THE ROLE OF DLL4 IN THE REGULATION OF ARTERIOGENESIS AND ANGIOGENESIS

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ABSTRACT

Vascular development is dependent on various growth factors and certain modifiers critical for providing arterial or venous identity, interaction with the surrounding stroma and tissues, hierarchical network formation, and recruitment of pericytes. Notch receptors and ligands (Jagged and Delta-like) play a critical role in this process in addition to VEGF. Dll4 appears to be the most important of the Notch ligands in the regulation of arterial specification and vessel maturation events, as well as modulating the angiogenic response by controlling the abundance of tip cells. In this review we present various evidence supporting these claims and suggest that they indicate Dll4 as a promising target for therapeutic intervention in adult angiogenesis.

INTRODUCTION

Primary vasculogenesis serves as the template from which a higher order of branching network is generated by the process defined as angiogenesis.[1-3] During angiogenesis, branching of arterial and venous components is orchestrated such that the capillaries from these two compartments fuse in symmetry, anchored in place by interaction with matrix proteins.[4] Vascular endothelial growth factor (VEGF) is indispensable for the formation of the primary vascular network and for secondary angiogenesis.[5] However, it requires the presence of precise quantities of several other constituents within well-defined temporal and spatial constraints to construct and remodel the vascular system. Specifically the Notch signaling pathway is necessary to provide signals for phenotypic determination of arteries and veins, and regulated vessel migration and branching leading to the vascular morphogenesis and remodeling.[6]

In mammals, the Notch family of proteins is composed of four single-pass transmembrane receptors (Notch1-4) and five membrane bound ligands (Jagged1, 2 and Dll1, 3, and 4). Mutations of Notch receptors and ligands in mice and humans lead to abnormalities in the vascular system.[7] The Notch pathway functions through cell-cell interaction such that the extracellular domain of cell membrane–bound ligand interacts with the extracellular domain of the receptor on an adjacent cell. Notch receptor activation requires cleavage of Notch intracellular domain (NICD) and translocation to the nucleus, and activation of target genes.[8]

Differentiation of vascular cells to arterial or venous compartments was previously thought to depend on physical factors such as blood pressure and oxygen concentration. Over the past few years, however, the differential and restricted expression of a number of genes in arterial or venous endothelial cells prior to the onset of circulation suggested the potential for genetic determination of the arterial and venous fate of primary endothelial cells. Among these genes are *Notch1*,[9] *Notch4*,[10] *Dll4*,[11] and the Dll4-Notch-regulated genes *EphB4* and *EfnB2* specifically expressed in venous[12] and arterial endothelial cells, respectively.[13,14]

Vascular expression of Dll4 and its cognate receptors, Notch1 and Notch4, is restricted to arterial endothelium. *Dll4* is one of the earliest genes expressed in arterial endothelial cells, is induced by VEGF-VEGFR signaling, and is essential for the establishment of the arterial endothelial cell fate.[14-16]

DLL4 HAPLOINSUFFICIENCY

We, along with two other laboratories, have recently shown that the Dll4 ligand alone is required in a dosage-sensitive manner for normal arterial patterning in development.[16,17,18]

The *Dll4* gene was inactivated by targeted disruption in embryonic stem (ES) cells with a targeting vector designed to replace the initiation codon and the first three coding exons with the β-galactosidase (*lacZ*) reporter gene.[16] When 100% germline transmitting male chimeras were crossed with wild-type ICR females only 21% of the agouti offspring was $Dll4^{+/-}$, rather than the expected 50% frequency, indicating the death *in utero* of a proportion of the $Dll4^{+/-}$ F1 embryos. Outcrossing these viable heterozygotes to outbred ICR mice still resulted in a reduced number of heterozygotes in subsequent generations. This effect was dependent on the genetic background, as no live $Dll4^{+/-}$ offspring were obtained when the 100% germ-line transmitter chimeric males were crossed with inbred 129/Sv-CP females.

To further characterize this incompletely penetrant haploinsufficiency, we investigated the phenotypes of $Dll4^{+/-}$ embryos. In pre-somite stages, *lacZ* expression was exclusively detected in trophoblast giant cells. Around the two-somite stage (E8.0), the *lacZ* reporter was expressed in the cardiac crescent and the primordia of the dorsal aortae (Fig. 1A). At the five- to 10-somite stage (E8.5), it was expressed in the heart, paired dorsal aortae, branchial arch arteries, internal carotid arteries, umbilical artery, vitelline artery, and in the posterior region of the yolk sac where the arteries are formed (Fig. 1B,C).

