



Lincoln's Inn Fields

Vascular Biology

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During angiogenesis, vascular networks form through guided sprouting, branching and establishment of new connections between sprouts. The density of the network is a result of the initial branching frequency and subsequent regulated regression. Our recent work illustrates that two conserved signalling pathways, the Notch and the Wnt pathway, are coordinated in endothelial cells at vascular branch points by the small ankyrin repeat protein Nrarp, and that this function is required to balance proliferation and maintain vessel stability during network formation. Together, Notch and Wnt-signalling determine whether to establish or release new vessel connections by regulating sprouting, proliferation and cell-cell contacts.

Make or break vascular connections

Blood vessel formation is triggered by physiological demands of metabolically active tissue. Undersupply, in particular of oxygen, leads to transcriptional activation of multiple genes that will help cells survive oxygen shortage, while initiating a cascade of blood vessel changes to re-establish adequate supply.

Hypoxic cells produce the vascular endothelial growth factor (VEGF-A), which stimulates formation of new vessel sprouts that branch, elongate and make connections to establish perfusion. Effective sprouting requires selection of an

endothelial tip cell that will extend filopodia to probe the environment for the VEGF-A gradient (Gerhardt *et al.*, 2003 *JCB* 161, 1163-1177). Migration speed of the tip cell is controlled by a gradient of VEGF-A. Our recent studies illustrate that the formation of new tip cells is the default response of endothelial cells to the stimulation by VEGF-A gradients (Bentley *et al.*, 2008 *J Theor Biol* 250, 25-36; Hellstrom *et al.*, 2007 *Nature* 445, 776-780). In order to form a functional sprout, this default response is inhibited in cells adjacent to the tip. These endothelial cells instead form the stalk cells, establish adhesion junctions and polarise to form the lumen. The tip cells inhibit their neighbours by producing Dll4, a transmembrane ligand of the Notch receptor. Dll4 activates Notch signalling, which suppresses sprouting (Hellstrom *et al.*, 2007). Experimentally, ectopic stimulation of Notch signalling in endothelial cells inhibits sprouting, whereas a loss of Notch signalling leads to excessive sprouting through increased tip cell formation.

The Notch-regulated ankyrin repeat protein (Nrarp) is induced in endothelial cells by Dll4/Notch signalling (Phng *et al.*, 2009 *Developmental Cell* in press). Nrarp expression is most prominent in the stalk cells and at branchpoints in nascent vessels. To address the function of Nrarp, we studied the effects of morpholino-based knockdown of Nrarp in zebrafish embryos and retinal angiogenesis in *Nrarp*-deficient mice. Loss of Nrarp function causes a substantial decrease in vessel density although vessel sprouting at the level of tip cells is not affected. Instead, reduced vessel density is caused by dynamic regression and loss of connectivity in newly formed vessels. Time-lapse confocal microscopy in zebrafish illustrates that vessels fail to maintain their connections in the absence of Nrarp. This failure is associated with reduced stalk cell proliferation. Vessels thus contain fewer endothelial cells, which nevertheless strive to bridge the same distance, leading to excessively thin, and fragile connections (Figure 1).

At the molecular level, Nrarp controls Notch signalling through a negative feedback loop. Nrarp binds and interacts with the transcriptional activator complex consistent of the

Notch intracellular domain (NICD), histone acetylases and the DNA binding molecule Rbpjk (also known as CSL). Nrarp destabilises this complex and leads to NICD degradation, thus limiting Notch signalling (Figure 2).

Inhibition of Notch signalling in the absence of Nrarp can restore vessel density through induction of new vessels. However, these new vessels also remain fragile and regress, suggesting that Nrarp performs additional functions to stabilise vessels.

Previous work in zebrafish neural crest development identified that Nrarp also interacts with the transcription factor lymphoid enhancing factor (Lef1) (Ishitani *et al.*, 2005 *Nat Cell Biol* 7, 1106-1112). Also in endothelial cells, Nrarp binds Lef1 and increases its transcriptional activity in the canonical Wnt signalling pathway (Phng *et al.*, 2009). Studying localisation and quantitative signalling activity *in vivo* and *in vitro*, we find that Nrarp and Wnt-signalling reporter co-localise in stalk cells, while loss of Nrarp suppresses endothelial Wnt-signalling. Downstream targets of Wnt-signalling include the cell cycle regulator cyclin D1. Whereas Notch signalling suppresses cell proliferation through retinoblastoma protein de-phosphorylation and down-regulation of p21^{CIP} (Noseda *et al.*, 2004 *MCB* 24, 8813-8822), Wnt-signalling promotes proliferation (Masckauchan *et al.*, 2006 *Physiology Bethesda*, Md 21, 181-188) through up-regulation of cyclinD1 (Shtutman, M *et al.*, 1999 *PNAS* 96, 5522-5527). The dual control of Notch and Wnt by Nrarp provides a mechanism for endothelial cell proliferation control. This may also explain why endothelial stalk cells readily proliferate although they receive strong Notch activation.

