Critical functions of *Reck* in mouse forebrain development

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**Abbreviations**

APCDD1: Adenomatosis polyposis coli down-regulated 1
BBB: blood-brain barrier
cKO: conditional knockout
CNS: central nervous system
Cx: cerebral cortex
ECs: endothelial cells
EndMT: endothelial-to-mesenchymal transition
FZ-CRD: Frizzled cysteine-rich domain
Gapdh: Glyceraldehyde-3-Phosphate Dehydrogenase
GE: ganglionic eminence
Glut1: Glucose transporter 1
GPI: glycosylphosphatidylinositol
GVM: glomeruloid vascular malformation
HE: hematoxylin and eosin
ip.: intra-peritoneal
ID1: Inhibitor of differentiation/DNA binding 1
IF: immunofluorescent staining
Itgb8: Integrin beta8
LiCl: lithium chloride
Mmp2: matrix metalloproteinase 2
NPCs: neural precursor cells
NVU: neurovascular unit
RECK: Reversion-inducing cysteine-rich protein with Kazal motifs
S.E.M.: standard error of mean
SM22α: smooth muscle protein 22α
Sox17: SRY-related HMG-box 17
TOP-Flash: TCL/LEF-Firefly luciferase
TuJ1/Tubb3: Neuronal class III beta-tubulin
Vegfa: vascular endothelial growth factor A
αSMA: α-smooth muscle actin
Abstract

Proper development and functioning of the brain is dependent on the reciprocal regulation between the neural and vascular systems, which comprise what is known as neurovascular unit (NVU); it is likely that several molecules expressed by neuroepithelium, vascular cells, of both contribute to the crosstalk between these two systems. Previously, RECK in neural precursor cells (NPCs) was found to support Notch-dependent neurogenesis. Recent studies implicate RECK expressed in endothelial cells (ECs) in WNT7-induced canonical WNT signaling required for angiogenesis in the central nervous system (CNS). Here, I report that selective inactivation of Reck in Foxg1-positive NPCs in mice results in an unexpected phenotype characterized by the death shortly after birth with severe hemorrhage in the forebrain. As expected, these mice also show a defect in neurogenesis characterized by precocious neuronal differentiation. The hemorrhage, on the other hand, is accompanied by vascular malformations that are similar (in morphology, location, and timing) to those found in EC-specific Reck knockout mice as well as Wnt7a/7b double knockout mice. Notably, the hemorrhage could be partially rescued by administration of LiCl, an activator of WNT signaling, into the parental pregnant female mice, suggesting that impairment of canonical WNT signaling is involved in this phenotype. The Reck-deficient NPCs showed reduced potency to active canonical WNT signaling when co-cultured with the WNT reporter (TOP-Flash) cells in vitro. In a model system using the transfectable HEK293 cell line, an activity of RECK in WNT7-producing cells to enhance canonical WNT signaling in reporter cells was detectable in co-culture assay but not in the experiments using conditioned media. These findings indicate that RECK in NPCs has a non-cell-autonomous function to promote forebrain angiogenesis, probably through contact-dependent enhancement of WNT signaling in ECs, and
provide a fresh insights into the functions of RECK and its role in brain development.
Chapter 1. Introduction

The brain comprises two major systems, neural and vascular, and they communicate with each other through a unique physical interface, called blood-brain barrier (BBB), which maintains the homeostatic environment of the brain and prevents unwanted factors to enter into this environment. Mature BBB is composed of multiple types of cells including specialized endothelial cells, pericytes, neurons, astrocytes, and microglia. The intimate contacts between these cells and the functional interactions and signaling between them result in the formation of a dynamic functional unit, known as the neurovascular unit (NVU) (Paredes et al., 2018). Although the molecular mechanisms of NVU development remain largely obscure, the involvement of several neuroepithelium-derived molecules, such as integrin αV (Bader et al., 1998; McCarty et al., 2005; McCarty et al., 2002), integrin β8 (Zhu et al., 2002), ID1/3 (Lyden et al., 1999), TGFBR2 (Hellbach et al., 2014) and WNT7A/B (Daneman et al., 2009; Stenman et al., 2008), has been reported.

RECK (Reversion-inducing cysteine-rich protein with Kazal motifs) encodes a glycosylphosphatidylinositol (GPI)-anchored glycoprotein which contains 971 amino acid residues with hydrophobic domains on both termini, five cysteine-rich modules in the N-terminal region, five N-glycosylation sites, a Frizzled-like cysteine-rich domain (FZ-CRD), two EGF-like domain, and three copies of so-called Kazal motif, a core sequence typically found in serine protease inhibitors (Takahashi et al., 1998). Reck-deficient mice die at around E10.5 with reduced tissue integrity, abdominal hemorrhage, and fragile neural tube (Oh et al., 2001). In normal mice at this stage, RECK is abundantly expressed in blood vessels (ECs and mural cells or pericytes) (Oh et al., 2001; Chandana et al., 2010) and NPCs (Muraguchi et al., 2007). Of note, when Reck is systematically inactivated from E11, the mice show vascular defects including forebrain hemorrhage by E15.5 and die before birth (Chandana et al.,
Recently, functions of RECK in ECs have been reported by our group (Almeida et al., 2015) and other groups working on zebrafish (Ulrich et al., 2016) or conditional knockout mice (Cho et al. 2017). These studies indicate that RECK, together with GPR124, function as a co-receptor to promote WNT7a/7b-specific signaling in ECs, and this function is required for CNS angiogenesis, including proper tip cell function, angiogenesis, and BBB formation.

More recently, a novel mechanism by which RECK promotes WNT signaling was proposed. Eubelen et al. reported that WNT7 selectively binds RECK through its divergent linker domain while it binds FZD through its conserved domains and that the ability of RECK to bind GPR124 enables the WNT7-RECK complex to recruit GPR124 to the vicinity of the FDZ-LRP5/6 complex (i.e., the classical WNT receptor) thereby facilitating the clustering of the intracellular Dvl protein and hence the formation of WNT signalosome complex; they call RECK as a “WNT decoding module” of the complex (Eubelen et al., 2018). On the other hand, Vallon et al. reported the function of RECK to bind and stabilize the active, lipid-modified WNT7 monomer thereby facilitating the ligand-receptor interaction (Vallon et al. 2018). These findings have uncovered the important cell-autonomous functions of RECK in WNT7-signaling in brain ECs that are required for CNS angiogenesis.

