Artery and vein size is balanced by Notch and ephrin B2/EphB4 during angiogenesis

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A mutual coordination of size between developing arteries and veins is essential for establishing proper connections between these vessels and, ultimately, a functional vasculature; however, the cellular and molecular regulation of this parity is not understood. Here, we demonstrate that the size of the developing dorsal aorta and cardinal vein is reciprocally balanced. Mouse embryos carrying gain-of-function Notch alleles show enlarged aortae and underdeveloped cardinal veins, whereas those with loss-of-function mutations show small aortae and large cardinal veins. Notch does not affect the overall number of endothelial cells but balances the proportion of arterial to venous endothelial cells, thereby modulating the relative sizes of both vessel types. Loss of ephrin B2 or its receptor EphB4 also leads to enlarged aortae and underdeveloped cardinal veins; however, endothelial cells with venous identity are mislocalized in the aorta, suggesting that ephrin B2/EphB4 signaling functions distinctly from Notch by sorting arterial and venous endothelial cells into their respective vessels. Our findings provide mechanistic insight into the processes underlying artery and vein size equilibration during angiogenesis.

KEY WORDS: Angiogenesis, Vascular morphogenesis, Notch, Ephrin B2/EphB4, Mouse, Arterial-venous differentiation

INTRODUCTION

Angiogenesis, or new blood vessel growth, is a principal biological process in embryonic development, cancer progression, tissue regeneration, ischemic recovery and many other physiological and pathological conditions (Carmeliet, 2005; Coultais et al., 2005; Folkman, 2007). New vessel segments are generated by the well-described process of capillary sprouting from pre-existing vessels. Coordination between the sizes of developing arteries and veins is crucial in establishing an interface between these vessels and for a functional vasculature; however, the cellular and molecular regulation of this parity is unknown (Jones et al., 2006; Gridley, 2007). Elucidating the cellular and molecular basis of arterial and venous specification and endothelial cell (EC) distribution would provide a conceptual advance in our understanding of angiogenesis.

In this study we examined the first artery and vein to develop in the body: the dorsal aorta (DA) and the cardinal vein (CV), respectively. Given that initial DA and CV development involves ECs and not adjacent mural cells, this model provides an experimental system in which to study the role of EC signaling in arteriovenous morphogenesis. The DA emerges prior to the CV, and its morphogenesis begins with the assembly of ECs into the DA primordium, a transient capillary plexus (Sabin, 1917; Coffin et al., 1991). Remodeling of this primitive network generates a lumenized vessel, which subsequently matures into the major artery of the body. The CV emerges slightly later, at which stage transient capillary channels develop between the DA and CV (Sabin, 1917; Gerety and Anderson, 2002) (see Fig. S1 in the supplementary material), suggesting that the two vessels may interact to establish the proper circulatory system.

The discovery of ephrin B2 (Ephb2), a gene encoding a transmembrane signaling molecule specifically expressed in arterial ECs prior to the onset of circulation, unveiled a genetic program of arteriovenous differentiation (Wang et al., 1998; Adams et al., 1999). These studies demonstrate that the ephrin B2 ligand and its venous-specific EphB4 tyrosine kinase receptor (Wang et al., 1998; Gerety et al., 1999) are important for vascular remodeling of primitive capillary networks into distinct arteries and veins. Despite its distinctive arterial expression, ephrin B2 does not determine arterial specification in ECs (Wang et al., 1998). The precise cellular mechanism underlying ephrin B2 function in ECs is unknown. Ephrin/Eph signaling mediates cellular behavior such as repulsion, adhesion and motility in neuronal, bone and other tissue types (Klein, 2004; Poliakov et al., 2004; Kuijper et al., 2007), raising the possibility that ephrin B2/EphB4 signaling functions in a similar fashion in ECs.