While at E8.5 there were no obvious differences between wild-type and $Dll4^{+/-}$ embryos, as development proceeded, however, some heterozygous embryos started to show developmental delay. At E9.5 only 31% of the $Dll4^{+/-}$ embryos were normal in body size and somite number as the rest showed growth retardation. At E10.5, two types of $Dll4^{+/-}$ conceptuses were found: normal-sized embryos with reduced vitelline circulation, and severely retarded embryos with reduced vitelline circulation and enlarged pericardial space, indicative of embryonic circulatory defects (Fig. 1D–F). The caliber of the major vitelline arteries and the arterial branching on the yolk sac was reduced in all of the heterozygous embryos (Fig. 1I, J). The umbilical artery and placental blood

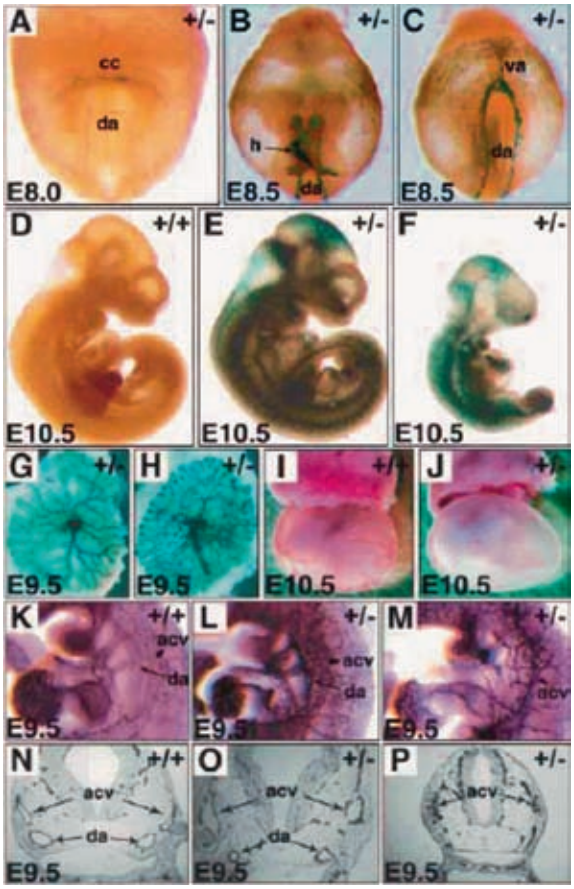

Fig. 1 – *Dll4* haploinsufficiency. (*A–H*) Whole-mount X-gal staining. (*K–P*) PECAM immunostaining: whole-mount (*K–M*) and sections (*N–P*). (*I, J*) Unstained specimens. (*A*) *Dll4* expression at two somite stage in the cardiac crescent (cc) and dorsal aortae (da) primordia. (*B,C*) At E8.5, *Dll4* expression is observed in all arterial vessels (da, dorsal aortae; va, vitelline arteries) and in the endothelial lining of the heart (h). (*D–H*) Variable penetrance of the hemizygous phenotype: littermate embryos (E10.5, *D–F*) and arterial vessels of the placenta (E9.5, *G, H*). (*I, J*) Morphology of E10.5 yolk sacs. Note the absence of large vitelline vessels and the poor circulation in the *Dll4*$^{+/-}$ yolk sac compared with wild-type. (*K–P*) At E9.5 two types of *Dll4*$^{+/-}$ embryos can be found: with reduced dorsal aorta but without defects in the anterior cardinal vein (*L,O*) and with more severe defects, with a very reduced dorsal aorta, enlarged heart, and the anterior cardinal vein reduced to a disorganized venous plexus (*M,P*).

vessels were also reduced in size in some *Dll4*$^{+/-}$ embryos (Fig. 2G,H). The variable phenotype of the placental vascular bed probably explains why some *Dll4*$^{+/-}$ embryos could survive to term, despite the poor vitelline circulation in all heterozygotes, since the dependence of the embryo on the vitelline circulation for gas and nutrient exchange is only transient until the placental circulation is established.