I was interested in neuroscience, and my initial goal of this project was to confirm and extend the previous finding that RECK was essential for neurogenesis in mouse embryos (Muraguchi et al., 2007). My strategy was to generate conditional knockout (cKO) mice in which Reck is selectively inactivated in Foxg1-positive NPCs. To my surprise, the most obvious phenotype of these mice was brain hemorrhage. I, therefore, attempted in this study to elucidate the mechanism by which this unexpected phenotype (i.e., vascular phenotype in neural Reck mutant) arises in these mice.
Chapter 2. Materials and Methods

2.1 Mice

All animal experiments were approved and conducted in accordance with its regulation by the Animal Research Committee, Graduate School of Medicine, Kyoto University. All mice were housed in the SPF environment of the animal facility of Graduate School of Medicine, Kyoto University. To generate Reck conditional knockout mice in neuronal precursor cells, Reck\textsuperscript{flex1/flex1} mice (Yamamoto et al., 2012) were crossed with Foxg1-Cre mice (Hebert and McConnell, 2000), the heterozygous Foxg1-Cre; Reck\textsuperscript{flex1/+} mice crossed again with the Reck\textsuperscript{flex1/flex1} mice to get homozygous Foxg1-Cre; Reck\textsuperscript{flex1/flex1} mice [Reck-cKO (Foxg1)] and heterozygous Foxg1-Cre; Reck\textsuperscript{flex1/+} mice of the same littermates as control. To generate Reck conditional knockout mice in endothelial cells, Reck\textsuperscript{flex1/flex1} mice were crossed with Tie2-Cre mice (Kisanuki et al., 2001), the heterozygous male Tie2-Cre; Reck\textsuperscript{flex1/+} mice crossed again with the female Reck\textsuperscript{flex1/flex1} mice to get homozygous Tie2-Cre; Reck\textsuperscript{flex1/flex1} mice [Reck-cKO (Tie2)] and heterozygous Tie2-Cre; Reck\textsuperscript{flex1/+} mice of the same littermates as control. The homozygous mTmG/mTmG reporter mice (Muzumdar et al., 2007) were crossed with Foxg1-Cre mice to evaluate the expression of Cre recombinant. Mice were mated between 4 pm to 9 am, and when the vaginal plug was found, that noon was considered as E0.5. The yolk sac of the embryo or the tail of the adult mice were used for genotyping.

Table 1 Primer for genotyping

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<tr>
<td>Reverse</td>
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<td>Forward</td>
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<tr>
<td>Reverse</td>
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</tbody>
</table>
2.2 Histology

Mouse embryos at indicated time points were harvested on ice, and the yolk sac was used for genotyping. The embryonic forebrain was dissected, fixed in 4% PFA/PBS solution at 4°C for 4-24 h (for E11.5-E15.5) and then immersed in serial sucrose solution (10%, 20% and 30% in PBS) for 24 hours each. The sample was then embedded in OCT compound, frozen, and stored at -80°C. For Hematoxylin and Eosin (HE) staining, 10μm-thick coronal sections of the forebrain were immersed in phosphate-buffered saline (PBS) for 5 min twice, stained with hematoxylin for 1 min, washed with tap water for 30 min, and treated with a series of alcohol solutions (50%, 70%, 90%), followed by eosin staining for several seconds, washing with limonene 5 min three times and mounting using permanent mounting medium.

For densitometry, the mean density of fluorescence signals per pixel in a square ROI (90 x 90 pixels) was determined using ImageJ. Five ROIs were randomly selected in the region adjacent to the ventricle (in the cases of Sox2 and Ki67) or outer surface of the brain (in the case of TuJ1) in microscopic images acquired under comparable conditions.

2.3 Immunofluorescent staining

For Immunofluorescent staining (IF), 5-μm thick coronal cryosections of the forebrain were used. Briefly, the sections were incubated with primary antibodies overnight at 4 degrees, washed in PBS three times, and incubated with indicated secondary antibodies for 2 h at room temperature, washed, and then mounted with DAPI Fluoromount-G (0100-20, Southern Biotechnology Associates). Primary antibodies included: rat monoclonal anti-PECAM/CD31 (BD Bioscience, 553370), rabbit polyclonal anti-NG2 (Merck Millipore, AB5320), Mouse monoclonal anti-Laminin(clone LAM-89) (Sigma-Aldrich, L8271), Rabbit polyclonal anti-Type IV Collagen (PROGEN Biotechnik, 10760), Goat polyclonal anti-Sox2 (Santa Cruz Biotechnology, sc-17320), Rabbit
polyclonal anti-Neuronal Class III beta-Tubulin/TUJ1 (COVANCE, PRB-435P), and Rabbit polyclonal anti-Ki67 (Leica Biosystems, NCL-Ki67p).

Secondary antibodies used: Goat anti-rat IgG(H+L) highly cross-adsorbed secondary antibodies (Alexa-555, Alexa-594, or Alexa-647 conjugates), and goat anti-rabbit IgG(H+L) highly cross-adsorbed secondary antibodies (Alexa-488 or Alexa-647 conjugates), Donkey anti-goat IgG(H+L) cross-adsorbed secondary antibodies (Alexa-647 conjugates), Donkey anti-rabbit IgG(H+L) cross-adsorbed secondary antibodies (Alexa-488 conjugates) (Thermo Fisher Scientific).

2.4 Primary NPC culture

NPCs were prepared according to the method of Kitani et al. (Kitani et al., 1991). Briefly, the forebrains of E11.5 embryos were cut into several pieces in DME/F12 medium. Eye cups, nasal plates, and other non neuroepithelial tissues were removed under a dissection microscope. The tissue pieces were treated with PBS containing 0.5% trypsin for 1 h at 37°C and then with DNase I to reduce viscosity. The cells were transferred to DME/F12 medium supplemented with 10% FBS on 12 well plates. After incubation for 7 h at 37°C in a CO₂ incubator, cells in the NPC-enriched fraction (floating cells) were collected, counted, and sediment by centrifugation. The cells and the supernatant were separated and used in TOP-Flash assays.

2.5 Lithium chloride (LiCl) administration

*Foxg1-Cre; Reck*<sup>flax1/+</sup> male mice and female *Reck*<sup>flax1/</sup><sub>flax1</sub> mice were time-mated, and LiCl solution was intraperitoneally injected (dose: 200 mg/kg) into pregnant female mice once a day for indicated period of time. The embryos were harvested at E15.5 for inspection.
2.6 TOP-Flash assay

A RECK-deficient HEK293 sub-line, ADA99-25 (Matsuzaki et al. unpublished), was generated using the CRISPR/Cas9 double nickase method (Ran et al., 2013). To establish the RECK-deficient TOP-Flash reporter, ADA99-25 cells were transfected with pGL4.49 (Clontech) using CalPhos Mammalian Transfection Kit (Clontech), selected in growth medium (DMEM supplemented with 10% fetal bovine serum and Pen Strep) containing 100 µg/ml Hygromycin B Gold (Invitrogen), and a clone (named HNM1) whose firefly luciferase activity shows robust response to 20 µM LiCl was selected. To establish a WNT7-selective TOP-Flash reporter (named LC20b), HNM1 cells were co-transfected with four expression vectors (expressing Fzd4, Lrp5, GPR124, and RECK) (Takahashi et al., 1998; Vallon et al., 2018) and two marker plasmids [pRL (Promega) and pUCSV-BSD (Kimura et al., 1994)], selected in growth medium containing 8 µg/ml Blasticidin-S, and a clone was isolated whose firefly luciferase activity showed a robust response when co-cultured with HEK293 cells producing WNT7A.

