

**The tumor suppressor Reck is critical for vascular
patterning and stabilization in mice**

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Abbreviations

ARA: aortic ring assay

Ang1: Angopoetin -1

CCM1: encodes Krev interaction trapped protein 1

CCM2: encodes malcavernin protein

CD31: cluster of differentiation 31

Cre: recombinase enzyme

ECM: extracellular matrix

ECs: endothelial cells

FN: fibronectin

GLUT-1 (glucose transporter 1)

HIF-a: Hypoxia induction factor

IL-8: Interleukin 8

JAM-B: Junctional adhesion molecule B

JAM-C: Junctional adhesion molecule C

LDH-A (lactate dehydrogenase-A),

Lama5: Laminin α 5

MMP: matrix metalloproteinase

MMP: metalloproteinase

MT1-MMP: membrane type 1-matrix metalloproteinase

PDGF: platelet-derived growth factor

PDGF $\beta\beta$: platelet-derived growth factor receptor Beta

PFKFB3 (6-phosphofructose-2 kinase/fructose-2,6-bisphosphatase-3)

Reck: reversion-inducing cystein-rich protein with kazal motifs

RhoA: Ras homolog gene family, member A

Rock: Rho-associated protein kinase

SIRT-1: silent mating type information regulation homologous

Sma: α -actin smooth muscle

Tie2: Tyrosine Kinase receptor

VEGF: vascular endothelial factor

Wnt: Wingless-related integration site

cKO: conditional knockout

vBM: vascular basement membrane

Abstract

Angiogenesis is essential for embryonic development as well as for a variety of physiological processes at postnatal stages, such as wound healing, blastocyst implantation, and menstrual cycle. *RECK* is a tumor suppressor frequently downregulated in a wide variety of cancers; it encodes a membrane-anchored matrix-metalloproteinase-regulator and regulates cell migration, invasion, and metastasis. Exact molecular mechanisms behind these biological effects, however, remain largely unknown. Mice deficient in *Reck* die *in utero*, demonstrating its essentiality for proper embryogenesis; however, the underlying causes of mid-gestation lethality remained unclear. I used *Reck* conditional knockout mice to address this question and found that the loss of *Reck* expression in vascular mural cells or mesenchymal-derived cells leads to mid-gestation lethality. Using cultured aortic explants (aortic ring assay), I found that *Reck* is essential for at least two events during sprouting angiogenesis; (1) correct association of mural and endothelial tip cells to the microvessels and (2) maintenance of fibronectin matrix surrounding the vessels. These findings demonstrate the importance of appropriate cell-cell interactions and extracellular matrix maintenance for angiogenesis and the involvement of *Reck* as a critical regulator of these events in sprouting angiogenesis.

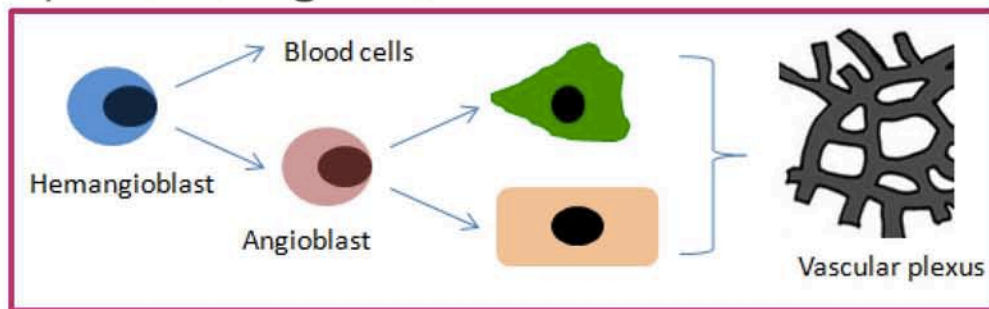
1. Introduction

1.1 Angiogenesis

Blood vessel formation is a relatively early event in mammalian embryogenesis. It begins with the formation of vascular plexus originated from haemangioblasts in the processes termed vasculogenesis (Figure 1a) ¹⁻³. The expansion of primary vessels throughout embryo tissue is known as angiogenesis. It originates from progenitor, stem or quiescent endothelial cells ³⁻⁵ by sprouting, pruning or intussusceptions (Figure 1b).

Blood vessels formation

a) Vasculogenesis



(Carmeliet, P. 2003; Munoz-Chapuli, 2009)

b) Angiogenesis



(Munoz-Chapuli, 2009; Chung and Ferrara, 2011)

Figure1. Biological processes involved in blood vessels formation. (a) Vasculogenesis give a rise to primary vascular plexus through the differentiation of multipotent progenitor cells, Hemangioblast. (b) Angiogenesis, the process by which new blood vessels are formed from preexisting ones. The cartoon illustrated three different angiogenic processes.

Angiogenesis ensures ovary cycling, placental formation, trophoblast implantation, embryonic development, wound healing⁶, and support organ homeostasis by delivering nutrition and cleaning metabolic residue ⁴. The imbalance between angiogenic stimulators and inhibitors acting on endothelial cells or/and peri-endothelial cells (also called mural cells which include smooth muscle cells and pericytes) triggers variety of diseases. Excessive angiogenic stimulators are associated with pathological events/conditions such as cancer metastasis, ocular disorders, asthma, diabetes, cirrhosis, and endometrioses, while reduced angiogenic stimulators are found in aneurysm, ischaemic heart disease, and neurodegeneration ⁴. Despite the ample studies on the roles of diffusible factors, such as cytokines, proteases, and angiogenesis inhibitors, in vascular development, the contributions of extracellular matrix to this process remain understudied ⁷.

1.2 Roles of MMPs in angiogenesis

The matrix metalloproteinases (MMPs) are a family of proteolytic enzymes critical for ECM remodeling and processing during development and physiological events in adult tissues. As a zinc-dependent endopeptidases, MMPs targets collagen, FN, laminin, cytokines and cell surface receptors to affect cellular behaviors. Expressed by endothelial cells and stromal cells, MMPs support morphogenesis and ensure successful angiogenesis ^{5,8-10}.

MMPs often modify ECM components rather than remove or degrade them. This

modification helps the cells to sense the environmental cues, for example, by cleaving and exposing cryptic cell-binding domains in fibrillar collagen ^{11,12}. Indeed, endothelial cells are capable of invading 3D collagen gel by proteolytically cleaving type I collagen. As a result, ECs migrate and populate the surrounds by guiding and generating new vessels. In this context, MMPs are important not only to promote migration, but also to drive cell invasion, morphogenesis, and perhaps cell survival ^{13,14}.

