Functional characterization of Klippel–Trenaunay syndrome gene AGGF1 identifies a novel angiogenic signaling pathway for specification of vein differentiation and angiogenesis during embryogenesis

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Specification of arteries and veins is a key process for establishing functional vasculature during embryogenesis and involves distinctly different signaling mechanisms. Vascular endothelial growth factor-A (VEGFA) is required for differentiation of arteries; however, the upstream angiogenic factor for vein specification is unknown. Klippel–Trenaunay syndrome (KTS) is a congenital vascular disease associated with capillary and venous malformations (VMs), but not with arterial defects. We have previously reported that upregulation of angiogenic factor AGGF1 is associated with KTS, but the molecular mechanism is not clear. Here, we show that AGGF1 is involved in establishing venous identity in zebrafish embryos. Overexpression of AGGF1 led to increased angiogenesis and increased lumen diameter of veins, whereas knockdown of AGGF1 expression resulted in defective vasculogenesis and angiogenesis. Overexpression of AGGF1 increased expression of venous markers (e.g. flt4), but had little effect on arterial markers (e.g. notch5). Knockdown of AGGF1 expression resulted in a loss of venous identity (loss of expression of flt4, ephb4 and dab2), but had no effect on the expression of arterial development. We further show that AGGF1 activates AKT, and that decreased AGGF1 expression inhibits AKT activation. Overexpression of constitutively active AKT rescues the loss of venous identity caused by AGGF1 downregulation. Our study establishes AGGF1 as an angiogenic factor with an important role in the specification of vein identity and suggests that AGGF1-mediated AKT signaling is responsible for establishing venous cell fate. We propose that increased AGGF1 expression leads to increased vein differentiation by inducing activation of AKT signaling, resulting in VMs in KTS patients.

INTRODUCTION

Klippel–Trenaunay syndrome (KTS) is a congenital vascular disease characterized by capillary malformations, venous malformations (VMs) or varicose veins and hypertrophy of the affected tissues (1–5). Most KTS cases are sporadic, although familial cases have been reported (1,4). Identification of de novo chromosomal mutations in KTS patients, including translocations and a supernumerary ring chromosome 18, suggested that genetic factors contribute to the pathogenesis of KTS.

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Molecular dissection of a KTS translocation involving chromosomes 5 and 11 [t(5;11)] led to the molecular cloning of a novel angiogenic factor gene AGGF1 (Angiogenic Factor with G-patch and forkhead-associated (FHA) domain) associated with KTS ([7]). Moreover, recent association studies established significant association between two genomic variants in AGGF1 and KTS ([8]).

The AGGF1 gene is located on chromosome 5q13.3 and encodes an angiogenic factor with a coiled-coil domain and an OCRE domain at the N-terminus, and a FHA domain and a G-patch domain at the C-terminus ([4, 7]), but function of these domains is unknown. Several sensitive assays showed that AGGF1 can be secreted outside endothelial cells (ECs), and a purified AGGF1 protein can promote EC adhesion in AGGF1-coated wells, EC capillary tube formation in an in vitro matrigel angiogenesis assay, and potentially promote angiogenesis in chick chorioallantoic membrane angiogenesis assays ([7]). Recently, we showed that transcription of AGGF1 was activated by transcriptional factor GATA1 ([9]). GATA1-specific siRNA reduced AGGF1 expression, and led to EC apoptosis and inhibition of EC migration and capillary vessel formation ([9]). These effects were rescued by a purified recombinant human AGGF1 protein ([9]), which further supports the role of AGGF1 as an angiogenic factor.

We previously linked increased AGGF1 expression to KTS ([7]). First, the KTS-associated gross chromosomal mutation, i.e. translocation t(5;11), increased AGGF1 expression by 3-fold ([7]). Second, we recently demonstrated that the gross chromosomal mutation increased AGGF1 expression by removing two repressor sites in the AGGF1 regulatory region ([9]). However, the mechanistic link between increased AGGF1 expression caused by the KTS mutation and the histological features of KTS remains missing. Furthermore, the in vivo physiological role of AGGF1 is unknown.

More than 70 human diseases are associated with abnormal vascular development and angiogenesis ([10]). Therapies targeted to blood vessels, including antiangiogenic therapy and therapeutic angiogenesis, have shown promising potentials in the treatment of most common diseases such as cancer, cardiovascular disease and macular degeneration. However, our understanding of the molecular mechanisms for vascular development and angiogenesis remains incomplete. Arteries and veins are structurally and functionally distinct organs critical to embryonic development and adult life, and the development of these vascular systems is one of the earliest steps in embryogenesis ([11–16]). Differentiation of arteries is determined by vascular endothelial growth factor-A (VEGFA), which acts upstream of the Notch signaling pathway ([11–16]). VEGFA expressed in the somites activates VEGFR2 on ECs, which in turn results in the activation of PLC-γ, PKC, Raf, MEK1/2, ERK1/2 and of the Notch pathway, leading to expression of ephrinB2 and arterial specification ([11–17]). A distinctively different genetic program has been proposed to specify vein identity, but little is known about the program and associated molecular signaling mechanism. Signaling molecule AKT was shown to be involved in the differentiation of veins, probably by blocking arterial Raf-MEK1/2-ERK1/2 signaling ([18–20]). However, it is important to note that factors upstream of AKT are not known for vein specification. Transcription factor COUP-TFII was shown to be involved in vein specification, but it is unknown whether it has any effect on AKT and vice versa ([21]). To date, no angiogenic factor has been identified for specification of venous cell fate (i.e. a similar molecule like VEGFA for arterial specification).