For TOP-Flash assay with NPCs, LC20b cells (3 x 10^4/well) were plated onto a white 96-well plate (BD 353296) on day 0. NPCs (3 x 10^4/well) or their culture supernatant were overlaid at day 1, and luciferase assay was performed at day 2 using Dual-Glo Luciferase Assay System (Promega) or twinlite Firefly & Renilla Luciferase Reporter Gene Assay System (PerkinElmer).

For TOP-Flash assay with HEK293 cells, we first established RECK-deficient HEK293 cells expressing either WNT7A or WNT7B by stably transfecting ADA99-25 cells with an expression vector (carrying the neo marker) expressing WNT7A or WNT7B (Vallon et al., 2018) followed by selection in growth medium containing 1 mg/ml G418. These WNT7 producer cells were transfected with either vacant vector (V) or RECK-expression vector (R) using FuGENE HD (Promega) and plated onto a white 96-well plate (1.5 x
10^4/well) at day 0 (Producer). In parallel, HNM1 cells were co-transfected with five expression vectors (pRL, Fzd4, Lrp5, GPR124, plus control or RECK-expressing vector) using FuGENE HD and plated onto regular dishes (Reporter). At day 1, media in all cultures were replaced with the fresh growth medium. At day 2, culture supernatant on white plate (Producer) were transferred to new wells (Conditioned media), and then the Reporter cells, suspended using trypsin and washed with growth medium, were plated (3 x 10^4/well) onto the wells containing either the Producer cells or the Conditioned media. After 1-day incubation, luciferase assay was performed using the twinlite system

2.7 qRT-PCR

Total RNA was extracted from the forebrains of embryos at E11.5, E12.5, or E13.5 using Nucleo Spin RNA kit (Macherey-Nagel). cDNA was prepared using PrimeScript II 1st Strand cDNA synthesis kit (Takara), and quantitative PCR was performed with the Stratagene Mx3005P (Agilent) using KAPA SYBR Fast Universal qPCR kit (Kapa Biosystems) and the primers listed in Table 2. The ratio of mRNA in Reck-cKO (Foxg1) and control samples were calculated using the delta-delta Ct method.

2.8 Statistics

Quantitative data are presented in the form of scatter dot plots representing mean and the standard error of mean (S.E.M.). The significance of difference between two groups was assessed using Student’s t-test.
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<td>Gapdh</td>
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<td>Mmp2</td>
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<td>Wnt7a</td>
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Chapter 3. Results

3.1 Selective inactivation of *Reck* in neural precursor cells in mice results in death shortly after birth with unexpected forebrain hemorrhage

To extend the previous findings on the important function of *Reck* in NPCs, I chose to investigate the functions of *Reck* in the forebrain development by the cell-type-selective Cre-loxP knockout strategy. Dr. Hanashima suggested us that *Foxg1-Cre* transgenic mouse was an ideal choice for this purpose, since it was reported that robust Cre-expression is found in the telencephalon of these mice from early embryonic stages (Hebert and McConnell, 2000) specifically in the neural cells (Hellbach et al., 2014).

In collaboration with Dr. Hanashima, I first tried to confirm the expression of *Foxg1-Cre* during the forebrain development by crossing the *Foxg1-Cre* mice with the mTmG reporter mice (Muzumdar et al., 2007). The *Foxg1-Cre* expressing cells (green) were found in telencephalon at E8.5, and the cells labeled with green fluorescence (*Foxg1-mG*) were most abundant in the forebrain from E9.5 onward (Figure 1). These results nicely support the original report (Hebert and McConnell, 2000).
Figure 1. Expression of Foxg1-Cre in Foxg1-Cre; mTmG mice at various embryonic stages.

Expression of Foxg1-Cre in mouse embryo as visualized in Foxg1-Cre; mTmG mice. Green signals represent Foxg1-Cre-expressed cells. Scale bar: 500 μm.

Next, I crossed the Foxg1-Cre mice with Reck\textsuperscript{flex1/flex1} mice and found that the heterozygous Reck\textsuperscript{flex1/+}; Foxg1-Cre mice were viable and fertile. I therefore decided to use these mice as a control. To generate homozygous Reck\textsuperscript{flex1/flex1}; Foxg1-Cre mice, which we call Reck cKO (Foxg1) in this paper, I crossed the heterozygous Reck\textsuperscript{flex1/+}; Foxg1-Cre mice with Reck\textsuperscript{flex1/flex1} mice. Interestingly, I have never obtained Reck cKO (Foxg1) mice in the adult littermates obtained from such cross, in which the expected ratio is 25%. Since global Reck knockout mice die around E10.5, I decided to trace back and determine when Reck cKO (Foxg1) mice die.

After repeated mating and careful examination, I found that Reck cKO (Foxg1) mice were alive until the day of birth (P0; Figure 2A) but they all died shortly after birth exhibiting massive hemorrhage in the forebrain (Figure 3B).

I was also curious to know when and where the hemorrhage started. In most cases, the forebrain hemorrhage in Reck cKO (Foxg1) mice became visible by E12.5, but at E13.5 and later time points, all Reck cKO (Foxg1) mice showed clear hemorrhage in the forebrain (Figure 2B and 3F, H). Histological examination, on the other hand, revealed that the hemorrhage of the forebrain can be observed as early as E11.5 (Figure 2B). The hemorrhagic lesions were mainly found in ganglionic eminence (GE) in early stages (Figure 3H) but became more severe in the cerebral cortex (Cx) at later stages (Figure 3B).
Figure 2. Survival of, and hemorrhage in, Reck cKO (Foxg1) mice.

(A) Reck-cKO (Foxg1) mice were found at the Mendelian ratio (~25%) from E9.5 to P0 but not among the adult mice.

(B) The proportion of the mice with cerebral hemorrhage among the Reck-cKO (Foxg1) mice (n>26) at the indicated stage obtained after mating between Reckflex1/+;Foxg1-Cre mice and Reckflex1/flex1 mice. Note that from E13.5 to P0, all Reck-cKO (Foxg1) mice exhibited cerebral hemorrhage.

Previous data obtained from Dr. Almeida in our group indicated that selective inactivation of Reck in ECs leads to the late-stage embryonic lethality also with forebrain hemorrhage (Almeida et al. 2015). Hence, a critical question was whether Foxg1-Cre is expressed in ECs or not. To answer this question, I performed the immunofluorescence staining of the forebrain section of the Foxg1-Cre; mTmG reporter mice with the EC marker CD31 and mural cell/pericyte marker NG2.
Figure 3. Hemorrhage in the forebrains of Reck-cKO (Foxg1) mice at E13.5 and P0.
(A, B) Typical dorsal views of the brains from Reck-cKO (Foxg1) mice and control mice at P0.