Although MMP family is composed of over 20 members, a few of them have angiogenic functions. For instance, initial angiogenesis is mainly dependent on MT1-MMP *in vitro* ¹³⁻¹⁵. Endothelial cells, culture in 3D collagen and fibrin matrices, produce MMPs to induce migration through VEGF/VEGFR2 signaling activation. This pathway triggers the endothelial cell-junctions detachment, basement membrane degradation, and collagenolysis. In order to create space in the gel and guide the tube growth, endothelial cells require MT1-MMP secretion. But when, this mechanism is inhibited by chemicals, such as GM600, endothelial cell sprouts are completely abrogated in ARA ¹⁵ and other 3D cultures ^{16,17}, suggesting this MMP as essential player in sprouting angiogenesis at early stages.

Accumulated evidence indicates the requirement of MMPs for several events during angiogenesis. Endothelial cell tube formation and vascular guidance are promoted primarily by MMP-1, MMP-10, MMP-2, MMP-9, and MT1-MMP ^{15,18-20}. Although the expression of these MMPs in endothelial cells is essential for normal vessels morphogenesis, their excessive expression leads to abnormal tube morphology, larger width, and earlier vessels regression ^{17,21}.

Considerable advances in the understanding of molecular mechanisms of EC tube and lumen formation have been achieved using *in vitro* models ^{22,23}, as well *in vivo*

models ²²⁻²⁶. Vascular morphogenesis depends on complex signaling. At upstream of lumen and tube formation, endothelial cells require JAM-B, JAM-C, MT1-MMP, and integrin $\alpha 2\beta 1$ (collagen receptor), integrin $\alpha 5\beta 1$, and integrin $\alpha v\beta 1$ (fibrin receptor). This complex activates PKC pathway which has as downstream EKR1/2 capable of inhibiting RhoA and ROCK ^{13,16}. This inactivation triggers cytoskeletal reorganization and cell alignment through specific control of transcription factor genes, such as CCM2 and CCM1, known to control tubulogenesis and vascular development ^{13,16}.

In contrast to the predominant MMP activities during early angiogenic events, later events require less or no MMP expression. After vessels have acquired tubular structure, endothelial cells need to suppress MMP activities to maintain the structure. Another pathway that endothelial cells regulate MMPs is through pericytes recruitment ^{17,18}. These mural cells not only protect endothelial cells from MMPs but also help deposit ECM components essential for the assembly of basement membrane, such as laminin, fibronectin, collagen type IV, nidogens, and perlecan ¹⁸.

1.3 RECK

RECK (Reversion-inducing cystein-rich protein with kazal motifs) is a GPI-anchored glycoprotein initially found as a cDNA clone that induced phenotypic reversion in fibroblast cells transformed by the *v-Ki-ras* oncogene ^{27,28}. RECK is downregulated in many types of human cancer and the levels of residual RECK expression in tumors correlate with better prognoses ^{29,30}. Although RECK has been studied as a molecule capable of suppressing malignant cellular behaviors of cancer cells, the molecule is also of interest in terms of embryogenesis, since Reck inactivation in mice results in mid-gestation lethality ³¹.

1.3.1 RECK at the molecular level

RECK is composed of 971 amino acids, 9% of which are cysteine; the N-terminal and C-terminal regions are both hydrophobic, whereas the middle region contains three serine protease inhibitor-like motifs, of which the first domain is a complete Kazal motif (CX₇-C-X₆-Y-X₃-C-X_{2,3}-C)²⁷(Figure 2a). *RECK* is conserved as a single gene from insect to mammals. The human *RECK* gene is located on the chromosomal bands 9p13-p12 and consists of 23 exons ³². The RECK protein forms a cowbell-shaped dimer (Figure 2b) and negatively regulates several members of the matrix metalloproteinase (MMP), family such as MMP2, MMP7, MMP9, and MMP14/MT1-MMP ^{27,31,33,34}, as well as other metalloproteinases such as ADAM10 and CD13 ^{33,35}. Importance of glycosylation for RECK's bioactivities has also been demonstrated ³⁶.

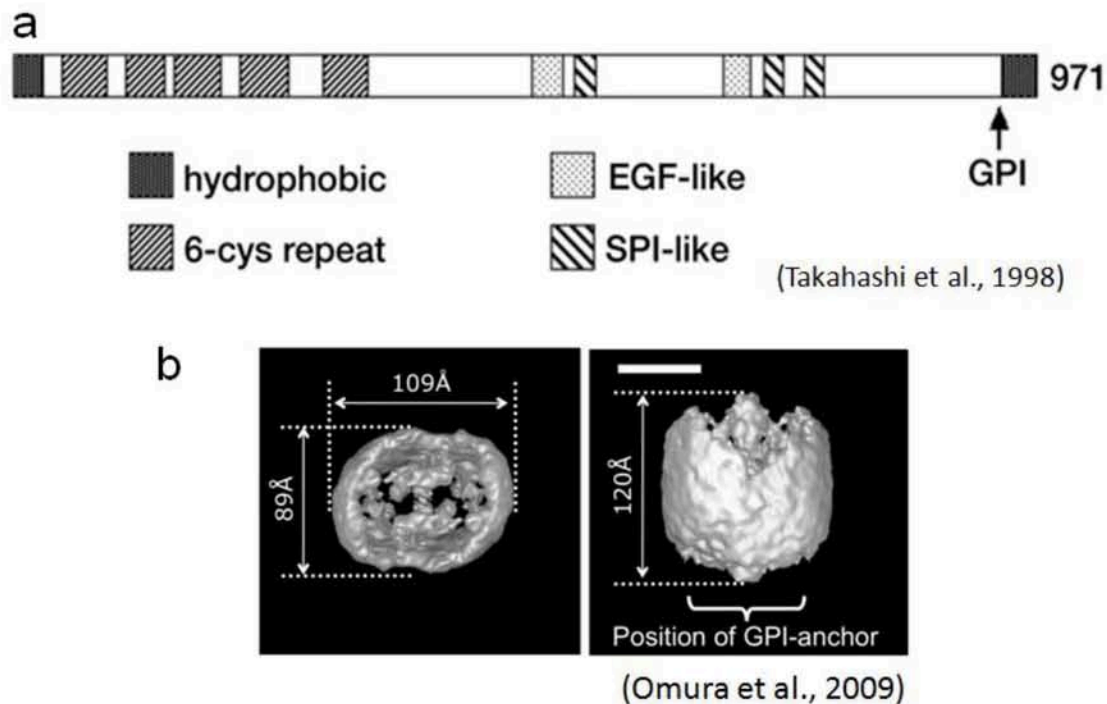


Figure 2. Structure of Reck protein. (a) The composition of the protein and its main domains. (b) The

reconstitute 3D of thousand electron microscopy image (TEM) showing that Reck protein has a Cowbell-Shaped like dimer.

