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Journal Club

Robo4

A guidance receptor that regulates angiogenesis

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During embryogenesis, neural and vascular networks undergo directed patterning, often following the same routes. Several guidance mechanisms, which evolved in order to connect the nervous system, have been adopted by blood vessels. One such system, Slit-Roundabout signaling was first identified by studying axonal growth cones.[1] The identification of an endothelial-specific Roundabout family member, Robo4, led Jones and colleagues to an a priori hypothesis proposing that Robo4 plays a role in regulating vascular patterning. However, they were able to revise this presumptive hypothesis by identifying a role for Robo4 in maintaining vascular stability.[2]

Blood vessel formation encompasses a wide assortment of cellular processes; proliferation, migration and tube formation to name a few. Generation of a functional vascular system requires these processes to be orchestrated between neighboring endothelial cells. Angiogenesis, for example, requires the formation of specialized endothelial 'tip' cells which navigate the blood vessels.[3] Analogous to axonal growth cones, tip cells repeatedly extend and retract several filopodia, sensing and guiding the path for trailing 'stalk' endothelial cells which maintain contact with the basal vessel. The initial response of a functional blood vessel to pro-angiogenic stimuli, such as VEGF, must be limited to a defined number of endothelial cells. If too many cells differentiate into tip cells, the contact provided by stalk cells to the basal vasculature would be lost resulting in blood vessel instability.[4] It has recently come to light that Δ-like 4 (Dll4)/Notch1 signaling regulates tip cell formation among contiguous endothelial cells. Inhibition of Notch1 signaling by γ-secretase inhibitors, genetic inactivation of one allele of the endothelial Notch1 ligand Dll4, or endothelial-specific genetic deletion of Notch1, all promote increased tip cell formation and vessel branching. Alternatively, activation of Notch1 signaling by soluble jagged1 peptide leads to a reduction of tip cell formation and vessel branching.[5]

By generating mice which carry alleles for a fusion Robo4 gene ($Robo4^{AP/AP}$, the ligand binding site of Robo4 was replaced with the human placental alkaline phosphatase reporter gene leading to a null allele) Jones et al. were able to examine the retinal vascular bed for $Robo4^{AP/+}$ expression.[2] They found that Robo4 was preferentially expressed by endothelial stalk cells and was not found in tip cells or smooth muscle cells of the retinal vascular bed. The authors inferred that Robo4 must have a distinct function that is different from its known function as a neuronal guidance molecule. They found that overexpression of Robo4 inhibits migration, and the nonfunctional $Robo4^{AP/AP}$ mutant showed increased vessel complexity when compared to wild type retinal vascular beds. This is strikingly similar to what is reported for Notch1 signaling. Unfortunately, Jones et al. did not consider the possibility of Robo4 functioning similarly to that of Notch signaling. Overexpression of DII4 has been shown to decrease endothelial cell migration and proliferation. Conversly, the inactivation of Notch1 increased vessel branching.[5,6] Perhaps the lack of Robo4 expression in tip cells is a factor in determining which endothelial cells initiate migration in response to pro-angiogenic stimuli (Fig. 1).

Since endothelial stalk cells mimic the phenotype of mature lumenized vascular tubes, the authors examined if Robo4 expression was maintaining vascular stability. Although lack of Robo4 had no effect on cell proliferation, cell migration and tube formation (Classical in vitro angiogenesis assays) induced by proangiogenic factor VEGF-165 was inhibited by Robo4 upon binding of its ligand Slit2. Furthermore, they provide evidence, which indicates Robo4/Slit2 signaling is able to prevent vascular leakage induced by VEGF-165. An intravitreal injection of VEGF-165 induced leakage of Evans Blue from retinal blood vessels and was suppressed by co-injection of Slit2 in wild type mice, but Slit2 was not inhibitory in the non-functional $Robo4^{AP/AP}$ mice. VEGF-165 is a known permeability inducer, that activates a cascade of signaling events through its tyrosine kinase receptor, VEGFR2.[7,8] With this in mind, Jones et al. sought out where in the VEGF signaling pathway Robo4/Slit was inhibiting signal transduction (Fig. 2). They provide evidence that Robo4/Slit2 signaling does not block the phosphorylation of VEGF-165 receptor, but inhibits the activation of downstream Src family nonreceptor tyrosine kinases (SFKs), thereby inhibiting VEGF-165 activity.

Macular degeneration, retinopathy of prematurity and diabetic retinopathy are all characterized by pathological angiogenesis and hallmarked by vascular leakage. VEGF-165 involvement in these disorders is highlighted by the recent success of antibodies to VEGF