Notch receptors and their ligands are transmembrane proteins that are primarily expressed in arteries and not veins (Villa et al., 2001). Notch signaling influences bi-potential cell fate decisions through cell-cell communication (Artavanis-Tsakonas et al., 1999). Studies in zebrafish and mice show that Notch activation promotes arterial characteristics in ECs (Lawson et al., 2001; Zhong et al., 2001; Torres-Vázquez et al., 2003; Shawber and Kitajewski, 2004). Carlson et al., 2005). Gain- and loss-of-function mutations in the Notch pathway lead to abnormal vascular development in mice (Krebs et al., 2000; Uyttendaele et al., 2001; Duarte et al., 2004; Fischer et al., 2004; Gale et al., 2004; Krebs et al., 2004). We have shown that expression of constitutively active Notch4 in a subset of ECs can cause prompt and massive arteriovenous malformations in adults (Carlson et al., 2005). In addition to its ability to promote arterial characteristics, Notch signaling also restricts capillary sprouting in normal and tumor angiogenesis (Noguera-Troise et al., 2006; Ridgway et al., 2006; Hellstrom et al., 2007; Siekmann and Lawson, 2007; Suchting et
al., 2007). However, the precise cellular function of Notch signaling in the establishment of arteriovenous distinction remains unknown.

We have combined mouse genetics and in vivo analysis to examine concurrently the effects of these pathways on DA and CV development and have found that the size of the developing DA and CV is coordinated. ECs are distributed between the DA and CV, and both Notch and ephrin B2/EphB4 signaling pathways are crucial for this coordination during vascular morphogenesis. Notch controls the proportion of ECs in the DA and CV by promoting arterial specification, thereby modulating their respective lumen size. The ephrin B2/EphB4 signaling pathway segregates arterial and venous ECs into their respective vessel. Our work suggests that the growth of arteries and veins during angiogenesis is inversely coordinated, and that the Notch and ephrin B2/EphB4 pathways are essential for balanced arteriovenous development during blood vessel formation.

MATERIALS AND METHODS

Mice

The Tie2-tTA, TRE-int3 and TRE-LacZ transgenic mice have been described (Carlson et al., 2005), as have the Efnb2-tauLacZ (Wang et al., 1998), Efnb2-H2BGFP (Davy and Soriano, 2006), EphB4-tauLacZ (Gerety et al., 1999), Efnb2^lox/lox^ (Gerety and Anderson, 2002), Notch1^+/–^ (Conlon et al., 1995), Notch1^floXfloX^ (Radkte et al., 1999) and Tie2-LacZ (Schlaeger et al., 1997) mice. Embryos were genotyped as described previously (Braren et al., 2006). All animals were treated in accordance with the guidelines of the UCSF Institutional Animal Care and Use Committee.

Immunofluorescence

Immunofluorescence was performed according to a previously described protocol. (Braren et al., 2006). Goat anti-EphB4 (1:50) was from R&D Systems (Minneapolis, MN), rabbit anti-βGal (1:200) was from MP Biomedicals (Irvine, CA) and Alexa 488 donkey anti-goat (1:1000) was from Invitrogen (Carlsbad, CA). Cy5 donkey anti-rabbit (1:500) was from Jackson ImmunoResearch Laboratories (Baltimore, MD).

EC counting

To quantitatively assess the distribution of ECs, we counted ECs in serial cross-sections of the trunk region between the otic vesicle and the heart of E8.75 embryos. ECs were identified by CD31 immunofluorescent staining, and total ECs included those in the DA, primordial anterior CV and capillaries in the vicinity. For Notch4 gain-of-function analysis, five pairs of controls and mutants at 15-16 ss were used, and between eight and twelve 10 μm frozen sections per embryo were analyzed. For Notch1 loss-of-function, four pairs of controls and mutants at 12-15 ss were used. Depending on the quality of the sections, two, six, nine and ten 10 μm frozen sections per embryo were analyzed. For Ephb2 loss-of-function, three pairs of controls and mutants at 15-17 ss were used, with 13 paraffin sections (5 μm) per embryo being analyzed. The number of sections analyzed between mutant and somite stage-matched littermate control was equal. The sum of ECs per mutant embryo (see Table S3 in the supplementary material) was normalized against that of its control, with controls expressed as 100%. Primordial CV compartment includes all ECs except those in the DA. The ratio of DA and primordial CV ECs was calculated over the total EC number.

Whole-mount lacZ staining, histology, and immunohistochemistry

lacZ staining, tissue embedding, histology and immunohistochemistry were performed as described (Carpenter et al., 2005), with modifications in fixation duration for lacZ staining: 40 minutes (E9.0), 45 minutes (E9.5), or 2 hours (E12.5) at 4°C. For imaging, E12.5 and E9.5 lacZ-stained embryos were cleared in benzyl alcohol and benzyl benzoate (1:2 ratio) after serial dehydration in 25, 50, 75 and 100% methanol, in 20 minutes intervals. Section positions were identified according to Kaufman (Kaufman, 1992).