(C, D) HE-stained coronal sections of the brains from Reck-cKO (Foxg1) mice (D) or control mice (C) at P0. Note the numerous microscopic hemorrhage in the Reck-cKO (Foxg1) mouse brains (black arrows in D) and larger ventricles (blue arrows in D) and smaller striatum (the area indicated by a blue dotted line) in the Reck-cKO (Foxg1) mouse at P0 (compare C to D).

(E, F) Lateral views, focusing on the head region, of Reck-cKO (Foxg1) (F) and control (E) embryos at E13.5. The typical red spot (arrow) above the eye, commonly found in Reck-cKO (Foxg1) embryos, corresponds to hemorrhage in GE.

(G, H) HE-stained coronal sections of the brains from Reck-cKO (Foxg1) mice (H) or control mice (G) at E13.5. Note the numerous microscopic hemorrhage in the Reck-cKO (Foxg1) mouse brains at GE (arrows in H). Scale bar: 500 μm.

The results indicate that Foxg1-Cre-positive cells are different from pericytes (mural cells) and ECs (Figure 4), which is consistent with the previous paper reporting that Foxg1-Cre is expressed in neuronal cells but not in vascular cells (Hellbach et al., 2014). These data strongly support the idea that the phenotype of Reck-cKO (Foxg1) mice results from the lack of RECK in NPCs rather than vascular cells.
Figure 4. *Foxg1-Cre* is not expressed in pericytes and ECs

Coronal sections of mTmG; *Foxg1-Cre* mice at E12.5 were immuno-stained (magenta) with antibodies against CD31 (EC marker, panel A) or NG2 (mural cell marker, panel B) followed by nuclear counterstain (blue). Note that magenta signals (vascular cells) and green signals (*Foxg1*-expressed cells) are essentially non-overlapped (A5 and B5). Scale bar: 50 μm.

3.2 Reck-cKO (*Foxg1*) embryos show vascular malformations

To better understand the mechanism of forebrain hemorrhage in Reck-cKO (*Foxg1*) mice, I first investigated the characteristics of the hemorrhage at multiple embryonic developmental stages, including E11.5, E12.5, and E13.5.

At E11.5, the hemorrhage was barely visible in the forebrain, but immunofluorescent staining with CD31 indicated that the vasculature in the forebrain had changed dramatically even at this early time point. The dense microvessels distributed in the cortex and GE, especially in the ventricular side in the control group. In the forebrain of cKO (*Foxg1*) mice, abnormal vessels with multiple layers of walls and large lumen were found in their GE, and the avascular zone was often found in the ventricular side of cortex and GE (Figure 5).

At E12.5, hemorrhage was readily visible in the forebrain of cKO (*Foxg1*) mice, and abnormal vessels with the features of glomeruloid vascular malformation (GVM) and those with large lumens were found together with the avascular zone and the zone of lower vessel density on the ventricular side in the GE and cortex (Figure 6).

At E13.5, very large GVM in the GE was found in cKO (*Foxg1*) mice (Figure 11B).

The integrity of vasculature can be disrupted in many ways, such as
disruption of the basement membrane and loss of pericytes or mural cells, which may lead to hemorrhage. To gain insights into the cause of hemorrhage, I investigated the vascular structure in the forebrain of mutant mice by immunohistochemical detection of relevant marker molecules.
Figure 5. Vascular phenotype of Reck-cKO (Foxg1) mice at E11.5

A coronal section of the brain from control (A, C and E) or Reck-cKO (Foxg1) mouse (B, D, and F) at E11.5 were double-stained with antibodies against CD31 (red) and NG2 (green) followed by nuclear counterstain (blue). Single-color images for CD31 (A, B) and NG2 (C, D), as well as three-color images (E, F), are shown. Note the abnormal vessels in GE (arrows in B) and that a line of regularly spaced small vessels, as found in the control mouse (arrowheads in A), are absent in Reck-cKO (Foxg1) mouse (arrowheads in B).

Scale bar: 200 μm
Figure 6. Vascular phenotype of Reck-cKO (Foxg1) mouse at E12.5

A coronal section of the brain from control or Reck-cKO (Foxg1) mouse at E12.5 was stained with anti-CD31 antibodies (red) followed by nuclear counterstain (blue). Note that typical GVMs are found in GE of Reck-cKO (Foxg1) mouse (arrows in B) and that a line of regularly spaced small vessels along the ventricular edge in the control mouse (arrows in A) are absent in Reck-cKO (Foxg1) mouse (arrowheads in B).

Scale bar: 200 μm

Figure 7. Characterization of abnormal vessels found in the cerebral cortex of Reck-cKO (Foxg1) mouse at E12.5

Coronal sections of the brain from control (A-D) and Reck-cKO (Foxg1) mice (E-H) at E12.5 were doubly stained with anti-CD31 (endothelial marker, green; panels C and G) and anti-Ki67 (proliferation marker, red; panels B and F) antibodies followed by nuclear counterstaining (blue; panels A and E). Merged images of the red, green, and blue fluorescence are also included (panels D and H). Note that CD31-positive vascular endothelial cells are largely Ki67-negative in control mice (arrowheads) while clusters of CD31-positive cells found in Reck-cKO (Foxg1) mice are often Ki67-positive (arrowheads), indicating proliferative nature of these cells.

Scale bar: 50 μm
Figure 8. Characterization of abnormal vessels found in the GE of Reck-cKO (Foxg1) mouse at E13.5

(A, B) Distribution of ECs and mural cells in vascular malformations found in Reck-cKO (Foxg1) mice. Coronal sections of the brain from Reck-cKO (Foxg1) mice at E13.5 were stained for endothelial marker (CD31, red; panel 2) and mesenchymal marker [SM22α (A, panel 3) or α-smooth muscle actin (αSMA; B, panel 3)], without (A) or with Ki67 (B, magenta, panel 4), followed by nuclear counterstaining (blue; panel 1). Note that the endothelial and mesenchymal signals are largely non-overlapped in vascular malformations in merged images (A4 and B5) and that Ki67-signals are associated with many CD31-positive cells (B6 and B7), again indicating the proliferative nature of these endothelial cell clusters.

(C) Distribution of basement membrane around the abnormal vessels found in Reck-cKO (Foxg1) mice. The brain sections were stained for the endothelial marker (CD31, red; panel 3) and two basement membrane markers, laminin (LN, green; panel 2) and type IV collagen (Col4, magenta; panel 4). Scale bar: 50 µm.
The multi-layered GVMs are accompanied by proliferative ECs as detected by Ki67/CD31 double-staining in the cortex at E12.5 (Figure 7), which become more prominent in the GE at E13.5 (Figure 8).

The pericytes marker NG2 was detectable in the less severe malformations at E11.5 (Figure 5D, F). The basement membrane-markers laminin and type IV collagen were also detectable around the abnormal vessels at E13.5 (Figure 8C).

