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Notch signaling is required for arterial-venous differentiation during embryonic vascular development

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SUMMARY

Recent evidence indicates that acquisition of artery or vein identity during vascular development is governed, in part, by genetic mechanisms. The artery-specific expression of a number of Notch signaling genes in mouse and zebrafish suggests that this pathway may play a role in arterial-venous cell fate determination during vascular development. We show that loss of Notch signaling in zebrafish embryos leads to molecular defects in arterial-venous differentiation, including loss of artery-specific markers and ectopic expression of venous markers within the dorsal aorta. Conversely, we find that ectopic activation of Notch signaling leads to repression of venous cell fate. Finally, embryos lacking Notch function exhibit defects in blood vessel formation similar to those associated with improper arterial-venous specification. Our results suggest that Notch signaling is required for the proper development of arterial and venous blood vessels, and that a major role of Notch signaling in blood vessels is to repress venous differentiation within developing arteries.

Movies available on-line

Key words: Artery, Notch, Vein, Zebrafish

INTRODUCTION

The differentiated identity of arterial and venous blood vessels was thought to arise in response to hemodynamic forces such as blood pressure and the direction of blood flow. However, recent data suggest that molecular differences between arterial and venous endothelial cells are apparent well before the onset of circulation. The transmembrane ligand ephrin-B2 is expressed in an artery-specific fashion within both extraembryonic and embryonic blood vessels before circulation (Adams et al., 1999; Wang et al., 1998). Homozygous targeted disruption of *ephrin-B2* results in defective remodeling of the yolk sac vascular plexus and decreased branching of cranial capillaries, as well as abnormal sprouting of intersomitic vessels and decreased vascularization of the neural tube. Expression of a *tau-lacZ* transgene recombined into the *ephrin-B2* locus is maintained in arterial endothelial cells in the absence of functional ephrin-B2 (Wang et al., 1998), suggesting that it is not necessary for the initial specification of arterial fate. EphB4, the proposed receptor for ephrin-B2 within the cardiovascular system, is expressed at higher levels on venous endothelial cells (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998) and mice lacking EphB4 exhibit vascular abnormalities similar to mice lacking ephrin-B2 (Gerety et al., 1999). These observations suggest that establishing arterial and venous identity is a crucial step during embryonic vascular development. However, despite the functional relevance of artery and vein restricted expression of ephrin-B2 and EphB4, respectively, little is known about the upstream factors that are responsible for defining the differences between arteries and veins. It has recently been reported that mice lacking the activin receptor-like kinase-1 (ACVRL1) fail to express ephrin-B2 within their arterial vessels, suggesting a possible role for TGFβ signaling during this process (Urness et al., 2000).

A number of signaling pathways have known roles in cell fate determination, including the Notch pathway. Notch is a large transmembrane receptor that is important in a wide array of developmental contexts for specifying cell populations with different fates and for defining the boundaries between them (Irvine, 1999; Muskavitch, 1994). In vertebrates, Notch signaling is required for normal neurogenesis, somite formation and lymphoid cell development (Chitnis et al., 1995; Conlon et al., 1995; Dornseifer et al., 1997; Ellisen et al., 1991; Pui et al., 1999; Takke et al., 1999). A variety of evidence suggests that Notch has an important role during blood vessel development. In the mouse, *Notch1*, *2* and *4* are expressed within endothelial cells (Del Amo et al., 1992; Uyttendaele et al., 1996; Zimrin et al., 1996). Targeted disruption of *Notch4* shows that it is dispensable for vascular development (Krebs et al., 2000), while expression of an activated form of Notch4 within the endothelium disrupts normal vascular development (Uyttendaele et al., 2001) and mice lacking Notch1 display defects in angiogenic remodeling (Krebs et al., 2000). There is

also evidence of a role for Notch signaling in vessel homeostasis. CADASIL (*C*erebral *A*utosomal *D*ominant *A*rteriopathy with *S*ubcortical *I*nfarcts and *L*eukoencephalopathy), a human disease characterized by early adult onset stroke and dementia, is caused by mutations in *Notch3* (Joutel et al., 1996) and its etiology has been ascribed to a vascular defect (Salloway and Hong, 1998).

Notch ligands are also expressed within the endothelium and are required for proper vessel development. *Jagged1* is expressed in the developing endothelium and targeted disruption of the *Jagged1* gene in mice results in defects in head and yolk sac angiogenesis, although the formation of the major vessels appears normal (Xue et al., 1999). In zebrafish, *deltaC* is expressed in migrating lateral mesodermal vascular progenitors, and at later stages its expression is restricted to the dorsal aorta (DA) (Smithers et al., 2000), while a recently described novel Notch ligand, *Dll4*, is expressed in a similar pattern in mouse (Shutter et al., 2000).

Potential downstream targets of Notch signaling have also been identified within the developing vascular system. The recently identified HRT proteins are *hairy*-related basic helix-loop-helix (bHLH) transcription factors that display vascular-specific expression in mice and humans (Chin et al., 2000; Nakagawa et al., 1999). Recently, mHRT2 was shown to be a direct target of Notch signaling in in vitro cultured cells (Nakagawa et al., 2000). A mutation in the zebrafish homolog of HRT2 was found to be responsible for the *gridlock (grl)* mutation (Zhong et al., 2000), which causes a localized vascular patterning defect within the DA (Weinstein et al., 1995). Interestingly, *grl* expression becomes restricted to the DA during blood vessel development (Zhong et al., 2000), suggesting a possible role in arterial-venous differentiation.

We provide evidence that Notch signaling is important for arterial-venous differentiation of blood vessels. We find that zebrafish *notch3* is expressed in the DA, but not the posterior cardinal vein (PCV), during embryonic vascular development. Embryos lacking Notch activity fail to induce arterial-specific *ephrinB2* (*efnb2a*) expression, and exhibit ectopic expression of venous markers within the DA. Furthermore, we show that activation of the Notch signaling pathway, either throughout the embryo or targeted to the endothelium, causes loss of venous-specific marker expression. Finally, perturbation of Notch signaling causes defects in vascular morphology similar to those observed in mice lacking ephrin-B2 or EphB4, including abnormal remodeling of the major trunk vessels and aberrant intersomitic vessel projection. Taken together, our results suggest that Notch signaling is required within the vasculature for proper arterial-venous differentiation and repression of venous cell fate.