In situ hybridization

A 2.7 kb Dll4 antisense probe was used at a final concentration of 1 μg/ml (probe plasmid kindly provided by D. Pleasure). After fixation in 4% PFA, followed by dehydration in methanol and rehydration in PBS, 0.1% Tween-20, E9.0 embryos were digested with 10 μg/ml Proteinase K for 3 minutes on ice. AP-conjugated digoxigenin-labeled RNA probes were prepared according to the manufacturer’s instructions (Roche, Indianapolis, IN), hybridized at 65°C overnight under stringent conditions (1.3× SSC, 50% formamide, 0.2% Tween-20, 5 mM EDTA, pH 8.0, 50 μg/ml Yeast RNA and 100 μg/ml heparin) and stained with BM purple (Roche). Stained embryos were embedded in paraffin and cross-sectioned (10 μm).

RT-PCR

Total mRNA was extracted from snap-frozen, pooled E9.5 embryos and yolk sacs using PolyATtract System (Promega, Madison, WI), and reverse-transcribed using oligo dT primers according to the manufacturer’s instructions (Superscript III RT, Invitrogen). The int3 cDNA was amplified with transgene-specific primers, CGGAGGAAAGGTGATGCTC (sense) and GGGTCATTGGTGATACAG (antisense), at 60°C annealing temperature. Primer sequences for Gapdh were AGCTTGTACACAGGGAAAG (sense) and GGATGCGAGGATGATGCTC (antisense), and for β-actin were ATGACAGTCTCGACCAGGG (sense) and TACCTGGCTGCAAGGAGC (antisense). For E8.5 embryos and yolk sacs, total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) for cDNA synthesis.

Ink injection analysis

Black ink (Staedtler, Nuernberg, Germany) diluted 1:4 in PBS, 0.1% Tween-20, was injected into the outflow tract of E9.5 embryos still attached to the yolk sac, using a micro-needle. Embryos were subsequently fixed in 4% PFA.

In vivo EC proliferation assay

Proliferating cells were labeled 2 hours before embryo collection by intraperitoneal injection of BrdU (Sigma, 100 μg/g body weight) into pregnant females 9 days post-coitum. EC proliferation was detected by double immunofluorescent staining for CD31 and BrdU in frozen cross-sections (10 μm) using a BrdU staining kit (Zymed Laboratories, South San Francisco, CA) in combination with fluorescent secondary antibodies. In each section, DA ECs were counted over an area spanning the otic vesicle to the heart, and the proportion of BrdU-positive ECs calculated.

Statistical analyses

Data bars represent the mean values and error bars the standard deviation. All cell counts were analyzed using two-tailed t-test.

RESULTS

Tet-regulatable endothelial-specific expression of constitutively active Notch4

To determine the effect of gain-of-function Notch on the developing vasculature, we used transgenic mice in which a constitutively active form of Notch4 (int3) is expressed specifically in ECs. Int3 is driven by a tetracycline (Tet) response element (TRE) (TRE-int3), which is activated by a Tet transactivator (tTA) that is, in turn, driven by the EC-specific Tie2 promoter (Tie2-tTA) (Carlson et al., 2005). We verified that tTA was active specifically in the ECs by examining β-galactosidase (β-gal) activity in embryos carrying both Tie2-tTA and TRE-LacZ reporter genes. β-gal activity was detected specifically in the vasculature of yolk sacs by E9.5 and more strongly at E12.5 (see Fig. S2A,C in the supplementary material), and as shown in embryo cross-sections, was restricted to a subset of the ECs of DA and CV at E9.5 while more uniformly at E12.5 (see Fig. S2B,D in the supplementary material).