Although I found abnormal distribution of the mural cell markers SM22α and αSMA in vascular malformations in the GE of cKO (Foxg1) mice, the cells positive for these markers were largely non-overlapped with the cells positive for the endothelial marker CD31, which does not support the idea that endothelial-to-mesenchymal transition (EndMT) is involved in these malformations (Figure 9).

Nevertheless, these findings indicate that Reck expressed in NPCs is critical for proper angiogenesis in the forebrain, especially in the GE and cortex.

### 3.3 Reck-cKO (Foxg1) embryos show precocious neuronal differentiation

As I initially intended, I also investigated the possible neuronal phenotype of cKO (Foxg1) mice. First, I performed immunofluorescent staining of sections of the forebrain of these mice at multiple development stages: E11.5, E12.5, and E13.5.
### E11.5

<table>
<thead>
<tr>
<th>Cortex</th>
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<tbody>
<tr>
<td>A</td>
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<td>SOX2</td>
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<td>KI67</td>
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<tr>
<td>CD31</td>
<td>CD31</td>
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<td>Nuc/KI67/CD31</td>
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### E12.5

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<td>Nuc/KI67/CD31</td>
<td>Nuc/KI67/CD31</td>
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Figure 9. The neural phenotype of Reck-cKO (Foxg1) mice at three embryonic stages

Coronal sections of the brains from control (panels 1-3) or Reck-cKO (Foxg1) mice (panels 4-6) at E11.5 (set [1]), E12.5 (set [2]), or E13.5 (set [3]) were doubly stained with anti-Sox2 (neural precursor marker, red; A, B, panels 1, 4)
and anti-TUJ1 (neuronal marker, green; A, B, panels 2, 5) antibodies or with anti-Ki67 (proliferation marker, red; C, D, panels 1, 4) and anti-CD31 (endothelial marker, green; C, D, panels 2, 5) antibodies followed by nuclear counterstain (blue). Merged images of the red, green, and blue fluorescence are also included (panels 3 and 6). Typical images focusing on the cerebral cortex (A, C) or GE (B, D) are shown. Symbols in [1]-[3]: asterisk, ventricle; arrowhead, signals of interest; bracket and double-pointed arrow, layer of positive cells, with some semi-quantitative information such as the thickness of the layer (length), intensity (line thickness), and sparseness (dotted line). Scale bar: 50 µm. The images in the set [3] A and B are at a lower magnification (x10 objective lens) than others (x20 objective lens). [4] Fluorescence density per unit area in images as shown in [1]-[3] was determined using ImageJ (see Methods for detail). The dot represents the density in one area, and brown horizontal bar the mean of 5 areas. The significance of difference is indicated by an asterisk(s): * P<0.05, ** P<0.01. Note that in Reck-cKO (Foxg1) mice, TUJ1-positive mature neurons tend to be more abundant and widely distributed while Ki67-positive cells are less abundant than the control at all three stages. At E13.5, decreased abundance of Sox2-positive cells becomes also apparent (bottom row, group 2 in A and B).
Figure 10. Typical precocious neuronal differentiation phenotype found in Reck-cKO (Foxg1) mouse at E12.5.

A coronal section of the brain from a control mouse (A) or Reck-cKO (Foxg1) mouse (B) at E12.5 was stained with anti-TUJ1 antibodies (green) followed by nuclear counterstain (blue). Note the expanded TUJ1-positive area in the Reck-cKO (Foxg1) mouse brain (B). Scale bar: 200 μm.

The reduced number and narrower distribution of the Ki67-positive proliferative cells and the Sox2-positive NPCs in GE, often accompanied by the increased number and widened distribution of TUJ1-positive differentiated neurons, were observed (Figure 9; Figure 10).

These results indicate that RECK in NPCs affects both neurogenesis and angiogenesis.

3.4 Reck cKO (Foxg1) and Reck cKO (Tie2) give rise to similar vascular phenotypes

I was curious to know whether we can find any difference in vascular phenotypes between NPC-selective Reck knockout mice [i.e., Reck-cKO (Foxg1)] and the EC-selective Reck knockout mice [i.e., Reck^flex1/flex1; Tie2-Cre], hereafter referred to as Reck cKO (Tie2). At E13.5, large GVMs are often found in the GE of both cKO (Foxg1) and cKO (Tie2) mice. Tissue damage at the central avascular zone in the GE and malformations in the cortex are also found in both lines (Figure 11).

In contrast, the abnormal distribution of TUJ1-positive neurons is not found in cKO (Tie2) mice (Figure 12), indicating that the RECK molecules in ECs and NPCs show different effects on neurogenesis.

Nevertheless, as far as the vascular phenotype is concerned, Reck-inactivation in NPCs and in ECs give rise to very similar vascular
abnormalities reminiscent of each other in terms of timing, location, and morphology.

Figure 11. Effects of Reck inactivation in NPCs and in ECs

(A, B) The vascular phenotype of Reck-cKO (Foxg1) mice at E13.5. A coronal section of the brain from control (A) or Reck-cKO (Foxg1) mouse (B) at E13.5 was fluorescently stained with anti-CD31 antibodies (red) followed by nuclear counterstain (blue). Note that large GVMs are found in GE of Reck-cKO (Foxg1) mouse (arrows in B) and that a line of regularly spaced small vessels along the ventricular edge found in the control mouse is absent in Reck-cKO (Foxg1) mouse (arrowheads in B). Instead, a few larger and irregularly located vessels are found in the cortex of mutant mice (arrowheads, B). Asterisk in B: tissue damage.

(C, D) The vascular phenotype of Reck-cKO (Tie2) mice at E13.5. A coronal section of the brain from control (C) or Reck-cKO (Tie2) mouse (D) at E13.5
was similarly stained. Note the phenotype very similar to that of Reck-cKO (Foxg1) mice, including large GVMs in GE (arrowheads in D), the absence of regularly spaced small vessels along the ventricular edge (compare C to D), and the tissue damage in the center of GE (asterisk in D). Scale bar: 200 μm.

Figure 12. Neural phenotype of Reck-cKO (Tie2) mouse at E13.5
A coronal section of the brain from control (A, B) or Reck-cKO (Tie2) mouse (C, D) at E13.5 was stained with anti-TUJ1 antibodies (neuronal marker, red) followed by nuclear counterstain (blue). Magnified views of the cortical area (dotted-line box) in panels A and C are shown in B and D, respectively. Note the less prominent TUJ1 signals (red) in the Reck-cKO (Tie2) mouse brain (panels C, D) than the control (panels A, B). Scale bar: 200 μm.
3.5 Altered gene expression in the forebrain of Reck-ckO (Foxg1) mouse

Previous studies indicated that the vascular phenotype of EC-selective Reck knockout mice could be attributed to the impairment of WNT7-triggered canonical WNT signaling (Cho et al., 2017). To gain insights into the mechanism by which Reck in NPCs affect the vascular development in the forebrain, I determined the levels of some mRNAs (relevant to brain angiogenesis and WNT signaling) expressed in the forebrain of these mice at three time points: E11.5, E12.5, and E13.5.