The nature and origin of the Wnt-ligands involved in this process remain to be determined. Premature vessel regression in mouse mutants of Lrp5 and following endothelial deletion of β -catenin suggest that the canonical Wnt-pathway is the key regulator of vessel stability (Phng *et al.*, 2009). The Notch and Wnt-signalling components identified in our research are induced under hypoxia and VEGF-A stimulation, suggesting that these pathways are mainly functional in endothelial cells during patterning of the angiogenic response.

Hypoxia responses, VEGF-A up-regulation, and formation of new blood vessels are important events during tumour progression and ischemia induced neo-vascularisation processes. It is tempting to speculate that a similar mechanism involving Notch and Wnt-signalling regulates vessel stability during adult neo-vascularisation. We therefore hope that our identification of the mechanism required for stability of newly formed blood vessels will open new possibilities to modulate blood vessel formation and function in disease.

Publications listed on page 122

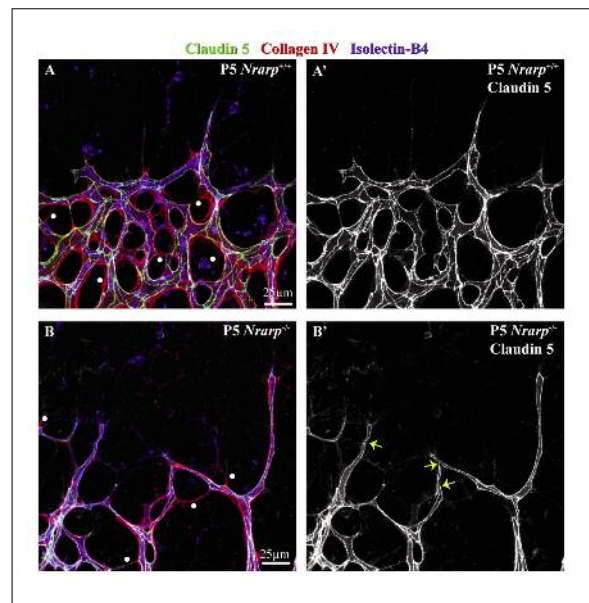


Figure 1. Increased vessel regression in Nrarp-deficient mice. Vascular front of P5 Nrarp^{+/+} (A) and Nrarp^{-/-} (B) retinas stained for the tight junction protein Claudin 5 (green), basement membrane protein Collagen IV (red) and endothelial cell surface epitope recognised by Isolectin-B4 (blue). Dots in A and B indicate empty Collagen IV sleeves. Arrows in B' indicate discontinuous inter-endothelial junctions. Loss of Nrarp results in decreased vessel density and increased vessel regression at the sprouting front of developing retina vessels.

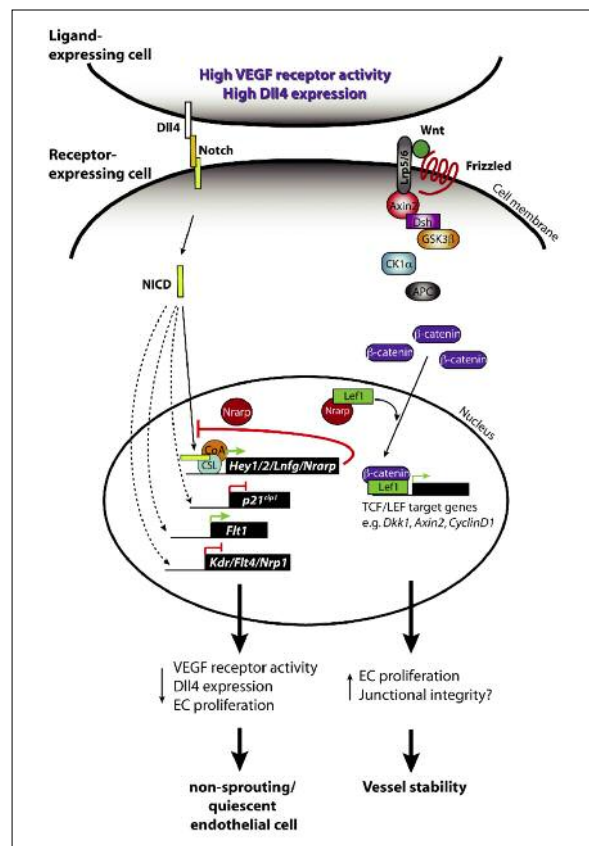


Figure 2. Proposed model illustrating integration of Notch and Wnt signalling pathways in promoting non-sprouting endothelial cells and stabilisation of nascent blood vessels.