We verified int3 expression by RT-PCR in pooled embryos and yolk sacs using transgene-specific primers that do not amplify the endogenous Notch4 gene. The int3 mRNA was detected in the Tie2-tTA,TRE-int3 mutant at E8.5 [9-12 somite stage (ss)] and E9.5 (22-
26 ss), at higher a level than the low basal level seen in TRE-int3 tissues, and was not detected in Tie2-tTA tissues (see Fig. S2E in the supplementary material). The Tie2-tTA;TRE-int3 embryos exhibited severe vascular abnormalities by E9.5 and ultimately died by E11.5. Characterization of the gross phenotype of the mutant embryos is described in Tables S1 and S2, and in Fig. S2F-H, in the supplementary material. As no obvious abnormalities were detected in either the TRE-int3 or Tie2-tTA embryos, they, along with the wild type, were included as controls.

**Constitutively active Notch elicits enlarged DA and underdeveloped anterior CV**

To examine the development of DA and CV, we performed CD31 immunostaining and found that the defects first appeared at E9.0 (15-19 ss) with larger DA and aortic arch arteries in all mutants compared with the controls (Fig. 1A-D). In addition, the mutant anterior CVs were less elaborate. Efnb2-taulacZ and EphB4-taulacZ reporter assays (Wang et al., 1998; Gerety et al., 1999) verified that int3 results in an enlarged anterior DA and an underdeveloped CV (Fig. 2A-D). We confirmed with serial cross-sections at E9.5 the enlargement of mutant DA and the underdeveloped mutant CVs displaying a primitive capillary structure lacking the well-defined lumen seen in controls (Fig. 1E,F). The morphological defects in the mutant DA and CV were accompanied by the development of arteriovenous shunting at E9.5, demonstrated by ink injection (Fig. 2E,F).

To determine whether alterations in smooth muscle cell (SMC) recruitment were involved in the DA enlargement, we performed CD31 and smooth muscle α-actin double staining in E9.0 (18 ss) embryos (Fig. 2G,H). At this stage, no SMCs were associated with either the control or mutant DA, yet the mutant DA was enlarged (Fig. 2H). This result suggests that enlargement of the DA occurred before, and thus independently of, the recruitment of SMCs.

**int3 does not affect absolute EC number**

To investigate the cellular mechanism underlying the reciprocal DA and CV size, we tested whether increased EC proliferation was associated with the enlarged DA. We performed in vivo BrdU-labeling combined by CD31 staining in embryos at E8.75 (13-15 ss), prior to apparent gross mutant abnormalities. CD31-positive and BrdU-positive proliferating ECs were counted in cross-sections of the DA (Fig. 3A). The mutant DA exhibited a 14.5% (±15.2) increase in EC number compared with controls, indicating an enlargement of DA. However, the number of proliferating ECs was indistinguishable between mutant and control at ~12% (±2.9, control versus ±3.2, mutant; Fig. 3B). This result suggests that the increase in DA size was not due to an increase in EC proliferation.

We also tested whether EC death was decreased in the mutant but did not detect any apoptotic ECs in either control or mutant DA by TUNEL assay and CD31 staining (data not shown), thus we could not evaluate the effect of int3 on EC apoptosis directly. We then counted the total ECs, including in the DA, CV and capillaries in the vicinity, from the cross-section of anterior E8.75 (15-16 ss) embryos labeled by immunofluorescent CD31 staining, and found no significant change in the absolute number of ECs between the mutant and control (0.3±9.4% increase in the mutant over the control; P=0.94, n=5). Because both total EC number and EC proliferation were not significantly affected, these results also suggest that int3 did not affect EC survival.

**Fig. 1. EC-specific gain-of-function allele of Notch4 elicits DA enlargement and CV underdevelopment.** (A,B) Whole-mount CD31 staining shows enlarged DA and underdeveloped CV in the trunk region of embryos expressing int3 in ECs at E9.0 (18 ss). Arrows, DA; arrowheads, anterior CV (ACV). (C,D) Higher magnifications of A, B, C, D. (E,F) CD31 staining (red) of cross-sections of A, B, C, D, respectively. Arrows and white brackets, DA; arrowheads and blue brackets, ACV. (E,F) CD31 staining (red) of cross-sections of A, B, respectively, confirms enlarged DA and underdeveloped CV in embryos expressing EC-specific int3. Arrows, DA; arrowheads, ACV. (G,H) Quantitative analysis of EC distribution. Total ECs, including those in the DA, primordial CV and capillaries, were counted from cross-sections of the anterior region of E8.75 (15-16 ss) embryos. A total of 3328 and 3334 ECs were counted in control and mutant embryos, respectively. Total EC number between mutant and control is comparable (n=5, P=0.94). The proportion of ECs in DA (da, red) to primordial ACV including capillaries (p-acv, blue) is significantly increased (n=5; *P=0.02) in mutants (H) when compared with controls (G). (I,J) Whole-mount lacZ staining of the Tie2-lacZ reporter identifies head vessels at E10.5. Females were treated with tetracycline water (500 μg/ml) until E7.5, and embryos were collected at E10.5. Internal carotid arteries (yellow arrows) are enlarged, and head veins are reduced (yellow arrowheads) in embryos expressing EC-specific int3 (J). Scale bars: 600 μm in B; 200 μm in D-F,J.
**int3 increases the ratio of arterial to venous ECs**

