Recent Advances in Research on Vascular Permeability to Establish Novel Therapeutic and Drug Delivery Strategies for Intractable Diseases

Review

Vascular Leakage Prevention by Roundabout 4 under Pathological Conditions

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Vascular permeability is regulated mainly by the endothelial barrier and controls vascular homeostasis, proper vessel development, and immune cell trafficking. Several molecules are involved in regulating endothelial barrier function. Roundabout 4 (Robo4) is a single-pass transmembrane protein that is specifically expressed in vascular endothelial cells. Robo4 is an important regulator of vascular leakage and angiogenesis, especially under pathological conditions. The role of Robo4 in preventing vascular leakage has been studied in various disease models, including animal models of retinopathy, tumors, diabetes, and endotoxemia. The involvement of Robo4 in vascular endothelial growth factor and inflammation-mediated signaling pathways has been well studied, and recent evidence suggests that Robo4 modulates endothelial barrier function *via* distinct mechanisms. In this review, we discuss the role of Robo4 in endothelial barrier function and the underlying molecular mechanisms.

Key words angiogenesis; endothelial permeability; Roundabout 4; vascular leakage

1. INTRODUCTION

Vascular endothelial cells present on the inner surface of blood vessels act as a barrier that restricts the movement of fluid, molecules, and immune cells. Endothelial barrier function is regulated by cell junctions and cytoskeleton molecules. At cell junctions, the adjacent cells are connected by adherens junction proteins such as vascular endothelial (VE)-cadherin and tight junction proteins such as claudin family proteins.¹⁾ The actin cytoskeleton is connected to cell junction proteins and generates a pulling force *via* reorganization.²⁾ Various molecules, such as growth factors and inflammatory cytokines, affect the integrity of the endothelial barrier.^{3–5)} The dynamics and plasticity of the endothelial barrier are essential for vascular homeostasis, vascular development, and immune function. Dysfunction of the endothelial barrier is associated with several diseases.⁶⁾

Roundabout 4 (Robo4), a single-pass transmembrane protein belonging to the Roundabout family of proteins. Other Roundabout proteins are predominantly expressed in neural cells and are involved in axon guidance.^{7–10)} Robo4 was identified using bioinformatics data mining as an endothelial cell-specific Roundabout family protein.¹¹⁾ Subsequent studies have confirmed the specific expression of Robo4 in vascular endothelial cells.^{12–16)} Robo4 stabilizes the vasculature, particularly under pathological conditions.^{17,18)} In cultured endothelial cells, Robo4 suppresses cell migration, proliferation, and tube formation.^{19,20)} Studies have also shown that Robo4 is required for proper vasculature development in zebrafish.^{13,21)} Although mice lacking Robo4 do not show apparent defects in developmental angiogenesis, loss of Robo4 induces pathological angiogenesis in various other disease models, including those of hypoxia-induced retinopathy^{22,23)} and cancer.²⁴⁾ Robo4 ex-

pression is upregulated under several pathological conditions, confirming the protective role of this protein.^{25–27)}

Several studies have confirmed the role of Robo4 in stabilizing vascular endothelial cells; however, the underlying molecular mechanisms remain controversial. Recent studies on the role of Robo4 in preventing vascular hyperpermeability using various pathological models demonstrated that Robo4 regulates vascular permeability *via* distinct mechanisms. This review discusses the present knowledge of Robo4-mediated regulation of vascular permeability and highlights the biological functions of this protein.

2. ROBO4 SUPPRESSES VASCULAR LEAKAGE UNDER PATHOLOGICAL CONDITIONS

Mice lacking Robo4 are viable and show slightly increased vascular permeability under basal conditions.^{22,28,29)} In several disease models, such as those of hypoxia-induced retinopathy,²²⁾ cancer,²⁴⁾ diabetes,³⁰⁾ and endotoxemia,^{29,31)} studies have shown that lack of Robo4 leads to more severe vascular leakage. Robo4 stabilizes endothelial permeability by regulating both adherens junctions and tight junctions in endothelial cells.^{29,32,33)} Adherens junctions are involved in the transmigration of cancer and immune cells,^{34–36)} and lack of Robo4 enhances lung metastasis of cancers^{24,37)} and diapedesis of immune cells.²⁹⁾ *In vitro* studies have shown that Robo4 suppresses endothelial hyperpermeability induced by various stimuli such as vascular endothelial growth factor (VEGF),^{22,38)} interleukin-1 β (IL-1 β),³¹⁾ tumor necrosis factor α (TNF- α),^{29,31)} and histamine.³⁸⁾ Recent evidence suggests that Robo4 regulates endothelial permeability through several distinct mechanisms.

