Development 133, 1529-1541 (2006) doi:10.1242/dev.02333

The TGFβ activated kinase TAK1 regulates vascular development in vivo

Joy L. Jadrich[1], Michael B. O'Connor[1,2,*] and Electra Coucouvanis[1,3,*,†]

TGFβ activated kinase 1 (TAK1) is a MAPKKK that in cell culture systems has been shown to act downstream of a variety of signaling molecules, including TGFβ. Its role during vertebrate development, however, has not been examined by true loss-of-function studies. In this report, we describe the phenotype of mouse embryos in which the *Tak1* gene has been inactivated by a genetrap insertion. *Tak1* mutant embryos exhibit defects in the developing vasculature of the embryo proper and yolk sac. These defects include dilation and misbranching of vessels, as well as an absence of vascular smooth muscle. The phenotype of *Tak1* mutant embryos is strikingly similar to that exhibited by loss-of-function mutations in the TGFβ type I receptor *Alk1* and the type III receptor endoglin, suggesting that TAK1 may be a major effector of TGFβ signals during vascular development. Consistent with this view, we find that in zebrafish, morpholinos to TAK1 and ALK1 synergize to enhance the *Alk1* vascular phenotype. Moreover, we show that overexpression of TAK1 is able to rescue the vascular defect produced by morpholino knockdown of ALK1. Taken together, these results suggest that TAK1 is probably an important downstream component of the TGFβ signal transduction pathway that regulates vertebrate vascular development. In addition, as heterozygosity for mutations in endoglin and *ALK1* lead to the human syndromes known as hereditary hemorrhagic telangiectasia 1 and 2, respectively, our results raise the possibility that mutations in human *TAK1* might contribute to this disease.

KEY WORDS: TAK1 (MAP3K7), Angiogenesis, ALK1 (ACVRL1), HHT, TGFβ

INTRODUCTION

Transforming growth factor β (TGFβ) signaling plays diverse roles in a variety of cellular processes during development (Massague, 1998). TGFβ ligands signal through a heteromeric serine/threonine kinase receptor complex consisting of both type I and type II receptors. Upon ligand binding, the two receptors are brought into close proximity and the type I receptor is phosphorylated and activated by the type II receptor (Shi and Massague, 2003). More recently, a type III TGFβ receptor, endoglin, has been isolated and is thought to assist in binding of the ligand to the receptor complex (Letamendia et al., 1998). Once activated, the type I receptor is able to phosphorylate and activate downstream signaling molecules, most notably, the SMAD intracellular transcription factors (Liu, 2003; Shi and Massague, 2003).

Proper vascular development requires precise regulation of many cellular processes, including proliferation, migration and differentiation, and has been shown to depend on TGFβ signaling (Lebrin et al., 2005; Pepper, 1997). During vasculogenesis, endothelial precursor cells (angioblasts) are formed from mesoderm and assemble into a primitive plexus (Risau and Flamme, 1995). Subsequently, angiogenesis remodels the primitive plexus through activation and resolution phases. During the activation phase, endothelial cells proliferate and migrate to form new vessels (Lebrin, 2005; Pepper, 1997). During the resolution phase, proliferation and migration cease, the basement membrane is reconstituted, and peri-endothelial cells such as smooth muscle cells and pericytes are recruited for stabilization of the vessel (Lebrin, 2005; Pepper, 1997).

Gene inactivation studies in mice have highlighted the specific roles of TGFβ signaling pathway elements in vascular development. Half of TGFβ1-null mice die at midgestation owing to defects in vasculogenesis and hematopoeisis of the yolk sac (Dickson et al., 1995). Likewise, loss-of-function mutations in the type I TGFβ receptors, *Alk1* (*Acvrl1* – Mouse Genome Informatics) and *Alk5* (*Tgfbr1* – Mouse Genome Informatics), and the type III receptor, endoglin, lead to vascular abnormalities indicative of defects in angiogenesis and result in lethality at midgestation (Arthur et al., 2000; Bourdeau et al., 1999; Larsson et al., 2001; Li et al., 1999; Oh et al., 2000; Urness et al., 2000). ALK1 and endoglin have been shown by several in vitro studies to act together downstream of TGFβ to regulate the activation phase of angiogenesis (Carvalho et al., 2004; Goumans et al., 2002; Lebrin et al., 2004). Loss of function mutations in *Smad5*, which has been shown to be activated by ALK1, result in defects in angiogenesis such as those seen in *Alk1* mutants (Yang et al., 1999).

In addition to the canonical SMAD pathway, TGFβ receptors can also activate members of the mitogen activated protein kinase (MAPK) pathway, including TGFβ activated kinase 1 (TAK1; MAP3K7 – Mouse Genome Informatics) (Mulder, 2000; Yamaguchi et al., 1995). TAK1 is a MAPKKK discovered in a suppressor screen designed to identify novel MAPK pathway members, and TAK1 kinase activity was shown to be rapidly induced in response to TGFβ (Yamaguchi et al., 1995). In vitro studies have demonstrated that expression of a dominant-negative version of TAK1 abrogates signaling downstream of TGFβ, while expression of a constitutively active version of TAK1 is able to elicit ligand-independent TGFβ responses (Ono et al., 2003; Yamaguchi et al., 1995; Zhang et al., 2000). TAK1 has also been shown to play an important role in signaling downstream of a

[1]Department of Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN 55455, USA. [2]The Howard Hughes Medical Institute, University of Minnesota, Minneapolis, MN 55455, USA. [3]Stem Cell Institute, University of Minnesota, Minneapolis, MN 55455, USA.

*Present address: R&D Systems, Inc., 614 McKinley Place, NE, Minneapolis, MN 55413, USA
†Authors for correspondence (e-mail: moconnor@mail.med.umn.edu and ecoucouvanis@gmail.com)

Accepted 9 February 2006

variety of other molecules in vitro, including BMP, WNT and IL1 (Ishitani et al., 2003; Ninomiya-Tsuji et al., 1999; Shibuya et al., 1998).

TAK1 function has been studied using a variety of model organisms. Ectopic overexpression of TAK1 in *Xenopus* embryos results in apoptosis, but when the apoptotic inhibitor BCL2 is co-expressed, embryos are ventralized. In this system, TAK1 activation is mediated by BMP signaling. Interestingly, further experiments in *Xenopus* showed that loss of TAK1 function, by overexpression of dominant-negative constructs, also blocks ventralization caused by ectopic expression of SMAD1 and SMAD5, suggesting a possible cooperation between the canonical SMAD pathway and TAK1 (Shibuya et al., 1998). Mutations in the Drosophila *Tak1* gene do not affect development. Mutant flies are fully viable and fertile but they do not produce antibacterial peptides and are highly susceptible to Gram-negative bacterial infection as a result of defects in rel/NF-κB-dependent innate immune responses (Park et al., 2004; Vidal et al., 2001). In *C. elegans*, loss of TAK-1 function results in a loss of endoderm owing to defects in WNT signaling (Meneghini et al., 1999).

We have previously shown that TAK1 is widely expressed during mammalian development (Jadrich et al., 2003); however, limited information is available regarding the role of mammalian TAK1 during development in vivo. Expression of constitutively active TAK1 in the heart was found to induce cardiac hypertrophy (Zhang et al., 2000), and expression of dominant-negative TAK1 in rat liver accelerates cell cycle progression following partial hepatectomy (Bradham et al., 2001). The degree to which TAK1 normally functions to regulate cardiomyocyte and hepatocyte function has not been determined. To identify potential functions for TAK1 in vertebrate development, we generated TAK1-deficient mice. We find that TAK1 is essential for proper embryonic development and provide evidence that it functions in conjunction with the TGFβ pathway in vivo. Specifically, loss of mouse TAK1 causes defects in developmental angiogenesis similar to those described for TGFβ pathway members ALK1, endoglin and SMAD5. We also demonstrate that in zebrafish, knockdown of TAK1 and the TGFβ receptor ALK1 synergistically enhance vascular defects, while overexpression of TAK1 is able to partially rescue the vascular phenotype caused by knockdown of ALK1. Taken together, our results provide the first evidence that TAK1 is required in vivo for proper development of the vasculature, possibly through a TGFβ responsive pathway.

MATERIALS AND METHODS

Generation of *Tak1*-null mice

We obtained ES cells containing a genetrap insertion in the first intron of the *Tak1* locus (Fig. 1A) from BayGenomics (BayGenomics cell line XB444) (Stryke et al., 2003). The genetrap construct contains a splice acceptor upstream of the β-galactosidase and neomycin resistance fusion gene, *βgeo*. The *βgeo* gene is linked via an internal ribosome entry site (IRES) to the placental alkaline phosphatase gene (PLAP). The insertion in *Tak1* is the only insertion we detected in XB444 cells by Southern blot (data not shown). Sequencing of the *Tak1* locus in XB444 cells revealed that the insertion was associated with a 12 bp deletion in the genomic sequence.

XB444 cells were injected into C57BL/6J blastocysts, and the resulting chimeric mice were outcrossed to C57BL/6J (Jax Laboratory) to test for germline transmission. Two chimeric male mice transmitted the *Tak1* genetrap allele through their germline and were used in the subsequent studies. No phenotypic differences were seen between descendents of the two founder chimeric mice.

Genotyping of the pups was performed by Southern blot or PCR. For Southern blotting, genomic tail or yolk sac DNA was isolated as described (Laird et al., 1991), digested with *Hin*dIII, and then hybridized with a probe corresponding to a 1 kb region of the *Tak1* locus just outside the insertion site (Fig. 1A). The probe was labeled with [^{32}P]-αATP using the Random Primed DNA Labeling kit (Roche). For PCR genotyping, a forward primer just upstream of the insertion site (5′-AGTCCCAGAATGTCGACCAC-3′) and a reverse primer just downstream of the insertion site (5′-AGTGGCCACCAATTTCACAT-3′) were used to detect the wild-type allele. The mutant allele was detected using the reverse primer from the wild-type PCR and a forward primer located within the end of the genetrap vector (5′-CCTCTTCGCTATTACGCC AG-3′). Both sets of primers were annealed at 58°C for 30 cycles. The wild-type and mutant primer pairs amplify 290 bp and 391 bp fragments, respectively (data not shown).

To analyze levels of wild-type *Tak1* and *Tak1*Δ/Δ fusion transcripts, cDNA was made from E9.5 embryos using the Thermoscript RT-PCR system (Invitrogen). Wild-type *Tak1* transcript was amplified using the following primers: Takexon1, 5′-CTTCTGCCAGTGAGATGATC-3′; Takexon2, 5′-TGAAAGCCTTCCTCTCAGAC-3′; *Tak1*Δ/Δ fusion transcript was amplified using the Takexon1 primer above; and βgeo1, 5′-TCTTCGCTATTACGCCAGCT-3′. Actin was amplified using the following primers: Actin1, 5′-GCTCCGGCATGTGCAA-3′; Actin2, 5′-AGGATGTTCATGAGGTAGT-3′. All three primer sets were annealed at 54°C for 35 cycles.

Western blotting

E10.5 embryos were dissected out in PBS and extra-embryonic membranes removed for genotyping. Dissected embryos were lysed in modified RIPA buffer (50 mM Tris-Cl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na orthovanadate, 1 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) for 15 minutes at 4°C. Lysates were cleared by centrifugation for 15 minutes at 4°C and quantitated with the BioRad Protein Assay reagent.

Protein (10 μg) was electrophoresed on a 4-12% gradient NuPAGE gel (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). Blots were blocked and then incubated with the following primary antibodies: α-full-length TAK1 (1:500; Santa Cruz Biotechnology), α-C-terminal TAK1 (3 μg/ml Upstate), α-βgalactosidase (1:1000 Promega) or α-β-tubulin (1:500; University of Iowa Hybridoma Bank), and then incubated with the following secondary antibodies: IRDye800 α-rabbit or IRDye700 α-mouse (1:8000; Rockland). The signal was visualized using the Odyssey Infrared Imaging System (Li-Cor).