Defects in vascular structures were also seen within the *Dll4*$^{+/-}$ embryos, most notably the reduction of the caliber of the dorsal aortae. At E9.0 the dorsal aortae were already thinner than normal in the majority of the embryos, a feature which was accentuated at E9.5 and E10.5. The constriction of the dorsal aortae was primarily observed rostrally between the cardiac level and the intersections with the branchial arch arteries (Fig. 1K–P), but some embryos exhibited longer zones of constriction extending caudally (data not shown). At both E9.5 and E10.5, 90% of the embryos displayed some degree of aortic constriction.

The potential impact of these arterial defects on the development of the venous system was also investigated. Reduced caliber and disorganization of the anterior and posterior cardinal veins were observed in the *Dll4*$^{+/-}$ embryos, but only in those cases where the dorsal aortae were severely affected, suggesting that the venous defect is a secondary response to a primary arterial restriction (Fig. 1, cf. L,O and M,P).

Surviving *Dll4*$^{+/-}$ mice were apparently normal, suggesting that in these animals the major defects caused by reduction of Dll4 are transient. This allowed the intercrossing of *Dll4*$^{+/-}$ mice and subsequent examination of the homozygous null phenotype.

DLL4 LOSS-OF-FUNCTION

As expected, the *Dll4*$^{-/-}$ embryos showed more severe and precocious vascular defects than heterozygotes.[16] Mutant embryos were morphologically normal until E8.5. The correct migration and aggregation of the angioblasts occurred to form the dorsal aortae, showing no disruption of the onset of vasculogenesis. However, the dorsal aortae already showed a clear reduction in diam-

eter by E8.75 (Fig. 2A, B). By E9.0 the homozygous null embryos were highly delayed and abnormal, with severe pericardial swelling, and drastically reduced dorsal aortic diameter in the anterior region (Fig. 3E–H). Not only were the major arteries abnormal; branching morphogenesis was also affected. *Dll4*$^{-/-}$ embryos showed an abnormal accumulation of *lacZ*+ endothelial cells in the apical portion of the intersomitic vessels and an abnormally dilated dorsal vessel in this region (Fig. 2, cf. G and H). This defect would result in abnormal blood circulation in the embryo, with misdirection from the dorsal aorta to the lateral vessels.

