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Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis

Jonathan D. Leslie[1,*], Linda Ariza-McNaughton[1], Adam L. Bermange[1], Ryan McAdow[2], Stephen L. Johnson[2] and Julian Lewis[1]

Notch signalling by the ligand Delta-like 4 (Dll4) is essential for normal vascular remodelling, yet the precise way in which the pathway influences the behaviour of endothelial cells remains a mystery. Using the embryonic zebrafish, we show that, when Dll4-Notch signalling is defective, endothelial cells continue to migrate and proliferate when they should normally stop these processes. Artificial overactivation of the Notch pathway has opposite consequences. When vascular endothelial growth factor (Vegf) signalling and Dll4-Notch signalling are both blocked, the endothelial cells remain quiescent. Thus, Dll4-Notch signalling acts as an angiogenic 'off' switch by making endothelial cells unresponsive to Vegf.

KEY WORDS: Notch, Delta-like 4, Angiogenesis, Endothelial, Motility, Zebrafish

INTRODUCTION

Both during development and in adult life, blood vessels undergo periods of sprouting and growth, as well as periods of quiescence. During these quiescent periods, the vascular bed remains static while blood circulates through it, whereas, during growth, new vessels are generated by the proliferation and migration of endothelial cells, which form sprouts from the sides of existing vessels under the influence of vascular endothelial growth factor (VEGF) and other signals (Carmeliet and Tessier-Lavigne, 2005; Coultas et al., 2005; Goishi and Klagsbrun, 2004; Weinstein, 2002). To create a vascular network with the right density of branches, this motile behaviour must be strictly controlled. What, then, is the mechanism that switches endothelial cells from motile to non-motile behaviour at the appropriate times and places?

Many previous studies have shown that the disruption of Notch signalling gives rise to abnormal patterns of blood vessels (Krebs et al., 2000; Shawber and Kitajewski, 2004; Uyttendaele et al., 2001), and that the Notch ligand Delta-like 4 (Dll4), in particular, is essential for normal vascular development. Mice homozygous for a knockout mutation of Dll4 die early, at around E10, and heterozygotes show severe vascular defects (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). However, what type of disrupted behaviour by individual endothelial cells is responsible for these vascular abnormalities has remained a puzzle. Some clues have come from studies in culture, where the blockade of Notch signalling favours vessel branching and endothelial cell proliferation (Liu et al., 2006; Sainson et al., 2005; Shawber et al., 2003). However, other evidence, obtained from tumour angiogenesis, suggests that Notch signalling may have an opposite effect and that, by targeting Dll4, one might be able to deprive tumours of their blood supply and stop their growth (Patel et al., 2005; Zeng et al., 2005).

[1]Vertebrate Development Laboratory, Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK. [2]Department of Genetics, Box 8232, Washington University Medical School, 4566 Scott Avenue, St Louis, MO 63110, USA.

*Author for correspondence (e-mail: jonathan.leslie@cancer.org.uk)

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The zebrafish embryo provides a unique opportunity to observe the behaviour of endothelial cells in the living organism (Weinstein, 2002). In this study, this model is used to show that Notch signalling governs the transition from active angiogenesis to quiescence. The Notch ligand Dll4 is expressed in arterial endothelial cells, which are responsible for primary angiogenesis. In the normal course of development, a subset of these cells becomes highly motile and forms angiogenic sprouts, which eventually join up to create vascular circuits (Childs et al., 2002). Once the connections have been made, the endothelial cells halt their motility. In embryos in which either Dll4 is lacking or Notch signalling is blocked, angiogenic sprouting begins normally. Later, however, as the sprouts join up to form vascular circuits, the cells in the defective embryos continue to proliferate and migrate. Artificial overactivation of the Notch pathway has opposite consequences. When Vegf signalling and Dll4-Notch signalling are both blocked, the excessive sprouting is prevented. Thus, while endothelial cells depend on Vegf from the environment to switch on angiogenic behaviour, they depend on Dll4-Notch signals exchanged with other endothelial cells to switch it off; and this 'off' switch works by making the cells unresponsive to Vegf.

MATERIALS AND METHODS

Animals

Zebrafish were raised and maintained under standard conditions.