Here, we employed zebrafish as a model system to demonstrate the important in vivo physiological role of AGGF1 in vascular development, which provides the missing link to the pathogenesis of KTS. Most importantly, we unexpectedly found that AGGF1 encoded the angiogenic factor that acted upstream of AKT in the specification of venous cell fate during zebrafish embryogenesis.

**RESULTS**

**Molecular cloning and expression of zebrafish AGGF1**

Using the human AGGF1 complementary DNA (cDNA) sequence, we searched for its homologous genes in the NCBI database (www.ncbi.nlm.nih.gov) and identified zebrafish AGGF1 gene (RefSeqDNA: NM_001079982.1). Its corresponding protein sequence (RefSeq peptide: NP_0010773451) showed a high degree of homology to human AGGF1 (NP_005016) (36% identity, 57% homology) and mouse AGGF1 (NP_079906) (65% identity, 81% homology) ([1A and Table 1]). Zebrafish AGGF1 is located on chromosome 21 and consists of 14 exons and 13 introns. Similar to human AGGF1, zebrafish AGGF1 protein contains 774 amino acids and has a FHA domain (amino acids 469–574) and a G-patch domain (amino acids 674–718) ([Fig. 1A]). Phylogenetic analysis showed that zebrafish AGGF1 was more closely related to chicken AGGF1 ([Fig. 1B]).

The expression profile of AGGF1 messenger RNA (mRNA) during zebrafish embryogenesis and in different adult zebrafish organs was analyzed by reverse transcription–polymerase chain reaction (RT–PCR) analysis. As shown in [Fig. 1C], AGGF1 mRNA was expressed throughout embryogenesis and in adult skeletal muscle, brain, heart, liver, gill and eye. We also determined the expression pattern of AGGF1 mRNA by using whole-mount in situ hybridization (WISH) ([Fig. 1D]). The AGGF1 expression was detected from one-cell-stage embryos, suggesting that AGGF1 is maternally expressed. From 2 hpf to 3.5 hpf stages, AGGF1 mRNA was localized in blastomeres. From the 6 hpf stage to 12 hpf stage, AGGF1 expression was ubiquitous in these embryos. By 24 hpf, the head region expressed a higher level of AGGF1 than other regions of the body ([Fig. 1D]). The AGGF1 expression is predominant in the brain at 48 hpf ([Fig. 1D]).

**Overexpression of zebrafish AGGF1 mRNA stimulates overgrowth of intersegmental vessels (ISVs) and promotes venous differentiation**

Because our earlier study linked increased AGGF1 expression to KTS, we investigated the effect of overexpression of AGGF1 on the in vivo role of AGGF1 in vascular development. Zebrafish AGGF1 mRNA was injected into Tg(kdrl:GFP) [1A16] zebrafish embryos. In these transgenic zebrafish embryos, ECs of the vasculature express GFP under the control of the EC-specific kdrl promoter, so that abnormal vascular
development can be easily visualized in morphant zebrafish. One typical vascular feature of KTS patients is capillary malformations. Consistently, overexpression of zebrafish AGGF1 induced oversprouting of ISVs at 24 hpf (asterisks in Fig. 2A), whereas uninjected embryos or embryos injected with control mCherry mRNA did not show oversprouting of ISVs. In addition, we observed induced filopodia formation (arrows in Fig. 2A) in AGGF1 overexpression embryos, but not in uninjected embryos or embryos injected with mCherry mRNA (Fig. 2A and B). Interestingly, embryos injected with zebrafish VEGFA165 mRNA did not induce oversprouting of ISVs (Fig. 2A), suggesting that AGGF1 is functionally distinguishable from VEGFA.

On the other hand, embryos injected with either zAGGF1 or VEGFA mRNA induced excessive branching of subintestinal vessels (SIVs) compared with uninjected embryos or embryos injected with mCherry mRNA at 72 hpf as revealed by alkaline phosphatase (AP) staining (Fig. 2C). These data suggest that AGGF1 and VEGFA share some similar functions during angiogenesis. No overt vascular abnormalities in vessels in brain areas were observed (Supplementary Material, Fig. S1). Injection of zAGGF1 mRNA did not alter the overall morphology of embryos (Supplementary Material, Fig. S2). VMs are also a vascular abnormality typically found in KTS patients. Therefore, we assessed the effect of AGGF1 overexpression on venous differentiation. As shown in Fig. 2D, injection of AGGF1 mRNA caused abnormal venous differentiation at 24 hpf compared with uninjected embryos or embryos.