Reck expression is downregulated by several oncogenes, including *ras*^{27,37}, as well as various environmental cues: for instance, ethanol, cadmium, low oxygen, cell sparsity, and growth factors³⁸. RECK promoter region has two Sp1-binding motifs, cEBPB, and CAAT box; the Sp1 sites have been implicated in transcriptional downregulation of RECK by *ras* and HER-2/neu^{37,39}. Reck transcription was also reported to be affected by histone deacetylase-1 and phosphorylation of Sp1 at Thr453 and Thr739⁴⁰.

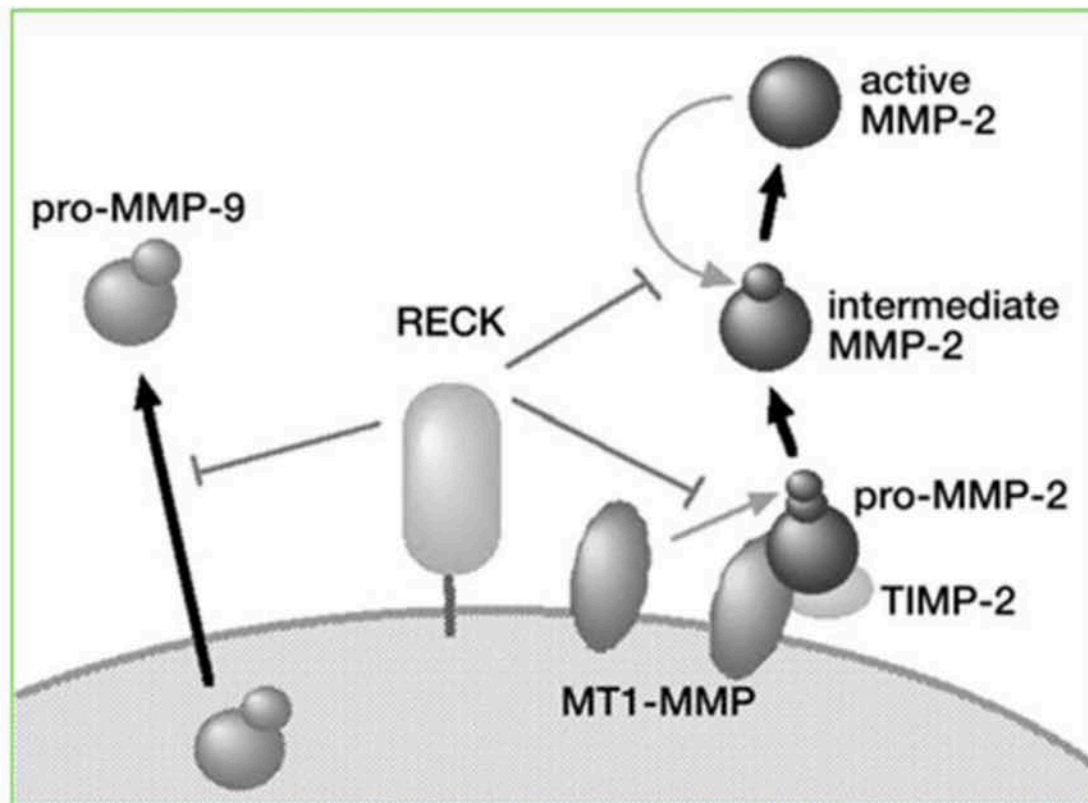
Frequency of RECK promoter methylation is increased in colon cancers ($n = 12$), and colon cancer cell lines treated with 5'-azacytidine resulted in suppression of invasive activity with concomitant upregulation of RECK⁴¹, suggesting involvement of promoter methylation in *RECK* downregulation in colon cancer. In a non-biased, genome-wide methylation study, Hill and colleagues detected RECK as one of several loci whose methylation showed significant inverse correlation with relapse-free survival among breast cancer patients⁴², providing compelling evidence for the significance of not only *RECK* promoter methylation but also *RECK* downregulation itself in breast cancer prognosis.

1.3.2. Reck at the cellular level

Reck is abundantly expressed in mesenchymal cells. Fibroblasts without Reck expression show reduced cell-substrate adhesion, elevated pericellular proteolysis, ambiguous polarization, high speed and low directional persistence in migration⁴³. The mutant cell phenotype was partially rescued by fibronectin (FN), suggesting that Reck

stabilizes focal adhesion by protecting ECM components. Although RECK expression is low in normal epithelial cells, it is induced after epithelial-mesenchymal transition (EMT). This properties is compromised in carcinoma-derived cells, and forced RECK expression results in suppression of malignant behaviors of cancer cells after EMT⁴⁴.

Acute expression of RECK in a colorectal cell line resulted in G1 arrest and senescence-associated phenotype, including SA- β -gal, SKP2 downregulation, and p27 upregulation. The cells tended to show similar molecular changes when plated on type I collagen, suggesting that RECK protects collagen from degradation thereby inducing these changes⁴⁵. Thus, RECK is capable of regulating cell cycle as well as cell migration.



(Takahashi et al., 1998; Oh et al., 2001; Noda and Takahashi , 2007)

Figure 3. Reck at cellular level. The cartoon illustrates the regulation of MMPs by Reck. Anchored to cell membrane, Reck can interact with MMP-9, MT1-MMP, and MMP-2, in both active and pro-active forms.

1.3.3 Reck at the tissue and systemic levels

RECK is essential for mouse embryogenesis playing roles in the development of the vascular system ^{31,46}, CNS ³⁵, and limbs ⁴⁷. It is also required for proper development of brain vasculature and dorsal root ganglia (DRG) in zebra fish^{48,49}.

