Commentary

Notch signaling during vascular development

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Formation of the cardiovascular system is one of the earliest and most important events during embryogenesis in mammals. During the early stages of vascular development in both the mammalian embryo and its extraembryonic membranes such as the yolk sac, endothelial cell precursors differentiate and proliferate *in situ* in a process termed vasculogenesis. These endothelial cells then coalesce and form the primary vascular plexus, a network of homogeneously sized primitive blood vessels. This vascular network is then remodeled by the process of angiogenesis, which involves the sprouting, branching, splitting, and differential growth of vessels in the primary plexus to form the large and small vessels of the mature vascular system (1–4). During this phase of angiogenic vascular remodeling, supporting cells such as pericytes and smooth muscle cells are recruited to the vessels to provide structural support and stability for the vascular walls. A number of different intercellular signaling pathways have been implicated in the control of these processes. These pathways include the vascular endothelial growth factor pathway, the transforming growth factor-β and platelet-derived growth factor pathways, the angiopoietin/Tie receptor pathway, and the ephrin/Eph receptor pathway (2–4). Recent work has added the Notch signaling pathway to this list. In this issue of PNAS, Uyttendaele *et al.* (5) add important new information to our understanding of the role that the Notch signaling pathway plays during vascular development in mice.

The Notch signaling pathway is an evolutionarily conserved intercellular signaling mechanism in which both ligands and receptors are Type 1 transmembrane proteins, which restricts the Notch pathway to regulating interactions between physically adjacent cells (6). To date, four Notch family receptors and five ligands have been described in mammals. Evidence that the Notch pathway plays a critical role in vascular development and homeostasis includes the specific expression of Notch pathway ligands and receptors in vascular endothelium or supporting cells (7–14), as well as the phenotypes of several targeted mutants in Notch pathway components. These mutants, which include mutations in genes encoding both ligands and receptors, die during embryogenesis from hemorrhaging because of defects in vascular morphogenesis (15–17). In adults, a role for the Notch pathway in vascular homeostasis has been demonstrated by the finding that the degenerative vascular disease CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) is caused by mutations in the *Notch3* gene (18).

Uyttendaele *et al.* (5) studied the effects of expressing an activated form of the Notch4 protein in endothelial cells. The *Notch4* gene was identified as a common proviral integration site (originally termed the *int-3* locus) in mouse mammary tumor virus-induced mammary tumors (19, 20). In these tumors, proviral integration leads to the production of a truncated *Notch4* transcript. This truncated transcript initiates within the 3′ long terminal repeat of the provirus and encodes a constitutively active form of the Notch4 protein (termed the Notch4/int-3 oncoprotein), in which most of the extracellular domain of the Notch4 protein is deleted (9, 20, 21). Uyttendaele *et al.* (5) used homologous recombination to introduce into embryonic stem (ES) cells a truncated *Notch4/int-3* cDNA clone. They introduced this construct into the *Flk1* locus (also known as the *Kdr* locus), which encodes a receptor for vascular endothelial growth factor. The *Flk1* gene is expressed in vascular endothelial cells and their precursors in the embryo and the yolk sac. ES cells heterozygous for this ''knock-in'' allele (referred to as the *Flk1/int-3* allele) were then aggregated with tetraploid recipient mouse embryos. Because tetraploid cells cannot contribute to embryonic tissues, this technique results in the production of mouse embryos in which both the embryo and the mesoderm of the yolk sac are entirely derived from the donor ES cells (22).

Uyttendaele *et al.* (5) found that the *Flk1/int-3* embryos died between 9.5 and 10.5 days of gestation, and exhibited substantial defects in the embryonic and extraembryonic vasculature. The vascular system in both the embryos and their yolk sacs was disorganized, and branching morphogenesis and patterning of the vascular network was disrupted. They observed similar defects on *in vitro* differentiation of the ES cells heterozygous for the *Flk1/int-3* allele, indicating that the embryonic vascular defect was intrinsic to the *Flk1/int-3*-expressing endothelial cells. Interestingly, although the *Flk1/int-3* allele should be expressed in the hemangioblast, the common precursor to the hematopoietic and endothelial cell lineages, Uyttendaele *et al.* detected no effect of expression of the *Flk1/int-3* allele on hematopoietic development.

The work of Uyttendaele *et al.* clearly demonstrates that appropriately regulated Notch signaling is required in the endothelial cell lineage.

The vascular defects observed in the *Flk1/int-3* embryos are similar in many respects to the defects observed in *Notch1* homozygous mutant and *Notch1/Notch4* double homozygous mutant embryos (17). Because the *Flk1/int-3* allele is a gain-of-function *Notch4* mutation, and the *Notch1* and *Notch4* targeted mutations are loss-of-function mutations, the similar vascular phenotypes observed in each case demonstrate that appropriate levels and regulation of Notch signaling are critical for proper development of the embryonic vasculature. Furthermore, the work of Uyttendaele *et al.* demonstrates that this appropriately regulated Notch signaling is required specifically in the endothelial cell lineage and its precursors. It currently is not known in the loss of function experiments whether Notch signaling is required in endothelial cells, in supporting cells, or in both the endothelial and supporting cell lineages. The work of Uyttendaele *et al.* clearly demonstrates that appropriately regulated Notch signaling is required in the endothelial cell lineage, although their work does not preclude a reciprocal requirement for Notch signaling in supporting cells.

See companion article on page 5643.

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Expression of the *Flk1/int-3* allele results in ligand-independent activation of Notch signaling in the endothelial cell lineage. An interesting experiment would be to use this system to test the specificity of Notch receptor activation in this gain of function situation. Mice homozygous for a null mutation of the *Notch4* gene do not exhibit an obvious mutant phenotype, apparently because of functional redundancy with the *Notch1* gene (17). It seems likely that expression in the endothelium of a constitutively activated form of the Notch1 protein would yield a similar phenotype to that observed in the *Flk1/int-3* embryos, but what of activated forms of other Notch receptors? In particular, it has been suggested that the Notch3 protein acts to suppress Notch1-mediated transcriptional activation of the hairy/enhancer of split 1 (*Hes1*) and *Hes5* genes (23). In support of the notion that the Notch3 protein may have different functions from those of the other Notch family receptors, transgenic mice expressing activated forms of the Notch1 or Notch3 proteins in thymocytes exhibit different phenotypes (24, 25). Given that mutations in the *Notch3* gene are causative for the degenerative vascular disease syndrome CADASIL (18), it would be interesting to compare the phenotype of an activated Notch3 protein expressed under the control of the *Flk1* locus. If the Notch3 protein acts to antagonize signaling by the Notch1/Notch4 class of receptors, embryos expressing activated Notch3 in endothelial cells may exhibit a different phenotype than the *Flk1/int-3* embryos. The gain-of-function assay system utilized by Uyttendaele *et al*., coupled with tissue-specific loss-of-function mutants generated with the Cre recombinase system (26), should help unravel the intricacies of Notch signaling during vascular development.

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