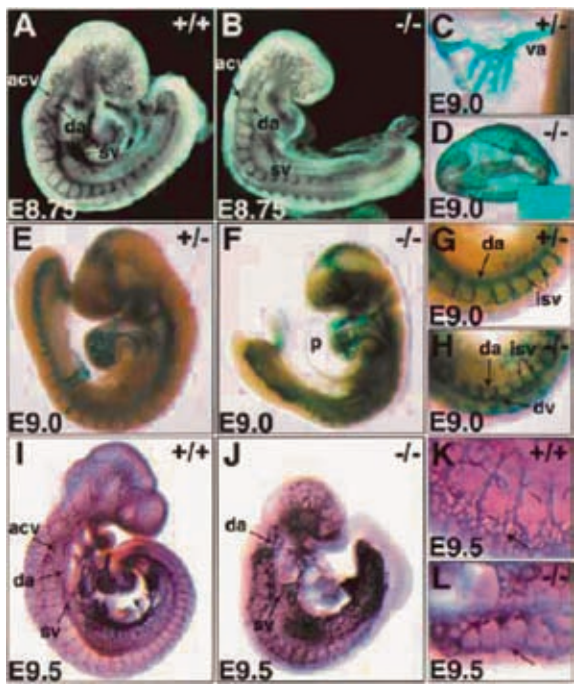

Fig. 2 – Defective arterial and venous remodeling in *Dll4*$^{-/-}$ embryos. Whole-mount PECAM (*A,B,I–L*) and X-gal staining (*C–H*) of control and *Dll4*$^{-/-}$ embryos. At E8.75, *Dll4*$^{-/-}$ embryos display reduced dorsal aorta (da), anterior cardinal vein (acv), and sinus venosus (sv). (*C,D*) Presence of vitelline arteries (va) in the yolk sac of a E9.0 *Dll4*$^{+/-}$ embryo contrasts with *Dll4*$^{-/-}$ yolk sac vessels, which appear stalled at the primary capillary plexus stage (*inset*); in *Dll4*$^{-/-}$ embryos the *Dll4-lacZ*reporter expression is observed throughout the yolk sac vessels. (*E–H*) By E9.0, *Dll4*$^{-/-}$ embryos exhibit growth retardation and arrested heart development with pericardial edema (p); the dorsal aorta appears further reduced, and there is an abnormal accumulation of *lacZ*-positive cells in the apical portion of the intersomitic vessels (isv) forming an enlarged dorsal vessel (dv). By E9.5, the *Dll4*$^{-/-}$ embryos are severely retarded with extremely reduced dorsal aortae, a reduced and almost indistinguishable venous structure, sinus venosus, and anterior cardinal vein. (*K,L*; higher magnification of *I, J*, respectively). In the more dorsal region, instead of an intricately branched network between arterial and venous intersomitic vessels, the *Dll4*$^{-/-}$ embryos display a single fused vessel that did not undergo angiogenic remodeling.

Yolk sac circulation was also highly abnormal. The vitelline artery was drastically reduced, and the yolk sac vascular plexus showed an angiogenic remodeling defect with persistence of the primary capillary bed. Interestingly, whereas in the heterozygous embryos the *lacZ* reporter was strictly expressed in the arterial (posterior) region of the yolk sac vasculature, in the null embryos, *lacZ* was expressed in all endothelial cells of the yolk sac plexus (Fig. 2C,D). Although vitelline arteries fail to form, *Dll4* expression is clearly activated in the yolk sac precursors, consistent with it being upstream of the genes that specify artery fate.

By E9.5 these phenotypic traits were accentuated, with more pronounced growth retardation and arterial atrophy, the dorsal aortae being absent or reduced to a rudimentary capillary plexus (Fig. 2I,J). The hearts in these embryos showed reduced atrial and ventricular chambers, and the ventricular trabeculation was markedly reduced.

The head vasculature consisted of a simple plexus of disorganized and fused vessels (Fig.2J). Venous development was also impaired in the null embryos. At E8.75 the anterior cardinal vein already appeared to have a reduced caliber and ectopic branching at some points, and the sinus venosus appeared smaller (Fig. 2A,B). By E9.0 the anterior cardinal vein was further reduced, and by E9.5, a distinct anterior cardinal vein was absent and the embryos showed a very reduced sinus venosus (Fig. 3I,J). Therefore, although the major arteries and veins of the embryo form in the absence of *Dll4*, their later development is severely disrupted. As *Dll4* expression is artery-specific, the venous defects are likely secondary to arterial patterning and growth problems.