Table 1. Analysis of homology between zebrafish AGGF1 protein and its homologous proteins from other species.

<table>
<thead>
<tr>
<th>Species</th>
<th>NCBI accession#</th>
<th>Identity to zebrafish AGGF1 (%)</th>
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Figure 2. Overexpression of AGGF1 stimulates oversprouting of ISVs, excessive branching of SIVs and PCV development. (A) Injection of 100 pg of zebrafish AGGF1 mRNA induced oversprouting of ISVs (asterisks) and filopodia formation (arrows) at the 24 hpf stage (lateral views with the head to the left). Injection of the same dosage of mCherry mRNA and VEGFA165 mRNA was used as controls. (B) Statistical analysis of filopodia formation in embryos (filopodia per ISV) injected with AGGF1 mRNA compared with control embryos (uninjected embryos or embryos injected with mCherry mRNA). The arrows in (A), which represent filopodia, were manually counted and plotted. (C) Injection of AGGF1 mRNA induced excessive branching of SIVs. Vessels were stained by AP at 72 hpf. Uninjected embryos or embryos injected with mCherry mRNA were used as negative controls, whereas VEGFA165 mRNA acted as a positive control. (D)
injected with mCherry mRNA. The diameter of the posterior cardinal vein (PCV) was significantly increased in embryos injected with AGGF1 mRNA compared with uninjected embryos or embryos injected with mCherry mRNA (Fig. 2D and E). Furthermore, we assayed the expression of arterial marker *notch5* and venous marker *flt4* in the DA and PCV regions using *in situ* hybridization, respectively. Embryos injected with AGGF1 mRNA showed upregulation of *flt4* expression in the PCV region compared with uninjected embryos or embryos injected with mCherry mRNA (Fig. 2F), but had little effect on *notch5* expression (Fig. 2G). The increased intensity of an *flt4* signal by overexpression of AGGF1 (Fig. 2F) may suggest that the number of venous ECs was increased by AGGF1. Together, these results suggest that AGGF1 overexpression increases capillary and vein differentiation, but does not impact arterial differentiation.

**AGGF1 is involved in *in vivo* angiogenesis in zebrafish**

To further determine the *in vivo* function of AGGF1 during early zebrafish embryonic development, we used two independent antisense morpholino oligos (MOs) to knock the expression of AGGF1 down *in vivo*, and examined the resulting vessel phenotypes. MO1 targets the first exon 1–intron 1 boundary, and MO2 targets the start codon ATG (Fig. 3A). The efficacy of MO1 was confirmed by RT–PCR analysis. MO1 created a new splice donor site, which results in an alternative AGGF1 transcript with addition of 39 nucleotides from intron 1, leading to frame shift and resulting in a premature stop codon (Fig. 3B and C). The effectiveness of MO2 was examined by injecting MO2 together with an AGGF1–EGFP reporter plasmid, which contains the start codon of AGGF1, some sequences flanking the start codon and the N-terminal coding region. MO2 markedly reduced the GFP signal (AGGF1 expression) by 85% (*n* = 69) compared with a control Std-MO (*n* = 81) (Fig. 3D).

We knocked AGGF1 expression down first in Tg(*kdrl:GFP*)*la116* embryos to examine its effect on vascular development. Compared with Std-MO-injected embryos, embryos injected with AGGF1 MO1 and MO2 did not show severe morphological defects (Supplementary Material, Fig. S3). We also analyzed the expression of *myoD* and *pax2.1* by WISH in 12 hpf and 26 hpf embryos injected with AGGF1 MO1. AGGF1 knockdown did not affect the expression level or pattern of *myoD* and *pax2.1*, further suggesting that knockdown of AGGF1 expression did not affect morphological development (Supplementary Material, Fig. S4).

Knockdown of AGGF1 by injection of either AGGF1 MO1 or MO2 impaired the formation of dorsal longitudinal anastomotic vessels (DLAVs) and ISVs compared with embryos injected with a control Std-MO at 26 hpf (Fig. 4A).

We then analyzed the effect of AGGF1 MOs on vascular phenotypes in a zebrafish AB strain using AP staining for AGGF1. Overexpression of zebrafish AGGF1 mRNA expanded the PCV at 24 hpf. Ten embryos from two independent experiments were scored for each treatment. (E) *In situ* hybridization of venous marker *flt4* at 24 hpf. The expression of *flt4* in the PCV was increased in AGGF1 overexpression embryos compared with uninjected embryos or embryos injected with mCherry mRNA. (F) *In situ* hybridization of arterial marker *notch5* at 24 hpf. The expression of *notch5* in DA was not affected in AGGF1 overexpression embryos. The white arrow indicates pronephric expression outside the focal plane (25), ns, not significant.