RECK expression is found in a wide variety of normal organs but frequently downregulated in various types of cancers. Higher levels of residual RECK expression in tumors correlate with better prognoses. Forced expression of RECK in cancer cells suppresses their malignant phenotypes, such as tumor angiogenesis, invasion, and metastasis in xenograft models ³¹ and their aberrant migratory behaviors in culture ^{43,44}. In hepatocellular carcinoma, balance between RECK expression in tumor tissue and surrounding non-tumorous tissues seem to be an informative prognostic indicator ⁵⁰. A number of other studies indicate reduced expression of RECK in cancer cells and reduced malignancy of cancer cells upon restored RECK expression ³⁰.

1.4 Aims of this study and my contribution

We tried to address the following two questions in this study:

- 1) What is the exact reason for the embryonic death of *Reck*-deficient mice?
- 2) What is (are) the exact role(s) of Reck in angiogenesis?

To address the first question, we generated tissue-specific conditional Reck knockout mice, in which collaboration with Dr. Mako Yamamoto was essential. To address the second question, I optimized the conditions for a technique called aortic ring assay (ARA) using aortae from mice of the ICR background; this assay was originally developed for rat aortae ⁵¹ and later adapted to mouse aortae ⁵². This assay allowed me to study the process of angiogenic sprouting in detail *in vitro* and to find new roles of Reck in this process.

Material and Methods

Preparation of transgenic mice

Animal experiments were approved by Animal Experimentation Committee, Kyoto University and conducted in accordance with its regulation. Generation and genotyping of mice carrying the *Reck*^{CrER} (also known as *Reck-CreER*^{T2} or *KI*), *Reck*^{E1fx} (also known as *R1*), *Reck*^Δ, or *Reck*^{Low} (also known as *R2neo*) allele has been described by Yamamoto et al.⁴⁷. To evaluate the roles of *Reck* in mural cells, *Reck*^{+Δ} mice were crossed with mice carrying *Sm22-Cre* transgene⁵³ to obtain *Reck*^{+Δ};*Sm22-Cre* male mice, which were then crossed with *Reck*^{E1fx/E1fx} female mice, and their pups harvested at various stages for genotyping and morphological studies.

To evaluate the roles of *Reck* in endothelial cells, *Reck*^{+Δ} mice were crossed with *Tie2-Cre* transgenic mice⁵⁴ to obtain *Reck*^{+Δ};*Tie2-Cre* male mice, which were crossed with *Reck*^{E1fx/E1fx} female mice, and their pups examined at various stages. To visualize cells expressing *Tie2*, *Sm22*, or *Reck*, female mice homozygous for the allele containing the *mTmG* transgene⁵⁵ were crossed with male mice carrying the *Tie2-Cre*, *Sm22-Cre*, or *Reck*^{CrER} transgene to obtain pups carrying both the *mTmG* and Cre transgenes.

To test the roles of *Reck* in microvessel formation *in vitro*, *Reck*^{+CrER};*mTmG/mTmG* male mice were crossed with *Reck*^{E1fx/E1fx} female mice (both in ICR background), and *Reck*^{E1fx/CrER};*mTmG* mice (*Reck* cKO) were selected. To generate a matched control, *Reck*^{+CrER} male mice were crossed with *mTmG/mTmG* female mice, and *Reck*^{+CrER};*mTmG* mice (Cont) were selected. Mice carrying the *Reck*^{CrER} transgene were treated four times by daily intra-peritoneal injections of tamoxifen (80 mg/kg), and then two days later, aortae were harvested for ARA.

Histology analyses

Mouse embryos were fixed, sliced (10 μ m-thick), and stained with Hematoxylin and Eosin⁴⁷, by immunohistochemistry with anti-CD31, anti- α SMA, or anti-laminin⁴⁶, or by Kluver-Barrera method⁵⁶ as described previously. Tissues from mice carrying the mTmG reporter were fixed, incubated overnight in 30% sucrose, embedded in O.C.T, frozen at -80°C, sliced (5 μ m-thick), and observed with a fluorescent microscope.

Aortic ring assay

ARA was performed following the protocol of Baker et al.⁵², with some modifications. The following conditions, optimized as shown in Figure 2, were used unless otherwise stated. Thoracic aortae were dissected from 5-week old transgenic mice after overnight starvation. The peri-aortic fibroadipose tissue and blood were carefully removed with fine microdissecting forceps, and aorta tunics were preserved without damage.

After cleaning, aortae were cut into 1 mm-thick rings, rinsed 3 times with PBS (-), and incubated overnight in Opti-MEM at 37°C. The rings were then embedded in a mound of gel prepared on ice by mixing 5 volumes of 3 mg/ml collagen type I-A (Nitta Gelatin, Osaka, Japan), 3 volumes of 3 mg/ml collagen type IV (Nitta Gelatin, Osaka, Japan), 1 volume of 10 $\times$ DMEM, and 1 volume of reconstitution buffer (2.2 g NaHCO₃ in 100ml of 0.05 N NaOH and 200 mM HEPES). For embedding, 50 μ l of the collagen mixture was placed into wells of 96-well plates and incubated at 37°C for 10 minutes to form a basal gel. Aortic rings were placed on the basal gel and covered with 10 μ l of cold collagen mixture. After 20-min incubation at 37°C, each well was fed with 200 μ l Opti-MEM (Invitrogen) supplemented with 20 ng/ml VEGFa (R & D systems), 5% fetal horse serum (Cell Culture Laboratories, Ohio, USA), 100 U/ml penicillin and 100

$\mu\text{g/ml}$ streptomycin. The dishes were tightly sealed with parafilm, kept 37°C for two weeks, and examined every day under an inverted microscope. Micrographs were taken every day up to day 14 for morphometric analyses. In some experiments (Figure 7), bovine plasma fibronectin (Sigma, F1141) was added to the gel at $1 \mu\text{g/ml}$.