To quantitatively assess the distribution of ECs between DA and CVs, we counted ECs in serial cross-sections of the anterior trunk E8.75 (15-16 ss). The proportion of DA ECs increased from 34.8% in controls (Fig. 1G) to 49.5% in mutants (P = 0.02, n = 5; Fig. 1H), reflecting the enlarged mutant DA. Conversely, EC proportion in the mutant CV including capillaries in the vicinity was reduced from 65.2% in controls to 50.5% in mutants, confirming the underdevelopment of CVs. These data show that int3 leads to an increase in the number of arterial ECs with a concomitant reduction in the number of venous ECs.

To analyze EC identity, we examined the expression of the arterial markers EphB2-tauLacZ and Dll4 and showed that ephrin B2- or Dll4-positive ECs were detected in the DA and not CVs in the control; however, they were ectopically present in the mutant CVs (Fig. 3C-H). Furthermore, we found ephrin B2-tauLacZ and EphB4-double positive ECs in mutant but not control CVs (Fig. 3E,F). These data show at single cell resolution that int3 promoted arterial identity, even in venous ECs. Taken together, our results suggest that int3 does not affect proliferation in the enlarged DA. Data were analyzed by t-test and results are reported as means ± s.d. (n = 3). A total of 2320 and 2074 ECs were counted in control and mutant embryos, respectively.
indicate that int3 leads to an increase in the number of arterial ECs at the expense of venous ECs, thus increasing the allocation of ECs in arteries over veins, without affecting the absolute EC number.

**int3 also elicits enlarged arteries and underdeveloped veins in the head**

To verify whether the reciprocal changes in arterial and venous size occurred at other locations, we analyzed the development of the head arteries and veins. The optimal time to analyze these vessels is ~E10.5, when Tie2-cre;TRE-int3 embryos were severely retarded from int3 expression. We thus optimized the timing of int3 expression by treating the pregnant females with tetracycline in water until day 7.5 of gestation, as we described previously (Carpenter et al., 2005). Embryos were collected at E10.5. Under these conditions, only a subset of mutant embryos was affected (51.2%; 22 out of 43 mutants), and four mutants analyzed for head vessels exhibited enlarged internal carotid arteries, which were often accompanied by smaller head veins (Fig. 1J). This finding suggests that int3 can also induce enlarged arteries along with underdeveloped veins in other organs.

**The CV primordium is expanded, while DA is smaller in Notch1−/− embryos**

It has been previously reported that the Notch1+ DA is smaller than wild-type DA (Krebs et al., 2000), and we have confirmed this finding (see Fig. S3B in the supplementary material). To analyze the CV structure in Notch1−/− mutants, we stained the embryos for both EphB4 and CD31 at E9.0 (15 ss) when the mutant embryos were affected. At this stage, when the control CV was still composed of capillary plexus, the Notch1−/− primordial CV was expanded (Fig. 4B, arrowheads). This phenotype is reciprocal to that of the Notch4 gain-of-function mutant.

To quantitatively assess the DA and CV sizes, we counted ECs in serial cross-sections of anterior E8.75 (12-15 ss) embryos. Total EC numbers, including those in the DA and primordial CV were comparable in mutants and controls (4.2±11.7% increase in the mutant over the control; P=0.52, n=4). However, the proportion of ECs in the DA was reduced in Notch1−/− (21.1%, compared with 47.7% in controls, P=0.0007; Fig. 4D). Concomitantly, the proportion of ECs in the CV region was significantly increased. These findings further suggest that the reduced DA size is accompanied by an increase in the CV size in the Notch1−/− embryos, reciprocal to that of the Notch4 gain-of-function mutant.