AGGF1 is involved in vein development

We carried out a detailed analysis of the effect of AGGF1 knockdown by MOs on the development of veins, specifically...
the PCV. The PCV in Tg(kdrl:GFP)la116 embryos injected with AGGF1 MO1 disappeared at 24 hpf, whereas embryos injected with a Std-MO showed the presence of both the DA and PCV (Fig. 5A–C). Similar results were obtained in transverse sections in embryos at 26 or 48 hpf (Fig. 5D–G). These results suggest that AGGF1 plays an important role in the differentiation of veins.

To further validate the functional role of AGGF1 in vein development, we analyzed the expression levels of artery-specific and vein-specific markers in zebrafish embryos. Expression of...
artery-specific markers notch5, ephrinB2 and grl (13) was shown to be restricted to the DA region. Compared to embryos injected with a control Std-MO, embryos injected with AGGF1 MO1 showed increased notch5 expression in the DA domain. No marked difference was detected on expression of ephrinB2 and grl in the DA domain (Fig. 5H–K). Injection of AGGF1 MO2 also led to upregulation of notch5 in the DA domain (Fig. 5P), but did not affect the expression of grl in the same region (Fig. 5Q).

In contrast, the expression of three vein-specific markers, flt4, dab2 and ephB4 (11,13), almost disappeared with knockdown of AGGF1 expression by AGGF1 MO1 (Fig. 5L–O). In addition, knockdown AGGF1 by AGGF1 MO2 also severely reduced expression of flt4 and dab2 in the PCV domain (Fig. 5S–U).

In addition to their effect on the development of the PCV, we found that AGGF1 MO1 and MO2 also inhibited the development of the midcerebral vein in the brain region compared with a Std-MO (Supplementary Material, Fig. S8).

The data above suggest that AGGF1 is required for establishing venous cell fate (versus arterial cell fate). To further show that AGGF1 is involved in vasculogenesis, we analyzed embryos at 12 hpf for expression of etsrp, a molecular marker of angioblasts. Compared with embryos injected with a Std-MO, those injected with AGGF1 MO1 or MO2 showed significantly reduced expression of etsrp (Fig. 6A–D). WISH of 12 hpf embryos with both etsrp and myoD probes excluded the possibility that the shorter etsrp signal was due to developmental delay (Fig. 6E).

Overexpression of constitutively activated AKT restores vascular defects caused by AGGF1 knockdown

To identify the molecular mechanism by which AGGF1 specifies vein differentiation, we next analyzed the phosphorylation of AKT, which was shown to be involved in venous specification (18–20). Western blot analysis showed that knockdown of AGGF1 expression inhibited activation (phosphorylation) of AKT, whereas injection of zebrafish AGGF1 mRNA induced activation of AKT in embryos (Fig. 7A and B). Similarly, whole-mount immunostaining with an anti-phosphorylated AKT antibody showed that knockdown of AGGF1 expression inhibited AKT activation, whereas injection of zebrafish AGGF1 mRNA increased AKT activation (Supplementary Material, Fig. S9).

Interestingly, co-injection of mRNA for a constitutively active human myrAKT (myristoylated AKT) with AGGF1 MO into Tg(kdrl:GFP)ja16 embryos could restore ISV defects caused by knockdown of AGGF1 expression at 36 hpf stage (Fig. 7C and F). In addition, co-injection of human myrAKT mRNA rescued the ISV defects caused by AGGF1 MO2 (ISVs were marked by flk1 expression) (Fig. 7H and J).

We have found that injection of AGGF1 MO in embryos caused near absence of expression of flt4, a specific marker for the PCV (11,13), but co-injection of myrAKT mRNA
Figure 5. AGGF1 specifies development of veins. (A and B) Injection of AGGF1 MO1 blocked the development of the PCV in Tg(kdrl:GFP)k116 transgenic embryos at 24 hpf (lateral views with the head to the left). DA, dorsal aorta (red bracket); PCV, posterior cardinal vein (blue bracket). (C) Statistical analysis of the percentage of embryos with defective PCV. (D and E) Transverse sections of 26 hpf embryos showing the normal DA and PCV in embryos injected with a Std-MO (D) and a single DA in AGGF1 morphants (E). (F and G) Transverse sections of 48 hpf embryos showing the normal DA and PCV in Std-MO injected embryos (F) and a single DA in AGGF1 morphants (G). (H–J) WISH of 26 hpf embryos with artery-specific markers notch5 (H), ephrinB2a (I), and grl (J) in AB embryos injected with a control Std-MO (left) or AGGF1 MO1 (right). (K) Statistical analysis of artery-specific genes expression levels in (H–J). (L–N) Similar analysis as in (H–J), but with vein-specific markers flt4 (L), dab2 (M) and ephB4 (N). The expression of these genes was nearly absent in the PCV domain. (O) Statistical analysis of vein-specific genes expression levels in (L–N). (P–Q) WISH of 26 hpf embryos with artery-specific markers notch5 (P) and grl (Q). (R) Statistical analysis of artery-specific genes expression levels in (P–Q). (S–T) The expression of venous markers flt4 and dab2 was dramatically decreased in 26 hpf embryos injected with AGGF1 MO2 compared with those with control Std MO in the PCV domain. (U) Statistical analysis of vein-specific genes expression levels in (S and T). Scale bar in H–T, 1 mm.
could restore the expression of flt4 in PCV (Fig. 7E, G, I and K). These results further demonstrate that AGGF1 determines venous specification by activating AKT.