In situ hybridization

Antisense digoxigenin labeled RNA probes were made as described (Hogan et al., 1994). The *Anf* (Bruneau et al., 2001) and *Gata4* (Molkentin et al., 1997) probes have been previously described. Whole-mount in situ hybridization was performed essentially as described previously (Hogan et al., 1994; Wilkinson and Nieto, 1993).

Immunohistochemistry and X-gal staining

X-gal staining of whole embryos was done as described (Hogan et al., 1994). Whole-mount immunohistochemistry with the α-PECAM (1:100; Pharmingen) and α-SMA (1:100; Sigma) primary antibodies was carried out on 4% paraformaldehyde fixed embryos as described (Corson et al., 2003; Hogan et al., 1994).

Section immunohistochemistry was performed on 4% paraformaldehyde fixed tissue that was embedded in paraffin and sectioned as described (Jadrich et al., 2003). Staining with α-SMA antibody and counterstaining in 0.5% Methyl Green were performed as previously described (Freemark et al., 1997; Jadrich et al., 2003).

Cloning of the zebrafish *tak1* homolog

cDNA was made from 2-day-old zebrafish embryos as described above. The zebrafish *tak1* homolog was amplified using the Expand High Fidelity PCR system (Roche) from this cDNA using the following primers: TAK1, 5′-TCATGAGGTGCCCTGTCTCTTCTGC-3′; TAK2, 5′-ATGTCTATGCCCTCCGCCGATATGC-3′. The primers were designed based on sequence alignment of the mouse *Tak1* gene with the predicted gene, ensdarg00000020469, in the Ensembl database.

The zebrafish *tak1* PCR fragment was subcloned into pT3TS for RNA synthesis (Hyatt and Ekker, 1999). Kinase-dead *tak1* (K52A*tak1*) was made by replacing lysine 52 with an alanine using the QuikChange Site-Directed

Mutagenesis kit (Stratagene) with the following primers: K52ATAKF, 5′-GCAGAGATGTGGCCATCGCGACTATAGAGAGTG-3′; K52ATAKR, 5′-CACTCTCTATAGTCGCGATGGCCACATCTCTGC-3′. For RNA synthesis of *Gfp*, the *Gfp*-coding sequence was subcloned from pEGFP (Clontech) into pT3TS.

Zebrafish injection

Gfp, *tak1*, or K52A*tak1* capped mRNA was made by in vitro transcription from the plasmids described above using the mMessage mMachine kit (Ambion). Both *tak1* and K52A*tak1* were found to cause significant lethality when injected alone at concentrations over 100 pg. At 100 pg, *tak1* and K52A*tak1* resulted in no significant lethality (data not shown).

Vbg and *Smad5* morpholinos were used as previously described (Roman et al., 2002; Lele et al., 2001). *tak1* morpholinos were designed to interrupt splicing between exons 6 and 7 with the sequences: TAK1MO, 5′-GGA-AAGTATTCAAAACTTGCCTTCG-3′; TAK1MOmis, 5′-GCAAACTAT-TGAAAACTGCCTTCG-3′ (the mismatch control morpholino used). All morpholinos were synthesized by Gene Tools. All morpholinos (resuspended in Danieau solution) and mRNAs were injected into the marginal zones of one-cell stage embryos from Fli::GFP; Gata::DsRed fish and embryos were allowed to develop 48 hours before analyzing the vascular phenotype by fluorescence microscopy. Statistical significance was calculated using the Student's *t*-test.

To detect interruption of splicing by the *tak1* morpholino, cDNA was made as described above from 24-hour morpholino-injected fish. The following primers were used to detect inclusion of intron 6: Takexon6, 5′-ACTGTCCTGAAGATCTGTGAC-3′; Tak1intron6, 5′-TCCATCATCTA-GACCAGGAAC-3′.

RESULTS

Embryos homozygous for the *Tak1* genetrap allele die by 12.5 days of gestation

To determine the effect of TAK1 protein loss during mouse development, we created mice carrying a genetrap insertion in the *Tak1* locus using embryonic stem cells from BayGenomics (Stryke et al., 2003). The *Tak1* locus consists of 16 exons; the insertion is located in the first intron, leading to a predicted in frame fusion transcript between the first exon of *Tak1* and full-length *βgeo* (Fig. 1A).

The genetrap-containing cells were used for blastocyst injections to create mice heterozygous for the *Tak1* genetrap allele. Mice were genotyped by Southern blot (Fig. 1B) or PCR (data not shown). We will refer to the genetrap allele as *Tak1*Δ.

To determine if the *βgeo* gene was under the control of the endogenous *Tak1* promoter, *Tak1*+/Δ embryos at different stages were stained for β-galactosidase activity using X-gal. The pattern of X-gal staining reproduced the previously reported ubiquitous expression pattern of TAK1 at E9.5 (data not shown) (Jadrich et al., 2003).

Homozygous mutant mice, on a mixed 129/C57BL/6J background, were produced by intercrossing *Tak1*+/Δ mice. Genotyping of the resultant progeny demonstrated that no *Tak1*Δ/Δ embryos survived to term out of 225 live births (Table 1).

To determine the stage at which the homozygous mutant embryos are lost, litters from *Tak1*+/Δ intercrosses were dissected out at various time points. Genotyping of embryos was performed by Southern blot of DNA extracted from the yolk sac. We found that the latest time *Tak1*Δ/Δ embryos could be recovered was E11.5 (Table 1).

Until E8.5, *Tak1*Δ/Δ embryos could not be distinguished from wild-type embryos (see Fig. S1 in the supplementary material). At E9.5, *Tak1*Δ/Δ embryos exhibited varying severity of phenotype. To reflect this we assigned *Tak1*Δ/Δ embryos to one of three classes. Class I embryos comprised 31% of *Tak1*Δ/Δ embryos, contained localized areas of necrosis, were reduced in size compared with wild-type and *Tak1*+/Δ embryos at this stage, and had small heads and truncated curved tails (Fig. 2A, +/Δ; Fig. 2C, Δ/Δ). Embryos of this class also had pericardial edemas (Fig. 2C, arrow). Fifty-five

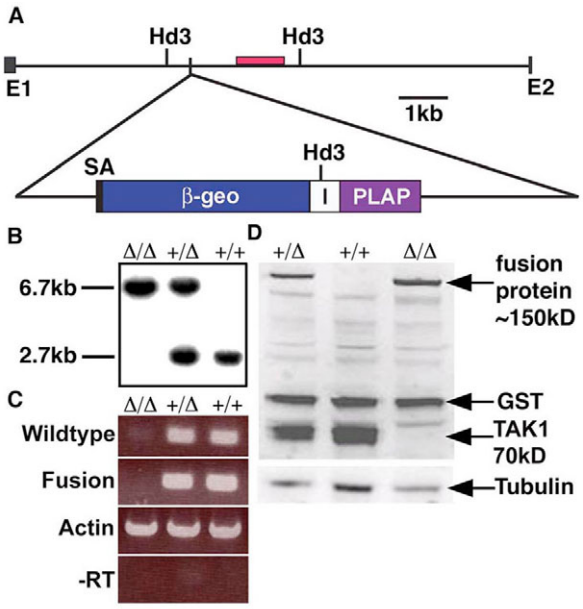

Fig. 1. Structure of the *Tak1* genetrap allele and western analysis of TAK1 protein. (**A**) The genetrap insertion in the *Tak1* locus. The insertion is between exon 1 (E1) and exon 2 (E2) of *Tak1*. The genetrap contains a splice acceptor (SA) upstream of the *βgeo* gene, which is linked via an internal ribosome entry site (I) to the placental alkaline phosphatase gene (PLAP). Genomic DNA was digested with *Hin*dIII (Hd3) for Southern blotting using a probe just outside the insertion site (red box). (**B**) Genotyping by Southern blot and transcript levels in *Tak1*Δ/Δ embryos. Southern blot of DNA extracted from yolk sacs of E9.5 embryos derived from *Tak1*+/Δ intercrosses. The wild-type and genetrap alleles result in probe hybridization to 2.7 kb and 6.7 kb bands, respectively. (**C**) rtPCR analysis of wild-type transcript (primers amplifying exon1 to exon 2 of *Tak1*), fusion transcript (primers amplifying exon1 to *βgeo*) and *actin* as a control for the amount of cDNA that was amplified in each sample. Samples that did not contain reverse transcriptase (–RT) were used as a control for DNA contamination. A small amount of wild-type transcript is detected in *Tak1*Δ/Δ samples. (**D**) Western blot analysis of lysates from E10.5 embryos derived from *Tak1*+/Δ intercrosses with a polyclonal antibody to full-length TAK1. A 70 kDa doublet is visible in *Tak1*+/+ and +/Δ samples, but is missing in the *Tak1*Δ/Δ sample. The TAK1 antibody used also detects a GST background band (labeled GST) and a band of 150 kDa in *Tak1*+/Δ and Δ/Δ samples, which is probably the predicted fusion protein between exon 1 of *Tak1* and *βgeo*. The amount of total protein per lane was determined by assaying β-tubulin levels.

Table 1. Genotyping of *Tak1*+/Δ intercrosses

Age	+/+	+/Δ	Δ/Δ	Resorbed
E8.5	28 (29)	39 (41)	29 (30)	0 (0)
E9.5	82 (25)	154 (48)	86 (27)	0 (0)
E10.5	27 (22)	57 (46)	40 (32)	0 (0)
E11.5	7 (24)	15 (52)	7 (24)	0 (0)
E12.5	3 (11)	17 (61)	4* (14)	4 (14)
Postnatal	84 (37)	141 (63)	0 (0)	N/A

All genotyping was carried out by Southern blot. Numbers in parentheses indicate % of total.
*Four out of the E12.5 embryos were in the process of resorption with yolk sac tissue intact for genotyping.

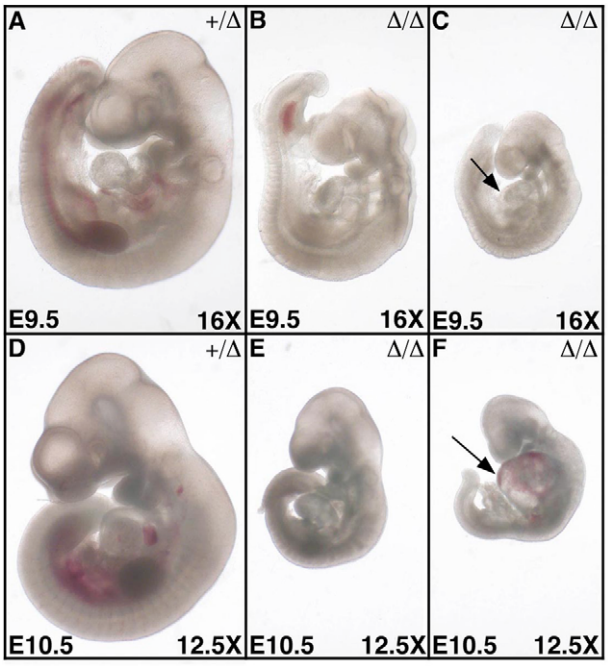

Fig. 2. *Tak1*Δ/Δ embryos are abnormal by E9.5. (**A-C**) E9.5 embryos. (**D-F**) E10.5 embryos. (A,D) *Tak1*+/Δ control embryos. (B,C,E,F) *Tak1*Δ/Δ embryos. The Class II *Tak1*Δ/Δ embryo at E9.5 (B) is slightly reduced in size. The Class I *Tak1*Δ/Δ embryo (C) is further reduced in size in comparison with the embryo in B, and has a pericardial edema (arrow). The less severely affected *Tak1*Δ/Δ embryo at E10.5 (E) is reduced in size and has a small head. The more severely affected *Tak1*Δ/Δ embryo at E10.5 (F) is severely reduced in size, and has localized areas of necrosis and a large pericardial edema (arrow).

percent of *Tak1*Δ/Δ embryos at E9.5 were designated class II, and were reduced in size and occasionally had small edemas (Fig. 2B). The remaining 14% (class III) could not be morphologically distinguished from wild-type embryos (data not shown). Class III presumably gives rise to the less affected class seen at E10.5, discussed below.