At E11.5, the NPC marker Nes was downregulated (Figure 13, group 5). At E12.5, the upregulation of Mmp2, Id3, and downregulation of Itgb8, Wnt7a, Apcdd1, and Sox17 were observed (Figure 13, group 3, 21, 15, 29, 38 and 41). Apcdd1 and Sox17 are authentic targets of canonical WNT signaling. At E13.5, upregulation of Mmp2, Vegfa, downregulation of Wnt7a, Wnt7b, Apcdd1, and Sox17 were detected (Figure 13, group 4, 27, 30, 33, 39 and 42).

Those results suggest that the levels of WNT7 ligands, as well as canonical WNT signaling, could be affected by the Reck deficiency in NPCs.

![Figure 13. Expression of genes involved in brain angiogenesis and canonical WNT signaling](image)

Changes in the levels of mRNAs encoding 15 proteins considered to be...
relevant to the vascular and/or neural phenotypes of Reck-cKO (Fogx1) mice and WNT/β-catenin signaling. Total RNA extracted from the forebrains of control mice and Reck-cKO (Fogx1) mice at E11.5, E12.5, and E13.5 were subjected to qRT-PCR using primer sets specifically amplifying indicated mRNAs and internal control (Gapdh or Mars). The level of mRNA relative to the control in each sample was calculated, and then the ratio of the values for Reck-cKO (Fogx1) mice and control mice is presented in dot plots with an open bar representing mean±SEM. The vertical axis is in the Log2 scale. *p<0.05, **p<0.01.

3.6 Vascular phenotype of Reck cKO (Fogx1) mice can be partially rescued by pharmacological treatment with LiCl

My data described so far strongly suggest the involvement of impaired canonical WNT signaling as a cause of vascular phenotype found in Reck-cKO (Fogx1) mice. If this is the case, then re-activation of WNT signaling might rescue the phenotype. After an extensive literature search, I chose to use a pharmacological approach to test this idea: daily injection of LiCl into pregnant mice followed by morphological examination of Reck cKO (Fogx1) embryos. LiCl is known to inhibit GSK-3β, thereby bypassing the ligand-receptor interactions and directly activating downstream canonical WNT signaling (Klein and Melton, 1996). The reagent has been used to demonstrate the involvement of canonical WNT signaling in a number of developmental processes (Briggs et al., 2016; Cambier et al., 2014; Cohen et al., 2009; Cohen et al., 2007; Cornett et al., 2013; Curtis and Griffin, 2012; Da Silva et al., 2017; Griffin et al., 2011; Kugimiya et al., 2007; Tian et al., 2010).

First of all, I confirmed that untreated Reck cKO (Fogx1) mice show conspicuous hemorrhage in both GE and Cortex at 100% penetrance at E15.5 (Table 3a; Figure 14),
Table 3. Suppression of forebrain hemorrhage by LiCl-administration

<table>
<thead>
<tr>
<th>Schedule for the daily administration of LiCl (200mg/kg/d, ip)</th>
<th>Number of embryos (hemorrhage at E15.5 / cKO embryos examined)</th>
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</thead>
<tbody>
<tr>
<td>a No treatment</td>
<td>GE 45/45 Cx 45/45</td>
</tr>
<tr>
<td>b</td>
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<td>e</td>
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<td>f</td>
<td>GE 3/3 Cx 3/3</td>
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ip, intraperitoneal injection; cKO, conditional knockout (Reck\textsuperscript{flex1/flex1}; Foxg1-Cre)

Figure 14. The vascular phenotype of Reck-cKO (Foxg1) mice at E15.5 after LiCl-treatment

Lateral (A, B) and dorsal (C, D) views, focusing on the head region, of Reck-cKO (Foxg1) embryos left untreated (A, C) or treated with LiCl daily from...
E10.5 to E14.5 (B, D) and harvested at E15.5. *, hemorrhage in the GE. #, hemorrhage in the cerebral cortex. Scale Bar: 1 mm.

Figure 15. Effects of LiCl on the vascular phenotype of Reck-cKO (Foxg1) mice

(A) The vascular phenotype of Reck-cKO (Foxg1) mice at E15.5 (untreated control). Panel 1: Lateral view of an intact, typical mutant embryo exhibiting cerebral hemorrhage (red arrow). Panel 2: an HE-stained coronal section with numerous hemorrhagic lesions (black arrows). Panel 3: magnified view of a cortical area shown by the dotted-line box in panel 2. Panel 4: a next section
subjected to immunofluorescence staining with anti-CD31 antibodies (red, vascular endothelial cells and some blood cells) followed by nuclear counterstain (blue). Panel 5: magnified view of a cortical area indicated by the dotted-line box in panel 4.

**(B, C)** Two Reck-cKO (Foxg1) embryos harvested at E15.5 from pregnant mice pretreated with a daily injection of LiCl from E10.5 to E14.5. Images in panels 1-5 were similarly acquired as those in A. Note that hemorrhagic lesions are greatly reduced in number and size in one case (C) and almost absent in the other case (B).

**(D)** A control embryo harvested at E15.5 from pregnant mice pretreated with a daily injection of LiCl from E10.5 to E14.5 (LiCl-treated control mouse). Scale bars: 200 μm.

After treatment with LiCl from E8.5 to E14.5, hemorrhage in the cortex could be rescue completely and the hemorrhage in GE also improved partially (Table 1, b). Then I tried to determine the minimum period of treatment required for the rescue by varying the treatment schedule (Table 3 and data not shown). The results indicate that the treatment from E10.5 to E14.5 was essential for the rescue. In most cases, the small hemorrhage was found in the GE but not in the cortex, which is in sharp contrast to the severe hemorrhage found in both GE and cortex in untreated mice. These results support the idea that RECK in NPCs supports forebrain angiogenesis by promoting canonical WNT signaling.

**3.7 RECK in NPCs can enhance contact-dependent activation of canonical WNT signaling in adjacent reporter cells**

Then the critical questions would be how RECK in NPCs promotes WNT signaling and whether this WNT signaling differs from the WNT7-triggered
WNT signaling known to be promoted by the cell-autonomous action of RECK in ECs. To address these questions, I attempted to utilize the luciferase reporter assay (called TOP-Flash assay) widely used to detect, and quantify the intensity of, canonical WNT signaling.

Prior to this study, Dr. Matsuzaki and Dr. Noda established RECK-deficient HEK293 cell lines, and Mr. Tamura and Dr. Noda used one of these lines (named ADA99-25) to establish RECK-deficient TOP-Flash reporter cells (named HNM1). Dr. Noda also established HNM1 stably expressing four WNT7 receptor components, FZD4, LRP5, GPR124, and RECK (named LC20b).