**DISCUSSION**

In this study, we used zebrafish as a model organism to characterize the in vivo physiological functions of AGGF1. We demonstrated that AGGF1 had a profound effect on the development of vasculature. Overexpression of AGGF1 by injection of AGGF1 mRNA into zebrafish embryos induced oversprouting of ISVs, increased filopodia formation from ISVs, excessive branching of SIVs and increased the diameter of the PCV during embryogenesis (Fig. 2). We also used a morpholino antisense oligo knockdown technology in zebrafish to characterize the in vivo physiological functions of AGGF1. Knockdown of AGGF1 expression by two independent AGGF1 MOs impaired the formation of DLAVs, and ISVs and resulted in a loss of SIVs (Fig. 4). These results indicate that AGGF1 plays an important role in vascular development in zebrafish.

We have previously identified genomic variants in AGGF1 which are associated with vascular disease KTS (7,8); however, the molecular mechanism by which these genomic variants increase the risk of KTS in vivo is unknown. Previously we reported that the t(5;11) chromosomal mutation in a KTS patient increased the expression of AGGF1 by about 3-fold in cultured human umbilical vein ECs or HEK293 cells (7,9), but the in vivo effect of increased AGGF1 expression is unknown. In this study, we directly demonstrate that increased AGGF1 expression in one- to two–cell-stage zebrafish embryos resulted in increased ISV oversprouting (Fig. 2). Increased AGGF1 expression caused important venous differentiation as marked by an increase in the diameter of the PCV and upregulation of flt4 expression in the PCV (Fig. 2). These results provide a pathogenic basis for KTS. A study of 36 KTS limbs and 30 normal limbs revealed that calf blood flow was significantly faster in KTS limbs than in normal limbs (3.67 ± 0.26 versus 2.40 ± 0.20 ml/min/100 ml tissue, P < 0.001) (22). Moreover, histological analysis revealed an increase in the number and diameter of small venules in affected KTS tissues compared with normal tissues (22). Together, both human studies and animal modeling studies lead to the conclusion that KTS is caused by increased angiogenesis mediated by increased AGGF1 expression.

One most important finding from this study is that AGGF1 plays an important role in the development of veins. Knockdown of AGGF1 expression by AGGF1 MOs inhibited the development of the PCV (Fig. 5). Consistent with this observation, the expression of three vein-specific markers flt4, dab2 and ephB4 almost disappeared in embryos with knockdown of AGGF1 expression, whereas no effect was observed for DA differentiation (Fig. 5). In contrast, overexpression of AGGF1 led to an increased diameter of the PCV and increased expression of venous marker flt4 (Fig. 2). Knockdown of AGGF1 by injection of AGGF1 MOs also inhibited the development of the midcerebral vein in the brain (Supplementary Material, Fig. S8). These results again
Figure 7. Critical function of AKT activation in AGGF1-mediated vascular development. (A) Western blot analysis to assess the effect of AGGF1 MO1 (2.5 ng) or overexpression of zebrafish AGGF1 mRNA (100 pg) on the phosphorylation level of AKT (p-AKT, Ser437). Total AKT (T-AKT) was used as the loading control. (B) The p-AKT level of AGGF1 MO1 or AGGF1 overexpression was compared with normal embryos by statistical analysis. (C) Rescue of ISV vascular defects in 36 hpf Tg(kdrl:GFP)al16 embryos injected with AGGF1 MO1 (2.5 ng) by overexpression of human myristoylated-AKT mRNA (100 pg). (D) WISH analysis showing rescue of defects in ISVs marked with fltl expression in embryos injected with AGGF1 MO1 (2.5 ng) by overexpression of human myristoylated-AKT mRNA (100 pg) (red arrows) at 26 hpf. (E) WISH analysis showing rescue of venous defects in fltl expression in embryos injected with AGGF1 MO1 (2.5 ng) by overexpression of human myristoylated-AKT mRNA (100 pg) (black arrow) at 26 hpf. (F) Graph showing the percentage of wild-type embryos and embryos with defective ISVs from C and D. (G) Graph showing the percentage of wild-type embryos and embryos with a defective PCV from E. (H) Co-injection with AGGF1 MO2 (5.0 ng) with human myristoylated-AKT mRNA (100 pg) recovered fltl expression in the ISV domain (red arrow) at 26 hpf. (I) Constitutively activated myrAKT rescued the PCV defects caused by AGGF1 MO2 (5.0 ng) at 26 hpf embryos (black arrows). WISH staining for fltl was performed to localize the PCV at 26 hpf embryos. (J) Graph showing the percentage of wild-type embryos and embryos with defective ISVs from H. (K) Graph showing the percentage of wild-type embryos and embryos with a defective PCV from I. Scale bar, 1 mm.
are consistent with the clinical findings in KTS patients. Since 1900, it has been a mystery why vascular disease KTS affects veins, but not arteries (23,24). The results in this study provide a molecular explanation why the pathogenesis of KTS does not involve arteries.