MATERIALS AND METHODS

Zebrafish

The wild-type line EK was obtained from Ekkwill Farms (Gibsonton, FL) and inbred for several generations. Fish heterozygous for *mib*^{ta52b} were provided by Julian Lewis (Imperial Cancer Research Fund, London). Fish and embryos were maintained as described previously (Westerfield, 1993). The *hsp70:Gal4* and *UAS:notch1a-intra* ICD transgenic lines are described elsewhere (Scheer and Campos-Ortega, 1999; Scheer et al., 2001). Hemizygous adult fish were set up in multiple matings and eggs collected the following morning. The development of embryos was delayed by placing in a chilling incubator overnight at 24°C. The following day, embryos were heat shocked at either the 15- or 18-somite stage by transferring to a capped 50 ml culture flask (Nalge Nunc International, Rochester, NY) and incubating at 40°C for 15 or 30 minutes. Embryos were then incubated at 28.5°C until the 28- to 30-somite stage, after which they were fixed and processed for in situ hybridization.

Plasmids and probes

Plasmids encoding zebrafish *tie1*and *flt4* were kindly provided by Leonard Zon (Children's Hospital, Boston). A plasmid for zebrafish *ephrinB2* was kindly provided by Michael Tsang (NICHD, Bethesda). A plasmid encoding *tbx20* was graciously provided by Dae Gwon Ahm and Robert Ho (Princeton University, Princeton). Plasmid pBS 30D1.C, containing a fragment of zebrafish *deltaC* was provided by Julian Lewis (Imperial Cancer Research Fund, London). A plasmid containing the coding sequence of *rtk5* was kindly provided by Julie Cooke (University College, London). A plasmid containing a fragment of the extracellular domain of *notch3* (C.-H. K., unpublished data) was linearized with *Nco*I and transcribed with SP6.

The pCSGreen plasmid was constructed by digesting pEGFP-C1 (Clontech, Palo Alto, CA) with *Nhe*I and ligating to oligonucleotide linkers (5′-CTAGGCTTGATTTAGGTGACACTATAGAATACAAG-CTACTTGTTCTTTTTGCAG and 5′-CTAGCTGCAAAAAGAACA-AGTAGCTTGTATTCTATAGTGTCACCTAAATCAAGC) containing the SP6 promoter and 5′ leader sequence found in pCS2+ (Rupp et al., 1994). An enhanced green fluorescent protein (EGFP)/XSu(H) DBM fusion construct was made by partially digesting pCS2+XSu(H)DBM (provided by C. Kintner, Salk Institute, San Diego) with *Eco*RI and X*ho*I. This fragment was cloned into pCSGreen digested with *Eco*RI and *Sal*I to yield pCSGSuDN. Plasmids were linearized with *Mlu*I and mRNA was synthesized using the mMessage mMaker kit (Ambion, Inc., Austin, TX).

An *Xba*I fragment of the zebrafish *fli1* promoter that includes 1 kb upstream of exon 1 and 6 kb of intron 1 was subcloned into pBluescript from a PAC obtained by hybridization to the 5′ end of the *fli1* cDNA (Brown et al., 2000). An oligonucleotide linker containing *Nhe*I, *Srf*I, and *Asc*I sites was cloned into an *Mlu*I site upstream of the *fli1* start codon to give pBS119d10L. A fragment encoding the intracellular domain (ICD) of zebrafish Notch3 was cloned in frame with the myc epitope in pCS3+MT. The cassette containing the myc-tagged Notch3 ICD and the polyA signal sequences was removed from pCS3 MTN3ICD by digesting with *Dra*I and *Not*I, filled in with Klenow and cloned into pBS119d10L digested with *Srf*I to give pflimTN3ICD.

In situ hybridization and immunostaining

Whole-mount in situ hybridization was performed as described previously (Hauptmann and Gerster, 1994). Immunostaining was performed as described previously (Westerfield, 1993) using monoclonal antibody 9E10 (Berkeley Antibody Co., Richmond, CA) that recognizes the myc epitope.

Videomicroscopy, microangiography and histological analysis

Embryos were raised at 28.5°C in 30% Danieau buffer containing 0.003% 1-phenyl-2-thiourea to prevent pigmentation. For videomicroscopy and microangiography individual embryos were anesthetized in 0.02% tricaine (Sigma, St. Louis, MO) and mounted in 5% methyl cellulose dissolved in 30% Danieau buffer. Videomicroscopic images were collected directly on videotape using a Zeiss Axioplan microscope equipped with an MTI-DAGE SIT-68 camera. Video clips were assembled, edited, and compressed into Quicktime movies using Adobe Premiere 5.1. Microangiography was performed as described (Weinstein et al., 1995). For histological analysis, embryos were embedded in JB4 plastic resin according to described protocols (Westerfield, 1993). Embedded embryos were

sectioned using a Leica microtome and sections were mounted on glass slides. Sections were stained with Hematoxylin and Eosin, coverslipped using Permount, and photographed under water immersion at 630× magnification using a Zeiss Axioplan microscope.

Microinjection

Capped mRNA was injected into 1-cell stage wild-type embryos as described (Xu, 1999). Expression was monitored at shield stage using a Leica MZFLII dissection microscope equipped for epifluorescence with an FITC filter set. Embryos not exhibiting EGFP expression at this point were removed and not included in later analyses. For DNA injection, plasmids were linearized by digestion with *Not*I followed by phenol:chloroform extraction and ethanol precipitation. Approximately 100-200 pg of linearized DNA was injected into early 1-cell stage embryos.

RESULTS

Markers of arterial and venous identity in zebrafish trunk vessels

We are interested in determining the factors required for arterial-venous specification during vascular development in the zebrafish, *Danio rerio*. We characterized the vascular expression patterns of the zebrafish homologs of ephrinB2 and its receptor, EphB4, in developing embryos by whole-mount in situ hybridization. Although a previous study had described the expression of zebrafish *ephrinB2* in presomitic mesoderm and in the posterior half of developing somites (Durbin et al., 1998), its expression in blood vessels was not reported. We found that, as in the mouse (Wang et al., 1998), *ephrinB2* is also expressed in the zebrafish vasculature and is restricted to the DA at the 30-somite stage (Fig. 1B,C). *ephrinB2* expression in the DA is initiated after migrating lateral mesodermal angioblasts reach the trunk midline (between the 16- and 18-somite stages; data not shown). Interestingly, we found that zebrafish *notch3* (Kim, C.-H. and Chitnis, A. B., unpublished; previously known as *notch5*; Westin and Lardelli, 1997) is also expressed within the DA but not the PCV (Fig. 1D,E). The other zebrafish notch homologs do not exhibit a similar expression pattern (Westin and Lardelli, 1997) (data not shown). Two putative homologs of EphB4, referred to as *rtk5* and *rtk8*, have also been reported in the zebrafish (Cooke et al., 1997). The vascular expression of these genes had also not been previously reported. We found that *rtk5* is expressed within the vasculature and is found at a higher level in the posterior cardinal vein (PCV) than in the DA (see Fig. 4C). *Rtk5* is also expressed within the hypochord (Fig. 4C), a structure that has been implicated in DA formation in *Xenopus laevis* (Cleaver and Krieg, 1998). The expression patterns of the receptor tyrosine kinase *flt4*, which becomes restricted to venous blood vessels by the 30-somite stage (Fig. 1F,G), and the ETS family transcription factor *fli1*, which is expressed in both the DA and PCV (Fig. 1H,I), are shown for reference.