Determining EC identity, we found that EphB4-positive ECs were exclusively located in the control CV primordium, and not in the DA (Fig. 4A). By contrast, EphB4-positive ECs clustered at the smaller, atretic DA in addition to the CV primordium (Fig. 4B). Quantitative analysis showed that the ratio of EphB4-positive to negative ECs in the DA region (as DAs were small and atretic in the mutant) was increased from 0.01 in the control to 0.15 in Notch1−/− (data not shown). As previously demonstrated (Fischer et al., 2004), we observed that the mutant DA ECs were devoid of ephrin B2 expression (data not shown). In addition, in situ hybridization revealed that the DA ECs express Dil4 in the control but not in Notch1−/− embryo (see Fig. S3C, in the supplementary material). These findings demonstrate that Notch1−/− DA may lose arterial identity, but harbor ECs with venous identity.

To determine whether lack of Notch1 in ECs is responsible for such defects, we used conditional mutants in which the Notch1fox/+ allele (Radtke et al., 1999) was excised in ECs by Cre recombinase under the control of Tie1 promoter: Tie1-Cre (Gustafsson et al., 2001). We have shown that Tie1-Cre is active in about 80% ECs and a minority of hematopoietic cells (He et al., 2008). CD31 staining reveals that these mutant embryos displayed similarly smaller, atretic DAs and enlarged CV primordia, at a similar developmental stage to Notch1−/− embryos (Fig. 4E-H). These results suggest that Notch1 in ECs is essential for the balanced growth of the DA and the CV. In summary, these data suggest that Notch loss- and gain-of-function mutants elicit reciprocal effects balancing DA and CV morphogenesis.
Enlarged DA and underdeveloped CV in Efnb2−/− embryos

The balanced distribution of ECs between the DA and CV led us to hypothesize that a cell-sorting mechanism would be involved. The ephrin B2/EphB4 system is known to mark these specific venous and arterial compartments, and has the potential to affect cell sorting. Twenty out of 29 Efnb2−/− embryos (average 17.2 ss) developed enlarged DA on both sides, which were accompanied by reduced CV primordial capillaries (Fig. 5A-F). The remaining nine mutants, at a later stage (average 19.6 ss) with more severe developmental defects, exhibited an enlarged left-anterior DA that was still accompanied by a reduction in number of CV capillaries. But the right-anterior DA was smaller and coincided with an increase in number of CV primordial capillaries (data not shown).

To quantitatively assess the vessel defects in these mutants, we counted ECs in serial cross-sections through the trunk region of E8.75 (15-17 ss) embryos. Total EC numbers, including those in the DA, CV and capillaries in the vicinity, were reduced in Efnb2−/− mutants by 20% compared with controls (19.5±5.6% decrease in the mutant over the control; \( P = 0.02, n = 3 \)), suggesting that loss of ephrin B2 may have affected EC proliferation and/or survival. Nevertheless, the proportion of DA ECs increased to 47.9% in mutants (\( P = 0.02, n = 3 \)) from 31.2% in controls (Fig. 5F). Therefore, Efnb2−/− embryos with enlarged DA and reduced CVs primarily resemble Tie2-tTA;TRE-int3 and not Notch1−/− embryos.

To determine the arterial-venous identity of the ECs, we stained cross-sections for EphB4 and CD31 and demonstrated that, in controls, EphB4-positive cells were present only in the veins at E8.75 (Fig. 5G). In Efnb2−/− mutants, however, EphB4-positive ECs were also present in the enlarged DA (Fig. 5H). The expression of another arterial marker, Dll4, absent in the Notch1−/− mutant, was unchanged in the Efnb2−/− mutant (data not shown), suggesting that lack of ephrin B2 did not affect overall EC identity. These data indicate that venous ECs may mislocalize to the DA when the embryo lacks ephrin B2.