It is well known that VEGFA is the determining factor for arterial specification in vertebrates (17,25,26). VEGFA binds to its receptors VEGFR2 and Nrp 1, activates the Raf/ERK signaling cascade and increases the expression of Notech, leading to arterial specification (12,13,20). In this study, we established AGGF1 as an angiogenic factor for venous specification. We also identified the corresponding angiogenic signaling pathway for establishing venous cell fate by AGGF1. We show that AGGF1 activates AKT, leading to venous specification. It is possible that two growth factors, VEGFA and AGGF1, act as two parallel pathways in artery–vein specification, which ultimately determines the development of either arteries or veins, respectively.

Little is known about the molecular mechanism for specification of veins. ERK1/2-AKT crosstalk was shown to play a crucial role in artery–vein differentiation in the mouse and zebrafish (18–20). ERK1/2 signaling mediated by VEGFA was shown to be involved in arterial morphogenesis, whereas AKT appeared to promote venous morphogenesis by suppressing ERK activation (18–20). However, the upstream regulatory factor for activation of AKT during venous specification, in particular an angiogenic factor like VGEFA for arterial specification, is unknown. Our findings in the present study suggest that venous specification requires activation of AKT by angiogenic factor AGGF1. Knockdown of AGGF1 expression reduced the activation level of AKT, whereas overexpression of AGGF1 increased the activation of AKT (Fig. 7). Furthermore, overexpression of a constitutively active form of AKT (myr-AKT) rescued the decreased expression level of vein marker flt4 as well as vascular defects caused by knockdown of AGGF1 expression (Fig. 7). However, the detailed molecular mechanisms by which AGGF1 activates AKT and promotes venous development need future studies.

It is interesting to note that the expression profile of AGGF1 differs dramatically between zebrafish and mice. We previously used immunostaining to demonstrate the strong expression of the AGGF1 protein in blood vessels embedded in various mouse organs (kidney, heart, limb, tail, etc.) (7). We failed to detect AGGF1 expression in vessels in 48 hpf zebrafish embryos by in situ hybridization (Fig. 1). These results may suggest that AGGF1 acts in a paracrine fashion in zebrafish. To substantiate this hypothesis, we investigated whether specific overexpression of AGGF1 in ECs (versus ubiquitous overexpression as control) could rescue the ISV defects caused by knockdown of AGGF1 MOs. We found that ubiquitous overexpression of AGGF1 using the CMV promoter rescued the AGGF1 MO defects, but EC-specific overexpression using the kdr promoter failed to rescue the defects. These data provide the support that AGGF1 acts by a paracrine mechanism. This conclusion is further supported by our recent finding that injection of a mammalian expression plasmid for human AGGF1 into skeletal muscles resulted in increased angiogenesis, increased blood flow in limbs and significant improvement of mobility in a mouse hindlimb ischemic model for peripheral artery disease (27). However, a caution should be taken not to over-interpret these data because the kdrl promoter may not be as strong as the CMV promoter in transcription activation. Previously, we found that in the mouse, an AGGF1 protein signal co-localized with both EC marker CD31 and vascular smooth muscle cell marker α-actin (7). Thus, an autocrine mode of signaling by AGGF1 remains a possibility in mammalian systems. Clearly, more studies are needed to resolve this issue.

The significance of the present finding is 2-fold. Because AGGF1 plays a critical role in the differentiation of veins, it is not only involved in the development of VMs and varicose veins present in KTS patients, but may be associated with more common VMs and varicose veins, which are probably the commonest disorder presenting to general surgeons and affect ~22.1% of female adults and 14.6% of males (28).

Second, for antiangiogenic therapy, a combination of anti-AGGF1 and anti-VEGFA agents may be superior to anti-VEGFA therapy alone. The well-known anti-VEGFA antiangiogenic therapy is encouraging, but less impressive than expected because a majority of patients do not respond, and the duration of response and overall survival are modest (29).

Resistance to the bevacizumab antiangiogenic therapy is also an issue. The tumor vasculature is highly heterogeneous (30). In fact, studies based on the morphological criteria before 2001 suggested that new vessel formation in tumor angiogenesis was all derived from postcapillary venules (venous origin), although later studies showed that some of the tumor vessels also had arterial origin (31,32). Inhibition of vessels from all origin, including anti-AGGF1 targeting veins, may be more effective for successful antiangiogenic therapy. In the same fashion, therapeutic angiogenesis combining various angiogenic factors, including AGGF1, may be more effective in treating ischemic heart disease.