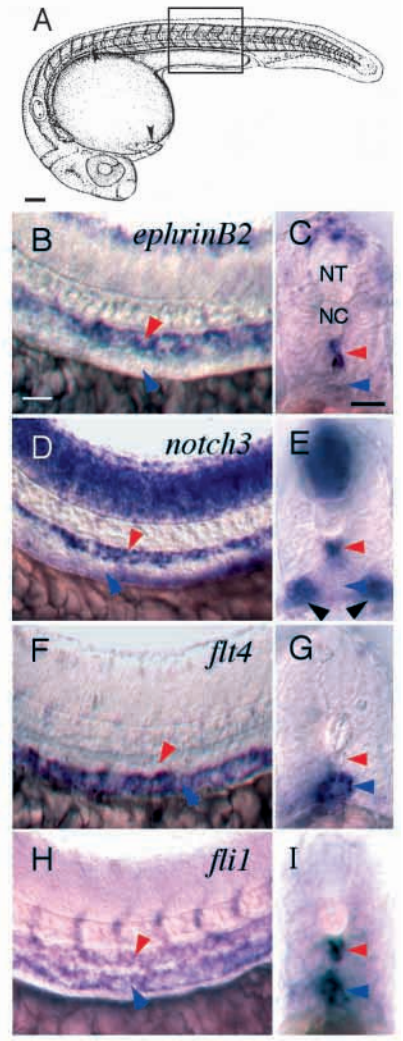

Fig. 1. In situ hybridization of 30-somite stage wild-type embryos using markers of arterial-venous specification. (A) Line drawing of a 30-somite stage embryo. The boxed region corresponds to the area shown in B,D,F and H (modified from Kimmel et al., 1995). (B,D,F,H) Lateral views of (B) *ephrinB2*, (D) *notch3*, (F) *flt4* and (H) *fli1* expression in the trunk vessels. (C,E,G,I) Thick sections (~35-50 μm) through the trunk of embryos showing expression of (C) *ephrinB2*, (E) *notch3*, (G) *flt4*, and (I) *fli1*. (B-I) The dorsal aorta (DA) is indicated by a red arrowhead and the posterior cardinal vein (PCV) by a blue arrowhead. (E) Pronephric duct expression of *notch3* is indicated by black arrowheads. NT, neural tube; NC, notochord. Scale bars, (A) 100 μm, (B,D,F,H) 40 μm, (C,E,G,I) 30 μm.

Loss of Notch signaling perturbs normal arterial-venous differentiation

The observation that *notch3* is expressed specifically within the DA suggested a potential role for Notch signaling in determining arterial cell fate during blood vessel development. To examine the role of the Notch pathway in the vasculature, we utilized a DNA binding mutant of a *X. laevis* homolog of the Suppressor of Hairless protein [XSu(H)DBM] that functions as an inhibitor of Notch signaling and elicits a neurogenic phenotype (Wettstein et al., 1997). A fusion protein was constructed between the enhanced green fluorescent protein (EGFP) and XSu(H) DBM, and mRNA encoding this fusion construct (EGFPSuDN) was injected into 1-cell stage embryos followed by in situ hybridization analysis for artery-specific gene expression. Embryos injected with 800 pg of EGFP expressed *ephrinB2* within the DA at 24 hours postfertilization (hpf; Fig. 2A). while those injected with EGFPSuDN mRNA (800 pg) showed reduced or absent *ephrinB2* expression within the DA (Fig. 2B). Expression of

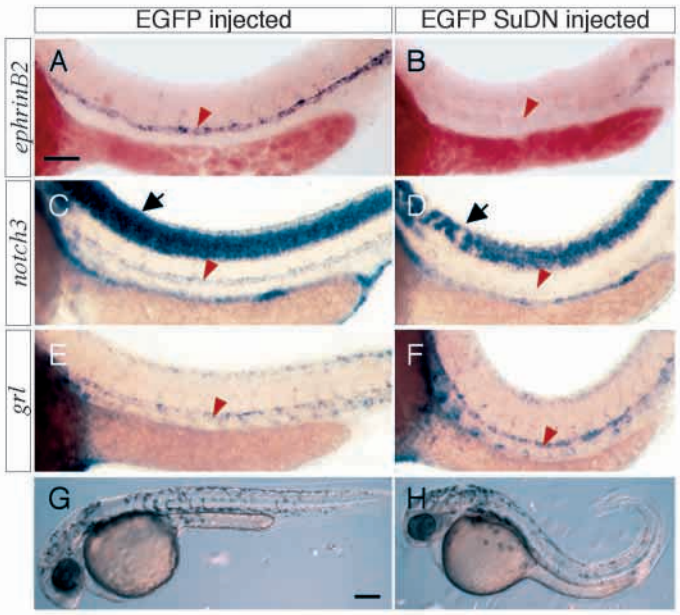

Fig. 2. Microinjection of EGFPSuDN perturbs arterial differentiation. (A,B) In situ hybridization of *ephrinB2* in injected embryos fixed at 24 hpf. (A) Embryos injected with 800 pg of EGFP express *ephrinB2* in the DA (red arrowhead). (B) In embryos injected with 800 pg of EGFPSuDN there is reduction of vascular *ephrinB2*. (C,D) In situ hybridization of *notch3* in injected embryos fixed at 24 hpf. (C) Embryos injected with 1 ng of EGFP express *notch3* within the neural tube (black arrow) and DA (red arrowhead). (D) *notch3* expression within the neural tube (black arrow) and the DA (red arrowhead) is reduced following injection of 1 ng of EGFPSuDN. (E,F) In situ hybridization of *grl* in injected embryos fixed at 24 hpf. (E) Embyros injected with 800 pg EGFP express *grl* within the DA; (F) *grl* expression is not affected by injection with 800 pg EGFPSuDN. (A-F) All embryos were injected with indicated amount of mRNA at the 1-cell stage. (G, H) Transmitted light images of living embryos at 36 hpf. (G) Wild-type embryo injected with 800 pg of EGFP mRNA. (H) Wild-type embryo injected with 800 pg of EGFPSuDN exhibiting trunk curvature. (A-F) Lateral views, anterior to the left. (A-F) Scale bars (A-F) 100 μm, (G, H) 200 μm.