To examine whether ephrin B2 in ECs is responsible for DA and CV development, we analyzed EC-specific conditional knockouts, using Tie1-Cre lines described above and the Efnb2fl/fl allele (Gerety and Anderson, 2002). The conditional mutant embryos developed similar phenotypes to the Efnb2−/− embryos at the same stage, suggesting that loss of ephrin B2 in ECs is responsible for the vascular defects (Fig. 5I-L). In summary, these results imply that ephrin B2 signaling within the ECs is responsible for the coordinated sizes of the developing DA and CV, in a manner similar to, but distinct from, Notch signaling.
Ephb4–/– embryos also exhibit enlarged DA and underdeveloped anterior CV

Because EphB4 is a putative receptor for ephrin B2, and Ephb4–/– embryos exhibit similar vascular phenotypes to the Efnb2–/– mutants (Gerety et al., 1999), we examined the Ephb4–/– DA and CV. Ephb4–/– embryos indeed developed enlarged DA along with underdeveloped anterior CV around E9.25 (20 ss) (Fig. 6A–H). In addition, the enlarged DA harbored ephrin B2-negative ECs (Fig. 6E,F) and EphB4-positive ECs, as judged by EphB4-tauLacZ promoter activity (Fig. 6G,H), not seen in the controls. These data demonstrate that Ephb4 deficiency led to similar DA enlargement and CV underdevelopment as with Efnb2 deficiency, and that the enlarged mutant DA contained mislocalized EphB4-positive, ephrin B2-negative (and thus likely venous) ECs. The CV size is reduced and its ECs express EphB4. The ratio of arterial to venous ECs is reduced. In both loss-of-function Efnb2 and Ephb4 mutants, the DA is enlarged whereas the CV is reduced. The enlarged DA bears some ECs with venous identity, ephrin B2– and EphB4+ (blue). The CV size is reduced and its ECs express EphB4. (B) Proposed model depicts the Notch and ephrin B2/EphB4 pathways as molecular regulators in the balanced growth of the DA and CV. Alterations in the size of one type of vessel are accompanied by reciprocal changes in the other. Notch signaling controls this equilibrium by promoting arterial differentiation, thereby dictating the ratio of arterial to venous ECs. Ephrin B2/EphB4 signaling regulates this balance by sorting differential ECs into the respective vessels.

DISCUSSION

To understand the molecular basis of arterial-venous growth, we conducted concurrent analysis of DA and CV morphogenesis in mouse Notch, Efnb2 and Ephb4 mutants (summarized in Fig. 7A).
Our findings lead us to propose that the sizes of the DA and CV are balanced through the reciprocal regulation of vessel growth (Fig. 7B). By promoting arterial differentiation, Notch balances the proportion of arterial to venous ECs without affecting their absolute number, thus regulating both artery and vein size. Ephrin B2/EphB4 signaling functions distinctly from Notch by sorting differentiated arterial or venous ECs into their respective vessels.

**Coordinated arterial and venous growth is achieved through a reciprocal balance**

Developing arteries and veins must coordinate both the number and size of their branches to generate a proper circulatory system. The cellular and molecular mechanisms underlying this regulation are poorly understood. One potential mechanism to achieve such equilibrium is interdependent vessel growth. In support of this hypothesis, we have provided quantitative evidence showing that an increase or decrease in DA size leads to a reciprocal change in CV size.

An expansion of the CV region with a concomitant loss of DA segments has been observed in zebrafish. In a subset of zebrafish embryos, inhibition of Notch signaling through high dose antisense constructs targeting the Notch downstream gene gridlock (grl), was shown to increase CV length or region but not lumen size, with loss of DA segments (Zhong et al., 2001). However, this phenotype did not occur in the majority of embryos injected with the high dose construct nor in embryos injected with low dose antisense DNA. In addition, even in the most severe zebrafish mindbomb mutant, a putative Notch loss-of-function mutant, the DA remained normal, although the expression of arterial markers was diminished (Lawson et al., 2001). Conversely, in gain-of-function mutants, induced by over-expression of grl or expression of Notch ICD, the size of the DA was not affected, despite increased ephrin B2 expression (Lawson et al., 2001; Zhong et al., 2001). Therefore, although these earlier studies show that Notch activity is necessary and sufficient for arterial marker expression, a role for Notch in balancing DA and CV lumen size has not been established. Our findings in both Notch gain- and loss-of-function mouse mutants suggest that Notch is critical in equilibrating both arterial and venous lumen size.