Gene sequence and in situ hybridization

The zebrafish ortholog of mammalian *Dll4* (*dll4*, Ensembl Gene ENSDARG00000053401) has recently come to light through the zebrafish genome sequencing project. Whole-mount in situ hybridization (ISH) was carried out as previously described (Ariza-McNaughton and Krumlauf, 2002). The DIG-labelled antisense probe for *dll4* corresponded to amino acids 73-232. Probes for *notch1b* (Westin and Lardelli, 1997), *notch3* (Westin and Lardelli, 1997), *efnb2a* (Durbin et al., 1998) and *ephb4a* (Cooke et al., 1997) were as previously described. After ISH, embryos were processed for histology either as whole mounts or as cryostat sections and were immunostained with anti-GFP antibody (Molecular Probes).

Morpholino injection and heat-shock constructs

Morpholino antisense oligonucleotides (Gene Tools) were diluted in Danieau buffer containing 0.2% phenol red. This solution (2 nl, which contained 5-10 ng oligonucleotide) was then injected into the yolk of 2- to 4-cell-stage embryos.

Morpholino sequences were:

for *dll4* splicing (MO[Dll4]), 5′-TAGGGTTTAGTCTTACCTTGG-TCAC-3′;

for MO[Dll4] mismatch control (mismatched bases in lowercase), 5′-TAcGGTTTAcTCTTAgCTTcGTgAC-3′;

for *sox32* (*casanova*) (MO[cas]), 5′-GCATCCGGTCGAGATACA-TGCTGTT-3′ (Sakaguchi et al., 2001);

for *notch1a*, 5′-GAAACGGTTCATAACTCCGCCTCGG-3′ (Lorent et al., 2004);

for *notch1b,* 5′-AATCTCAAACTGACCTCAAACCGAC-3′ (Milan et al., 2006);

for *notch2*, 5′-AGGTGAACACTTACTTCATGCCAAA-3′ (Lorent et al., 2004);

and for *notch3*, 5′-ATATCCAAAGGCTGTAATTCCCCAT-3′ (Lorent et al., 2004).

For the misexpression of N^{ICD}, we used fish carrying *hsp70:Gal4* and *UAS:*N^{ICD} (*UAS:myc-notch1a-intra*) constructs, as described (Scheer et al., 2001), and heat-shocked them at 37°C for 30 minutes.

Drug treatments

DAPT or SU5416 (both Merck) was dissolved in DMSO, stored at –20°C and added to aquarium water to give a final concentration of 100 μM DAPT or 2 μM SU5416 plus 0.2% or 0.01% DMSO, respectively. Aquarium water containing DMSO alone was used as a control.

Fluorescence microscopy

fli1:EGFP embryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C and were then washed in PBS at room temperature. Embryos were permeabilized in PBS containing 10% goat serum, 2% BSA, 0.5% Triton X-100 and 10 mM NaN_3 for 1-3 hours at room temperature, washed in PBS containing 1% Triton X-100 and then mounted in SlowFade Gold (Invitrogen) containing 0.2 μg/ml DAPI (Sigma). Specimens were examined with a Zeiss LSM510 confocal microscope. Endothelial cells were counted from projections of Z-series of optical sections. The region counted in each specimen spanned two somites [three intersegmental vessels (ISVs) and the accompanying segments of the dorsal aorta (DA) and dorsal longitudinal anastomotic vessel (DLAV)].

Live imaging

Living *fli1:EGFP* embryos were anaesthetized in aquarium water containing 320 μg/ml Tricaine (Sigma) and were then mounted on a glass-bottom dish (MatTek) in a drop of aquarium water containing 0.2% agarose. Images were captured with a Zeiss LSM 510 confocal microscope. Frames for movies were collected every 30 seconds for 20 minutes. Each frame is the projection of a Z-series of optical sections.