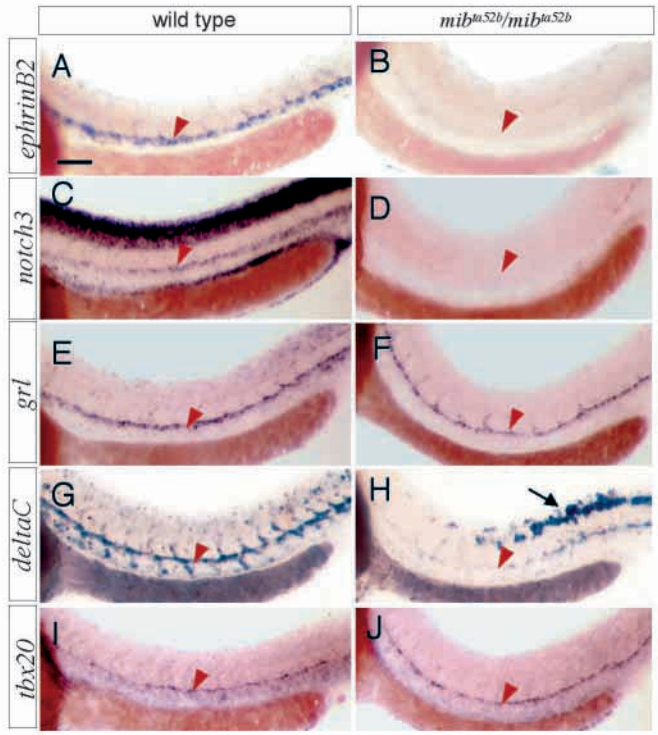

Fig. 3. In situ hybridization of artery markers in 30-somite stage *mib*^{ta52b} mutant and wild-type sibling embryos. (A) In wild-type siblings, *ephrinB2* expression is apparent in the DA (red arrowheads in A-J). (B) In *mib*^{ta52b} mutant embryos this expression is absent. (C) In wild-type sibling embryos *notch3* is expressed within the DA. (D) In *mib*^{ta52b} mutant embryos *notch3* expression is absent from both vascular and non-vascular tissues. (E) In wild-type sibling embryos *grl* is expressed specifically in the DA. (F) In *mib*^{ta52b} mutant embryos the expression of *grl* is not affected. (G) In wild-type sibling embryos *deltaC* is expressed within the DA. (H) In *mib*^{ta52b} mutant embryos expression of *deltaC* within the DA is decreased in approximately half of the mutant embryos. Excess neuronal expression is apparent (black arrow). (I) In wild-type siblings the T-box transcription factor *tbx20* is expressed normally within the roof of the DA. (J) In *mib*^{ta52b} mutant embryos this expression is normal. Scale bar (A-J) 100 μm.

ephrinB2 in the eye, forebrain and midbrain was not affected by injection of EGFPSuDN (data not shown). We also determined the effect of EGFPSuDN on *notch3* expression within the DA. Embryos injected with 1 ng of EGFP display *notch3* expression within the DA and neural tube (red arrowhead and black arrow, respectively, in Fig. 2C). However, *notch3* expression is reduced in the DA and neural tube in embryos injected with a comparable amount of EGFPSuDN (Fig. 2D). Since the mouse homolog of *grl* has recently been identified as a downstream target of Notch signaling, we assayed for its expression in EGFPSuDN-injected embryos. Embryos injected with 800 pg of EGFP showed normal *grl* expression within the DA (Fig. 2E). Surprisingly, all embryos injected with EGFPSuDN also exhibited expression of *grl* within the DA at normal levels (Fig. 2F). No effect was seen on DA-specific *grl* expression with EGFPSuDN injections up to 1 ng (data not shown). Embryos injected with 800 pg (Fig. 2G) or 1 ng (data not shown) of EGFP were morphologically normal, while embryos injected with the same amount of EGFPSuDN exhibited curved trunk and tail (Fig. 2H) as well as abnormal circulatory patterns (see below). In embryos injected with 1 ng EGFPSuDN we observed more severe trunk curvature and kinked notochords (data not shown).

To further investigate the role of Notch signaling in arterial-venous differentiation, we utilized the zebrafish mutant *mindbomb* (*mib*), which displays a hallmark neurogenic phenotype typical of defective Notch signaling (Appel et al., 1999; Jiang et al., 1996; Riley et al., 1999; and see Discussion). For these studies we used the most severe allele of *mib* (*mib*^{ta52b}), although we observed similar phenotypes with less severity in embryos mutant for the weaker *mib*^{m178} allele (data not shown). To confirm that *mib*^{ta52b} embryos exhibited defects in arterial-venous differentiation, we performed in situ hybridization using artery-specific markers. While wild-type siblings exhibited normal expression of *ephrinB2* (Fig. 3A), embryos homozygous for *mib*^{ta52b} failed to express *ephrinB2* within the DA (Fig. 3B) as was observed in EGFPSuDN-injected embryos (Fig. 2B). In addition, *notch3* is apparent within the

DA as well as the neural tube of wild-type embryos (Fig. 3C) while its expression is severely reduced or absent in *mib*^{ta52b} embryos (Fig. 3D). We also found that *grl* expression does not appear to be affected in the DA of *mib*^{ta52b} mutant embryos (Fig. 3E, F), in agreement with our observations concerning EGFPSuDN-injected embryos. *DeltaC*, a recently described Notch ligand (Smithers et al., 2000), is also expressed within the DA in wild-type embryos (Fig. 3G). An increase in neural expression of *deltaC* is evident in *mib*^{ta52b} mutant embryos (Fig. 3H) while *deltaC* expression within the DA is reduced in approximately half of the mutant embryos at the 30-somite stage (Fig. 3H). Similar to *grl*, expression of the T-box gene *tbx20*, which is normally restricted to the dorsal roof of the DA (Ahn et al., 2000; Griffin et al., 2000), is not affected in *mib*^{ta52b} mutant embryos (Fig. 3I,J).