At E10.5, wild-type and *Tak1*+/Δ embryos had grown significantly in size from E9.5 and had blood visible throughout the vasculature (Fig. 2D). By contrast, 88% of *Tak1*Δ/Δ embryos were severely reduced in size, and had large pericardial edemas (Fig. 2F). Occasionally, edemas were also seen along the body wall (data not shown). These embryos presumably reflect classes I and II combined from E9.5. The remaining 12% were less severely affected, but were reduced in size and in most cases these embryos also had pericardial edemas (Fig. 2E). This 12% presumably reflects class III from E9.5. At E11.5, all *Tak1*Δ/Δ embryos were dead.

At all stages examined, *Tak1*+/Δ embryos were indistinguishable from wild-type embryos. For the subsequent analysis of the *Tak1*Δ/Δ phenotype, embryos with significant areas of necrotic tissue were not used, to ensure that the effects observed were not secondary to dying tissue.

No wild-type TAK1 protein is produced from the genetrap allele

To determine if any functional TAK1 protein could be produced from the genetrap allele, we performed PCR on cDNA derived from *Tak1*Δ/Δ embryos to detect if any wild-type transcript is made. We found that the *Tak1*Δ/Δ embryos do make an extremely low level of wild-type transcript, although we cannot rule out that this may be due to contaminating maternal tissue (Fig. 1C). Additionally, it is unlikely that this is sufficient for TAK1 signaling as others have created a null allele of *Tak1*, which results in the same time of death as we see in *Tak1*Δ/Δ embryos (Sato et al., 2005). To determine if any wild-type TAK1 protein is made from the genetrap allele, *Tak1*Δ/Δ embryos were analyzed by western blotting using a polyclonal antibody that recognizes epitopes along the entire TAK1 protein. We detected an apparent doublet in wild-type and *Tak1*+/Δ lysates corresponding to TAK1, which was not visible in *Tak1*Δ/Δ lysates (Fig. 1D). No difference in the amount of TAK1 protein was seen between the different classes of mutants (data not shown).

The location of the genetrap insertion would predict an in-frame fusion protein between the first exon of *Tak1* and full length *βgeo* (Fig. 1A). In *Tak1*+/Δ and *Tak1*Δ/Δ lysates, a band was seen at ~150 kDa. This same band was recognized by antibodies raised against the C-terminal end of β-galactosidase, but was not recognized by an antibody specific for the C-terminal end of TAK1 (data not shown), indicating that the 150 kDa band represents the predicted fusion protein between exon 1 of *Tak1* and *βgeo*. Exon 1 contains the first 40 amino acids of the TAK1 protein, which includes the first 10 amino acids of the kinase domain, and lacks 10 out of the 11 conserved regions required for kinase function (Hanks et al., 1988). Therefore, we conclude that this fusion protein is unlikely to retain any TAK1 function.

Analysis of heart development in *Tak1*Δ/Δ embryos

The pericardial edema seen in the *Tak1*Δ/Δ embryos prompted us to focus our analysis on the development of the heart and vascular system. The *Tak1*Δ/Δ hearts at E8.5 were morphologically indistinguishable from wild-type hearts. Cardiac looping and chamber specification appeared normal in *Tak1*Δ/Δ embryos (Fig. 3). Sections of *Tak1*Δ/Δ hearts were immunostained with an antibody to smooth muscle α-actin (SMA), to label the myocardium. At E9.5, *Tak1*Δ/Δ hearts showed areas in which the compact myocardium and trabeculae appeared thin and disorganized in comparison with wild-type hearts (compare Fig. 3A with 3B).

To determine whether the heart was correctly specified and patterned, we looked at two markers for heart development by in situ hybridization. First, we looked at expression of the zinc-finger transcription factor, *Gata4*. GATA4 is one of the earliest markers for cardiac specification and is required for the formation of ventral heart structures (Arceci et al., 1993; Kuo et al., 1997). *Gata4* expression in the extension of the primary heart field and inflow tract in E9.5 *Tak1*Δ/Δ embryos appeared normal (Fig. 3D). Next, we looked at the later cardiac specification marker atrial natriuretic factor (ANF). ANF is downstream of early cardiac specification markers Nkx2.5 and GATA4, and is expressed in the atrium and ventricle of the mouse embryo starting at E8.0 (Durocher et al., 1996; Zeller et al., 1987). At E9.5, the localization and intensity of *Anf* in the ventricle and atrium within the *Tak1*Δ/Δ heart was normal. We noted a slightly smaller field of expression in the ventricle, as well as a significant overall decrease in *Tak1Δ/Δ* heart size, probably owing to developmental delay (Fig. 3F). Additionally, protein expression levels (as assayed by immunohistochemistry) of platelet endothelial cell adhesion molecule (PECAM) in the endocardium (Fig. 5B, asterisk) and SMA in the myocardium (Fig. 3B) were normal. We conclude that TAK1 is not essential for early cardiac specification.

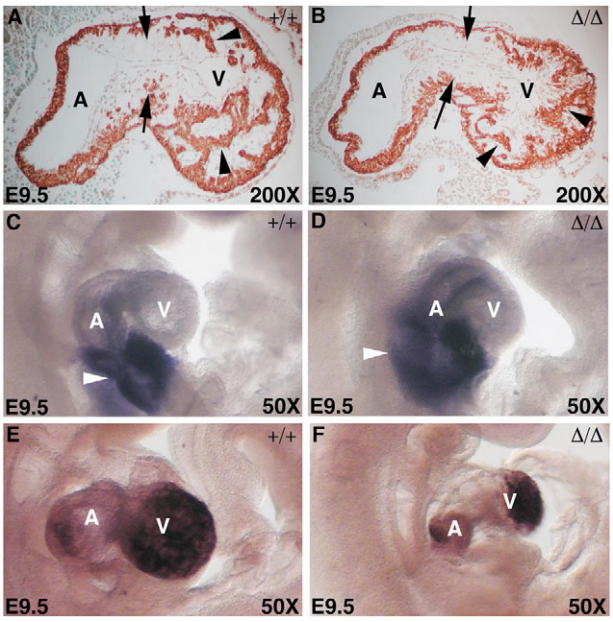

Fig. 3. *Tak1*Δ/Δ embryonic heart phenotype. (A,C,E) Wild-type E9.5 embryos. (B,D,F) E9.5 *Tak1*Δ/Δ embryos. (**A**,**B**) Sagittal sections immunostained with an antibody to smooth muscle α actin, labeling the myocardium (brown). Arrowheads indicate trabeculation within each ventricle. Endocardial cushion formation takes place normally in both embryos (arrows) and the curved myocardium in both panels indicates that looping has taken place. (**C**,**D**) Embryos labeled with an antisense probe to *Gata4*. *Gata4* labels the extension of the primary heart field and inflow tract at this stage (arrowhead). (**E**,**F**) Embryos labeled with an antisense probe to *Anf*, marking the atrium and ventricle. A, atrium; V, ventricle.

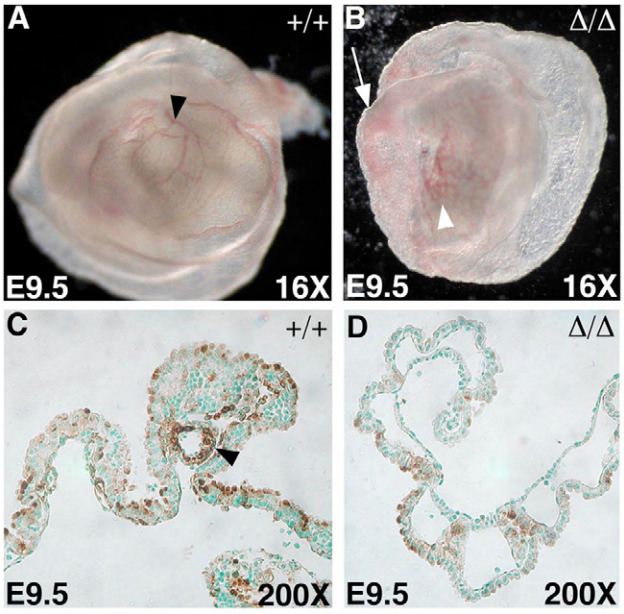

Fig. 4. The yolk sac vasculature of the *Tak1*Δ/Δ embryos fails to undergo angiogenesis. (**A**,**B**) E9.5 yolk sacs of *Tak1*+/Δ and *Tak1*Δ/Δ embryos, respectively. The yolk sac vascular plexus in A has undergone angiogenesis to create a branched network of mature vitelline vessels (arrowhead). The yolk sac vascular plexus in B has not remodeled but has retained its honeycomb-like structure of blood islands (arrowhead). The vessels in B are also prone to rupture, causing pooling of blood in the yolk sac (arrow). (**C**,**D**) Cross-sections of *Tak1*+/Δ and *Tak1*Δ/Δ yolk sacs, respectively, that are immunostained for vSMCs with an antibody to SMA (brown). The *Tak1*+/Δ yolk sac in C contains a mature vitelline vessel surrounded by a layer of smooth muscle (arrowhead). The *Tak1*Δ/Δ yolk sac in D has no mature vitelline vessels, the existing vessels are extremely dilated and have little SMA expression.

Extra-embryonic vasculature is abnormal in *Tak1*Δ/Δ embryos

We next examined the vasculature of *Tak1*Δ/Δ embryos. Proper vascularization of the placenta is important for a variety of functions, including nutrient exchange between the embryo and mother (Rossant and Cross, 2001). We analyzed placental structure by histology at E9.5 and found no obvious differences between wild-type and *Tak1*Δ/Δ embryos (data not shown). As embryos are not dependent on placental exchange at E9.5, even undetected placental abnormalities would be unlikely to cause the defects seen in *Tak1*Δ/Δ embryos at this stage (Cross et al., 1994).

The blood islands of the yolk sac are the first sites of vasculogenesis and hematopoiesis in the mouse embryo (Flamme et al., 1997). Blood islands are small pockets of endothelial cells surrounding primitive red blood cells. These pockets fuse to give rise to the primitive vascular plexus of the yolk sac (Hirakow and Hiruma, 1981). In wild-type embryos, beginning around E9.0, this primitive vascular plexus undergoes a remodeling process in which the initial honeycomb-like pattern of vasculature is remodeled into a more branched pattern of mature vitelline vessels (Risau, 1997). In *Tak1*Δ/Δ yolk sacs, this remodeling did not take place and the vessels remained in the honeycomb-like pattern (compare Fig. 4A with 4B). Sections of *Tak1*Δ/Δ yolk sacs revealed that the vasculature was dilated and mature vitelline vessels were not present (compare Fig. 4C, arrowhead indicates mature vitelline vessel in wild-type yolk sac, with Fig. 4D). In some cases, the extreme dilation of the yolk sac vasculature resulted in rupture and pooling of blood inside the yolk sac (Fig. 4B, arrow). *Tak1*Δ/Δ embryos formed blood islands normally, suggesting that hematopoiesis was initiated in the mutant embryos (Fig. 4B, arrowhead). Interestingly, in contrast to embryonic defects, the defects seen in *Tak1*Δ/Δ yolk sacs were the same in all classes of mutants.