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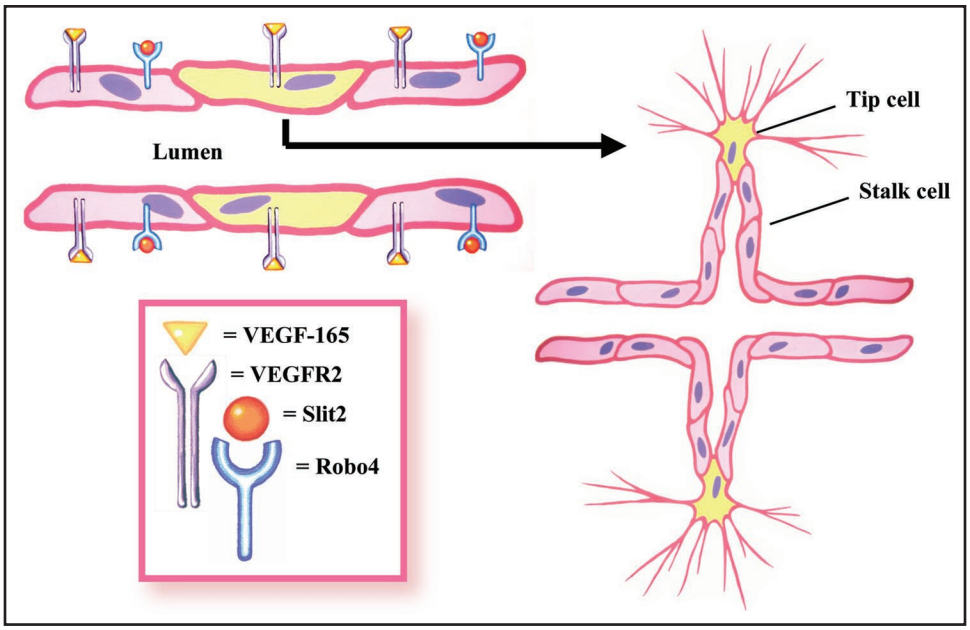

Figure 1. Possible role of Robo4 in tip cell selection. Findings of Jones et al. indicate that Robo4 may negatively regulate tip cell formation. Perhaps Robo4/Slit2 signaling is functioning similar to that of Notch/Dll4. Endothelial cells of a capillary tube that lack expression of Robo4 may be more likely to be transformed into tip cell upon VEGF stimulation.

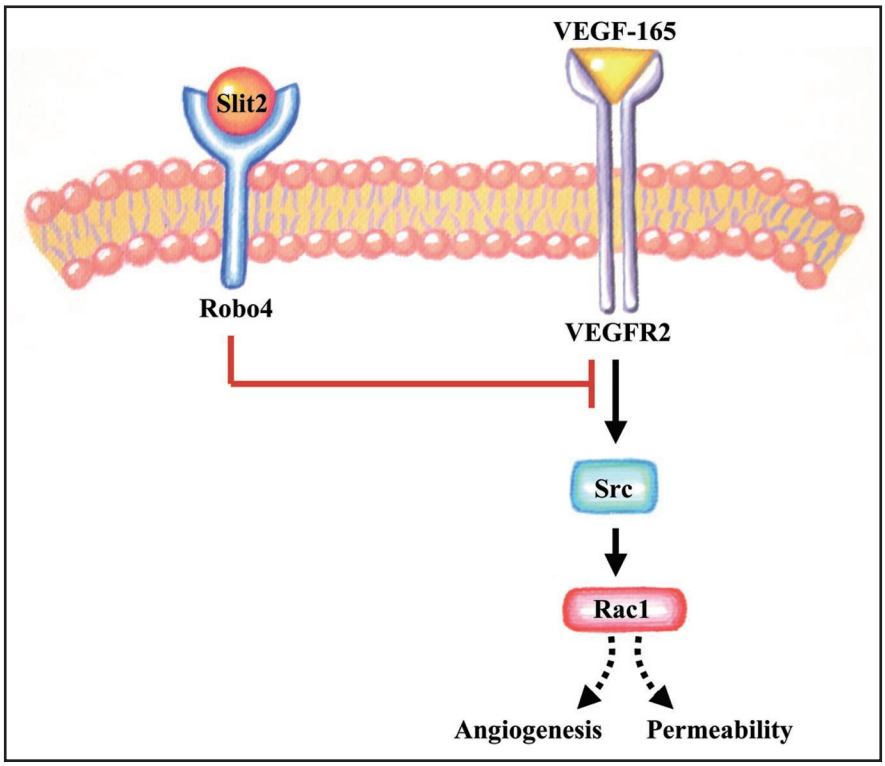

Figure 2. Crosstalk between Robo4/Slit2 and VEGF signaling pathways. Jones et al. suggests that Robo4/Slit2 signaling can negatively regulate angiogenesis. Activation of VEGFR2 by VEGF-165 leads to the phosphorylation of SFK family member Src, which in turn stimulates downstream effector, Rac1 leading to angiogenesis and increased vascular permeability. Binding of Slit2 to Robo4 leads to signaling that blocks the VEGFR2 signaling pathway.

in clinical studies.[9] Perhaps the most significant feature of Jones and colleagues work lies in their use of mouse models which mimic the phenotypes of various retinopathies. By utilizing mouse models of oxygen-induced retinopathy (OIR) and laser-induced choroidal neovascularization (CNV), the authors showed a clear reduction in pathological angiogenesis upon the activation of Robo4 signaling by Slit2 intravitreal injection. They also showed that tonic levels of Robo4 signaling stabilized the retinal blood vessels by comparing $Robo4^{+/+}$ and $Robo4^{AP/AP}$ mice after hyperoxic exposure. $Robo4^{+/+}$ mice displayed a much smaller degree of neovascularization.