In addition to decreased expression of artery-specific markers in *mib*^{ta52b} mutant embryos, changes were also noted using venous-specific markers. The venous endothelial marker *flt4*, which normally becomes restricted to the PCV within the trunk by the 30-somite stage (Fig. 4A) is expressed ectopically within the DA in mutant embryos (Fig. 4B). The putative ephrinB2 receptor *rtk5* is expressed at a higher level in the PCV than in the DA in wild-type embryos (Fig. 4C) while in mutants, expression is reduced within the PCV and ectopic expression in the DA is detected (Fig. 4D).

Embryos mutant for *mib*^{ta52b} also exhibited morphologic characteristics similar to embryos injected with EGFPSuDN. While wild-type sibling embryos were normal (Fig. 4E), *mib*^{ta52b} mutant embryos exhibited trunk curvature and lack of posterior trunk pigmentation (Fig. 4F) as has been described previously (Jiang et al., 1996).

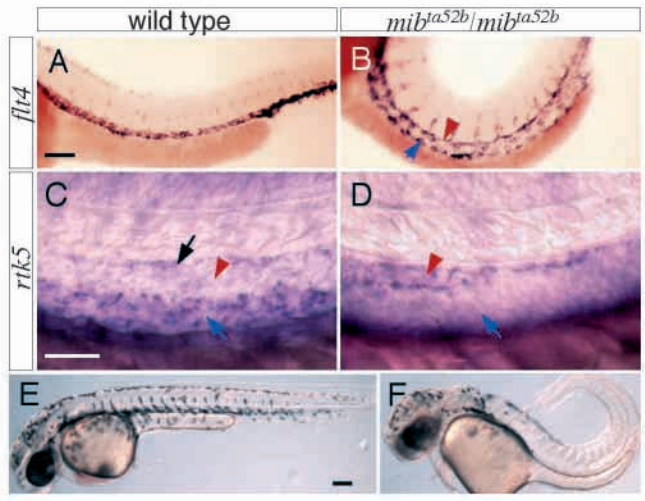

Fig. 4. Ectopic expression of venous markers in *mib*^{ta52b} mutant embryos. (A) In wild-type siblings *flt4* expression is restricted to the PCV by the 30-somite stage. (B) In *mib*^{ta52b} mutant embryos expression persists within both the PCV (blue arrow) and the DA (red arrowhead) at this stage. (C) In wild-type siblings *rtk5* is expressed within the hypochord (black arrow), and is detected at a higher level within the PCV (blue arrow) than the DA (red arrowhead). (D) In *mib*^{ta52b} mutant embryos *rtk5* expression is absent from the PCV, and regions of ectopic *rtk5* expression are noted in the DA (red arrowhead). (E,F) Transmitted light images of (E) wild-type and (F) *mib*^{ta52b} mutant living sibling embryos at 36 hpf. (F) The curled tail and other characteristics (see text) are apparent in the mutant embryo and are similar to embryos injected with EGFPSuDN (see Fig. 2H). Scale bars (A,B) 100 μm, (C,D) 50 μm, (E,F) 200 μm.

Activation of the Notch pathway suppresses venous cell fate

Determining the role of Notch during organogenesis is difficult because of the wide range of cell types responsive to Notch signaling throughout development (Artavanis-Tsakonas et al., 1999). Injection of mRNA encoding an activated form of Notch3 into zebrafish embryos results in defects in somitogenesis and notochord formation as well as the absence of anterior structures (data not shown). Thus, mRNA injection could result in indirect effects on arterial-venous differentiation. To eliminate the early effects of Notch activation, we utilized a system that allows for temporal control of a transgene of interest by placing the yeast transactivator GAL4 downstream of the zebrafish heat shock promoter. Adult *hsp70:Gal4* zebrafish (Halloran et al., 2000; Scheer et al., 2001) were crossed with *UAS:notch1a-intra* fish (Scheer and Campos-Ortega, 1999) and embryos derived from these crosses were heat shocked and processed as described in Materials and Methods. Since the parental lines are hemizygous for each transgene, only one quarter of the embryos will inherit both components of the GAL4-UAS system. Therefore, embryos were stained with an anti-myc antibody following in situ hybridization to confirm expression of Notch1a-intra.

To determine the effect of activated Notch on artery specification, embryos were stained for the DA-specific markers *ephrinB2*, *notch3*, *deltaC* and *grl*. Surprisingly, there was no observable difference in heat shocked embryos with respect to the expression of any of these arterial markers, including *grl*, within the vasculature (Fig. 5A,B and data not shown). Earlier induction at the 15-somite stage also failed to result in ectopic expression of any artery markers (data not shown).

We next determined if activating the Notch pathway affected the identity of venous blood vessels by assaying embryos for *flt4* expression. In heat-shocked sibling embryos without ectopic Notch1a-intra expression, *flt4* expression can be seen within the primordial midbrain channel (PMBC, arrows in Fig. 5C) and midcerebral vein (MCeV, arrowhead in Fig. 5C), as well as the PCV within the trunk (Fig. 5E). Embryos staining positive for the myc-tagged Notch1a-intra exhibited a decrease in *flt4* expression within these venous vessels (Fig. 5D, F). To determine whether the absence of *flt4* staining was due to a decrease in expression or the loss of cells within the vein itself, heat shocked embryos were stained with the endothelial marker *tie1*. In embryos that do not express Notch1a-intra, *tie1* can be seen in all blood vessels of the head (Fig. 5I) and trunk (Fig. 5K). *Tie1* expression within all blood vessels appears normal in embryos expressing Notch1a-intra (Fig. 5J, L).