Aortic Ring Assay (ARA)

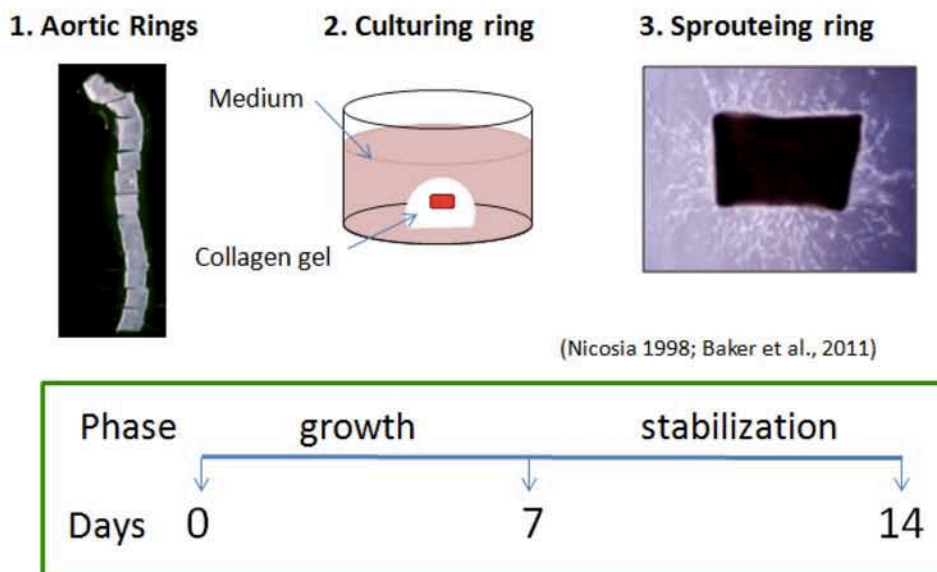


Figure 4. Cartoon illustrating the aortic ring assay. Aorta is prepared and sectioned in ring, then embedded in collagen gel, where vascular cells migrate out of the rings during the incitial growth phase.

Morphometry analyses

Microscopic images were recorded with a digital camera at various time points and magnification, depending on the properties to be assessed: $\times 40$ on day 3 to 7 for microvessel growth (number and length), $\times 100$ on day 8 and 9 for microvessel width, and $\times 200$ on day 10 to 14 for the cells associated with microvessels. The images were analysed using ImageJ to determine the length, width, and anastomosis of microvessels and the area covered by them. The numbers of microvessels were counted manually,

following the criteria described by Aplin et al.⁵⁷ where outgrowth constituted by singular cells such as fibroblasts, pericytes, and macrophages or segmented sprout structures were not included. AngioTool⁵⁸ was also employed to quantify microvessel area, branching points (total junctions), total length, end points, and lacunarity.

Immunofluorescent staining

Aortic rings extending microvessels were fixed with 4% formalin, permeabilised with 0.25% Triton-X for 15 min and incubated in PBS (+) containing 10% goat serum for 1 h at room temperature to block non-specific binding. After being rinsed with PBS (+) for 5 min they were then incubated overnight with primary antibody diluted in PBLEC⁵². After rinsing with PBS (+) (3 x 10 min), samples were then incubated for 30 min at 37°C in a cocktail of Alexa Fluor (488, 555, or Cy5) conjugated with appropriate secondary antibodies in PBLEC. Samples were rinsed then incubated (1 min) with Hoechst33342 and mounted using Fluoromount (Diagnostic BioSystems). Images were recorded using a fluorescence microscope (OlympusX70 or Keyence BZ-9000). The following primary antibodies were used: laminin (Progen 10765), CD31 (BD Pharmingen 550274), Reck, RECK-F (polyclonal rabbit antibodies; Matsuzaki et al., in preparation) and 5B11D12²⁷; α -Sma (DAKO MO85), fibronectin (BD Biosciences, 610078), PDGFR-beta (Santa Cruz Biotechnology sc-432), Flk-1 (Santa Cruz Biotechnology sc-625), LAMA5 (Sigma-Aldrich SAB4501720) and Ki67 (Leica Biosystems NCL-Ki67p).

Statistics

Quantitative data are presented in the form of mean $\pm$ sem in bar graphs. Significance of difference between two sets of data were assessed by Student's t-test

using the TTEST function in Excel (Microsoft), and the results are presented by symbols: *P<0.05, **P<0.01.

Results

3.1 Cell type-selective inactivation of *Reck* in vivo

Previous authors reported the essentiality of *Reck* for embryonic development in mice^{31,35,46}. Together with Dr. Mako Yamamoto, we tried to unveil functions of *Reck* in the vascular system. A series of engineered alleles of *Reck* prepared by several members of Noda laboratory, including Drs. Tomoko Matsuzaki and Mako Yamamoto in particular, with the help of RIKEN (the facilities of Dr. Shigeyoshi Itohara and of Dr. Shin-ichi Aizawa) are listed in Figure 5a: (1) original global Knockout³¹, (2) *Reck-CreERT2*, driving expression of Cre recombinase-estrogen receptor fusion protein (Matsuzaki et al., in preparation), (3) *Elfx*, exon1 flanked by two lox-P sites, (4) a novel null-allele that lacks proximal promoter exon-1, and (5) Low, a hypomorphic allele expressing *Reck* at lower levels, about 20% to 30% compared with *wt* allele⁴⁷.

To see which cells express *Reck*, Dr. Yamamoto prepared the mice carrying the *mTmG* reporter transgene plus one of the three Cre transgenes: *Sm22-Cre*, *Tie2-Cre*, or *Reck-CreER^{T2}*. The *mTmG* allele expresses a membrane-bound red fluorescent protein, named membrane Tomato (mT), before Cre expression or tamoxifen-induced translocation of CreER^{T2} to the nucleus, whereas after these events, this allele expresses a membrane-bound green fluorescent protein (mG). In this paper, we abbreviate mG-positive cells generated by *Reck-CreER^{T2}* expression followed by tamoxifen-treatment *Reck-mG*, those generated by *Sm22-Cre* expression *Sm22-mG*, and those generated by *Tie2-Cre* expression *Tie2-mG*. Thus, *Reck-mG* represents

Reck-expressed cells; *Sm22-mG* represents *Sm22*-expressed cells (mural lineage); and *Tie2-mG* represents *Tie2*-expressed cells (endothelial lineage) (Fig. 5b).