In addition to venous specification, our data showed that AGGF1 knockdown also affected the development of ISVs and the DLAV (Fig. 4). ISVs are sprouted from the DA and the DLAV is sprouted from ISVs, and both ISVs and DLAVs clearly express arterial markers gtl and ephrinB2 (33). Thus, AGGF1 plays an important role in angiogenesis and we cannot exclude the possibility that AGGF1 may play a role in the development of some arterial structures. In addition, we found that AGGF1 knockdown inhibited an accumulation of PL cells (parachordal lymphangioblasts) (34) (Fig. 4B), and it suggests that AGGF1 may also be involved in the development of lymphatic vessels. Future studies are needed to clarify these two issues.

In conclusion, we have found that AGGF1 plays an important role in vascular development and identified an angiogenic factor, AGGF1, for establishing venous cell fate. Our results demonstrate that AGGF1 activates AKT to promote the development of veins in zebrafish embryogenesis. These results reveal a novel molecular signaling mechanism responsible for establishing venous cell fate and suggest that independent angiogenic pathways are evolved to control the differentiation of arteries and veins, i.e. VEGFA for arteries and AGGF1 for veins, respectively.
MATERIALS AND METHODS

Zebrafish stocks and fish care
Wild-type AB zebrafish and Tg(kdrl:GFP)Ja116 transgenic zebrafish lines (35) (a kind gift from Dr Wuhan Xiao) were used. Zebrafish was grown and maintained at 28.5°C under standard conditions. Embryos were raised and maintained using standard protocols (36). This study was approved by the ethics committee of Huazhong University of Science and Technology and Institute of Hydrobiology of the Chinese Academy of Sciences.

Identification and homology analysis of zebrafish AGGF1
The full-length sequence of zebrafish AGGF1 gene was identified by searching the NCBI database (http://www.ncbi.nlm.nih.gov). The homology analysis of the zebrafish AGGF1 protein sequence with other protein sequences was carried out using the online program ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Microinjection of morpholin antisense oligos (MOs)
MOs were synthesized by GeneTools (Philomath, Oregon). Two AGGF1-specific MOs were used in the study. One MO is an antisense oligo affecting AGGF1 splicing (MO1, 5′-GCCCTGCTACCTGCTGTCGGAGAT-3′), and the other MO blocks the translation of the AGGF1 protein (MO2, 5′-CGCATCAATAGGGAGCAACCGATG-3′). The negative control is a standard control (Std) morpholino (5′-CCTCTTACCCTCAGTTACAATTTATA-3′).

MOs were injected into the yolk of one- to two-cell-stage zebrafish embryos using a pneumatic picopump (AGGF1 MO1, 2.5 ng; AGGF1 MO2, 5.0 ng; Std-MO, 5.0 ng; dissolved in water) as described by Hyatt et al. (37). The embryos were then maintained and analyzed at different developmental stages.

Preparation and microinjection of mRNAs
A cDNA clone with the full-length zebrafish AGGF1 was purchased from OpenBiosystems. The zebrafish AGGF1 coding region was then PCR-amplified from the cDNA clone, subcloned into a pGEM-T easy vector (Promega) and verified by DNA sequence analysis. The zebrafish AGGF1 cDNA was then subcloned into the pSP64 vector, resulting in pSP64-zAGGF1.

The cDNA for human AGGF1 (hAGGF1) was amplified by PCR analysis from a mammalian expression plasmid for human AGGF1 described by us previously (7). The PCR product was subcloned into the pSP64 vector, resulting in plasmid pSP64-hAGGF1.

The cDNA for zebrafish VEGFA165 was amplified using RT–PCR as described for zAGGF1, and subcloned into the pSP64 vector, resulting in pSP64-zVEGFA.

The cDNA for the coding region of the mCherry gene (negative control) was amplified by PCR analysis from the pmCherry-N1 vector (Clontech Laboratories, Inc) using primers listed in Supplementary Material, Table S1. The PCR product was subcloned into the pSP64 vector, resulting in plasmid pSP64-mCherry.

The cDNA for constitutively active myristoylated human AKTI (myrAKTI) was obtained using RT–PCR analysis with HEK293 RNA samples with a forward primer (5′-GTC GAC ATG GTG AGC AAC AAG AGC CCC AAG GAT GCC AGC CAG CGG ATG AGC GAT GCT ATT GTG AA-3′) containing Src myristoylation sequences at 5′ end (underlined) (38–40) and a reverse primer (5′-GGA TCC TCA GCC GTG GCC GCT GGC CG-3′). The cDNA for myrAKTI was subcloned into the pSP64 vector, resulting in pSP64-myrAKTI.