RESULTS AND DISCUSSION

In the zebrafish, *dll4* transcript was detectable by reverse transcriptase (RT)-PCR analysis by 8 hours post-fertilization (hpf), with expression levels increasing through to the third day of development (Fig. 1A). Whole-mount in situ hybridization showed that all endothelial cells in the dorsal aorta (DA) and intersegmental vessels (ISV) expressed *dll4*, but no *dll4* transcript was detectable in the posterior cardinal vein (PCV) or caudal vein (Fig. 1B-D,F-H). Thus, zebrafish *dll4*, like *Dll4* in the mouse (Duarte et al., 2004; Gale et al., 2004; Mailhos et al., 2001; Rao et al., 2000; Shutter et al., 2000), is expressed in arterial, but not venous, endothelial cells, as is another member of the delta gene family, *deltaC* (*dlc*) (Lawson et al., 2001; Smithers et al., 2000). *dll4* was also detectable in a small subset of cells within the neural tube (Fig. 1B,D) and in the two developing sensory patches of the ear (data not shown). Endothelial expression of *dll4*, like that of *dlc*, was lost in *mind bomb* mutants (Fig. 1E), in which arterial cells are partially converted to a venous character as a result of a failure of Notch signalling during the initial determination of endothelial lineages (Lawson et al., 2001).

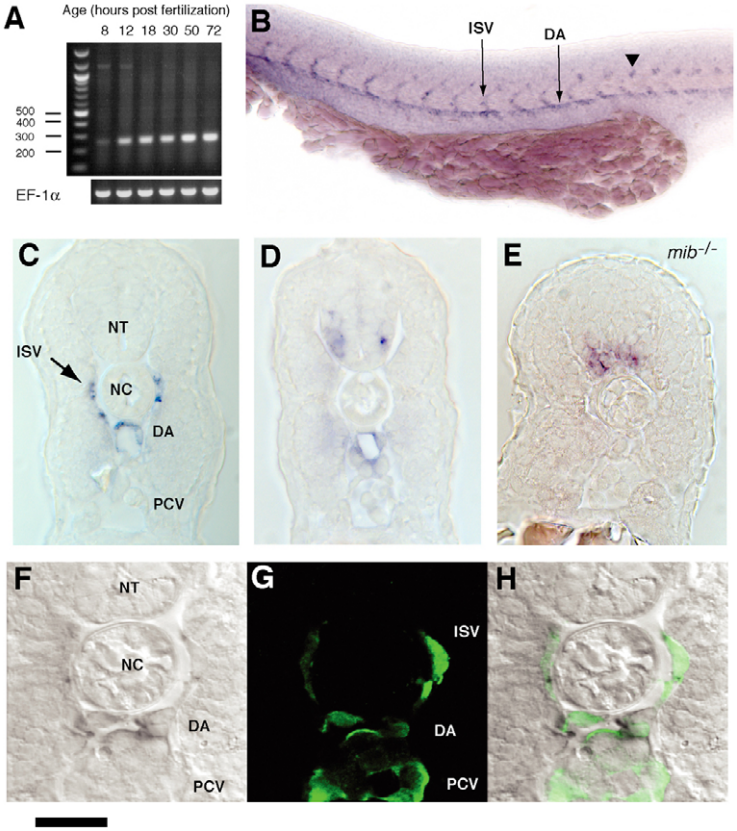

Fig. 1. Expression of *dll4* in wild-type and mutant zebrafish embryos. (**A**) RT-PCR analysis of whole homogenized embryos, showing weak *dll4* expression as early as 8 hpf, becoming steadily stronger thereafter; *EF-1α* is a control. (**B**) Side view of a wild-type 25-hour-old embryo stained by in situ hybridization. *dll4* is expressed in the dorsal aorta (DA) and intersegmental vessels (ISV), and in a subset of neurons in the neural tube (arrowhead). (**C**) Cross section through the trunk of a similar specimen showing *dll4* expression in endothelial cells of the DA and ISVs but not in those of the posterior cardinal vein (PCV). (**D**) Similar section at a slightly different level relative to somite boundaries does not pass through any ISVs but reveals cells expressing *dll4* in the ventrolateral neural tube (NT), as well as in the DA. (**E**) Cross section through the trunk of a 25-hour-old *mib*-mutant embryo. *dll4* expression is increased and extended in the neural tube, but is undetectable in the endothelial cells. The expanded expression in the neural tube (and ear, for which data not shown) indicates that, in these tissues, *dll4*, like other delta genes, is regulated by lateral inhibition, so that its expression increases when Notch signalling fails. The loss of expression in the endothelial cells probably reflects their partial conversion to a venous character as a result of the failure of Notch signalling at an early stage (Lawson et al., 2001). The gross anatomy is also somewhat disturbed. (**F-H**) The neighbourhood of the notochord (NC) in a *fli1:EGFP* transgenic embryo, comparing *dll4* expression (visible in the DA and ISVs by in situ hybridization; F,H) with the endothelium-specific expression of EGFP (green, visible by immunofluorescence in the DA, ISVs and PCV; G,H). Scale bar: 100 μm in B; 70 μm in C-E; 35 μm in F-H.