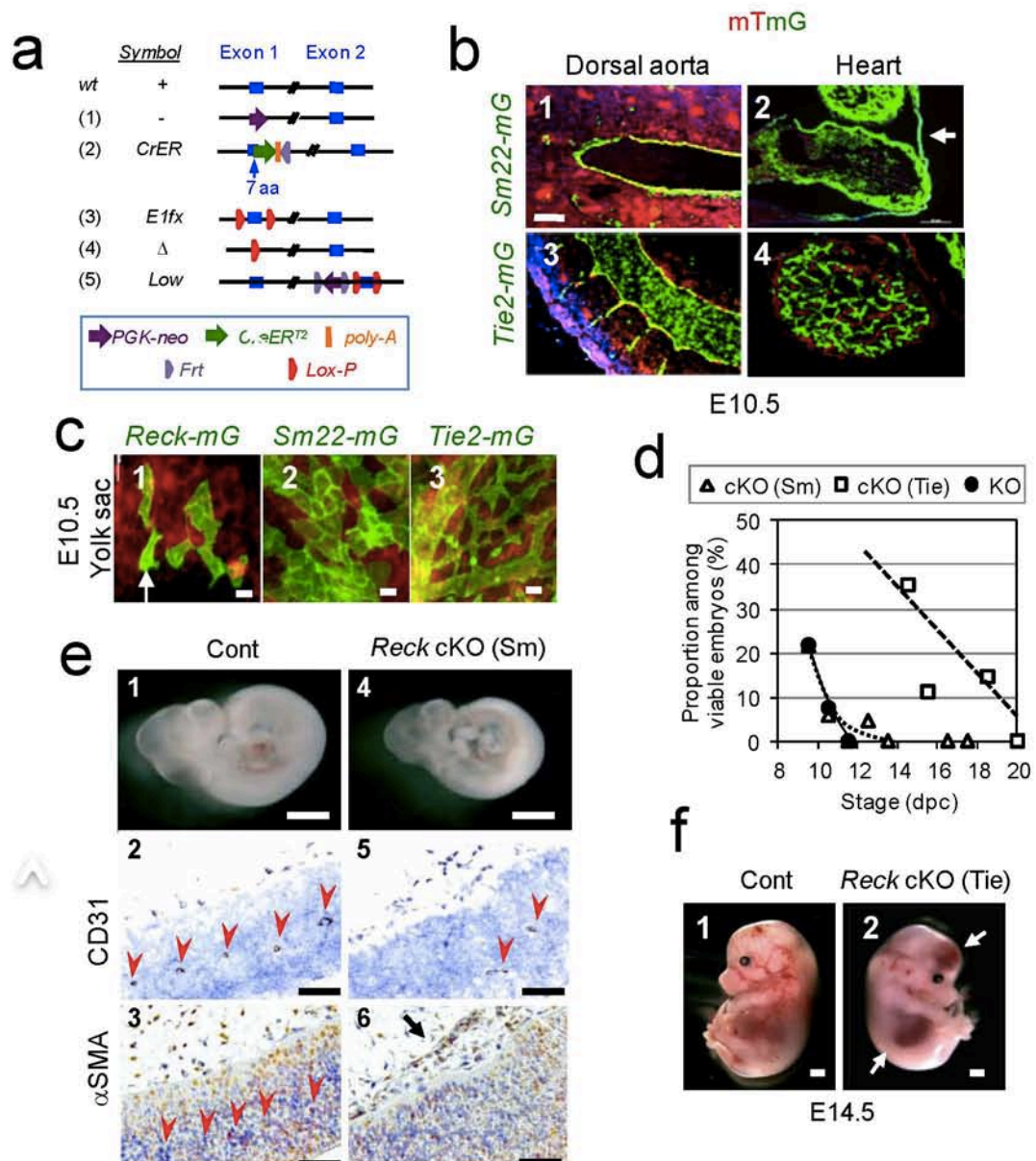
As we collected the yolk sacs of E10.5 embryos and compared the morphology of mG-positive cells (Fig. 5c), we found that most *Reck-mG* cells showed cobble stone-like alimnet similar to *Sm22-mG* cells but some *Reck-mG* cells show dendritic morphology resembling those of *Tie2-mG* cells (Figure 5c-1 arrowhead). These observations are consistent with previous findings by Oh et al. with electron microscope³¹ that *Reck* is abundantly expressed in mural cells and those of Chandana et al by immunohistochemistry that *Reck* is also expressed in some endothelial cells⁴⁶.

Next, we assessed the roles of *Reck* in mural cells and endothelial cells by selectively inactivating *Reck* in these cell types. This was achieved by generating mice carrying both *Reck*^{EIFx/Δ} and *Sm22-Cre* or *Tie2-Cre*, which we call *Reck* cKO (*Sm*) and *Reck* cKO (*Tie*), respectively. Dr. Yamamoto determined their survival curves and, Dr. Yamamoto and I prepared and examined their sections.

Interestingly, we found that loss of *Reck* affects mural cells and endothelial cells at different stages and tissues. *Reck* cKO (*Sm*) die around E10.5 (Fig.5d) with smaller body sizes (Figure 7e-4), frequent abdominal hemorrhage, and abnormal vessels (Figure 5d-5 and 6) reminiscent of those found in *Reck*-null (KO) mice³¹. Although *Reck* cKO (*Sm*) embryos were not so fragile as *Reck* KO embryos (Fig. 5g-1, 4 and 7, arrowhead), they shared similar defects: aorta disruption (Fig. 5g-2, 5 and 8, arrowhead) and frequent rupture of pericardial membrane (Figure. 5g-3, 6 and 9, arrowhead), suggesting that embryonic lethality of *Reck* KO mice may largely be attributable to the loss of *Reck* expression in mural cells.

In contrast, *Reck* cKO (*Tie*) mice survived longer (Fig. 5d, squares) and showed

major defects in the head starting around E14.5 with cranial hemorrhage (Figure. 5f-2; Fig. 5f-6), and later the cortical vasculature and cytoarchitecture were severe compromised (Fig. 5h, panels 7-10). Thus, *Reck* in mural cells is required in mid-gestation embryogenesis (~E10.5) while *Reck* in endothelial cells is required for brain development after E14.5.