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Fig. 1. Robo4 Regulates Endothelial Permeability by Acting as a Co-receptor for Robo1

Activation of VEGFR2 by VEGF increases the cell permeability by destabilizing the VE-cadherin. SLITs-Robo1/2 is required for the activation of VEGFR2 in response to VEGF. In the presence of Robo4, SLITs-Robo1/2 causes internalization of the VEGFR2, thus, preventing VEGFR2-VEGF interaction. Robo4 interacts with Robo1 and modulates its function. Therefore, Robo4 possibly enhances SLITs-Robo1/2-mediated internalization of the VEGFR2, resulting in decreased vascular permeability even in the presence of VEGF. Abbreviations: Robo, Roundabout; VE, vascular endothelial; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2. (Color figure can be accessed in the online version.)

3. MOLECULAR MECHANISM UNDERLYING ROBO4-MEDIATED REGULATION OF VASCULAR PERMEABILITY

3.1. Robo4 and Robo1 Cause Internalization of VEGF Receptor 2 (VEGFR2) The first proposed model to explain the mechanism underlying Robo4-mediated regulation of vascular permeability suggests that Robo4 acts as a co-receptor. SLIT family proteins (SLITs) were initially proposed as potential ligands of Robo4,^{12,22,31,39,40} as they are well-known ligands for Robo1 and Robo2 in the central nervous system.⁸ In the vascular system, SLITs play both pro- and anti-angiogenic roles *via* VEGFR2.^{22,41–44} VEGFR2 is a central player in the VEGF signaling pathway that promotes angiogenesis and induces vascular leakage.^{45,46} Robo1 and Robo2 are responsible for the pro-angiogenic functions of SLITs *via* enhanced VEGF-VEGFR2 signaling, whereas Robo4 does not participate in the pro-angiogenic functions of SLITs.^{42–44,47} In contrast, Robo1, Robo2, and Robo4 regulate the anti-angiogenic functions of SLITs *via* VEGFR2 internalization.^{22,42} Previously, the interaction between Robo4 and SLITs was studied using cell-based assays.¹² However, *ex vivo* studies using recombinant proteins demonstrated that Robo4 does not directly interact with SLIT2.^{28,42,48} Structural analysis also revealed that Robo4 lacks essential amino acid residues for SLIT2 binding.⁴⁹ These studies suggest that Robo4 indirectly modulates signaling cascades triggered by SLITs. Robo family receptors interact with each other to form multimers.^{50,51} Robo4 binds to Robo1 and affects SLIT2-mediated Robo1 function.^{20,52} Therefore, it is possible that Robo4 modulates the anti-angiogenic functions of SLITs by binding to Robo1 (Fig. 1).

3.2. Robo4 Suppresses VEGF Phosphorylation by Activating Unc5B The second model assumes that Robo4 acts as a membrane-associated ligand of Unc5B. Although Robo4-Fc, a chimeric protein of the Robo4 extracellular domain and Fc region of human immunoglobulin G, suppresses angiogenesis in pathological conditions, it does not bind to SLITs.⁴⁸ This finding indicates that the Robo4 extracellular domain binds to unknown partners. A high-throughput screening assay identified Unc5B as an interacting partner of the Robo4 extracellular domain.²⁸ Unc5B is a member of the Unc5 receptor family, which regulates axon guidance by binding to Netrin-1.⁵³ Unc5B is predominantly expressed in the vascular system, and interfering with Unc5B expression causes vascular defects during developmental and adult stages.^{28,54,55} The Robo4 extracellular domain binds to Unc5B and induces an intracellular signaling cascade to suppress VEGF-mediated vascular leakage.^{23,28} The Robo4-Unc5B axis suppresses the activation of VEGFR2 by reducing the phosphorylation of VEGFR2 Y951 (Y949 in mouse) but not Y1175.^{23,28} Phosphorylation of VEGFR2 Y951 is responsible for vascular hyperpermeability *via* the T cell-specific adaptor (TSAd) and Src.^{56,57} On the other hand, phosphorylation of VEGFR2 Y1175 plays an essential role in developmental vasculogenesis and angiogenesis.⁵⁸ These limited effects of Robo4 on VEGFR2 activation may explain why Robo4 does not affect vasculogenesis and angiogenesis during development. To address the role of Robo4-Unc5B signaling *in vivo*, mice expressing the mutant Robo4 lacking the intracellular domain were developed. Interestingly, this Robo4 mutant attenuated the vascular leakage induced by VEGF, but not histamine.²³ Taken together, these studies suggest that Robo4 functions as a ligand of Unc5B and selectively attenuates VEGF-VEGFR2 signaling (Fig. 2).