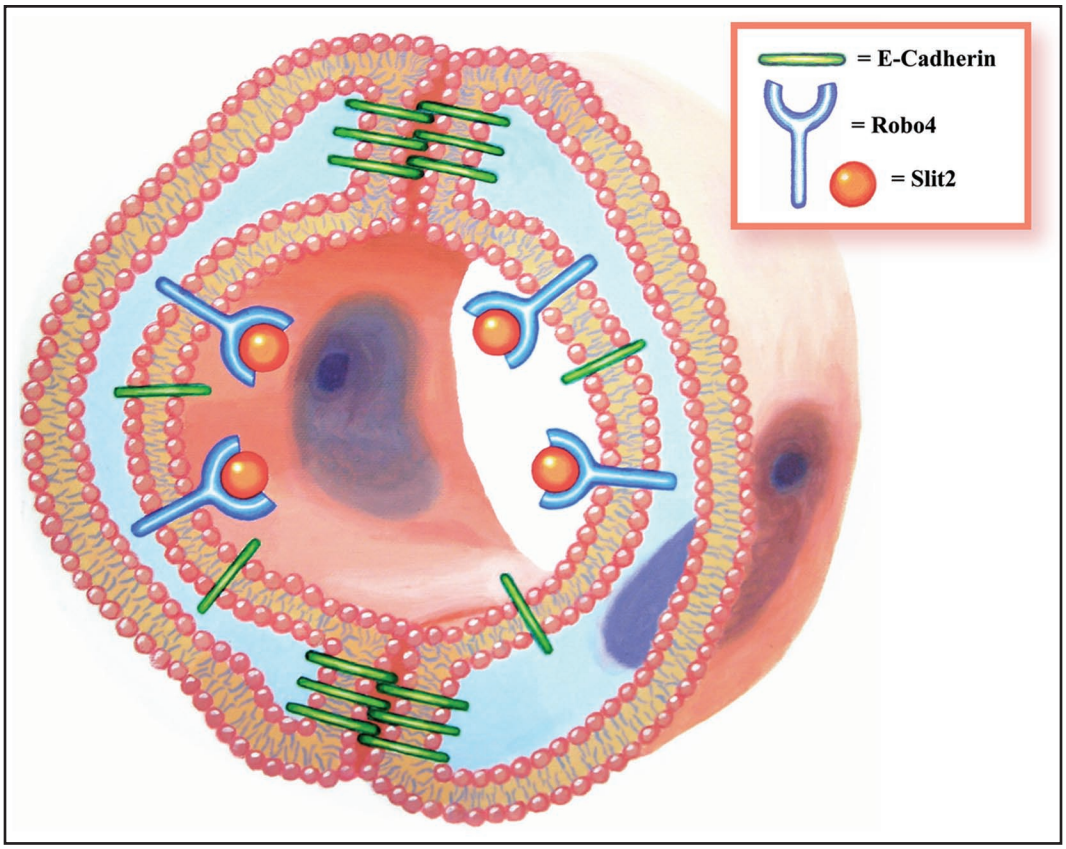

Figure 3. Schematic representation of endothelial stalk cell lumen formation. As in *Drosophila melanogaster* cardioblasts the expression of Robo4 may be limited to endothelial stalk cells. Repulsive signaling through Robo4/Slit2 binding may prevent E-cadherin mediated cell-cell adhesion along the apical surface of stalk cells leading to vascular lumen formation and maintenance.

Significance of this work lies in its development of a new paradigm for Robo4 acting not just as a nervous system guidance molecule, but a regulator of the vasculature. Their data suggests that SFKs may be the downstream target for Robo4/Slit2 signaling, but it falls short in identifying the point of intersection of these two pathways. The role of SFKs in VEGF-165 induced angiogenesis and permeability is well demonstrated.[8] To completely understand how Robo4 activation truncates the VEGF pathway the specific SFK family members whose phosphorylation is blocked needs to be identified. This may be necessary due to the multiple bands identified by the p-Src antibodies used in their western blotting. The antibody may not be specific enough to distinguish between Src and other family members. Further experimentation would be needed to determine if the effects of Robo4/Slit2 on vascular stability are simply a manifestation of its ability to control tip cell formation similar to that of the Notch/DII4 signaling pathway. It may also be intriguing to determine if Robo4/Slit2 signaling is acting on vessel lumen formation. Perhaps it is required to prevent cell adhesion at the apical surfaces of adjacent endothelial cells in newly formed angiogenic sprout. Recent findings published by two separate groups indicating Robo/Slit signaling involvement in *Drosophila melanogaster* heart tube lumen formation support this idea.[10,11] *Drosophila melanogaster* cardiac morphology comprises a simple linear tube, which is strikingly similar to vertebrate capillaries. It is therefore plausible that a repulsive signaling from Robo4/Slit promotes tube formation by inhibiting E-cadherin-mediated cell adhesion at the apical surface (Fig. 3). Nevertheless clinical trials of Robo4 activation in human retinopathy subjects must be the next step in translating this research to the bedside.

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