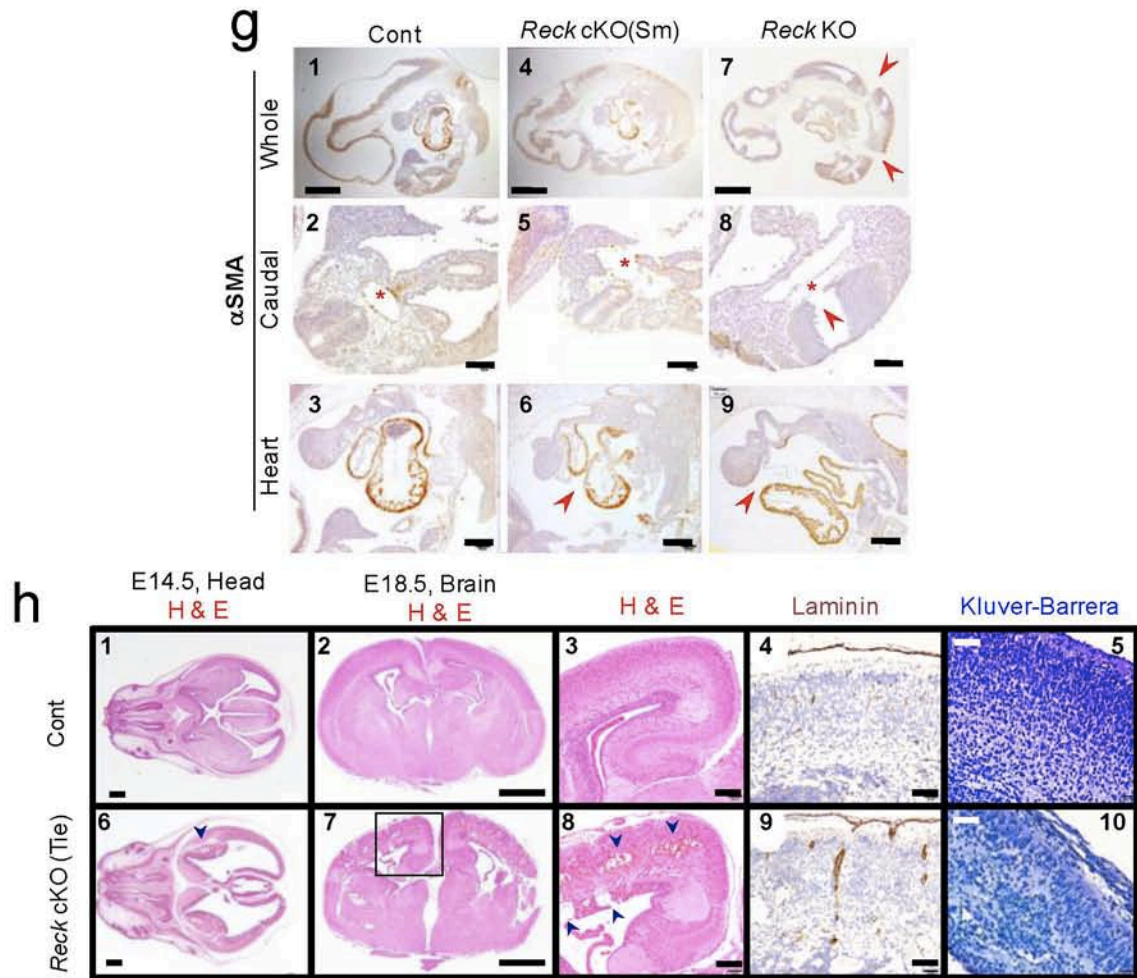


Figure 5 | Cell type-selective inactivation of *Reck* in Vivo using *Sm22-Cre* or *Tie2-Cre* mice. (a) Five *Reck* alleles used in this study. (b) *Sm22-mG* detected as a thicker layer surrounding mT cells in dorsal aorta and greater component in *bulbus acordis* (1, 2). *Tie2-mG* detected progenitor blood cells, in the dorsal aorta and *truncus arteriosus* (3,4) at E10.5 embryos. Tie2 is known as a hemangioblast marker which is also expressed in early hematopoietic progenitor cells¹. Red and blue signals represent untagged cells and cell nuclei, respectively. Arrow indicates pericardial membrane. Scale bar: 100 μ m. *Tie2-mG* cells are found in hematopoietic cells as well as vascular endothelial cells at this stage. (c) Cells expressing mG were specific to *Reck* (1), or *Sm22* (2, mural cells), or *Tie2* (3, endothelial cells) emitting green fluorescence in the yolk sac of E10.5 embryos. For *Reck*, a pregnant mouse was injected with tamoxifen at 8.5 dpc. Arrow indicates a cell with ambiguous morphology. (d) Viability of global *Reck* KO mice (*Reck*^{-/-}, filled circles with solid line) or tissue-selective *Reck* knockouts, *Reck* cKO (Sm) [*Reck*^{E1f Δ} ; *Sm22-Cre*, triangles] or *Reck* cKO (Tie) [*Reck*^{E1f Δ} ; *Tie2-Cre*, square. (e) Morphology of control (*Reck*^{E1f Δ}) and *Reck* cKO (Sm) mice at E10.5. Whole embryo (panels 1, 4) and the dorsal, peri-neural area of serial sagittal sections, immunostained for CD31 (panels 2, 5) or α SMA (panels 3, 6),

are shown. Brown signals indicate immunoreactivity. Arrows indicate abnormal peri-neural vessel. Arrowheads highlight CD31-positive small vessels within the neural tube. **(f)** Gross morphology of the control (*Reck*^{E1f^Δ/Δ}) and *Reck* cKO (Tie) mice at E14.5. Arrow indicates intra-cranial hemorrhage. **(g)** Distribution of αSMA-immunoreactivity in the sagittal sections of control, *Reck* cKO (Sm), and *Reck* KO mice at E10.5. Whole section (top row), caudal area containing a cross-sectional view of the neural tube and dorsal aorta (second row), and the heart (third row) are shown. Arrowheads show broken sites in panels 7, 8 and missing pericardial membrane in panels 6, 9. Asterisks highlight dorsal aorta. **(h)** Brain morphology in sections of control (upper panels) or *Reck* cKO (Tie) mice (lower panels) at E14.5 (panels 1, 2, 6, 7) or E18.5 (3-5, 8-10) that were subjected to hematoxylin and eosin (panels 1-3, 6-8), anti-laminin (panels 4, 9), or Kluver-Barrera (panels 5, 10) staining. Arrowheads indicate intra-cranial hemorrhage. Scale bar: (b) 20 μm; (c) 200 μm; (d) 1 mm (1, 4), 50 μm (other panels); (f) 1 mm; (g) 1 mm (1, 4, 7), 100 μm (2, 5, 8), 200 μm (3, 6, 9); (h) 1 mm (1, 2, 6, 7), 200 μm (3, 8), 100 μm (4, 9), 50 μm (5, 10).