To block the production of Dll4, we designed a splice-blocking morpholino antisense oligonucleotide, MO[Dll4] (see Fig. 1 in the supplementary material). We injected MO[Dll4] into fertilized eggs and followed vascular development in the morphant embryos up to 5 days post-fertilization (dpf). In normal zebrafish, blood flow in the trunk and head was readily observable by 30 hpf, and by 2.5 dpf the DA, PCV and ISVs carried robust circulation (Fig. 2A). However, in MO[Dll4]-injected embryos at this age, the flow of blood was severely reduced: circulation in the DA and PCV was slowed and blood flow was undetectable in the majority of ISVs (Fig. 2B). Embryos injected with a control morpholino generally had normal circulation (Fig. 2C).

Because Notch signalling is reported to regulate arterial versus venous identity (Duarte et al., 2004; Krebs et al., 2004; Lawson et al., 2001; Lawson et al., 2002; Lawson and Weinstein, 2002a), we examined the expression of genes that mark differences between arterial and venous endothelial cells: *notch3*, *efnb2a* and *ephb4a* were all unaffected in the MO[Dll4] morphants (data not shown). Because *dlc* is also found in the embryonic vasculature (Lawson et al., 2001; Smithers et al., 2000) and could function redundantly with *dll4*, we repeated the Dll4-knockdown experiment in $dlc^{bea/bea}$ mutants, in which Dlc is defective. Again, we found no effect on vessel identity (data not shown). This suggests that some Notch ligand other than Dll4 or Dlc is responsible for the control of vessel identity, although it is also possible that our morpholino allowed some residual *dll4* expression, which was sufficient to maintain arterial identity even though other aspects of endothelial behaviour were abnormal.

To clarify the reasons for the circulatory defects, we examined in detail the development and maintenance of the pattern of ISVs using *fli1:EGFP* zebrafish, in which all endothelial cells are labelled with a cytosolic EGFP reporter (Lawson and Weinstein, 2002b). In normal embryos, the ISVs originate at somite boundaries as sprouts from the DA. These sprouts are first seen at 20-24 hpf and grow dorsally, reaching the level of the top of the neural tube at approximately 30 hpf. There, they bifurcate to form two branches, which extend, in a T-shape, along the body axis (Fig. 3A). These longitudinal sprouts join up to form the dorsal longitudinal anastomotic vessel (DLAV), which can be seen carrying blood by 48 hpf. Over the next 3 days of development, the pattern of connections between the ISVs and the DLAV remain essentially static.

