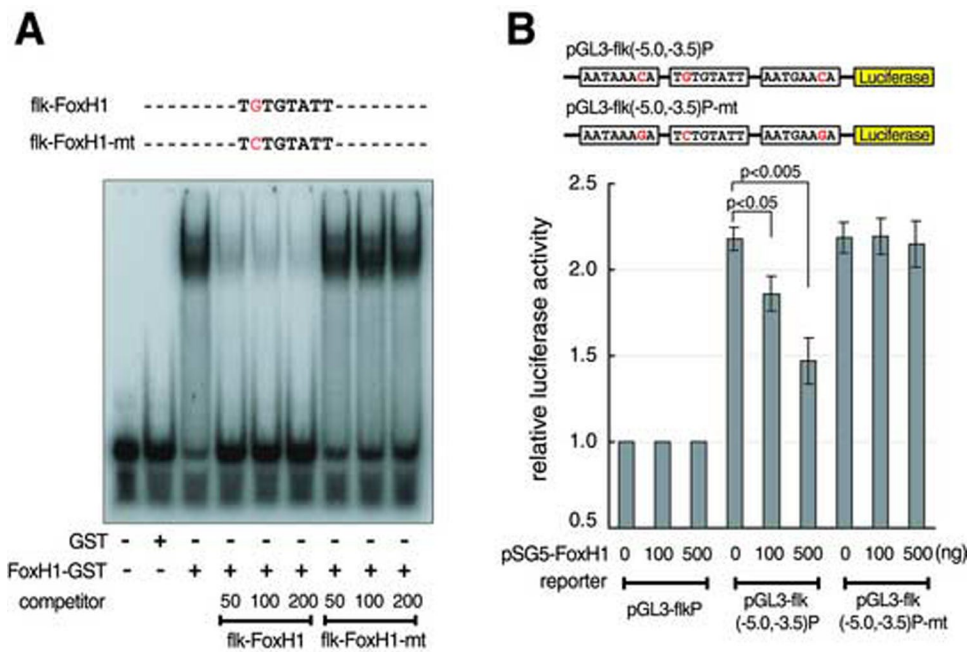


Fig5.

FoxH1 regulates *flk1* expression at the level of transcription. (A) FoxH1-GST fusion protein were purified and used in a gel shift assay with a radiolabeled probe (flk-FoxH1) corresponding to the *flk1* enhancer. Unlabeled probe and unlabeled flk-FoxH1-mt, oligonucleotides carrying a G to C substitution in the consensus sequences of the FoxH1 binding site were used as competitors in the gel shift assay at 50-, 100- or 200-fold molar excess. A representative experiment is shown. All assays were performed at least three times with comparable results. (B) HEK293T cells were transiently transfected with the indicated luciferase reporters and an expression vector encoding FoxH1. Values are relative to the luciferase activity of cells transfected with pGL3-flkP alone. Results of an average of four independent experiments are shown.