*Tak1*Δ/Δ embryos have vascular defects

To determine if the vasculature of the embryo proper was also abnormal, we performed whole-mount immunohistochemistry using an antibody to PECAM to visualize the vasculature. PECAM is one of the earliest endothelial cell markers expressed during mouse development (Baldwin et al., 1994). At E8.5, the pattern of PECAM staining in *Tak1*Δ/Δ embryos was indistinguishable from that in wild-type embryos (see Fig. S1 in the supplementary material). This result indicates that vasculogenesis was normal.

At E9.5, wild-type and *Tak1*+/Δ embryos displayed a continuous dorsal aorta with uniform width along the length of the embryo (Fig. 5A, arrowheads). By contrast, in the majority of *Tak1Δ/Δ* embryos from class II, the anterior dorsal aorta and branchial arch arteries appeared collapsed and discontinuous (Fig. 5C, arrowheads). The posterior dorsal aorta in these embryos was dilated (Fig. 5C, arrow) in comparison with wild type (Fig. 5A, arrow). Rarely, we saw *Tak1*Δ/Δ embryos of class II with a severely dilated anterior but

normal posterior dorsal aorta (Fig. 5B, arrowheads). It is possible that the dilated anterior dorsal aorta is a precursor to the collapsed dorsal aorta more commonly seen at this stage.

To analyze the structure of the abnormal vessels seen in the *Tak1*Δ/Δ embryos more thoroughly, PECAM-stained embryos were sectioned and examined histologically. This analysis confirmed the lack of integrity of the dorsal aorta and also showed severe dilation of the cranial vasculature (Fig. 5E,F,H,I). In most samples, the wall of the dorsal aorta was not continuous and in some sections it was missing entirely (Fig. 5I). Sections also revealed that the cardinal vein became dilated and in very rare cases fused to the dorsal aorta (data not shown).

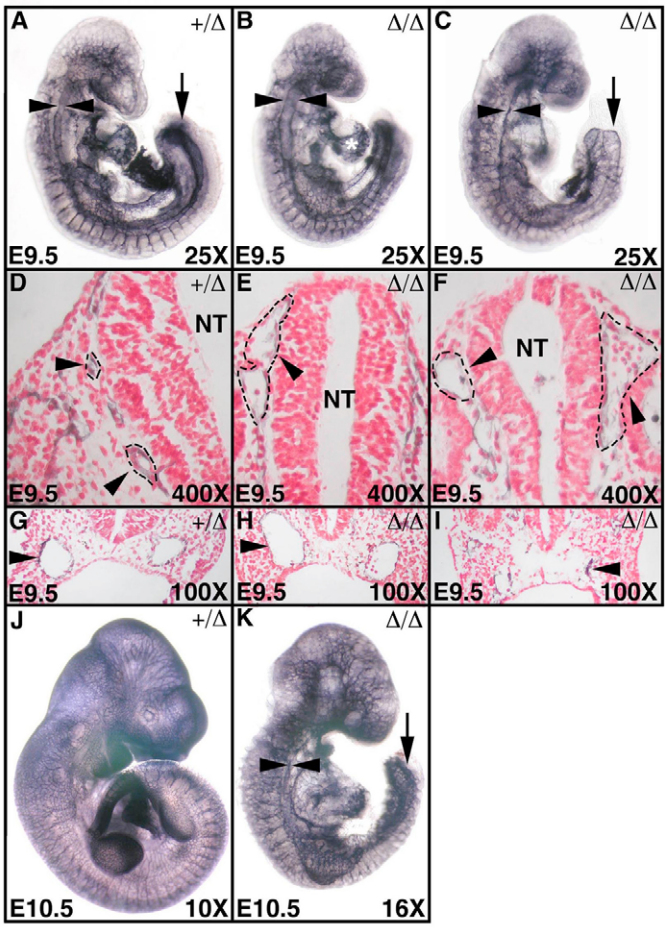

Fig. 5. The vascular architecture of *Tak1*Δ/Δ embryos is abnormal. In all panels, the developing vasculature of embryos was visualized by whole mount immunohistochemistry using an α-PECAM antibody. (**A**) *Tak1*+/Δ at E9.5. (**B**,**C**) *Tak1*Δ/Δ at E9.5. Two different vascular abnormalities are seen in *Tak1*Δ/Δ embryos. The embryo in B has an extremely dilated anterior dorsal aorta (arrowheads) in comparison with the *Tak1*+/Δ dorsal aorta in A (arrowheads). The embryo in C has a collapsed anterior dorsal aorta (arrowheads) and a dilated posterior dorsal aorta (arrow) in comparison with the posterior dorsal aorta in A (arrow). (**D-F**) Cranial sections of the heads shown in A-C, respectively. Arrowheads and broken lines indicate examples of cranial vessels that are extremely dilated in *Tak1*Δ/Δ embryos (E,F) in comparison with heterozygous embryos (D). (**G-I**) Cranial sections through the dorsal aorta of the embryos in A-C, respectively. Arrowheads indicate the dilated dorsal aorta in H and collapsed dorsal aorta in I in comparison with the heterozygote (arrowhead in G). (**J**) E10.5 *Tak1*+/Δ embryo exhibiting extensive remodeling of the vessels, most visibly in the head. (**K**) *Tak1*Δ/Δ embryo at E10.5, which has much less remodeling in comparison with the embryo in J (compare the tree-like branching vasculature of the head in J with the more honeycomb-like vasculature in K). The embryo in K also has a collapsed anterior (arrowhead) and posterior (arrow) dorsal aorta.

By E10.5, the vascular phenotypes were more pronounced. Wild-type and heterozygous embryos at this stage exhibited extensive branching and complexity in the vasculature (Fig. 5J). In the majority of E10.5 *Tak1*Δ/Δ embryos, the vasculature appeared to be disintegrating and the dorsal aorta was no longer visible (data not shown). Less severely affected *Tak1*Δ/Δ embryos at E10.5 looked similar to the class I and II E9.5 *Tak1*Δ/Δ embryos, and displayed a collapsed anterior dorsal aorta (Fig. 5K, arrowheads). Whereas at E9.5 we saw a primarily collapsed anterior and dilated posterior dorsal aorta, at E10.5 both the anterior and posterior dorsal aorta appeared collapsed (Fig. 5K, arrow), further indicating that dilation may be a precursor to the collapse of *Tak1*Δ/Δ vessels.

In addition to the abnormalities in vessel size and structure, we also saw vascular branching defects in *Tak1*Δ/Δ embryos. This was most easily visible in the head vasculature. In wild-type and *Tak1*+/Δ embryos, the primary capillary plexus initially set up by vasculogenesis is remodeled from a honeycomb-like pattern into a characteristic tree-like pattern. In *Tak1*Δ/Δ embryos, this remodeling was defective. Some vascular remodeling in the head was visible in less severely affected embryos, but the majority of vessels retained their more primitive honeycomb-like pattern (compare Fig. 5J with 5K).

*Tak1*Δ/Δ embryos display vascular smooth muscle defects

To further explore potential angiogenic defects in the *Tak1*Δ/Δ embryos, we looked for the presence of vascular smooth muscle cells (vSMC). vSMC are accessory cells that provide support and contractility to vessels (Owens et al., 2004). We analyzed vSMC by immunohistochemistry using an antibody specific for smooth muscle α-actin (SMA), one of the earliest known markers for smooth muscle in the developing mouse embryo (Takahashi et al., 1996).

At E8.5, there was little to no SMA staining in the vasculature of wild-type and *Tak1*Δ/Δ embryos (data not shown). In wild-type and *Tak1*+/Δ embryos at E9.5 and E10.5, SMA expression was observed in the myocardium and progressively extending from anterior to posterior surrounding the dorsal aorta (Fig. 6A,D). In *Tak1*Δ/Δ embryos at E9.5 and E10.5, SMA expression was extremely reduced or absent from the vasculature (Fig. 6B,C,E,F). At E9.5, no vascular SMA expression was seen in any class of *Tak1*Δ/Δ embryo (Fig. 6B,C). In the least affected class at E10.5, some SMA-positive cells were loosely associated with the dorsal aorta (Fig. 6E, arrowheads).

SMA expression was also abnormal in the yolk sac of mutant embryos. Upon sectioning, mature vitelline vessels lined with smooth muscle, as assayed by SMA expression, were easily visible in wild-type embryos (Fig. 4C, arrowhead). No smooth muscle was visible in *Tak1*Δ/Δ yolk sac sections (Fig. 4D).

Tak1 genetically interacts with *Alk1* in zebrafish

Loss-of-function mutations in the TGFβ type I receptor, *Alk1* and the type III receptor endoglin (Arthur et al., 2000; Bourdeau et al., 1999; Li et al., 1999; Oh et al., 2000; Urness et al., 2000) are phenocopied by the *Tak1*Δ/Δ embryos described above, suggesting that TAK1 could be acting downstream of TGFβ and ALK1 to regulate vascular development in vivo.

To test this hypothesis, we performed genetic interaction and rescue experiments using a zebrafish model. Roman et al. (Roman et al., 2002) have previously characterized mutations in the zebrafish

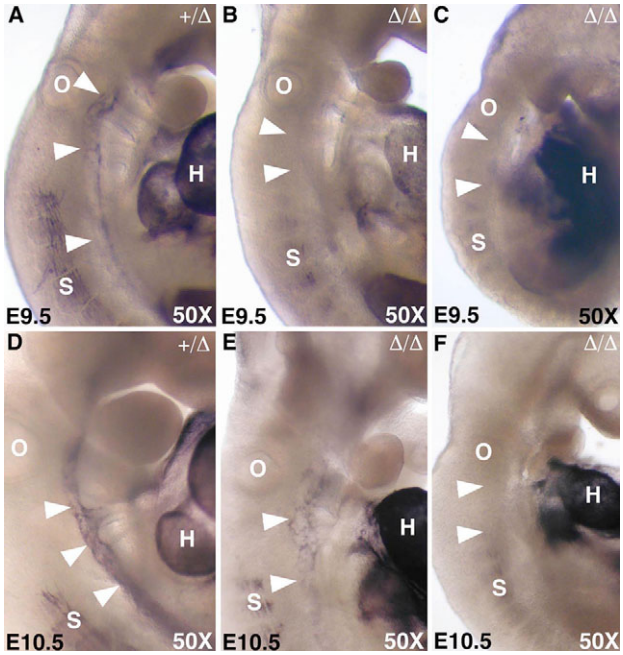

Fig. 6. *Tak1*Δ/Δ embryos have defects in vascular smooth muscle. To visualize smooth muscle, embryos were stained by immunohistochemistry using an antibody to SMA (purple staining in all panels). (A-C) E9.5 embryos. (D-F) E10.5 embryos. At E9.5 the *Tak1*+/Δ control embryo has a layer of SMA-expressing cells beginning along the dorsal aorta (**A**, arrowheads). The class II (**B**) and class I *Tak1*Δ/Δ (**C**) embryos have no SMA expression along their dorsal aorta (arrowheads). At E10.5, the *Tak1*+/Δ control embryo (**D**) shows a continuous layer of SMA expression all along the dorsal aorta (arrowheads). The more severely affected *Tak1*Δ/Δ embryo (**F**) has a complete absence of SMA expression in the dorsal aorta (arrowheads) and the less severely affected embryo (**E**) has only a few loosely associated expressing cells (arrowheads). All the embryos display staining of the somites and heart. S, somites; O, otic vesicle; H, heart.

Alk1 ortholog *violet beauregard* (*vbg*; *acvrl1* – Zebrafish Information Network). Loss-of-function mutations in *vbg* cause severe dilation of the cranial vasculature leading to loss of blood flow to the trunk and tail. This phenotype can be recapitulated through antisense morpholino injections specific to the *vbg* sequence (Roman et al., 2002). If TAK1 functions in conjunction with TGFβ signaling, then we expect that loss of *Tak1* might synergize with loss of *Alk1* to produce vascular defects. In addition, if TAK1 is downstream of ALK1, we might expect that overexpression of TAK1 might rescue the *vbg* phenotype.