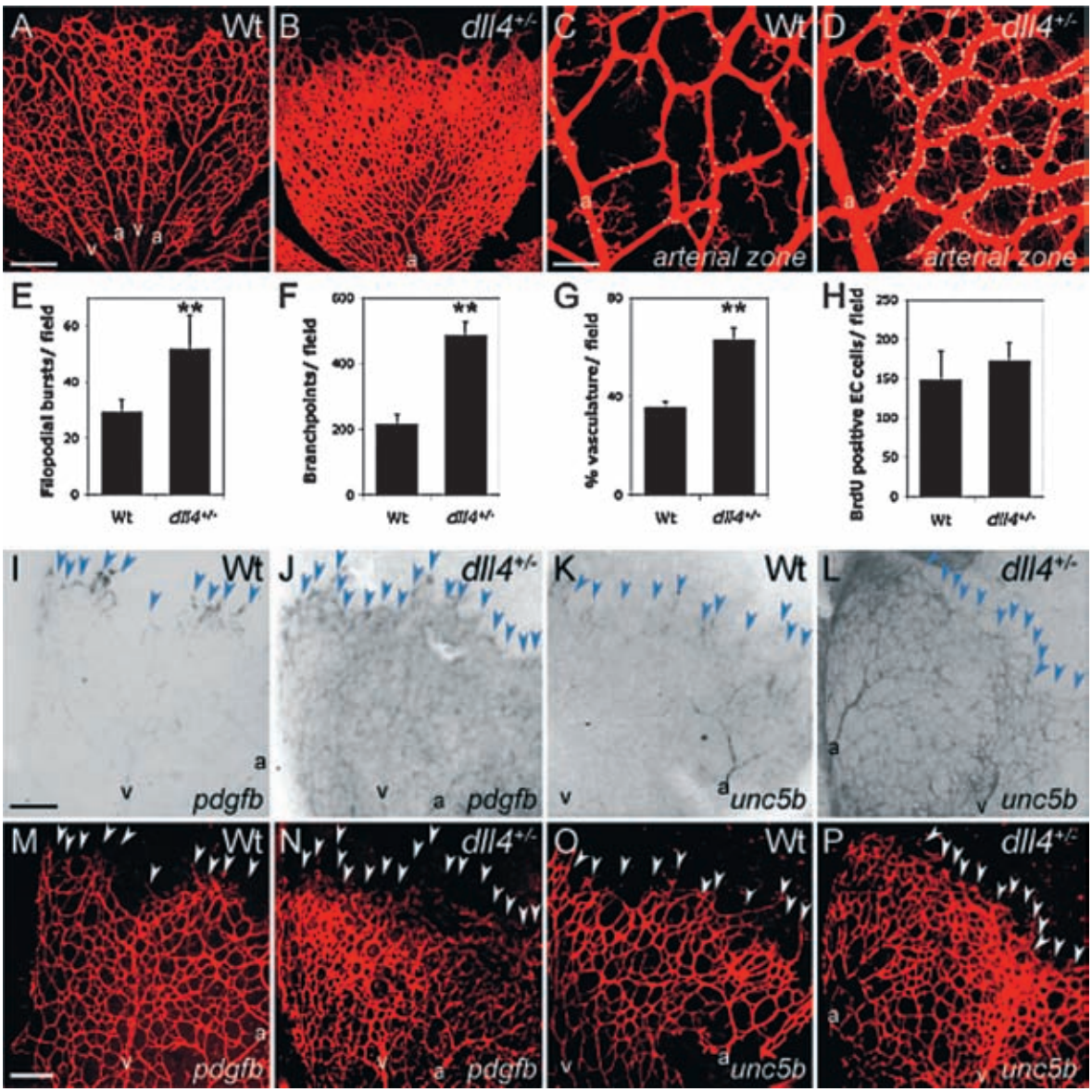

Fig. 3 – Increased tip cell formation in *Dll4*$^{+/-}$ retinal vessels. (*A* and *B*) Isolectin B4-stained P5 *Dll4*$^{+/-}$ retinal vessels show a hyperfused plexus compared with wild-type (a, artery; v, vein). (*C* and *D*) *Dll4*$^{+/-}$ vessels extend many more filopodia within the vascular plexus compared with wild-type (arterial zone shown; dots indicate filopodia extensions). (*E–H*) Quantification of filopodial bursts at the vascular front, branchpoints in the vascular plexus, percentage of retina covered by vessels, and BrdU-labeled retinal ECs in wild-type and *Dll4*$^{+/-}$. (*I–P*) Whole-mount ISH shows expanded expression of tip cell marker genes *Pdgfb* and *Unc5b* in *Dll4*$^{+/-}$ compared with wild-type (arrowheads indicate tip cell expression at vascular front). (*M–P*) Corresponding isolectin B4 staining of retinal vessels. Error bars, SD; ******, $P < 0.001$, Mann–Whitney *U* test. [Scale bars, 250 μm (*A* and *B*); 40 μm (*C* and *D*); and 100 μm (*I–P*).]

In zebrafish, Notch signaling has been implicated in the specification of arterial endothelial cells by suppressing the venous cell fate.[14] To investigate whether the disruption of vascular development in *Dll4* mutants could be at least partially attributable to abnormal identity of the vascular endothelial cells, we carried out RNA *in situ* hybridization and immunostaining to determine the expression of arterial and venous markers. In *Dll4*$^{-/-}$ embryos with residual intact dorsal aortae,