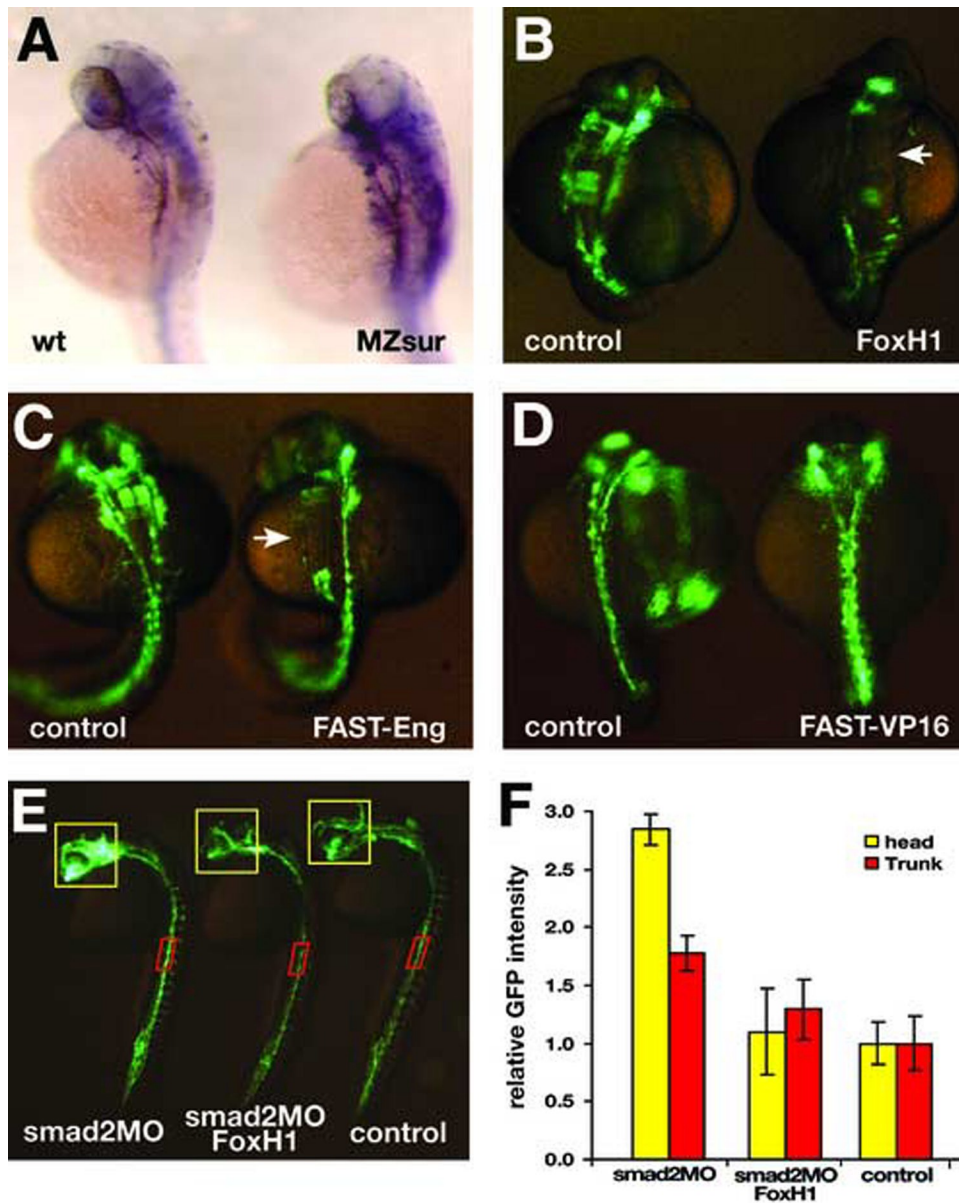


Fig6.
FoxH1 modulates vascular formation in zebrafish. (A) *flk1* is expressed in the developing vasculature of embryos at 32hpf (left). The expression level of *flk1* is elevated in MZsur embryos (right). (B) Injection of FoxH1 mRNA disrupts zebrafish vascular formation. Embryos were analyzed at the 18-somite stage. (C) Injection of mRNA encoding the FAST-Eng chimeric protein disrupts vascular formation (right), resembling the phenotype of FoxH1 overexpression. Embryos were analyzed at the 23-somite stage. (D) Injection of mRNA encoding FAST-VP16 chimeric protein does not disrupt the GFP expression pattern in *TG(flk1:GFP)^{la116}* embryos. Embryos were analyzed at the 18-somite stage. Arrows point to patches of endothelial cells missing in FoxH1 or FAST-Eng mRNA injected embryo. (E) Lateral view of the un-injected (right), *smad2MO* and FoxH1 mRNA co-injected (middle) and *smad2MO* injected (left) *TG(flk1:GFP)^{la116}* embryos at 24hpf. (F) Graph represents relative GFP intensities within the region of interests (indicated by the yellow and red boxes in panel E).