We identified a zebrafish TAK1 putative ortholog by sequence comparison with mouse TAK1 using the Ensembl database. Zebrafish TAK1 is 71% identical and 79% similar to mouse TAK1 at the amino acid level (Fig. 7A). We used primers specific to *tak1* sequence to amplify the full length cDNA from 48-hour wild-type zebrafish embryos.

To test if TAK1 is acting in a pathway with ALK1, we first looked for synergism between antisense morpholinos against *tak1* and *Alk1*. We used an antisense morpholino to the *tak* sequence predicted to interrupt splicing between exons 6 and 7. Morpholinos against splice junctions have been shown to alter splicing and exons 6 and 7 are predicted to encode essential regions of the TAK1 kinase domain (Hanks et al., 1988; Mann et al., 2001). At high doses (above 12 ng), the *tak1* morpholino injection led to a high incidence of death, apparently owing to nonspecific toxicity. Below 12 ng, no effects could be seen from the *tak1* morpholino injection. To confirm splicing interruption from the 6 ng dose of *tak1* morpholino injection, primers to exon 6 and intron 6 were used to detect inclusion of intron 6 in the *tak1* transcript. We found an increase in the amount of transcript containing intron 6 in the *tak1* morpholino-injected embryos in comparison with uninjected embryos (Fig. 7D).

To determine if the *tak1* and *alk1* morpholinos synergized, 3 ng of *alk1* morpholino, which leads to a very low incidence of cranial dilation in comparison to the 22.5 ng dose (see below), and 6 ng of *tak1* morpholino were injected individually and in conjunction at the one-cell stage. The zebrafish embryos used in these studies contained two reporter transgenes. The DsRed reporter was driven by the *Gata1* promoter, resulting in red fluorescent labeling of blood cells, and the eGFP reporter was driven by the *fli* promoter, fluorescently labeling endothelial cells green (Eckfeldt et al., 2005). At ~48 hours post fertilization, embryos were scored by fluorescence microscopy for the presence of the dilated cranial vasculature characteristic of the *vbg* morpholino phenotype as described by Roman et al. (Roman et al., 2002) (compare wild-type cranial vasculature in Fig. 7B with the dilated vasculature in 7C). We found that injection of the two morpholinos together was able to significantly increase the number of embryos displaying the *Alk1* phenotype in comparison with injection of either individually (47.3% in comparison to 7.3%; Fig. 7E). To ensure specificity of the *tak1* morpholino effect, a morpholino with four base mismatches was used at the same dose. This morpholino showed a significantly reduced interaction with the *Alk1* morpholino when compared with the wild-type *tak1* morpholino (Fig. 7E).

Smad5 has also been implicated as a factor downstream of ALK1 and TGFβ for proper vascular development (Yang et al., 1999; Goumans et al., 2002). As the TAK1 and SMAD pathways have previously been shown to cooperate (Shibuya et al., 1998; Monzen et al., 2001; Ohkawara et al., 2004), we sought to determine if SMAD5 is also playing a role in zebrafish vascular development. Injection of a low dose (2.5 ng) of an antisense morpholino specific to *Smad5*, which does not cause a phenotype alone, is able to synergize with a low dose of *Alk1* morpholino in a similar manner to *tak1* morpholino (Fig. 7E). We conclude from these studies that both TAK1 and SMAD5 are important mediators of vascular development in vivo.

To determine if overexpression of TAK1 is able to rescue *alk1* defects, one-cell stage zebrafish embryos were injected with morpholinos to *vbg* alone, or co-injected with morpholinos to *vbg* and 100 pg of mRNA encoding either the zebrafish homolog of *tak1* (*tak1*) or, as a control, *Gfp*. Injection of high levels of the *vbg* morpholino alone caused cranial vessel dilation in an average of 59% of embryos, and co-injection of *Gfp* mRNA resulted in a similar incidence of dilation (58%). By contrast, injection of *tak1* mRNA with *vbg* morpholino rescued this phenotype so the average percentage of affected embryos was reduced to 34.5% (Fig. 7F).

As an additional control, a kinase-dead version of *tak1* (K52A*tak1*) was created by replacing the conserved lysine at position 52 in the ATP-binding pocket with an alanine. Mutation of this residue has been shown to render the TAK1 kinase catalytically inactive (Yamaguchi et al., 1995). Co-injection of the K52A*tak1* with *vbg* morpholino failed to reduce the number of affected fish, demonstrating the specificity of the effect seen with *tak1* mRNA (Fig. 7F). These results are consistent with a model in which TAK1 acts downstream of ALK1 in conjunction with SMAD5 to regulate vascular development.

DISCUSSION

We have investigated the in vivo role for the MAPKKK TAK1 during mouse development. We find that TAK1 is required for angiogenesis and that the TAK1 loss-of-function phenotype closely resembles the phenotypes of other TGFβ pathway elements, including, ALK1, endoglin and SMAD5. Taken together, our results show that TAK1 probably acts in a TGFβ-responsive pathway in vivo to regulate vascular remodeling in mice and fish.

TAK1 is required for angiogenesis in extra-embryonic and embryonic tissues

*Tak1*Δ/Δ embryos do not survive beyond E10.5 and display a phenotype characteristic of defects in cardiovascular development. Heart development in these embryos appears relatively normal, with mild defects in heart structure and normal expression of marker genes, *Gata4* and *Anf*. Although we cannot rule out the possibility that these mild cardiac defects contribute to the phenotype and death

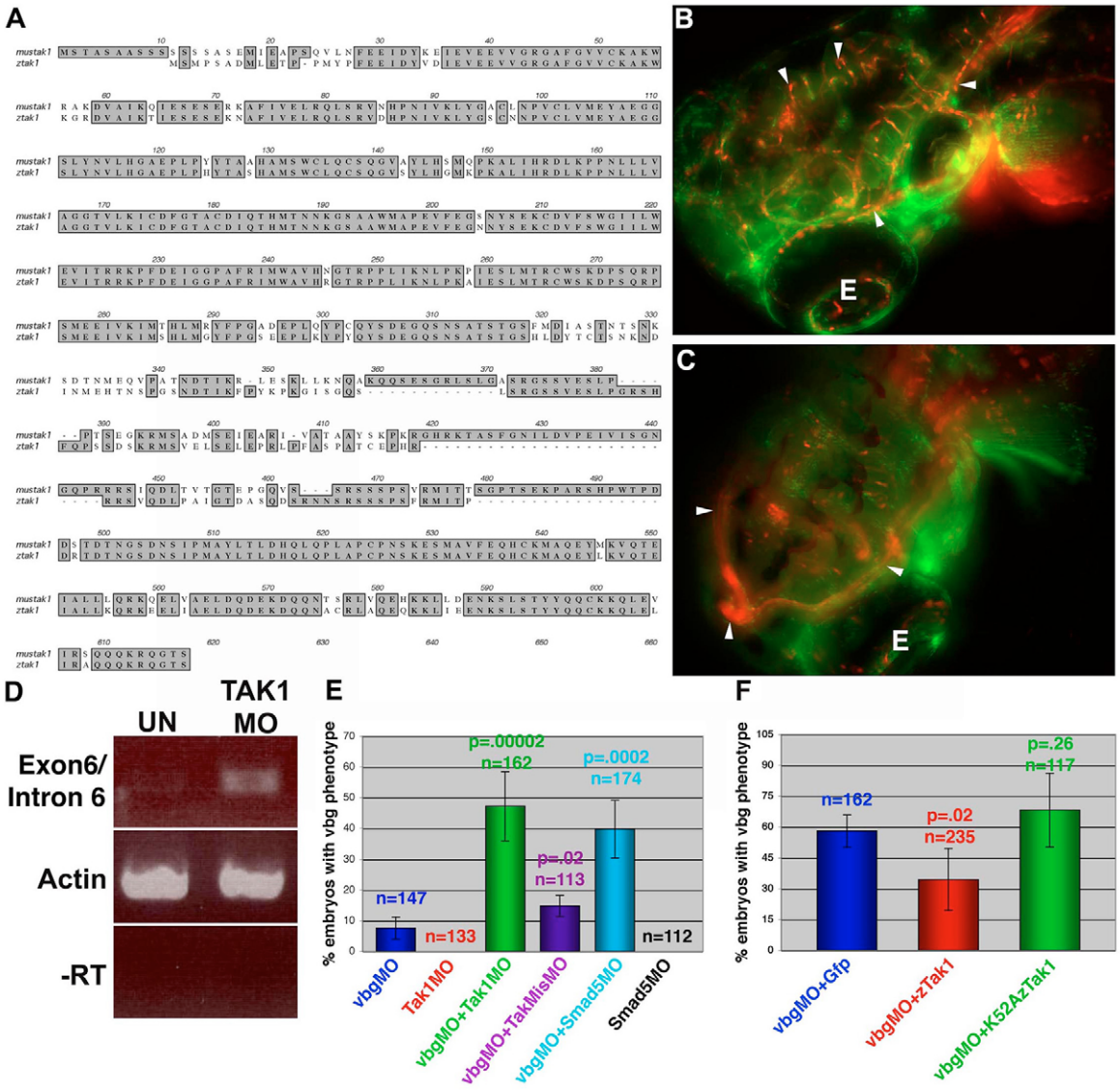

Fig. 7. Overexpression of TAK1 rescues knockdown of ALK1 in zebrafish vascular development. (**A**) The alignment of zebrafish TAK1 and mouse TAK1 proteins. (**B**) Wild-type cranial circulation in zebrafish containing the Gata1:DsRed and Fli:GFP reporter transgenes. Red, the circulating blood cells; green, the endothelium of the vasculature. Arrowheads indicate the complex meshwork of cranial circulation. E, eye. (**C**) Cranial circulation in *vbg* morpholino-injected fish. Arrowheads indicate the large dilated vessels that shunt blood away from the complex meshwork of vessels resulting in a dilated loop of circulation. (**D**) rtPCR analysis to detect inclusion of intron 6 in *tak1* transcripts upon *tak1* morpholino injection. A forward primer in exon 6 and a reverse primer in intron 6 detect an increase in inclusion of intron 6 in *tak1* transcripts in *tak1* morpholino-injected embryos (TakMO) in comparison with uninjected embryos (UN). Primers to *actin* were used as a control for the amount of cDNA in each reaction, and samples lacking reverse transcriptase (–RT) were used as a negative control. (**E**) The average percentage of fish displaying the dilated cranial vasculature after injection with *vbg* morpholino, *tak1* morpholino (or both), *vbg* morpholino with *Smad5* morpholino or *Smad5* morpholino alone. Each bar represents the average of four or five sets of injections. (**F**) Summary of the average percentage of injected fish displaying the *vbg* morpholino phenotype shown in C after injection with morpholino plus *Gfp*, *tak1* or L52Az*Tak1* mRNA. Each bar represents the average percent of embryos over 6 sets of injections. Although *tak1* RNA injection led to rescue in each injection, the level of rescue varied from 11-62%, which is the cause of the large error bars. Error bars in E and F represent the confidence interval for each set. Total number of fish injected is indicated above each bar and the *P*-value for each condition in comparison with vbgMO (3 ng) alone (E) or with vbgMO+Gfp (F) is displayed above each bar.

of the mutant embryos, they are unlikely to be the primary defect as similar heart abnormalities in embryos mutant for other genes are not associated with the extensive vascular defects and early lethality such as what we see in *Tak1*Δ/Δ embryos (Parlakian et al., 2004). In apparent conflict with our results, expression of dominant-negative TAK1 inhibited cardiomyocyte differentiation in vitro, and decreased expression of cardiac markers, including GATA4 (Monzen et al., 1999; Monzen et al., 2001). However, it is not unusual for dominant-negative mutations to produce more severe phenotypes than null mutations, perhaps explaining this discrepancy.