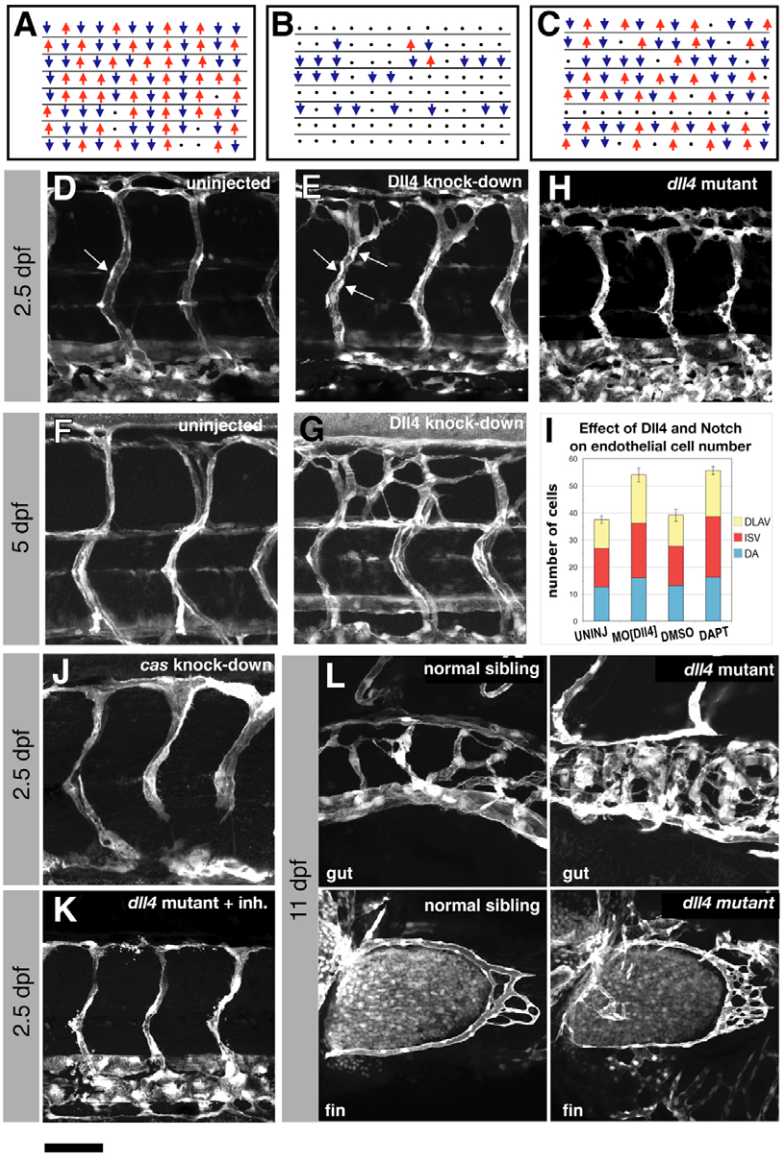

Fig. 2. Effects of Dll4 knockdown on circulation and vascular pattern. (**A**) Blood flow in intersegmental vessels (ISVs) of wild-type living embryos at 3 dpf. Each line represents a set of ISVs on one side of one embryo, scored for direction of blood flow: upward arrows (red) denote flow from ventral to dorsal; downward arrows (blue), denote flow from dorsal to ventral; dots represent vessels carrying no flow. (**B**,**C**) Blood flow in intersegmental vessels of living embryos at 3 dpf injected with either 10 ng MO[Dll4] to knockdown Dll4 (B) or with 10 ng of a 5-base mismatch control morpholino (C). (**D-G**) *fli1:EGFP* embryos at 2.5 dpf (D,E) or 5 dpf (F,G) either uninjected (D,F) or injected with 10 ng MO[Dll4] (E,G). White blobs (arrows in D,E) are endothelial cell nuclei. (**H**) Similar region of a 2.5-dpf *dll4*-homozygous-mutant embryo (carrying the *fli1:EGFP* transgene). (**I**) Endothelial cell counts in the DAs, ISVs and DLAVs of embryos at 3 dpf. Embryos lacking Dll4 or treated with 100 μm DAPT have more cells than uninjected or DMSO-treated control siblings. Both effects are significant at the P=0.001 level (t-test; $n \geqslant 6$ specimens for each treatment; error bars represent s.e.m.). (**J**) *fli1:EGFP* embryo at 2.5 dpf injected with 10 ng of a morpholino targeted against *casanova* (*sox32*) to block circulation in the trunk. (**K**) *fli1:EGFP*-positive *dll4*-mutant embryo at 2.5 dpf treated from 48 hpf with 2 μM SU5416 to block Vegf signalling. (**L**) The gut and pectoral fin vasculature of an *fli1:EGFP*-positive *dll4* mutant and a wild-type (normal) sibling embryo, both at 11 dpf. Scale bar: 50 μm in D-H,J,K; 40 μm in L.