To confirm that decreased *flt4* expression was due to endothelial cell autonomous activation of the Notch signaling pathway, we utilized the zebrafish *fli1* promoter to drive expression of a myc-tagged Notch3 ICD (MTN3 ICD). We have isolated a 7 kb fragment of the *fli1* promoter that is sufficient to drive transgene expression within the cells of the head and trunk vasculature (unpublished observation). One-cell stage embryos were injected with linearized pfliMTN3ICD and assayed for *flt4* and MTN3 ICD expression. An example of an embryo injected with fliMTN3 ICD is seen in Figs 5G

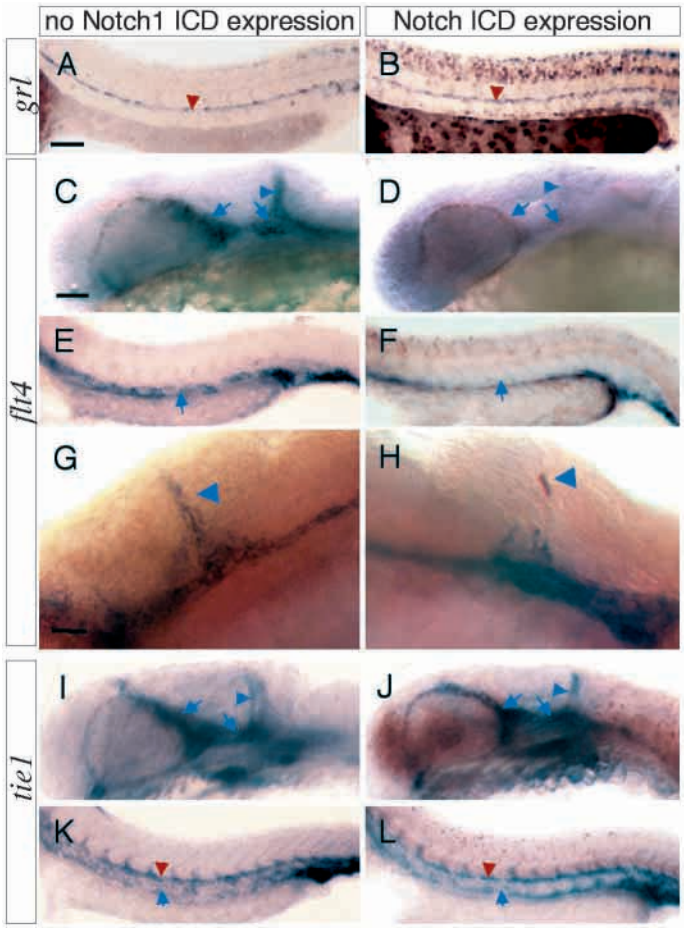

Fig. 5. Activation of Notch signaling represses venous cell identity. (A,B) Expression of *grl* in *hsp70:Gal4;UAS:notch1a-intra* heat shocked embryos. (A) *grl* expression in heat shocked embryos not expressing Notch1a-intra. (B) *grl* is not ectopically induced within the vasculature by Notch1a-intra. (C-F) *flt4* expression in *hsp70:Gal4; UAS:notch1a-intra* embryos heat shocked at the 18-somite stage. *flt4* is expressed within venous vessels of the head (C) and trunk (E) at the 28- to 30-somite stages in embryos that do not express Notch1a-intra. (D,F) Notch1a-intra represses *flt4* expression in venous blood vessels of the head (D) and trunk (F). (G,H) Targeted expression of Notch3 ICD to the endothelium using the *fli1* promoter, represses *flt4* expression. (G) An embryo injected with pfliMTN3ICD showing normal *flt4* expression within the midcerebral vein (blue arrowhead) in the absence of ectopic Notch3 ICD; anterior is to the right. (H) Opposite side of embryo in G, showing a myc-positive cell (blue arrowhead) within the midcerebral vein that fails to express *flt4*; anterior is to the left. (I-L) Expression of *tie1* in *hsp70:Gal4;UAS:notch1a-intra* heat shocked embryos. (I,K) In embryos not expressing ectopic Notch1a-intra, *tie1* is expressed in all blood vessels at the 28- to 30-somite stage. (J,L) *Tie1* expression within all vessels is maintained in embryos expressing Notch1a-intra. (C, D) Embryos are shown prior to staining with anti-myc antibody. (A,B,E-L) Embryos were subjected to whole-mount immunostaining with an anti-myc antibody to visualize ectopic expression of the (A,B,E,F,I-L) Notch1-intra or (G,H) Notch 3 ICD. (E,F,I,J) Arrows and arrowheads indicate the positions of the primordial midbrain channel and mid-cerebral vein, respectively. (A,B,K,L) Red arrowheads and blue arrows indicate the position of the DA and PCV, respectively. Scale bar (A,B,K,L) 50 μm, (C,D,I,J) 100 μm, (G,H) 200 μm.

and H. On the left side of the head at approximately the 30-somite stage the MCeV has sprouted to its dorsal-most point and expresses *flt4* in the absence of ectopic MTN3 ICD (confirmed by the absence of myc-positive nuclei; Fig. 5G). However, the MCeV on the opposite side of the embryo expresses MTN3 ICD as indicated by a myc-positive nucleus (Fig. 5H, blue arrowhead) and *flt4* transcript is undetectable in this cell (Fig. 5H).

Consequences of defective Notch signaling on vascular morphogenesis

Since we found that the Notch pathway affects the identity of blood vessels during development, we determined the consequences of these effects on vascular morphogenesis. While wild-type siblings exhibited normal circulation at 60 hpf (Fig. 6A), cranial hemorrhage (Fig. 6B) was evident in *mib*^{ta52b} mutant embryos (80 out of 84 mutant embryos in 3 different clutches). Additionally, more than half of the homozygous *mib*^{ta52b} mutant embryos completely lacked circulation by 48 hpf (50 out of 84 in 3 clutches) and most of those that retained circulation exhibited arterial-venous (A-V) shunts (27 out of 34 mutant embryos with shunts from three clutches). Embryos injected with 800 pg EGFPSuDN also exhibited A-V shunts (47 out 167 in three experiments), while all embryos injected with a comparable amount of EGFP were normal. The location and behavior of arterial-venous shunts were similar in EGFPSuDN-injected embryos (data not shown) and embryos mutant for *mib*^{ta52b} (see below).

To analyze the nature of the A-V shunts in more detail, we utilized real time differential interference contrast (DIC) videomicroscopy to examine blood flow patterns and vascular morphology in *mib*^{ta52b} mutant embryos that retained circulation. Normally, blood flows into the zebrafish trunk via the lateral dorsal aortae, which fuse to become a single medial DA running the length of the trunk (Fig. 6C). The DA continues into the tail, becoming the caudal artery (CA). At the caudal end of the CA, blood flow reverses direction and returns rostrally through the tail via the caudal vein (CV), and then through the trunk via the posterior cardinal vein (PCV), which empties into the large venous Ducts of Cuvier (DC) before re-entering the heart. DIC videomicroscopy of wild-type embryos at 48 hpf revealed DA and PCV vessel walls with well-demarcated boundaries between the two vessels (Fig. 6D; Movie 1 available on-line). While direct connections between the DA and PCV were never observed in wild-type siblings, *mib*^{ta52b} mutant embryos displayed shunts linking these vessels that resulted in premature venous return and little or no circulation through the CA and CV (Fig. 6C,E; Movie 2 available on-line). Histological analysis of the mutant embryo in Fig. 6E revealed significant disorganization of the DA and PCV (Fig. 6G) compared to wild-type sibling embryos (Fig. 6F), in addition to disruption of the vascular wall at the location of the shunt (indicated by a black arrow in Fig. 6G). Mutant embryos exhibited disorganized boundaries between the DA and the PCV compared to their wild-type siblings (indicated by black arrows in Fig. 6H) and the PCV often failed to be remodeled into a single vessel (Fig. 6H and data not shown).