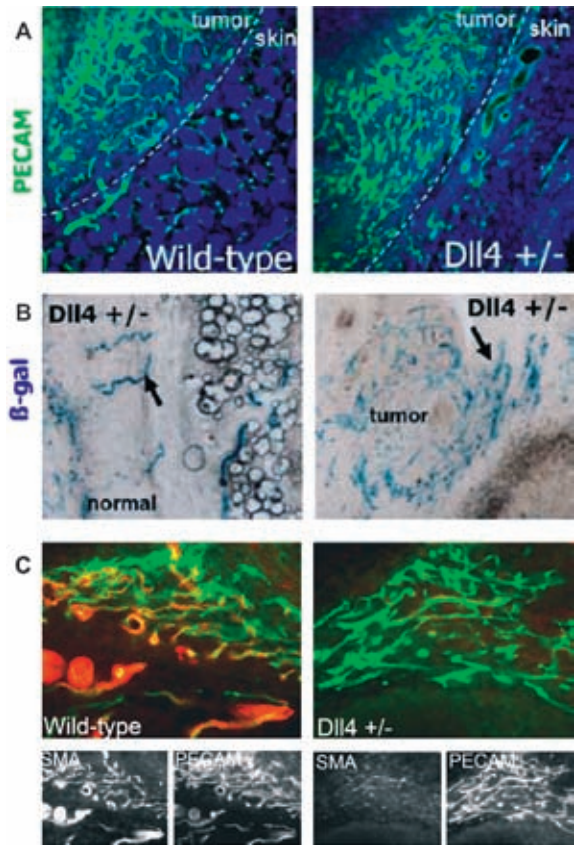

Fig. 4 – Increased tumor branching angiogenesis in *Dll4*$^{+/-}$ mutant mice: (A) Vascular response in ***Dll4***$^{+/-}$ adult mice was examined after tumor implantation. Wild-type mice showed organized vascular proliferation in the tumor (left half), while mutant mice showed markedly increased vascular response. **(B)** Expression of Dll4 in tumor and normal regions in Dll4+/- mutant mice was examined by ß-gal staining. Dll4 expression was observed in a few discrete vessels in the normal tissue, while the tumor region showed many ß-gal positive vessels of similar appearance, indicative of *Dll4* induction in tumor vessels. **(C)** Pericyte coverage around newly forming vessels was examined by α-SMA localization. In wild type mice, the vessels showed co-localization of PECAM and α-SMA (left panel). In *Dll4*$^{+/-}$ mice tumor vessels however, the number of α-SMA positive cells lining the endothelial cells was profoundly reduced (right panel).

the VEGF receptor, Flk1, was normally expressed in both arteries and veins, and the *Dll4-lacZ* reporter was expressed in the arteries. However, none of the downstream arterial markers studied (EphrinB2, Connexin37, and Connexin40) were expressed in the endothelium (data not shown).

This is consistent with the proposed pathway for zebrafish, where VEGF signaling upstream of Notch signaling promotes arterial cell fate.[14] In addition to loss of arterial markers, the venous marker EphB4 was ectopically expressed in the dorsal aortae as well as in the cardinal veins (data not shown). In some null embryos, from E9.0, the dorsal aorta fused with the anterior cardinal vein at the level of the sinus venosus (data not shown), consistent with possible loss of separate identity of the two vessels. These data strongly suggest an involvement of the Notch signaling pathway, mediated through the Dll4 ligand in a cell-autonomous manner, in the establishment of the endothelial arterial cell phenotype in mice.

The similarity of the *Dll4*$^{-/-}$ phenotype to that of the *Notch1/4* double mutants[9] is consistent with this, as is the contrasting effect of endothelial specific activated Notch expression.[19] Loss of arterial vascular identity in *Dll4*$^{-/-}$ mutants, in turn, could cause angiogenic defects leading to a generalized disruption of the vasculature and embryonic death.

The strain-dependent haploinsufficiency of *Dll4* was surprising because no other component of the Notch pathway has shown such an effect on vascular development. Indeed, this is the first reported lethal haploinsufficiency for any Notch signalling component in mammals. However, vascular development-related haploinsufficiency has also been reported for the VEGF gene[5] which appear to lie upstream of Notch signaling in arterial development.[14] This suggests that the development and patterning of the arterial system may be controlled by levels of availability of critical ligands. Interestingly, both VEGF and Dll4 have been shown to be up-regulated by hypoxia,[20, 21] which is one of the environmental factors that can impinge on vascular patterning and growth. Presumably, exquisite sensitivity to ligand levels helps to ensure appropriate vascular responses to changing external environments. Sensitivity of the embryonic vasculature to Dll4 levels raises the possibility that Dll4 might be a good target for intervention in adult neovascularization.