The pSP64-derived plasmids were linearized and used for the preparation of capped mRNAs for mCherry, zAGGF1, hAGGF1, myrAKT1 and zVEGFA using SP6 RNA polymerase and the mMESSAGE Mmachine system (Ambion, Austin, TX). Capped RNA samples (75–100 pg) were injected into the yolk of one- to two-cell-stage zebrafish embryos as described for MOs above.

Expression profiling of zebrafish AGGF1 using RT–PCR analysis
The expression profile of zebrafish AGGF1 was analyzed using RT–PCR analysis as described (41–43). Total RNA samples were extracted from 50 embryos at different developmental stages or different adult tissue samples harvested from 10 AB zebrafish using TRizol (Invitrogen). The RNA samples were converted into cDNA and used for RT–PCR analysis with forward primer 5′-AAT TAA AGA TGG GAG AAA -3′ and reverse primer 5′-CTC CGA GTG GTA TTA AAA TGA A -3′ as described (43–47).

Whole-mount staining of blood vessels using alkaline phosphatase
Alkaline phosphatase was used to stain vessels as described (48,49). Zebrafish embryos were processed routinely for AP at 72 hpf. In brief, embryos were fixed with 4% PFA (paraformaldehyde) in phosphate-buffered saline (PBS) buffer overnight at 4°C, dechorionated, treated with methanol for 2 h at −20°C and three times of 5 min rinsing with PBS with Tween20 (PBST) at room temperature, and digested with protease K (10 µg/ml in PBST) for 3 min at room temperature. The embryos were then re-fixed in 4% PFA for 1 h at room temperature, rinsed with PBST for 5 min (three times) at room temperature and equilibrated with AP buffer (100 mM NaCl, 50 mM MgCl2, 100 mM Tris pH 9.5, 0.1% Tween20) for 5 min for three times (3). The embryos were then stained with NBT/BCIP for 10–15 min and imaged under a microscope (Olympus SXZ16).

Whole-mount in situ hybridization
For generation of antisense RNA probes for WISH, we first used the PCR to amplify a 300–600 bp fragment specific for each gene under study using primers listed in Supplementary Materials, Table S1 (some of the probes for WISH were provided by Bo Hu). The gene fragments were then subcloned into a pGEM-T easy vector. The pGEM-T easy-derived plasmids were linearized. Antisense RNA probes were transcribed from linearized templates by T7 or SP6 polymerases.
(Promega) in the presence of DIG-labeled nucleotides (Roche, Mannheim, Germany).

WISH was carried out as previously described (50) using the antisense RNA probes prepared as above. Hybridization signals were detected using anti-digoxigenin-AP (Roche Mannheim, Germany) and by staining with BCIP/NBT (Promega).

**Western blot analysis**

Western blot analysis was carried out as described (9,46,51,52). Protein extracts were prepared from 24 hpf stage zebrafish embryos (n = 50) by homogenization in 100 µl of lysis buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol), fractionated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to PVDF membranes and probed with a primary polyclonal antibody. The membranes were then incubated with a goat anti-rabbit HRP-conjugated secondary antibody (Bio-Rad), which was followed by incubation with a SuperSignal West Pico chemiluminescent substrate (Thermo). The membranes were scanned with a FluorChem imager (Alpha Innotech) and analyzed. The primary antibodies used include an anti-phosphorylated-AKT (Ser473) antibody and an anti-AKT antibody from cell Signaling.

**Whole-mount immunostaining**

Whole-mount immunostaining for detecting the activity of AKT (phosphorylation levels) in 24 hpf zebrafish embryos was carried out as described (53).

**Rescue of ISVs defects associated with AGGF1 MOs by EC-specific or ubiquitous overexpression of AGGF1**

To develop an expression plasmid that drives overexpression of a zAGGF1–mCherry fusion protein specifically in ECs, we amplified the full zAGGF1 coding region from a zAGGF1 cDNA clone (OpenBiosystems) using PCR analysis and subcloned it into a mutant vector pmCherry-N1 without the CMV promoter, resulting in pAGGF1–mCherry. We then amplified a 6.4 kb kdr promoter fragment (35) using PCR and zebrafish genomic DNA, and subcloned it into pAGGF1–mCherry, resulting in pkdrl–AGGF1–mCherry. We also created a control expression plasmid, pCMV–AGGF1–EGFP, that drives ubiquitous expression of AGGF1 in the whole embryos. The full zAGGF1 coding region (starting with ATG) was PCR-amplified as described above and subcloned into pEGFP-N1, resulting in pCMV–AGGF1–EGFP.

We injected 100 pg of an expression plasmid together with 2.5 ng of AGGF1 MO1 or 5.0 ng of AGGF1 MO2 in single-cell-stage zebrafish embryos. The embryos were raised to 26 hpf and analyzed for ISV development by WISH with a fli1 probe.

**Statistical analysis**

An unpaired Student’s t-test was used to compare the means from two different groups. The data were presented as means ± SD. A P-value of ≤0.05 was considered to be significant.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** Q.W. declared acting as a consultant for Merck & Co., Inc.

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