Endothelial cells are able to differentiate and assemble into a primitive vascular plexus in the *Tak1*Δ/Δ embryos, indicating that vasculogenesis is normal. Once vasculogenesis has taken place, vessels are remodeled and stabilized by the two phases of angiogenesis. Balance between the activation and resolution phases of angiogenesis is crucial to obtain the appropriate number of stable vessels for delivery of nutrients to all areas of the developing embryo (Lebrin et al., 2005; Risau, 1997). Angiogenesis is clearly abnormal in *Tak1*Δ/Δ embryos; however, additional in vitro studies will be required to determine whether activation, resolution or some other aspect of angiogenesis, such as specification of the arterial versus venous system, is affected. Understanding the normal regulation of these processes is important for identifying defects in the control of vessel growth and stabilization during diseased states, such as hereditary hemorrhagic telangiectasia (HHT), discussed below.

Mechanisms of TAK1 and TGFβ signaling in the vasculature

The defects seen in the *Tak1*Δ/Δ embryos are remarkably similar to the phenotypes that result when loss-of-function mutations are made in a variety of other members of the TGFβ signaling pathway. Mutations in TGFβ1 or the TGFβ type II receptor are lethal at E10.5, resulting from defects in initial vascular tube formation (vasculogenesis) in the yolk sac. These mice also have defects in hematopoiesis that result in a reduction of erythroid cells (Dickson et al., 1995; Oshima et al., 1996). The TGFβ1 mutants do not have vascular defects in the embryo proper, and this is thought to be due to rescue by another TGFβ family ligand (Dickson et al., 1995). The extent of vascular development in the embryo proper of the TGFβ type II receptor mutants has not been reported.

Mutations in the canonical TGFβ type I receptor *Alk5* also lead to defects in vascular development. These embryos are able to initiate the yolk sac vasculature normally, unlike the TGFβ1 and TGFβ type II receptor mutants, but are unable to remodel the yolk sac vasculature into mature vitelline vessels. The *Alk5* mutants also display an absence of vascular smooth muscle in the yolk sac and embryo proper, although the vessel structure in the embryo appears normal (Larsson et al., 2001).

Null mutations in the TGFβ type I receptor *Alk1* and the TGFβ type III receptor endoglin cause lethality at E10.5, with morphological characteristics similar to those we see in *Tak1*Δ/Δ embryos, including a small head, retarded posterior development and an enlarged pericardium. As in *Tak1*Δ/Δ embryos, these embryos fail to remodel the yolk sac vasculature into mature vitelline vessels, leading to extreme dilation of the yolk sac vasculature. Unlike the TGFβ1 mutants, the *Alk1* and endoglin mutants also display vascular defects in the embryo proper. These embryos, like *Alk5* mutants, have an absence of vascular smooth muscle, which we also see in *Tak1*Δ/Δ embryos. Unlike the *Alk5* mutants, *Alk1* and endoglin mutants have defects in vessel structure and remodeling resulting in extreme dilation and decreased complexity of branching, similar to that described for the *Tak1*Δ/Δ embryos. In addition to the dilation of vessels, arteriovenous malformations resulting in fusion of the dorsal aorta and cardinal vein are also seen in *Alk1* and endoglin mutants (Arthur et al., 2000; Bourdeau et al., 1999; Li et al., 1999; Oh et al., 2000; Urness et al., 2000) and occasionally in *Tak1*Δ/Δ embryos. Loss-of-function mutations in *Smad5* also display similarities in phenotype to *Tak1*Δ/Δ embryos. *Smad5* mutants do not undergo angiogenesis in the yolk sac, have dilation and reduced branching of the cranial vessels, and a reduction in vascular smooth muscle (Yang et al., 1999).

The similarities in phenotype between mutations in TGFβ signaling components and *Tak1* mutants, together with the observation that in some tissues and cells TGFβ rapidly activates TAK1 (Mulder, 2000; Yamaguchi et al., 1995), suggests that TAK1 is likely to be a direct downstream effector of TGFβ signaling that, together with SMAD5, regulates angiogenesis (Fig. 8A). Our experiments using the zebrafish system are consistent with this view. The zebrafish model system is much better suited to genetic epistasis analysis due to its rapid development time. Mutations in the zebrafish ortholog of *Alk1*, *violet beauregard* (*vbg*) exhibit increased proliferation of the cranial endothelial cells resulting in extreme dilation of the cranial vasculature (Roman et al., 2002). Using this system, we have found two lines of evidence for TAK1 acting in a pathway with ALK1. First, we demonstrate that co-injection of low doses of morpholinos to *Alk1* and *tak1* act synergistically to cause the *Alk1* phenotype. Second, overexpression by mRNA injection of the zebrafish homologue of TAK1, is able to rescue morpholino knockdown of ALK1.

Though statistically significant, *tak1* RNA injection did not rescue 100% of *Alk1* morphant embryos. There are several possible reasons for this observation. First, instability of RNA during injections may be a cause of variability. Second, as it is not clear how TAK1 kinase is activated, simply injecting *tak1* RNA may not be sufficient to achieve full activation of downstream targets. In mammals, deletion of the N-terminal 22 amino acids of TAK1 leads to constitutive activation of the kinase domain (Yamaguchi et al., 1995). However, we found little conservation in this region of zebrafish TAK1 making it unclear how to produce a similarly activated protein. Third, and perhaps the most likely explanation of partial rescue, is that both TAK1 and SMAD5 contribute to vascular development by impinging on common target genes. Such a mechanism appears to regulate cardiomyocyte differentiation in vitro and *Xenopus* mesoderm specification in vivo (Monzen et al., 2001; Ohkawara et al., 2004). In addition, in vitro studies have shown that the TAK1 and

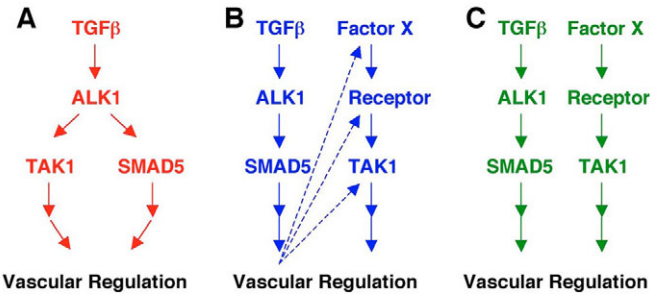

Fig. 8. Three possible models for TGFβ signaling in the developing vasculature. (**A**) TGFβ could activate both the SMAD and TAK1 pathways, which would then converge on a common target. (**B**) TGFβ could signal through SMADs, leading to the activation of TAK1. (**C**) The TGFβ/SMAD and TAK1 pathways could be acting independently, but synergistically, to regulate vascular development.

SMAD pathways are able to cooperate by converging on a common transcriptional target (Hanafusa et al., 1999; Monzen et al., 2001; Sano et al., 1999). Transcription factors activated by the TAK1 pathway (i.e. JUN) are able to form a complex with the SMAD transcriptional complex for transcriptional regulation (Zhang et al., 1998). Additionally, in vivo studies in *Xenopus* have shown that TAK1 activity is required for ventralization by SMAD1 and SMAD5 downstream of BMP signaling (Shibuya et al., 1998). Thus, during vascular development, the TAK1 and SMAD pathways may also converge on a common set of target genes.

In this scenario, as morpholino knockdown of *Alk1* probably does not result in a null situation, it is possible that enough ALK1 activity remains to maintain some SMAD5 activation, and that overexpression of TAK1 produces partial rescue by boosting up signal through the parallel signaling branch (Fig. 8A). Interestingly, recent studies have suggested that activation of two alternative downstream signaling branches may be triggered by the state of receptor oligomerization (Nohe et al., 2002). When preformed receptor complexes are used for signaling, the SMAD pathway is used for downstream signaling, whereas if the ligand recruits receptors into a complex, non-canonical downstream pathways (i.e. TAK1) are used.

Although we favor the view that both the SMAD and TAK1 pathways are activated by TGFβ, the genetics are also consistent with two alternative models (Fig. 8B,C) in which, although TAK1 signaling ultimately collaborates with the SMAD signal, the activation of TAK1 is more indirect. In model B, TAK1 activation is downstream of SMAD, while in model C TAK1 is activated independently of TGFβ but its signal cooperates with the SMAD signal to regulate common target genes.

Possible targets of TAK1 signaling

There are also several possible mechanisms for signaling downstream of TAK1. TAK1 could signal through the canonical MAPK pathway. TAK1 has been shown to activate the stress-activated protein kinases p38 and JNK through several MAPKK family members (Hanafusa et al., 1999; Moriguchi et al., 1996; Shirakabe et al., 1997). These downstream kinases in turn activate several different transcription factors, including p38 and JNK, whose downstream targets have been implicated in the regulation of angiogenesis. Loss-of-function mutations in the MAPK p38α cause defects in placental angiogenesis (Adams et al., 2000; Mudgett et al., 2000). p38α mutants do not have defects in angiogenesis of the embryo proper or yolk sac, but additional isoforms expressed in the embryo and yolk sac may be acting redundantly with p38α in these tissues (Ihle, 2000). Angiogenesis did not appear affected in the placentas of *Tak1*Δ/Δ embryos, although additional MAPKKKs may be compensating for its function in this tissue.

In addition to p38, JNK and its target transcription factor JunD, have been shown to play an essential role in the upregulation of urokinase-type plasminogen activator receptor (uPAR) downstream of TGFβ (Yue et al., 2004). Binding of uPAR by its ligand leads to degradation of the extracellular matrix, which is important for migration of endothelial cells during the activation phase of angiogenesis (Pepper, 2001; Yue et al., 2004). JNK and p38 have also been implicated in regulation of vascular smooth muscle. Expression of dominant-negative JNK or p38 is able to inhibit smooth muscle proliferation and migration in response to arterial balloon injury, an in vivo model of vascular remodeling (Kim and Iwao, 2003). Expression studies have shown that molecules upregulated during vascular remodeling in response to injury are similar to those upregulated during embryonic development (Carson-Walter et al., 2001; St Croix et al., 2000).

As an alternative to the canonical MAPK signaling pathway, TAK1 may be directly inhibiting SMADs 2/3 downstream of ALK5 during the activation phase. In vitro studies have shown that signaling downstream of ALK1 inhibits signaling downstream of ALK5 but does not affect the phosphorylation state of SMADs 2/3 (Goumans et al., 2003). Recent reports have shown that TAK1 is able to bind and inhibit SMAD function downstream of SMAD activation by the receptor complex (Benus et al., 2005; Hoffmann et al., 2005). Inhibition of signaling downstream of ALK5 by TAK1 may be essential for regulating the switch between the activation and resolution phases of angiogenesis. Further analysis using *Tak1*Δ/Δ isolated endothelial cells will help determine which of these molecules are working downstream of TAK1 during vascular development.