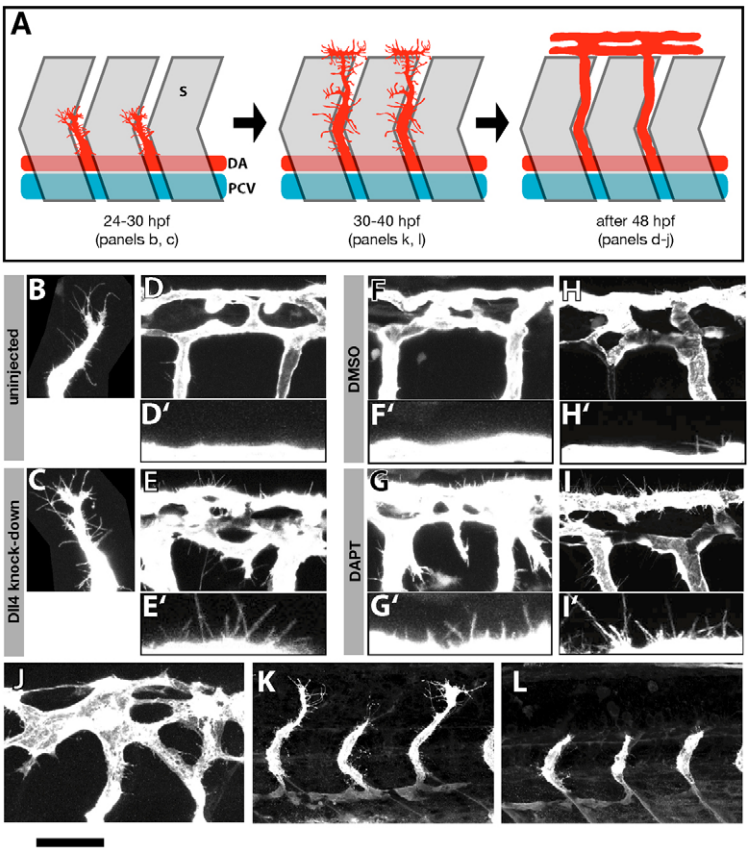

Fig. 3. Effects of Dll4 knockdown on endothelial cell behaviour. (**A**) Schematic depiction of ISV and DLAV formation and the concomitant changes in endothelial cell morphology. (**B**,**C**) Leading cell of an ISV sprout at 30 hpf in a living uninjected embryo (B) or in a living embryo injected with 10 ng MO[Dll4] (C). (**D**,**E**) DLAV and dorsal ISVs at 2.5 dpf in a living uninjected embryo (D) or in a living embryo injected with 10 ng MO[Dll4] (E). (**F-I**) Similar region at 2.5 dpf of living embryos treated with 100 μm DAPT (G,I) or control embryos treated with DMSO (F,H). Treatment with DMSO or DAPT began at either 33 hpf (F,G) or at 48 hpf (H,I) and continued through the course of the experiment. Higher magnifications of D-I are shown in **D′-I′**. (**J**) DLAV and ISVs of a 3-dpf embryo injected with 7 ng of a morpholino targeted against Notch1b. (**K**,**L**) ISV sprouts at 30 hpf of a normal sibling (K) and a *hsp70:Gal4;UAS:N*^{ICD} embryo (L). Embryos in K and L were given a heat shock at 24 hpf. S, somite. Scale bar: 50 μm in B-I; 17 μm in D′-I′; 40 μm in J; 70 μm in K,L.

In the morphant embryos, formation of the ISVs began normally, and the DLAV was able to form as normal. However, by 2.5 dpf, striking abnormalities were visible. Instead of a simple T-junction between each ISV and the DLAV, we observed a network of aberrant interconnected branches (Fig. 2D,E), which became more profuse over the following few days (Fig. 2F,G). The abnormality was reminiscent of that seen in the vasculature of mouse embryos lacking one or both copies of *Dll4* (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004), and implies a failure of a mechanism that normally arrests the formation of new vessels. Blocking Vegf signalling with the inhibitor SU5416 (Fong et al., 1999) both blocked normal endothelial sprouting (data not shown) (see also Covassin et al., 2006) and the additional sprouting observed in embryos lacking Dll4 (Fig. 2K). Thus, Dll4-Notch signalling affects angiogenesis by interfering with a response to Vegf.

Endothelial cells could be counted from the fluorescent specimens. Their number was greater, by approximately 30%, in Dll4 morphants compared with control siblings: the numbers in the DA, ISVs and DLAV were all increased by a similar factor (Fig. 2I). These data suggest that Dll4 acts in vivo to restrict both the migration and the proliferation of endothelial cells, a function that has been proposed previously based on in vitro studies using cultured endothelial cells (Leong et al., 2002; Liu et al., 2006; Sainson et al., 2005; Williams et al., 2006). Further experiments will be needed to establish whether the effect on endothelial cell numbers in the zebrafish reflects an increase of division rate or a decrease of cell death.