I prepared the NPCs from the forebrains of cKO (Foxg1) mice and control mice at E11.5 following Tomooka’s method (Kitani et al., 1991). These NPCs and their conditioned media were overlaid onto the WNT7-selective TOP-Flash reporter cells (LC20b), incubated for 24 h, followed by luciferase assay to detect canonical WNT signaling activity. First of all, NPCs could activate the WNT signaling in the reporter cells when co-cultured, but the conditioned media failed to do so (Figure 16B), indicating that contact-dependent, WNT7-mediated activation of WNT signaling can be detected in this assay. Importantly, when the same number of cells were applied, the NPCs derived from cKO (Foxg1) mice tend to show lower activity as compared to the control NPCs in this assay (Figure 16). These results suggest that RECK in NPCs may somehow increase or facilitate WNT7-triggered canonical WNT signaling in adjacent cells.

In addition, Dr. Almeida attempted to test the effects of forebrain tissue explants to stimulate vascular sprouting in aortic ring assay, but no clear difference between the two groups could be detected (data not shown).

Taken together, these data seem to better support the idea that it is the efficiency of contact-dependent WNT signaling affected by RECK.
Figure 16. The ability of NPCs and their conditioned media to activate canonical WNT signaling in the adjacent reporter cells.

(A) The ability of NPCs to activate canonical WNT signaling in the reporter cells in mixed culture. NPCs prepared from the forebrains of control embryos (n=4) or Reck-cKO (Foxg1) embryos (n=6) were co-cultured for 24 h with WNT7-selective TOP-Flash reporter cells followed by dual-luciferase assay. Horizontal lines in each group represent mean (red) and SEM (blue). *P<0.05. Note that NPCs from Reck-cKO (Foxg1) mice show significantly lower activity to stimulate WNT signaling in the reporter cells.

(B) The ability of NPCs and their conditioned media to activate canonical WNT signaling in the reporter cells. NPCs and their conditioned media were subjected to TOP-Flash assay as described in A. Note that the activity of conditioned media was very low in this assay, indicating that the activity of NPCs to stimulate canonical WNT signaling in the reporter cells is contact-dependent.
3.8 RECK in WNT7-producing cells can enhance contact-dependent activation of canonical WNT signaling in adjacent reporter cells

**Figure 17.** The effect of RECK in the reporter cells or producer cells to active canonical WNT signaling.

(Group 1-6) The effect of RECK in the reporter cells on, and contact-dependence of, the WNT7-triggered canonical WNT signaling. HEK293 cells producing WNT7A (groups 1, 2) or WNT7B (groups 3, 4) or their conditioned media (groups 5, 6) were added to the RECK-deficient TOP-Flash reporter cells transfected with either vacant vector (groups 1, 3) or RECK-expression vector (groups 2, 4-6). After a 24-h incubation, the dual-luciferase assay was performed. **P<0.003.** Note that RECK in the reporter cells greatly enhances WNT signaling in this co-culture assay (groups 2, 4) and that the stimulation could not be achieved with conditioned media.
(groups 5, 6), demonstrating a cell-autonomous effect of RECK to enhance the contact-dependent, WNT7-stimulated WNT signaling.

**Group 7-10** The effect of RECK in WNT7-producing HEK293 cells to stimulate WNT signaling in the reporter cells in mixed culture. RECK-deficient HEK293 cells stably expressing WNT7A (groups 7, 8) or WNT7B (groups 9, 10) were transfected with either control vector (V) or RECK-expression vector (R), incubated for 48 h, and then co-cultured for 24 h with TOP-Flash reporter cells lacking RECK, followed by dual-luciferase assay (in triplicate). *P<0.05. Note that RECK in WNT7-producing cells has an ability to enhance (1.6-1.7 fold) WNT signaling in the RECK-null reporter cells (i.e., non-cell-autonomous effect). The experiments were repeated three times with similar results.

To gain more insights into how RECK could facilitate WNT signaling, Dr. Noda performed a series of experiments using HEK293 model system. First, he established the RECK-deficient HEK293 cells (ADA99-25) stably expressing WNT7A or WNT7B. He then transfected a control vector or the vector expressing RECK into these cells and subjected these WNT7 producing cells (Producer) to the WNT7-selective TOP-Flash assay in co-culture with the reporter cells without or with RECK expression (Reporter) (Figure 17). When RECK was expressed in Reporter, the Producer could induce robust WNT signaling, demonstrating the cell-autonomous activity of RECK to promote WNT signaling (Figure 17, groups 2 and 4). Importantly, when RECK was absent in Reporter, RECK in Producer shows a small but significant effect to increase the WNT signaling in co-cultured Reporter (Figure 17, groups 8 and 10), demonstrating that RECK in ligand-producing cells may enhance the contact-dependent activation of WNT signaling in target cells in a non-cell-autonomous fashion.

GPR124 is known to be expressed in brain endothelial cells and cooperates with RECK to promote WNT7-signaling in a cell-autonomous fashion (Vanhollebeke et al., 2015). In contrast, GPR124 in Producer does not
cooperate with RECK in enhancing Wnt signaling in this assay (Figure 18, groups 3, 4, 7, 8).

These results support the idea that RECK in ligand producing cells (e.g., NPCs) may facilitate contact-dependent WNT7-signaling in target cells (e.g., ECs), that such effect does not require GPR124 in ligand producing cells.

Figure 18. Effects of RECK and GPR124 in WNT7-producing HEK293 cells on WNT signaling in the reporter cells in mixed culture

RECK-deficient HEK293 cells stably expressing WNT7A (groups 1-4) or WNT7B (groups 5-8) were transfected with either control vector (V), RECK-expression vector (R), GPR124-expression vector (G), or both expression vectors (R+G), incubated for 48 h, and then co-cultured for 24 h with TOP-Flash reporter cells lacking RECK, followed by dual-luciferase assay (in triplicate). The cells without WNT7-expression (groups 9, 10) and wild type NPCs (group 11) were also included for comparison. *P<0.05, **P<0.01. Note
that GPR124 suppressed, rather than enhanced, the activity of RECK to enhance WNT signaling in adjacent reporter cells. The experiments were repeated three times with similar results.
Chapter 4. Discussion

During the course of this study, a series papers have been published demonstrating that RECK in ECs is required for vascular development in mouse CNS and that RECK and GPR124 in ECs cooperate to enhance WNT7A/B-mediated canonical WNT signaling (Almeida et al., 2015; Cho et al., 2017; Eubelen et al., 2018; Ulrich et al., 2016; Vallon et al., 2018; Vanhollebeke et al., 2015). My study using tissue-specific knockout mice has revealed that RECK in NPCs is also essential for proper angiogenesis in the forebrain. I also attempted to gain insights into the mechanisms by which RECK in NPCs supports brain angiogenesis and made several interesting observations. First, I found similarity in the phenotype between Reck ckO (Foxg1) mice and Reck cKO (Tie2) mice, as described here (Figure 11), as well as Wnt7a/7b double knockout mice and EC-selective β-catenin knockout mice, as described in the literature (Daneman et al., 2009; Liebner et al., 2008; Stenman et al., 2008). Second, I found the effect of LiCl, an activator of canonical WNT signaling, to partially rescue the vascular phenotype of Reck cKO (Foxg1) mice. Third, I detected the downregulation of two targets of WNT signaling, Apcdd1 and Sox17, in the forebrain of Reck cKO (Foxg1) mice. Fourth, I with the help of Dr. Noda found that NPCs derived from cKO (Foxg1) mice show reduced activity in a WNT7-selective TOP-Flash assay in co-culture. Taken together, these findings support the idea that RECK in NPCs facilitates the cell-contact-dependent stimulation of WNT7 signaling in ECs in a non-cell-autonomous fashion.