During videomicroscopic analysis of *mib*^{ta52b} embryos, we also noted improper blood flow patterns in the region of the intersomitic vessels (ISVs). To further investigate the nature of these defects we performed confocal microangiography

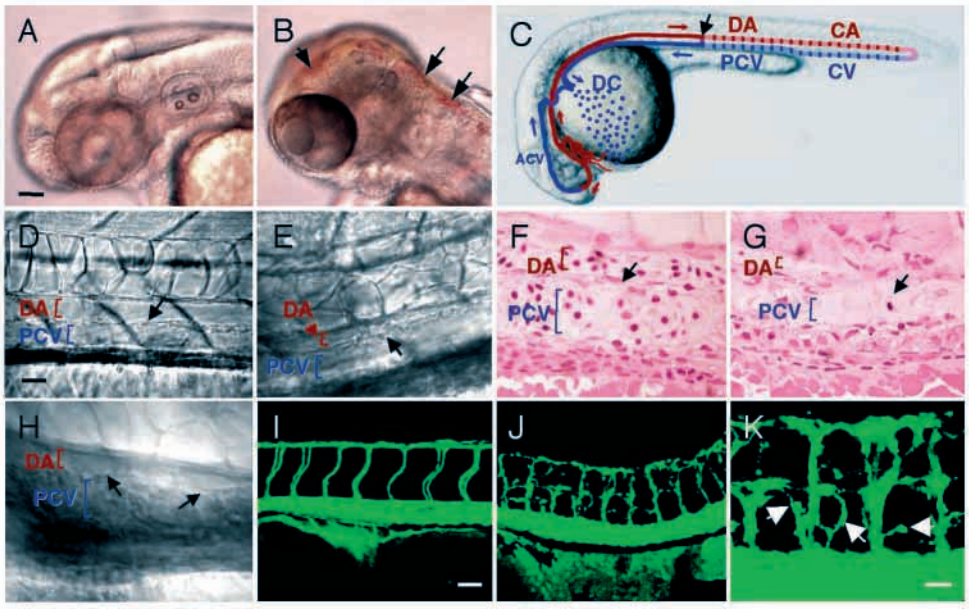

Fig. 6. Vascular defects in *mib*^{ta52b} embryos. (A) Head of a wild-type *mib*^{ta52b} sibling at 60 hpf. (B) Cranial hemorrhage in a *mib*^{ta52b} mutant embryo at 60 hpf (black arrows). (C) Schematic drawing of the trunk circulatory loop in a zebrafish embryo. Red and blue are arterial and venous vessels respectively, with the direction of blood flow indicated. The black arrow shows the position of the shunt in the mutant embryos in (E,G). (D) DIC image of the mid-trunk of a live wild-type *mib*^{ta52b} sibling at 55 hpf. A well-formed DA (red bracket) and PCV (blue bracket) are visible (see Movie 1, available on-line). The black arrow points to the endothelial cell walls clearly demarcating the boundary between the DA and PCV. (E) DIC image of a mutant *mib*^{ta52b} embryo displaying a shunt (black arrow) between the DA and PCV (see Movie 2, available on-line). (F) Longitudinal histological section through the trunk of a wild-type embryo showing the DA (red bracket) and PCV (blue bracket). Blood cells are present within the lumen of each vessel. The black arrow indicates the endothelial cell wall between the DA and PCV. (G) Longitudinal histological section through the trunk of the same *mib*^{ta52b} mutant embryo shown in E, also in the region of the shunt (black arrow). (H) DIC image of a *mib*^{ta52b} embryo showing a disorganized boundary between the DA and PCV (arrows) and lack of a properly remodeled PCV. (I) Confocal microangiogram of the trunk of a wild-type *mib*^{ta52b} sibling at 55 hpf. Intersomitic vessels appear regularly at the vertical myosepta. (J) Confocal microangiogram of the trunk of a *mib*^{ta52b} mutant at 55 hpf. Disorganization of the normal intersomitic vessel structure is apparent. (K) Higher magnification confocal microangiogram of the trunk of the *mib*^{ta52b} mutant in J. Ectopic sprouts from the main branches of the intersomitic vessels are visible (white arrows) penetrating the somite. Scale bars (A,B,I,J) 50 μm, (D-H,K) 25 μm. DA, dorsal aorta; CA, caudal artery; ACV, anterior cardinal vein; PCV, posterior cardinal vein; CV, caudal vein.

(Weinstein et al., 1995) on mutant and wild-type sibling embryos at 55 hpf, to directly visualize the patterns of patent vessels. In wild-type embryos, ISVs are completely formed by 55 hpf. These vessels appear regularly at successive vertical myoseptal boundaries, joining together above the neural tube as paired dorsal longitudinal anastomotic vessels (Fig. 6I). No patent vessels are ever observed within somitic tissue in wild-type embryos at this stage. Although the initial sprouting of the ISVs appears normal in *mib*^{ta52b} embryos (data not shown), ectopic sprouts penetrating the somitic tissue are apparent at 55 hpf (Fig. 6J, white arrows in Fig. 6K).

DISCUSSION

We have utilized the zebrafish to study arterial and venous differentiation during embryonic blood vessel formation. As in mouse and *Xenopus*, we find molecular differences between arteries and veins can be demonstrated before circulation in zebrafish embryos by the expression of the transmembrane ligand EphrinB2 and the EphB4 homolog *rtk5* (Cooke et al., 1997), respectively. We find that *notch3* is also expressed specifically within the vasculature in the DA beginning at the 18-somite stage, similar to the temporal expression pattern of *ephrinB2*. This artery-restricted pattern of expression and the known role of Notch signaling in cell fate decisions led us to investigate the role of this pathway during arterio-venous differentiation.