DLL4 IN THE REGULATION OF ANGIOGENESIS

All *Dll4*$^{-/-}$ embryos examined showed branching defects but with varying severity, indicating that unknown genetic modifiers also influence the *Dll4*$^{-/-}$ phenotype. To exclude the possibility that the defects observed in vessel branching were secondary to other defects in the embryo, *e.g.*, disrupted blood flow, we cultured aortic explants from *Dll4*$^{-/-}$ and wild-type mice in collagen gels.[22] Sprouting from *Dll4*$^{-/-}$ aortas occurred earlier and more profusely than from wild-type aortas (data not shown), indicating that the *Dll4*$^{-/-}$ vessel defect is intrinsic to endothelial cells (ECs) and independent of alterations to flow or cardiac output.

To establish the underlying cause of the *Dll4*$^{-/-}$ vessel branching defects, we performed high-resolution confocal microscopy on isolectin B4-stained *Dll4*$^{-/-}$ and wild-type E11.5 hindbrains.[22] Staining for β-gal in hindbrains showed expression in virtually all ECs (data not shown). Vessel branching was significantly increased in *Dll4*$^{-/-}$ compared with wild-type hindbrains (data not shown). Strikingly, vessels from *Dll4*$^{-/-}$ hindbrains extended numerous filopodia from the whole length of the vessel surface, whereas vessels from wild-type hindbrains extended only a few filopodia at scattered points.

Filopodia extension is a morphological characteristic of specialized ECs called ''tip cells,'' which are lumenless ECs present at the leading edge of vascular sprouts that integrate directional cues from their environment and so define the direction in which the new sprout grows.[23] Tip cells also find and create connections with adjacent sprouts and so generate functional vascular networks.[24] The profusion of filopodia in *Dll4*$^{+/-}$ hindbrains suggested that most, if not all, ECs in the hindbrain vessels were acting like tip cells, leading to increased connections between adjacent vessels (*i.e.*, branching).

To further evaluate whether loss of *Dll4* leads to increased endothelial tip cell formation, we analyzed postnatal retinal vascular development of surviving *Dll4*$^{+/-}$ and wild-type pups.[22] The retina vasculature at postnatal day (P) 4–6 allows for simultaneous visualization of angiogenic sprouting at the vascular front (where most endothelial tip cells are located) and remodeling of the nascent vasculature within the vascular plexus.[25] In wild-type retinas, *Dll4* expression was detected predominantly in arteries and in tip cells at the vascular front, with lower levels observed in vessels within the vascular plexus (data not shown). In *Dll4*$^{+/-}$ vessels, lower levels of *Dll4* were detectable by *in situ* hybridization and quantitative PCR (qPCR; 51% of wild-type levels), although the expression pattern was comparable to wild-type.

As with hindbrains, *Dll4*$^{+/-}$ retinal vessels showed severe patterning defects, forming a hyperbranched, hyperfused plexus behind the vascular front (Fig. 3 *A* and *B*). Numerous filopodia extended from *Dll4*$^{+/-}$ vessels at the vascular front and also within the vascular plexus in both arterial and venous zones (Fig. 3*D*). In contrast, wild-type vessels extended few filopodia in regions away from the vascular front (Fig. 3*C*). Quantification showed 75% more filopodia extensions at the vascular front, 125% more branchpoints within the vascular plexus, and an 80% increase in the area covered by vessels in *Dll4*$^{+/-}$ compared with wild-type (Fig. 3 *E–G*). This increase in the vessel coverage suggested that *Dll4*$^{+/-}$ vessels may also have proliferation defects. Quantification of BrdU-labeled (S-phase cells) or phospho-histone-H3-stained ECs (M-phase cells) indicated a modest (1.16-fold) non-significant increase in proliferating ECs in *Dll4*$^{+/-}$ vessels compared with wild-type (Fig. 3*H*). Taken together, these observations suggest that *Dll4*$^{+/-}$ retinal vessels are functional, and that the major defect in vessel patterning is due to inappropriate and excessive sprouting.