TAK1 requirement in endothelium versus smooth muscle

Our results do not establish whether TAK1 is required solely in endothelial cells, smooth muscle cells, or both. Expression of TAK1 is ubiquitious through E10.5 and so does not provide clues as to which tissue(s) require TAK1 function (Jadrich et al., 2003). The similarity of phenotype between *Tak1*Δ/Δ embryos and *Alk1* mutant mice, as well as our zebrafish rescue experiments, suggest that TAK1 is working downstream of ALK1 to regulate vascular development. ALK1 is expressed in endothelial cells and has not been shown to be expressed in vascular smooth muscle progenitor cells (Seki et al., 2003). In vitro culture of endothelial cells has also shown that ALK1 is required in endothelial cells to regulate their proliferation and migration (Goumans et al., 2002; Lebrin et al., 2004). Additionally, dilation and misbranching of the cranial vasculature in *Tak1*Δ/Δ embryos occurs prior to recruitment of smooth muscle cells to the endothelium. These studies suggest that TAK1 is acting in endothelial cells to regulate vascular development, although conditional removal of TAK1 expression specifically in endothelium will be needed to answer this question definitively. It is also possible that TAK1 is acting in both endothelial and smooth muscle cells. Recent studies have indicated that ALK5 is not expressed in the endothelium but is highly expressed in smooth muscle, although it is possible that ALK5 is expressed at very low levels in the endothelium (Seki et al., 2006). This result opens up a new possibility for signaling by TGFβ in the vasculature. Because signaling through ALK1 and endoglin has been shown to be required for TGFβ paracrine signaling (Carvalho et al., 2004), and TGFβ has been shown to be important for the differentiation of smooth muscle (Hirschi et al., 1998), ALK1/endoglin acting through TAK1 and its downstream components could upregulate TGFβ release by the endothelium. This TGFβ might then signal to the smooth muscle progenitors, affecting their subsequent differentiation. Tissue-specific inactivation of these receptors, as well as TAK1, will definitively determine whether they are required in endothelial cells in vivo. From our present studies, we cannot determine whether TAK1 may also act downstream of ALK5. Searches of the current zebrafish genome produced no functional homolog of ALK5 for use in epistasis experiments.

A potential role for TAK1 in human vascular disease

Mutations in both endoglin and *ALK1* (*ACVRL1* – Human Gene Nomenclature Database) have been shown to be the cause of the autosomal dominant disorders HHT 1 and HHT 2, respectively (van den Driesche et al., 2003). Individuals with HHT display arterio-venous malformations, epistaxis and telangiectasias, owing to weak

vascular walls (Plauchu et al., 1989). Some of the mice heterozygous for endoglin or *Alk1* mutations have recurrent epistaxis and telangiectasias, similar to what is seen in the human disorder (Bourdeau et al., 1999; Srinivasan et al., 2003). Recently, families with HHT have been identified in which the *Alk1* and endoglin loci are normal (Wallace and Shovlin, 2000). Mutations in the human *TAK1* (*MAP3K7* – Human Gene Nomenclature Database) gene are possible candidates for the cause of HHT in these individuals and deserve further exploration.

Although our results clearly implicate TAK1, in conjunction with TGFβ signaling, as a mediator of vascular development in vivo, it is likely that TAK1 has other in vivo roles given its wide expression pattern and the diversity of in vitro studies describing functions for TAK1 in numerous other signaling pathways. Indeed, recent studies using a knockout model similar to ours have demonstrated that TAK1 is required for proper activation of inflammatory signaling pathways (Shim et al., 2005). Conditional inactivation of TAK1 in other tissues and at later stages should provide insight into additional requirements for TAK1 function during development.

We thank Dr S. Ekker for assistance with zebrafish experiments and critical review of the manuscript. We thank Dr W. Shawlot for *Gata4* and *Anf* probes, and critical review of the manuscript. We thank M. Shimmell, Dr M. Serpe and Dr A. Luttun for critical review of the manuscript, and Drs J. Lohr and B. Martinsen for helpful discussions. We thank Matthias Hammerschmidt for the *Smad5* morpholino. The monoclonal α-β-Tubulin antibody developed by M. Klymkowsky was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of NICHD and maintained at the University of Iowa, Department of Biological Sciences, Iowa City, Iowa 52242. The genetrap ES cell line was obtained from the National Heart, Lung, and Blood Institute-funded Program for Genomics Applications at BayGenomics, which is supported by Grants HL66621, HL66600 and HL66590. J.J. was supported in part by the Developmental Biology Training Grant (T32 HD007480). M.B.O. is an investigator with the Howard Hughes Medical Institute.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/8/1529/DC1

References

Adams, R. H., Porras, A., Alonso, G., Jones, M., Vintersten, K., Panelli, S., Valladares, A., Perez, L., Klein, R. and Nebreda, A. R. (2000). Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. *Mol. Cell* **6**, 109-116.

Arceci, R. J., King, A. A., Simon, M. C., Orkin, S. H. and Wilson, D. B. (1993). Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol. Cell. Biol.* **13**, 2235-2246.

Arthur, H. M., Ure, J., Smith, A. J., Renforth, G., Wilson, D. I., Torsney, E., Charlton, R., Parums, D. V., Jowett, T., Marchuk, D. A. et al. (2000). Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Dev. Biol.* **217**, 42-53.

Baldwin, H. S., Shen, H. M., Yan, H. C., DeLisser, H. M., Chung, A., Mickanin, C., Trask, T., Kirschbaum, N. E., Newman, P. J. and Albelda, S. M. (1994). Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31): alternatively spliced, functionally distinct isoforms expressed during mammalian cardiovascular development. *Development* **120**, 2539-2553.

Benus, G. F., Wierenga, A. T., de Gorter, D. J., Schuringa, J. J., van Bennekum, A. M., Drenth-Diephuis, L., Vellenga, E. and Eggen, B. J. (2005). Inhibition of the TGFβ pathway by IL-1β Is mediated through TAK1 phosphorylation of SMAD3. *Mol. Biol. Cell* **16**, 3501-3510.

Bourdeau, A., Dumont, D. J. and Letarte, M. (1999). A murine model of hereditary hemorrhagic telangiectasia. *J. Clin. Invest.* **104**, 1343-1351.

Bradham, C. A., Hatano, E. and Brenner, D. A. (2001). Dominant-negative TAK1 induces c-Myc and G(0) exit in liver. *Am. J. Physiol. Gastrointest. Liver Physiol.* **281**, G1279-G1289.

Bruneau, B. G., Nemer, G., Schmitt, J. P., Charron, F., Robitaille, L., Caron, S., Conner, D. A., Gessler, M., Nemer, M., Seidman, C. E. and Seidman, J. G. (2001). A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* **106**, 709-721.

Carson-Walter, E. B., Watkins, D. N., Nanda, A., Vogelstein, B., Kinzler, K. W. and St Croix, B. (2001). Cell surface tumor endothelial markers are conserved in mice and humans. *Cancer Res.* **61**, 6649-6655.

Carvalho, R. L., Jonker, L., Goumans, M. J., Larsson, J., Bouwman, P., Karlsson, S., Dijke, P. T., Arthur, H. M. and Mummery, C. L. (2004). Defective paracrine signalling by TGFβ in yolk sac vasculature of endoglin mutant mice: a paradigm for hereditary haemorrhagic telangiectasia. *Development* **131**, 6237-6247.

Corson, L. B., Yamanaka, Y., Lai, K. M. and Rossant, J. (2003). Spatial and temporal patterns of ERK signaling during mouse embryogenesis. *Development* **130**, 4527-4537.

Cross, J. C., Werb, Z. and Fisher, S. J. (1994). Implantation and the placenta: key pieces of the development puzzle. *Science* **266**, 1508-1518.

Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S. and Akhurst, R. J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* **121**, 1845-1854.

Durocher, D., Chen, C. Y., Ardati, A., Schwartz, R. J. and Nemer, M. (1996). The atrial natriuretic factor promoter is a downstream target for Nkx-2.5 in the myocardium. *Mol. Cell. Biol.* **16**, 4648-4655.

Eckfeldt, C. E., Mendenhall, E. M., Flynn, C. M., Wang, T., Pickart, M., Grindle, S. M., Ekker, S. C. and Vervaillie, C. M. (2005). Functional analysis of human hematopoietic stem cell gene expression using zebrafish. *PLoS Biol.* **3**, e254.

Flamme, I., Frolich, T. and Risau, W. (1997). Molecular mechanisms of vasculogenesis and embryonic angiogenesis. *J. Cell. Physiol.* **173**, 206-210.

Freemark, M., Driscoll, P., Maaskant, R., Petryk, A. and Kelly, P. A. (1997). Ontogenesis of prolactin receptors in the human fetus in early gestation. Implications for tissue differentiation and development. *J. Clin. Invest.* **99**, 1107-1117.

Goumans, M. J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P. and ten Dijke, P. (2002). Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J.* **21**, 1743-1753.

Goumans, M. J., Valdimarsdottir, G., Itoh, S., Lebrin, F., Larsson, J., Mummery, C., Karlsson, S. and ten Dijke, P. (2003). Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. *Mol. Cell* **12**, 817-828.

Hanafusa, H., Ninomiya-Tsuji, J., Masuyama, N., Nishita, M., Fujisawa, J., Shibuya, H., Matsumoto, K. and Nishida, E. (1999). Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. *J. Biol. Chem.* **274**, 27161-27167.

Hanks, S. K., Quinn, A. M. and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52.

Hirakow, R. and Hiruma, T. (1981). Scanning electron microscopic study on the development of primitive blood vessels in chick embryos at the early somite-stage. *Anat. Embryol. (Berl.)* **163**, 299-306.

Hirschi, K. K., Rohovsky, S. A. and D'Amore, P. A. (1998). PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J. Cell Biol.* **141**, 805-814.

Hoffmann, A., Preobrazhenska, O., Wodarczyk, C., Medler, Y., Winkel, A., Shahab, S., Huylebroeck, D., Gross, G. and Verschueren, K. (2005). Transforming growth factor-beta-activated kinase-1 (TAK1), a MAP3K, interacts with Smad proteins and interferes with osteogenesis in murine mesenchymal progenitors. *J. Biol. Chem.* **280**, 27271-27283.

Hogan, B., Beddington, R., Costantini, F. and and Lacy, E. (1994). *Manipulating the Mouse Embryo – A Laboratory Manual*. New York, NY: Cold Spring Harbor Laboratory Press.

Hyatt, T. M. and Ekker, S. C. (1999). Vectors and techniques for ectopic gene expression in zebrafish. *Methods Cell Biol.* **59**, 117-126.

Ihle, J. N. (2000). The challenges of translating knockout phenotypes into gene function. *Cell* **102**, 131-134.

Ishitani, T., Kishida, S., Hyodo-Miura, J., Ueno, N., Yasuda, J., Waterman, M., Shibuya, H., Moon, R. T., Ninomiya-Tsuji, J. and Matsumoto, K. (2003). The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signaling. *Mol. Cell. Biol.* **23**, 131-139.

Jadrich, J. L., O'Connor, M. B. and Coucouvanis, E. (2003). Expression of TAK1, a mediator of TGF-beta and BMP signaling, during mouse embryonic development. *Gene Expr. Patterns* **3**, 131-134.

Kim, S. and Iwao, H. (2003). Stress and vascular responses: mitogen-activated protein kinases and activator protein-1 as promising therapeutic targets of vascular remodeling. *J. Pharmacol. Sci.* **91**, 177-181.

Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C. and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev.* **11**, 1048-1060.

Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R. and Berns, A. (1991). Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.* **19**, 4293.

Larsson, J., Goumans, M. J., Sjostrand, L. J., van Rooijen, M. A., Ward, D., Leveen, P., Xu, X., ten Dijke, P., Mummery, C. L. and Karlsson, S. (2001).

Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. *EMBO J.* **20**, 1663-1673.
Lebrin, F., Goumans, M. J., Jonker, L., Carvalho, R. L., Valdimarsdottir, G., Thorikay, M., Mummery, C., Arthur, H. M. and Dijke, P. T. (2004). Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *EMBO J.* **23**, 4018-4028.
Lebrin, F., Deckers, M., Bertolino, P. and Ten Dijke, P. (2005). TGF-beta receptor function in the endothelium. *Cardiovasc. Res.* **65**, 599-608.
Lele, Z., Bakkers, J. and Hammerschmidt, M. (2001). Morpholino phenocopies of the swirl, snailhouse, somitabun, minifin, silberblick, and pipetail mutations. *Genesis* **30**, 190-194.
Letamendia, A., Lastres, P., Botella, L. M., Raab, U., Langa, C., Velasco, B., Attisano, L. and Bernabeu, C. (1998). Role of endoglin in cellular responses to transforming growth factor-beta. A comparative study with betaglycan. *J. Biol. Chem.* **273**, 33011-33019.
Li, D. Y., Sorensen, L. K., Brooke, B. S., Urness, L. D., Davis, E. C., Taylor, D. G., Boak, B. B. and Wendel, D. P. (1999). Defective angiogenesis in mice lacking endoglin. *Science* **284**, 1534-1537.
Liu, F. (2003). Receptor-regulated Smads in TGF-beta signaling. *Front. Biosci.* **8**, s1280-s1303.
Mann, C. J., Honeyman, K., Cheng, A. J., Ly, T., Lloyd, F., Fletcher, S., Morgan, J. E., Partridge, T. A. and Wilton, S. D. (2001). Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. *Proc. Natl. Acad. Sci. USA* **98**, 42-47.
Massague, J. (1998). TGF-beta signal transduction. *Annu. Rev. Biochem.* **67**, 753-791.
Meneghini, M. D., Ishitani, T., Carter, J. C., Hisamoto, N., Ninomiya-Tsuji, J., Thorpe, C. J., Hamill, D. R., Matsumoto, K. and Bowerman, B. (1999). MAP kinase and Wnt pathways converge to downregulate an HMG-domain repressor in Caenorhabditis elegans. *Nature* **399**, 793-797.
Molkentin, J. D., Lin, Q., Duncan, S. A. and Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev.* **11**, 1061-1072.
Monzen, K., Shiojima, I., Hiroi, Y., Kudoh, S., Oka, T., Takimoto, E., Hayashi, D., Hosoda, T., Habara-Ohkubo, A. and Nakaoka, T. et al. (1999). Bone morphogenetic proteins induce cardiomyocyte differentiation through the mitogen-activated protein kinase kinase TAK1 and cardiac transcription factors Csx/Nkx-2.5 and GATA-4. *Mol. Cell. Biol.* **19**, 7096-7105.
Monzen, K., Hiroi, Y., Kudoh, S., Akazawa, H., Oka, T., Takimoto, E., Hayashi, D., Hosoda, T., Kawabata, M. and Miyazono, K. et al. (2001). Smads, TAK1, and their common target ATF-2 play a critical role in cardiomyocyte differentiation. *J. Cell Biol.* **153**, 687-698.
Moriguchi, T., Kuroyanagi, N., Yamaguchi, K., Gotoh, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, K., Nishida, E. and Hagiwara, M. (1996). A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J. Biol. Chem.* **271**, 13675-13679.
Mudgett, J. S., Ding, J., Guh-Siesel, L., Chartrain, N. A., Yang, L., Gopal, S. and Shen, M. M. (2000). Essential role for p38alpha mitogen-activated protein kinase in placental angiogenesis. *Proc. Natl. Acad. Sci. USA* **97**, 10454-10459.
Mulder, K. M. (2000). Role of Ras and Mapks in TGFbeta signaling. *Cytokine Growth Factor Rev.* **11**, 23-35.
Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z. and Matsumoto, K. (1999). The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature* **398**, 252-256.
Nohe, A., Hassel, S., Ehrlich, M., Neubauer, F., Sebald, W., Henis, Y. I. and Knaus, P. (2002). The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. *J. Biol. Chem.* **277**, 5330-5338.
Oh, S. P., Seki, T., Goss, K. A., Imamura, T., Yi, Y., Donahoe, P. K., Li, L., Miyazono, K., ten Dijke, P., Kim, S. and Li, E. (2000). Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc. Natl. Acad. Sci. USA* **97**, 2626-2631.
Ohkawara, B., Shirakabe, K., Hyodo-Miura, J., Matsuo, R., Ueno, N., Matsumoto, K. and Shibuya, H. (2004). Role of the TAK1-NLK-STAT3 pathway in TGF-beta-mediated mesoderm induction. *Genes Dev.* **18**, 381-386.
Ono, K., Ohtomo, T., Ninomiya-Tsuji, J. and Tsuchiya, M. (2003). A dominant negative TAK1 inhibits cellular fibrotic responses induced by TGF-beta. *Biochem. Biophys. Res. Commun.* **307**, 332-337.
Oshima, M., Oshima, H. and Taketo, M. M. (1996). TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev. Biol.* **179**, 297-302.
Owens, G. K., Kumar, M. S. and Wamhoff, B. R. (2004). Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol. Rev.* **84**, 767-801.
Park, J. M., Brady, H., Ruocco, M. G., Sun, H., Williams, D., Lee, S. J., Kato, T., Jr, Richards, N., Chan, K., Mercurio, F., Karin, M. and Wasserman, S. A. (2004). Targeting of TAK1 by the NF-kappa B protein Relish regulates the JNK-mediated immune response in Drosophila. *Genes Dev.* **18**, 584-594.
Parlakian, A., Tuil, D., Hamard, G., Tavernier, G., Hentzen, D., Concordet, J. P., Paulin, D., Li, Z. and Daegelen, D. (2004). Targeted inactivation of serum response factor in the developing heart results in myocardial defects and embryonic lethality. *Mol. Cell. Biol.* **24**, 5281-5289.
Pepper, M. S. (1997). Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev.* **8**, 21-43.
Pepper, M. S. (2001). Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. *Arterioscler. Thromb. Vasc. Biol.* **21**, 1104-1117.
Plauchu, H., de Chadarevian, J. P., Bideau, A. and Robert, J. M. (1989). Age-related clinical profile of hereditary hemorrhagic telangiectasia in an epidemiologically recruited population. *Am. J. Med. Genet.* **32**, 291-297.
Risau, W. (1997). Mechanisms of angiogenesis. *Nature* **386**, 671-674.
Risau, W. and Flamme, I. (1995). Vasculogenesis. *Annu. Rev. Cell Dev. Biol.* **11**, 73-91.
Roman, B. L., Pham, V. N., Lawson, N. D., Kulik, M., Childs, S., Lekven, A. C., Garrity, D. M., Moon, R. T., Fishman, M. C., Lechleider, R. J. and Weinstein, B. M. (2002). Disruption of acvrl1 increases endothelial cell number in zebrafish cranial vessels. *Development* **129**, 3009-3019.
Rossant, J. and Cross, J. C. (2001). Placental development: lessons from mouse mutants. *Nat. Rev. Genet.* **2**, 538-548.
Sano, Y., Harada, J., Tashiro, S., Gotoh-Mandeville, R., Maekawa, T. and Ishii, S. (1999). ATF-2 is a common nuclear target of Smad and TAK1 pathways in transforming growth factor-beta signaling. *J. Biol. Chem.* **274**, 8949-8957.
Sato, S., Sanjo, H., Takeda, K., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Matsumoto, K., Takeuchi, O. and Akira, S. (2005). Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat. Immunol.* **11**, 1087-1095.
Seki, T., Yun, J. and Oh, S. P. (2003). Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling. *Circ. Res.* **93**, 682-689.
Seki, T., Hong, K. H. and Oh, S. P. (2006). Nonoverlapping expression patterns of ALK1 and ALK5 reveal distinct roles of each receptor in vascular development. *Lab Invest.* **86**, 116-129.
Shi, Y. and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.
Shibuya, H., Iwata, H., Masuyama, N., Gotoh, Y., Yamaguchi, K., Irie, K., Matsumoto, K., Nishida, E. and Ueno, N. (1998). Role of TAK1 and TAB1 in BMP signaling in early Xenopus development. *EMBO J.* **17**, 1019-1028.
Shim, J., Xiao, C., Paschal, A. E., Bailey, S. T., Rao, P., Hayden, M. S., Lee, K., Bussey, C., Steckel, M., Tanaka, N., Yamada, G., Akira, S., Matsumoto, K. and Ghosh, S. (2005). Tak1, but not TAB1 or Tab2, plays an essential role in multiple signaling pathways in vivo. *Genes Dev.* **19**, 2668-2681.
Shirakabe, K., Yamaguchi, K., Shibuya, H., Irie, K., Matsuda, S., Moriguchi, T., Gotoh, Y., Matsumoto, K. and Nishida, E. (1997). TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-Jun N-terminal kinase. *J. Biol. Chem.* **272**, 8141-8144.
Srinivasan, S., Hanes, M. A., Dicken, T., Porteous, M. E., Oh, S. P., Hale, L. P. and Marchuk, D. A. (2003). A mouse model for hereditary hemorrhagic telangiectasia (HHT) type 2. *Hum. Mol. Genet.* **12**, 473-482.
St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B. and Kinzler, K. W. (2000). Genes expressed in human tumor endothelium. *Science* **289**, 1197-1202.
Stryke, D., Kawamoto, M., Huang, C. C., Johns, S. J., King, L. A., Harper, C. A., Meng, E. C., Lee, R. E., Yee, A. and L'Italien, L. et al. (2003). BayGenomics: a resource of insertional mutations in mouse embryonic stem cells. *Nucleic Acids Res.* **31**, 278-281.
Takahashi, Y., Imanaka, T. and Takano, T. (1996). Spatial and temporal pattern of smooth muscle cell differentiation during development of the vascular system in the mouse embryo. *Anat. Embryol. (Berl.)* **194**, 515-526.
Urness, L. D., Sorensen, L. K. and Li, D. Y. (2000). Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat. Genet.* **26**, 328-331.
van den Driesche, S., Mummery, C. L. and Westermann, C. J. (2003). Hereditary hemorrhagic telangiectasia: an update on transforming growth factor beta signaling in vasculogenesis and angiogenesis. *Cardiovasc. Res.* **58**, 20-31.
Vidal, S., Khush, R. S., Leulier, F., Tzou, P., Nakamura, M. and Lemaitre, B. (2001). Mutations in the Drosophila dTAK1 gene reveal a conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses. *Genes Dev.* **15**, 1900-1912.
Wallace, G. M. and Shovlin, C. L. (2000). A hereditary haemorrhagic telangiectasia family with pulmonary involvement is unlinked to the known HHT genes, endoglin and ALK-1. *Thorax* **55**, 685-690.
Wilkinson, D. G. and Nieto, M. A. (1993). Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol.* **225**, 361-373.
Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N.,

Taniguchi, T., Nishida, E. and Matsumoto, K. (1995). Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. *Science* **270**, 2008-2011.
Yang, X., Castilla, L. H., Xu, X., Li, C., Gotay, J., Weinstein, M., Liu, P. P. and Deng, C. X. (1999). Angiogenesis defects and mesenchymal apoptosis in mice lacking SMAD5. *Development* **126**, 1571-1580.
Yue, J. and Mulder, K. M. (2000). Requirement of Ras/MAPK pathway activation by transforming growth factor beta for transforming growth factor beta 1 production in a smad-dependent pathway. *J. Biol. Chem.* **275**, 30765-30773.
Yue, J., Sun, B., Liu, G. and Mulder, K. M. (2004). Requirement of TGF-beta receptor-dependent activation of c-Jun N-terminal kinases (JNKs)/stress-activated protein kinases (Sapks) for TGF-beta up-regulation of the urokinase-type plasminogen activator receptor. *J. Cell. Physiol.* **199**, 284-292.
Zeller, R., Bloch, K. D., Williams, B. S., Arceci, R. J. and Seidman, C. E. (1987). Localized expression of the atrial natriuretic factor gene during cardiac embryogenesis. *Genes Dev.* **1**, 693-698.
Zhang, D., Gaussin, V., Taffet, G. E., Belaguli, N. S., Yamada, M., Schwartz, R. J., Michael, L. H., Overbeek, P. A. and Schneider, M. D. (2000). TAK1 is activated in the myocardium after pressure overload and is sufficient to provoke heart failure in transgenic mice. *Nat. Med.* **6**, 556-563.
Zhang, Y., Feng, X. H. and Derynck, R. (1998). Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. *Nature* **394**, 909-913.