We determined the effect on vascular development of both loss and gain of Notch activity during embryonic development. We are able to effectively inhibit the Notch pathway using a dominant negative form of Su(H) that fails to bind DNA (Wettstein et al., 1997). In embryos expressing EGFPSuDN we find decreased arterial expression of both *ephrin-B2* and *notch3*. In addition, EGFPSuDN-injected embryos display circulatory shunts between the DA and PCV within the trunk, a phenotype that has been associated with decreased *ephrinB2* expression in mice lacking ACRVL-1 (Urness et al., 2000). We have noted identical, yet more severe phenotypes in embryos mutant for the *mib*^{ta52b} locus. Although the identity of the *mib* gene is unknown, several lines of evidence suggest that it is an important component of the Notch signaling pathway (Jiang et al., 1996). *Mib* mutant embryos display a severe neurogenic phenotype similar to that described in *Drosophila* mutants lacking components of the Notch signaling pathway (Jiang et al., 1996; Xu et al., 1990). In addition, zebrafish embryos with a mutation in the gene encoding the Notch ligand *deltaA* exhibit many of the phenotypes described in *mib* mutant embryos (Appel et al., 1999; Riley et al., 1999). Our results provide further evidence supporting the importance of *mib* in Notch signaling, by demonstrating that wild-type embryos expressing EGFPSuDN exhibit many of the molecular and morphologic vascular defects observed in *mib*^{ta52b} mutants. Embryos that are mutant for *mib*^{ta52b} display loss of *ephrinB2* and *notch3* expression within the DA and exhibit circulatory shunts similar to that seen in wild-type embryos injected with mRNA encoding EGFPSuDN. We have also noted aberrant intersomitic vessel projection and defective remodeling of the major vessels in *mib*^{ta52b} mutant embryos, phenotypes that have been noted in mouse embryos lacking ephrin-B2 (Adams

et al., 1999; Gerety et al., 1999; Wang et al., 1998) or EphB4, as well as *Xenopus* embryos injected with a dominant negative form of EphB4 (Helbling et al., 2000). Although embryos lacking Notch activity display defects in arterial differentiation, expression of other artery-specific markers, such as *tbx20* and *grl*, is not affected in mutant embryos. The proper expression of these markers as well as endothelial markers such as *flk1*, *fli1* and *tie1* in *mib*^{ta52b} mutant embryos (unpublished observations) indicates the presence of endothelial cells at the position of the DA. These results suggest that Notch signaling is required for a specific step during arterial differentiation and is dispensable for the initial positioning of the major trunk vessels.

In addition to defects in arterial differentiation, we find that the venous markers *flt4* and *rtk5* become ectopically expressed within the DA in *mib*^{ta52b} mutant embryos suggesting that a major role of Notch signaling is to repress the venous fate within the developing arterial primordium. This model is consistent with the expression patterns of both *notch3* and *flt4*. *Flt4* is initially expressed in angioblasts beginning at the 12-somite stage (Thompson et al., 1998), at which time *notch3* is not apparent in these cells (unpublished observation). As *notch3* expression becomes detectable within the DA at the 18-somite stage, *flt4* expression decreases in all presumptive arterial vessels and is restricted to veins by the 25-somite stage. The loss of *rtk5* expression within the PCV of *mib*^{ta52b} mutant embryos is possibly due to the absence of a positive autoregulatory loop that has been proposed to be responsible for EphB4 expression in the mouse vasculature (Gerety et al., 1999) as mice lacking EphB4 also fail to express a *tau-lacZ* transgene from the *EphB4* locus. Thus, the loss of *ephrinB2* expression in *mib*^{ta52b} mutant embryos likely results in the failure to activate the Rtk5 receptor and maintain its own expression within venous vessels.

We find further support for the role of Notch signaling in repressing venous cell fate from the ectopic expression of an activated form of Notch during embryonic development. Activation of Notch signaling in *hsp70:Gal4;UAS:notch1a-intra* embryos at the 18-somite stage leads to a decrease in *flt4* expression in all venous vessels by the 30-somite stage. Expression of *tie1* is unaffected in *hsp70:Gal4;UAS:notch1a-intra* heat-shocked embryos, indicating an effect on *flt4* expression rather than the loss of cells within venous vessels. Despite the effects on venous cell fate, activation of the Notch pathway throughout the embryo failed to induce an arterial cell fate ectopically, suggesting that Notch signaling may cooperate with other pathways to mediate arterial differentiation.

The observation that *notch3* is expressed within the DA suggests that the Notch pathway is acting at the level of the endothelial cell itself. We confirmed this by expressing Notch3 ICD using the zebrafish *fli1* promoter. We find that cells within venous vessels that express a myc-tagged form of Notch3 ICD from the *fli1* promoter failed to properly express *flt4*, confirming a cell-autonomous effect on endothelial cell identity. Thus, *notch3* is functioning within the endothelial cells of the DA to repress a venous cell fate

During neurogenesis, activation of the Notch pathway leads to expression of bHLH factors, such as the *hairy*-like genes of the *Enhancer of split* complex, that repress the action of proneural genes and inhibit a neural fate (Artavanis-Tsakonas et al., 1999). Interestingly, *grl* is a *hairy*-like factor and its mouse homolog, HRT2, has been shown to be a transcriptional repressor that is downstream of Notch signaling in vitro (Nakagawa et al., 2000). Thus, *grl* would seem to be a strong candidate for a downstream effector of venous repression. However, *grl* does not appear to be downregulated in EGFPSuDN-injected embryos or *mib*^{ta52b} mutant embryos as would be expected for a Notch target gene. Furthermore, activation of Notch signaling throughout the embryo fails to cause ectopic expression of *grl* within venous vessels, although *flt4* is reduced within these vessels under the same conditions. We have observed increased *grl* expression in the forebrain and optic tectum and ectopic expression within the lens following heat shock (unpublished ovservation) indicating that, in these tissues, *grl* can be downstream of Notch signaling in concordance with in vitro studies (Nakagawa et al., 2000). Although *grl* is clearly important for development of the DA, our data suggest that it does not function by mediating Notch-induced repression of venous fate. Consistent with these observations, expression of *flt4*, as well as *ephrinB2*, is unaffected in *grl*^{m145} mutant embryos (unpublished observation).

Until recently, the processes by which arterial and venous blood vessels acquire their identity were generally attributed to physiologic cues, but it is now apparent that genetic factors underlie this process. We propose a model in which the Notch pathway acts at a specific step in arterial-venous differentiation to repress a venous cell fate program within the presumptive DA. Notch activity seems to be dispensable for earlier steps of arterial-venous differentiation such as the positioning of the DA and PCV primordia, suggesting that the process of defining arteries and veins is a complex cascade of differentiation governed by signaling pathways that have yet to be identified.

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