In addition to *Dll4*, other genes are also expressed at high levels in endothelial tip cells in the retina, including *Pdgfb*[23] and *unc5b*[26]. Com-

pared with wild-type, *Dll4*$^{+/-}$ retinal vessels expressed *Pdgfb* (Fig. 3 *I*, *J*, *M*, and *N*) and *Unc5b* (Fig. 3 *K*, *L*, *O*, and *P*), over an expanded area, especially in the hyperfused plexus. Thus, vessels from *Dll4*$^{+/-}$ retinas display genetic as well as morphological (filopodia) and behavioral (hyperfused vessels) indicators of an expansion in the number of ECs that have a "tip cell" phenotype, suggesting that Dll4 normally functions to suppress tip cell formation in growing vessels.

DLL4 IN TUMOR NEOANGIOGENESIS

As mentioned earlier, the high sensitivity of the embryonic vasculature to Dll4 levels raises the possibility that it may constitute a good target for intervention in adult neovascularization, both in proangiogenic as well as in anti-angiogenic settings, such as inhibiting tumor growth by targeting its vasculature.

To try to understand how Dll4 levels influence tumor vasculature, we studied vascular response and remodeling in tumors transplanted to adult *Dll4*$^{+/-}$ mutant and wild-type mice[27]. Mice received implants of S180 tumor cells. Tumor and adjacent tissue harvested after 10 days was examined for vascular response by PECAM, and α-SMA immunolocalization. Wild-type mice showed increased vascular response in the tumor (Fig. 4B) and the vessels displayed an organized network. In comparison, *Dll4*$^{+/-}$ mice showed an even greater increase in the vascular response (1.5-fold increase, $P < 0.05$). Furthermore, the vessels showed lack of architecture and loss of hierarchy. Thus vascular response was increased but maturation was lacking. Maturation of newly forming vessels accompanies the recruitment of pericytes. We hypothesized that newly forming vessels in *Dll4*$^{+/-}$ mice may be defective in pericyte recruitment. Thus localization of pericytes with α-SMA antibodies showed abundant signal in tumor vessels in wild-type mice, whereas tumor vessels in *Dll4*$^{+/-}$ mice showed a profound deficiency in pericyte coverage. Reduced recruitment of pericytes may contribute to the lack of vascular hierarchy observed in the tumor vessels of *Dll4*$^{+/-}$ mice. Furthermore, these findings reveal a novel function of *Dll4* in the recruitment of pericytes to newly forming vessels. We next wished to determine if defective vascular response in adult mice leads to alteration in gene expression, in particular *Dll4*. To this end, we used the *LacZ* reporter included in the targeting vector used to generate mutant mice to observe *Dll4* promoter activity. *Dll4*$^{+/-}$ mutant mice showed highly structured *LacZ*--expressing vessels in the normal tissue adjacent to the tumor, whereas LacZ activity was markedly increased in vessels within the tumor vessels (Fig. 4C), indicative of *Dll4* activation in the tumor vasculature. PECAM localization in serial sections of the tumor vessels was done to determine the extent of *Dll4* activation in tumor vasculature, showing that it is expressed in the majority but not in all tumor vessels.

CONCLUSION

Dll4 appears to act in endothelial cells in at least two different ways, establishing arterial endothelial cell fate in development and regulating the intensity of the angiogenic response. The potential to therapeutically modulate angiogenesis through this signaling pathway constitutes a promising research avenue. The available data suggest a model in which Dll4, expressed in endothelial tip cells, inhibits the angiogenic response of adjacent ECs to VEGF stimulation, most likely through Notch signaling. This mechanism would permit an asymmetric cellular response to VEGF stimulation during vascular sprouting by allowing some ECs to respond to a local VEGF gradient by forming a sprout, while, through upregulation of *Dll4* expression, inhibiting adjacent cells from also forming sprouts. When even a single *Dll4* allele is absent, or when Notch signaling is blocked, this suppression is lost, resulting in increased sprout formation

and tip cell filopodia. This mechanism provides an elegant negative feedback system intrinsic to ECs to control their response to VEGF and suggests that vascular network formation is coordinated by VEGF and Dll4/Notch signaling. In addition, Dll4 appears to be involved in pericyte recruitment and therefore, in the stabilization of newly formed vascular branches. We are currently testing the effect of modulators of vascular Notch signaling *in vivo* to address the feasibility of its use in therapeutic intervention in angiogenesis.

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