Consistent with our findings, prior studies have shown that the DA is small and atretic in Notch loss-of-function mutants (Krebs et al., 2000; Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). However, these previous reports did not elaborate on CV development. Enlarged and reduced vessel sizes have been reported in Notch4 gain-of-function mutants (Uyttendaele et al., 2001), further suggesting that Notch controls vessel size. However, this earlier study did not specify coordinated changes in arteries and veins. We have combined mouse genetics and in vivo imaging to examine the effects of Notch on both the DA and the CV concurrently. We report here that Notch signaling regulates coordinated growth of both DA and CV in mice by balancing the ratio of arterial versus venous ECs.

It is unclear at present whether this balanced regulation is a universal mechanism during angiogenesis. Our evidence from the carotid arteries and the head veins supports the notion that it occurs in other developing arteries and veins. Furthermore, VEGF, a molecule genetically upstream of Notch (Lawson et al., 2002; Mukouyama et al., 2002), dictates the ratio of arterial and venous blood vessel types during angiogenesis in cardiac muscle (Visconti et al., 2002), suggesting that this mechanism of angiogenesis may be universal. In this study, ∼50% of capillaries in control animals were ephrin B2 positive. In the VEGF over-expressing mutant, nearly 90% of capillaries were ephrin B2 positive and fewer than 10% of capillaries were EphB4 positive. Similarly, a recent study reports that the Tie2-Cre conditional deletion of Smad4, a component of TGF-β signaling, yields a small DA and an enlarged CV at E9.5 (Lan et al., 2007). This result also lends support to the reciprocal regulation of arterial and venous size, which, together with our findings, suggests that the reciprocal relationship between growing arteries and veins may be a general process.

**Arterial-venous differentiation, not cell proliferation, is crucial for the balanced growth of the DA and CV**

Our data demonstrate that the cellular mechanism underlying the interdependence between arterial and venous size is a balanced allocation of ECs between these vessels. Balanced differentiation of one cell type at the expense of another by Notch during cell fate decisions has been observed in C. elegans ventral uterine precursor/anchor cells in the gonad, Drosophila neural versus epidermal precursor cells in the ventral ectoderm (Artavanis-Tsakonas et al., 1999), and T versus B cells in the mouse immune system (Pear and Radtke, 2003). Our quantitative data at cellular resolution suggest that the role of Notch in the balance between two cell types seems to extend into the mouse vasculature, where it similarly regulates the balance between arterial and venous ECs.

Although changes in cell proliferation could lead to differential size, we demonstrate that the proliferation of ECs was not affected by int3. Thus, Notch regulates EC allocation by dictating arterial specification, thereby controlling the ratio of arterial to venous ECs. Coincident with defective DA and CV size is evidence of abnormal vascular perfusion and arteriovenous shunting. We show that ink injected into the heart leaks from the DA into the CV compartment in Notch gain-of-function mutants. Others have similarly demonstrated DA and CV shunting in embryos lacking Notch1 (Gridley, 2007). These studies suggest the importance of proper EC allocation between arteries and veins in the establishment of a functional circulatory system.

**The reciprocal size changes between the mutant DA and CVs are unlikely results of aberrant blood flow**

It is well established that increase in blood flow induces enlargement, whereas a decrease leads to reduction in vessel diameter (Korshunov and Berk, 2003). Such observations raise the issue of whether the reciprocal DA and CV size changes are secondary to hemodynamic changes. As it is currently not feasible to measure blood flow changes in early mouse embryos, it is difficult to address this question empirically. However, evidence suggests that the reciprocal DA and CV sizes are likely to be primary effects of genetic perturbation and not of blood flow changes. First, the phenotypes were apparent at E8.75-E9.0, shortly after E8.5, when blood pressure is irregular and minimal, and unlikely to cause such defects (Jones et al., 2004). We have intentionally analyzed the defects early to avoid flow influence, and mutants were compared with size and somite-stage matched littermate controls. Second, the inverse size change does not fit the well-established flow theory. If the observed size changes in the DA were due to changes in flow, then CV size would coincide, as opposed to the reciprocal phenotype we observed. By contrast, both arteries and veins were reduced in a Myc (c-myc) mutant specifically harboring flow defects, as predicted by the flow theory (He et al., 2008). In this mutant, Myc (c-myc) was
Notch and ephrin B2 balance vessel size

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/22/3755/DC1

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