3.3. Robo4 Stabilizes Cellular Junctions by Regulating the Subcellular Localization of TNF Receptor-Associated Factor 7 (TRAF7) Several reports have demonstrated that Robo4 is involved in the endothelial response to inflammation.^{29,31,59,60} Robo4 prevents vascular leakage in an endotoxemia mouse model *via* TRAF7.²⁹ Unlike other TRAF family proteins, TRAF7 lacks the conserved TRAF-C domain, which

is responsible for binding to TNF receptors and instead contains a WD40 repeat domain.^{61,62} TRAF7 modulates the activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B) and activator protein 1 (AP-1) in response to inflammatory stimuli.^{63–67} Robo4 relocates TRAF7 near the nucleus *via* the Robo4 intracellular domain, which is essential for preventing endothelial permeability.²⁹ Previous reports have shown that Robo4 shuttles between the plasma membrane and endosome-like compartments.^{20,23,28} These results indicate that Robo4 regulates the subcellular localization of TRAF7 by intracellular shuttling, subsequently controlling its function.

The role of TRAF7 in vascular permeability is not fully understood. TRAF7 potentially regulates vascular permeability *via* ubiquitination of binding proteins. TRAF7 contains the RING finger domain, which is common to several ubiquitin E3-ligases. Previous reports have demonstrated that TRAF7 is involved in the ubiquitination of NF- κ B pathway molecules, NF- κ B essential modulator (NEMO), p65, and several transcription factors, including cellular FADD-like IL-1 β -converting enzyme-inhibitory protein (c-FLIP), c-Myb, and krüppel-like factor 4 (KLF4).^{65,66,68,69} Furthermore, proteomic studies have identified several TRAF7-interacting proteins, including Mitogen-activated protein kinase kinase 3 (MEKK3) and Rac1.^{66,69} Interestingly, these substrates and interacting proteins are involved in regulating vascular leakage.^{70–72} These results suggest that Robo4 regulates vascular permeability by modulating the activity of these TRAF7-related proteins (Fig. 3).

4. ROBO4 AS A POTENTIAL THERAPEUTIC TARGET

VEGF signaling is one of the targets that suppress patho-

Fig. 2. Robo4 Regulates Endothelial Permeability by Acting as a Membrane-Associated Ligand for Unc5B

The extracellular domain of Robo4 interacts with and activates Unc5B. Unc5B activation attenuates the phosphorylation of VEGFR2 Y951, but not Y1175, in response to VEGF, resulting in decreased vascular leakage. Abbreviations: Robo, Roundabout; VE, vascular endothelial; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2. (Color figure can be accessed in the online version.)

Fig. 3. Robo4 Regulates Endothelial Permeability *via* TRAF7

The intracellular domain of Robo4 interacts with TRAF7, thus sequestering and anchoring it close to the nuclei in endothelial cells. Robo4 suppresses the destabilization of VE-cadherin induced by TNF α in a TRAF7-dependent manner. Abbreviations: Robo, Roundabout; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TRAF7, tumor necrosis factor receptor-associated factor 7; VE, vascular endothelial. (Color figure can be accessed in the online version.)

logical vascular leakage and angiogenesis. In fact, stabilization of the pathological vasculature by inhibiting VEGF signaling is an effective treatment strategy against various diseases, including cancer,^{73,74} ocular diseases,⁷⁵ and diabetic nephropathy.⁷⁶ However, because VEGF signaling regulates vascular development and maintenance under physiological conditions, complete inhibition of VEGF signaling by the antibodies against VEGF and VEGFR2 induces adverse effects in the cardiovascular system.⁷⁷ In contrast, Robo4 stabilizes the pathological vasculature without affecting vascular development and maintenance. Thus, Robo4 could be a useful therapeutic target that specifically suppresses VEGF signaling under pathological conditions.

Several therapeutic studies have reported the use of molecules that activate Robo4 signaling. Robo4-Fc, which activates Unc5B signaling, suppresses basic fibroblast growth factor-driven angiogenesis in a mouse subcutaneous sponge model.⁴⁸ A humanized antibody against Robo4, DS-7080a, which possibly modulates Robo4 function, ameliorates neovascular age-related macular degeneration in cynomolgus monkeys⁷⁸ Annexin A2, as a potential ligand for Robo4, controls blood-brain barrier integrity.⁷⁹ These reports suggest that the development of molecules targeting Robo4 signaling will provide a new therapeutic strategy for the treatment of pathological vascular angiogenesis and permeability.

5. CONCLUSION AND PERSPECTIVES

Robo4 stabilizes the vasculature under pathological conditions by reducing vascular permeability and promoting angiogenesis. Furthermore, Robo4 stabilizes blood vessels by interacting with multiple proteins, including SLITs-Robo1, Unc5B, and TRAF7. Robo4 has been suggested to modulate the activity of interacting proteins in a context-dependent manner. Robo4 could be a potential therapeutic target for stabilizing the pathological vasculature. Further studies on Robo4 function and its activating molecules will accelerate the development of novel drugs against various vascular diseases.

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