Because MO[Dll4]-injected embryos have reduced circulation, one might suggest that the observed vessel-branching phenotype simply reflects a normal physiological response to hypoxic conditions, to reduced blood flow or to reduced pressure. To investigate this possibility, we blocked all trunk circulation using a morpholino (MO[cas]) targeted to the *sox32* (*casanova*, *cas*) transcript, which is required for the development of a normal, beating heart (Sakaguchi et al., 2001). Although MO[cas]-treated embryos showed some dilations of their vessels, there was no sign of the ectopic sprouts observed in Dll4-morphant and -mutant embryos (compare Fig. 2J with Fig. 2E,H), indicating that the observed ISV-branching phenotype is indeed a direct effect of Dll4 knockdown and not a secondary effect resulting from compromised circulation.

We obtained further confirmation of the role of Dll4 in angiogenesis from a *dll4* mutant, *j16e1*. This was discovered through its dominant, haplo-insufficient phenotype in a study of fin-morphology mutants, and subsequent mapping and positional cloning revealed a glycine to serine (G270S) substitution at a conserved site in an EGF-like domain of the zebrafish *dll4* gene on LG20. Homozygous embryos survived for up to 2 weeks and showed a vascular phenotype similar to, but less severe than, that seen in Dll4 morphants (Fig. 2H). At 11 dpf, a stage that cannot be studied by morpholino knockdown, mutant embryos showed excessive angiogenesis in other sites in addition to in the ISVs (Fig. 2L). Thus, Dll4-Notch signalling is required to keep angiogenesis under proper control, not only in the embryo, but also at later stages and in multiple tissues.

To discover what kind of disrupted endothelial cell behaviour underlies this aberrant pattern of vessels in Dll4 morphants and mutants, we examined living embryos at high resolution using time-lapse confocal microscopy. During ISV sprouting and migration, we could see the exploratory endothelial cells at the tips of growing ISVs rapidly extending and retracting filopodia; this behaviour was similar in morphants and control embryos (Fig. 3A-C). However,

differences became apparent later in development. In normal embryos, the leading cells of adjacent ISVs joined up and formed a DLAV with a lumen, the endothelial cells ceased their exploratory behaviour, and filopodia became few and far between (Fig. 3A,D and see Movie 1 in the supplementary material). By striking contrast, we found that endothelial cells comprising the DLAV and dorsal ISVs of embryos lacking Dll4 continued to extend and retract filopodia, and readily advanced into ectopic locations (Fig. 3E and see Movie 2 in the supplementary material).

Evidently, Dll4 is required for endothelial cells to make the transition from exploratory behaviour to quiescence. This could reflect a cell-autonomous influence of Dll4 on filopodium formation and motility, or could depend on cell-cell signalling via Notch. To shed light on this issue, we blocked Notch activation using the gamma-secretase inhibitor DAPT, starting at 33 hpf (when ISVs begin to connect), and examined the behaviour of endothelial cells at 54 hpf. As in embryos lacking Dll4, endothelial cells in DAPT-treated embryos had greatly increased filopodial activity and exploratory behaviour compared with DMSO-treated control siblings (Fig. 3F,G and see Movies 3,4 in the supplementary material). They also showed increased endothelial cell numbers (Fig. 2I). An increase of filopodial activity was also observed in 54-hpf embryos in which DAPT treatment was initiated at 48 hpf (after the DLAV has formed; Fig. 3H,I).

These findings strongly suggest that Dll4 acts via Notch. To identify the relevant receptor, we tested the effects of morpholinos targeted against each of the four known zebrafish notch genes. Notch1b morphants showed a clear phenotype resembling that of Dll4 morphants, Dll4 mutants and DAPT-treated embryos (Fig. 3J); we were unable to detect any such effect in Notch3 morphants. Thus, it appears that signalling by Dll4 via Notch1b is required to restrict the exploratory behaviour of endothelial cells and to reduce their proliferation once the capillary sprouts have connected to form a vascular circuit.

To test whether ectopic activation of Notch would, conversely, block the normal exploratory behaviour of endothelial cells, we used a GAL4-UAS technique to trigger the overproduction of the active intracellular fragment of Notch (N^{ICD}) via heat shock (Scheer et al., 2001). Heat shocking during the early stages of ISV formation severely reduced the filopodial activity of the pioneering endothelial cells and inhibited the extension of the ISV sprouts (Fig. 3K,L). The strong suggestion, therefore, is that Notch signalling acts as a switch to control the transition between motility and quiescence in endothelial cells exposed to Vegf. Previous work has shown that expression of the *vegfr3* gene (*VEGF receptor 3*, also called *flt4*) is required for normal extension of ISVs (Covassin et al., 2006) and is downregulated in response to Notch activation (Lawson et al., 2001). It seems likely, therefore, that Notch signalling inhibits motility by blocking the production of the receptors needed for a response to Vegf to occur.