Direct interaction between RECK and WNT7 has recently been demonstrated (Eubelen et al., 2018; Vallon et al., 2018). In their models, however, the fact that RECK forms dimer (Omura et al., 2009) was ignored. Of note, the configuration of dimeric Wnt3a-Fzd8-Lrp6 complex has recently been solved by Takagi’s group (Hirai et al., 2019). Vallon et al. have discovered the
activity of RECK to bind and stabilize active forms of WNT7A/B (lipid-modified monomers) that tend to quickly form inactive aggregates in the absence of RECK (Vallon et al., 2018). Taking these new findings into consideration, I propose a feasible model to explain my observations (Figure 19). The RECK dimer expressed by WNT7-producing cells (such as NPCs) holds and presents bioactive ligands (WNT7A/B) on the cell surface; this facilitates the transfer of short-lived ligands from the producer cells to the target cells (such as ECs). This model may provide a fresh insight into the hand-over problem in the intercellular WNT transport process (Routledge and Scholpp, 2019). To gain further insight into the role of RECK in brain angiogenesis and WNT7 signaling, knowledge on the 3D structures of the RECK forming complex with GPR124 and/or WNT7A/B should be useful.

Figure 19. A hypothetical model to explain how RECK on WNT7-producing cells (e.g., NPC) could enhance contact-dependent WNT7 signaling in adjacent cells (e.g., EC). The WNT7 receptor complex contains RECK, GPR124, FZD4, and LRP5; the
direct interaction between WNT7 and RECK has been demonstrated (Eubelen et al., 2018; Vallon et al., 2018). Since RECK is GPI-anchored and forms cowbell-shaped dimer (Omura et al., 2009), it may serve as a divalent docking site for WNT7A/B on the cell surface. This model proposes that RECK might work in this way on both ligand-producing (upper side) and signal-receiving (lower side) cells, and the latter facilitates ligand-transfer from one cell to the other. Ligand-transfer may also be facilitated by some other, more indirect mechanisms, such as protection of WNT7A/B from proteolysis and stabilization of cell-adhesion molecules to bring the cell surfaces of two cells closer to each other. Note that the 3D structures of RECK and GPR124 have not been solved so that the exact configuration among RECK, GPR124, and WNT7A/B is tentative. The configuration of dimeric WNT-FDZ-LRP complex is based on the crystal structure of the Wnt3a-Fzd8-Lrp6 complex recently reported by Takagi’s group (Hirai et al., 2019).

The gene expression data indicate the upregulation of Mmp2 in the forebrains of Reck-cKO (Foxg1) embryos (Figure 13, groups 3, 4). Although the relevance of this phenomenon to the mutant phenotype is unclear, the upregulation of Mmp2 mRNA was also observed in Gpr124 knockout mice (Cullen et al., 2011). The downregulation of the NPC marker Nes at E11.5 (Figure 13, group 5) may reflect the precocious neuronal differentiation (Figure 10), and yet upregulation of Tubb3/Tuj1 was not so prominent. With regards to the neuronal differentiation, the ID family is also of interest, since Id1/Id3 double knockout mice die by E13.5 with brain hemorrhage and precocious neuronal differentiation (Lyden et al., 1999), which is similar in nature to, but different in timing of death from, the Reck mutant mice (Almeida et al., 2015; Chandana et al., 2010b; Muraguchi et al., 2007; Oh et al., 2001). However, it is unclear whether the observed upregulation (rather than downregulation) of Id3 at E12.5 (Figure 13, group 21) contributes to the phenotype of Reck-cKO (Foxg1) mice.
Decreased expression of Apcdd1 and Sox17 (Figure 13, groups 37-42) are indicative of attenuated WNT/β-catenin signaling in the forebrain of Reck-cKO (Foxg1) mice. Since the products of these WNT-inducible genes are known to inhibit WNT/β-catenin signaling, their roles as negative feedback regulators have been postulated. Known properties and biological functions of these proteins, however, are intriguing in the present context. For instance, APCDD1 is a membrane-bound, cysteine-rich glycoprotein expressed in multiple tissues (including the nervous and vascular system as well as mesenchyme of several developing organs); it interacts with WNT3A and LRP5 and possibly prevents WNT-FZD binding (Cruciat and Niehrs, 2012; Shimomura et al., 2010). Hence, APCDD1 shares many similarities with RECK except that it inhibits (rather than promotes) Wnt/β-catenin signaling. Importantly, APCDD1 was found to coordinate vascular remodeling and barrier maturation of retinal blood vessels (Mazzoni et al., 2017). On the other hand, SOX17 is a transcription factor that forms a complex with β-catenin as well as several TCF/LEF family transcription factors and promotes their degradation (Sinner et al., 2007; Zorn et al., 1999). Of note, Sox17 bridges WNT and NOTCH signaling to promote arterial specification (Corada et al., 2013; Morini and Dejana, 2014) and blood-brain barrier maturation (Corada et al., 2019). Contribution of the reduction of these proteins to the vascular and/or neural phenotype of Reck-cKO (Foxg1) mice is an interesting possibility to be tested in future studies.

Wnt7a, Wnt7b, and Itgb8 are known to be critical for CNS angiogenesis (Daneman et al., 2009; Proctor et al., 2005; Stenman et al., 2008; Zhu et al., 2002). Their downregulation from E12.5 or E13.5 in Reck-cKO (Foxg1) mice (Figure 13, groups 29, 30, 33, 15) may also a contribution to the progression of vascular phenotype in these mice, although the mechanisms by which these genes get downregulated remain unclear. The Vegfa upregulation found at E13.5 could be a consequence of hypoxia due to the halted vascularization, especially in the bulky tissues such as the GE. The tissue damage at the center of GE (Figure 11, asterisk) seems to support this idea. Since VEGF
overexpression has been implicated in GVM (Sundberg et al., 2001) and intracranial hemorrhage (Yang et al., 2013), Vegfa upregulation may also contribute to the progressive nature of vascular defects in these mice.

In summary, I found that RECK on both endothelial and neuroepithelial components are essential for proper forebrain angiogenesis in mice. My findings raise an interesting possibility that RECK mediates neurovascular communication by facilitating contact-dependent WNT signaling triggered by the short-range ligands WNT7A/B.
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