Although studies in mice have shown that the loss of *Dll4* produces a malformation of the vascular bed (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004), ours is the first account to show directly in a living organism how Notch signalling affects endothelial cell behaviour and how this relates to vascular malformations. We have demonstrated that the removal of Dll4 results in a persistence of angiogenesis at a time when endothelial cells should normally make a transition to quiescence. Our findings agree with in vitro studies demonstrating that Notch signalling suppresses endothelial proliferation and branching (Leong et al., 2002; Liu et al., 2006; Sainson et al., 2005; Shawber et al., 2003; Williams et al., 2006). Given that *Dll4* is strongly expressed in developing vasculature, both in embryos and in tumours (Claxton and Fruttiger, 2004; Mailhos et al., 2001; Rao et al., 2000; Shutter et al., 2000), it seems that it forms part of an essential mechanism to keep angiogenesis under control at sites where the angiogenic program has been activated. Although Notch in mammalian endothelial cells may also be activated by Notch ligands of the Jagged family in neighbouring non-endothelial cells, with a stimulatory effect on angiogenesis (Patel et al., 2005; Zeng et al., 2005), our data clearly indicate that, in our system, the more important signal is from Dll4 expressed in endothelial cells and that its effect is to inhibit angiogenesis.

Our findings help to show how the functions of VEGF and Notch signalling are interwoven in the control of angiogenesis. In the continued presence of VEGF, angiogenesis may proceed or halt according to the level of activation of the Notch pathway, and this effect appears to be exerted through the regulation of VEGF-receptor expression by Notch signalling. In mammalian cells (Liu et al., 2003; Patel et al., 2005; Williams et al., 2006), VEGF has been shown to induce *dll4* expression, which, in turn, apparently by activating Notch, inhibits the expression of VEGF receptors (Liu et al., 2003; Patel et al., 2005; Williams et al., 2006). In this way, VEGF acting on a given cell, while inducing *dll4* in that cell, will tend to reduce VEGF-receptor expression in its neighbours and thereby downregulate their expression of *dll4*, creating a form of lateral inhibition that could serve to single out a subset of the endothelial population as motile tip cells (Hellström et al., 2007). The same effect could, however, also be achieved more directly by the standard Notch-dependent lateral-inhibition mechanism: that is, a cell strongly expressing *dll4* will activate Notch in its neighbours, and the activated Notch could directly inhibit their expression of *dll4*, enabling the first cell to escape Notch activation.

In the fish, we have not found evidence of any influence of the Vegf signalling pathway on the expression of *dll4*: blocking Vegf signalling with SU5416 had no visible effect on *dll4* expression (data not shown). Neither have we detected any obvious difference in *dll4* expression between the leading cells of the ISV sprouts and the cells that follow behind them. Moreover, both classes of cells normally show intense filopodial activity. When filopodial activity ceases upon formation of the DLAV, it ceases throughout the population of intersegmental endothelial cells. It is tempting to suggest that the trigger is the contact between the leading endothelial cells of the sprouts that meet, which could lead to mutual activation of Notch; however, it remains to be seen how the switch in behaviour of the leading endothelial cells leads to quiescence throughout the cell population – this might be coordinated by signals via the Notch pathway, or by some other means.

However this may be, our data indicate that Dll4-Notch signalling provides a way to halt endothelial motility and to downregulate endothelial proliferation in a coordinated way once the endothelial cells have become linked together into a vascular circuit. Thus, VEGF- and Notch-signalling may represent two distinct controls of endothelial cell behaviour, with VEGF signalling [and perhaps Jagged-Notch signalling (Zeng et al., 2005)] conveying information from the tissues that require a blood supply, and Dll4-Notch1b signalling conveying information about the relationship of an endothelial cell to its neighbours.

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Note added in proof

Shortly before submission of this manuscript, two related papers were published (Noguera-Troise et al., 2006; Ridgway et al., 2006).

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/5/839/DC1

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