3.2 Optimizing conditions for aortic ring assay using ICR transgenic mice

To understand how *Reck* affects angiogenesis at the cellular level, I used ARA. Although the C57BL/6 genetic background is recommended for the mouse version of ARA^{52,57,60}, we preferred to use ICR line because of its fecundity. I found four factors that influence the efficiency of microvessel formation.

First, excessive cleaning of aortae abolished their potential of forming microvessels (Fig. 6a), suggesting a contribution of some components in the outer most aortic layer, adventitia tunica, as previously suggested in rat aorta^{61,62}. Thus, damage in this layer may repress the endothelial sprouting and microvessel formation.

Second, the nature of embedding material affected sprouts and their morphogenesis. I tested two collagens and their mixture and found that the mixture yielded better response (Figure 6b).

Third, I tested the effects of medium supplements, such as fetal bovine serum (FBS), fetal horse serum (FHS), and VEGFa, and found that FHS supported efficient

activation of initial sprouts (Figure 6 c-3) and subsequent microvessel formation (Figure 6c-graphic). Forth, the age of animals had significant impact on the efficiency of angiogenic responses. I tested aortas from 4- to 8-weeks old mice and noticed two things: a) aortae from 4- to 5-weeks old mice produced more microvessels, and b) rings from the middle portion of aortae were most likely to generate microvessels (Figure 6-d).

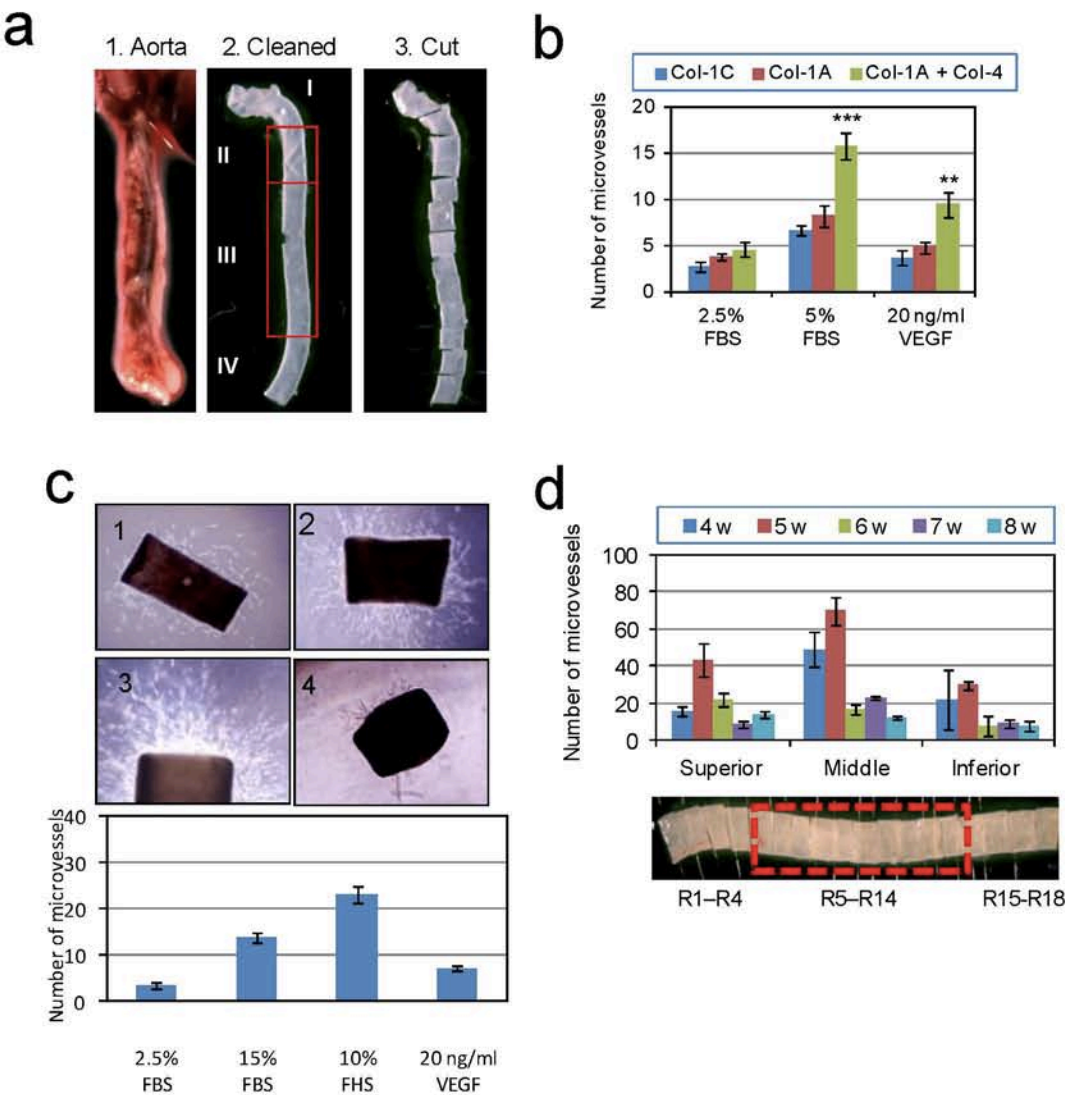


Figure 6. Optimizing ARA to use aortic ring from ICR mice. (a) Preparation of thoracic aorta from *Reck-CreRT2;mTmG* ICR mice. Fresh aorta covered by adipose tissue (1) was cleaned carefully to preserve the adventitia tunica (2), cut into the rings of about 1-mm thick (3), and subjected to ARA. Four regions of aorta are indicated in panel 2: ascendant aorta (I), superior descendent aorta (II), middle descendent aorta (III), and inferior descendent aorta (IV). (b) Effects of gel composition and medium supplements. Freshly prepared aortic rings were embedded in semi-solid medium containing collagen of three different compositions (type 1C, type 1A, and type 1A plus 4), fed with medium supplemented with fetal bovine serum (FBS, two different concentrations) or vascular endothelial growth factor-A (VEGFa; R & D Systems), and incubated for 7 days with medium changes at days 5 and 10, followed by microvessel counting. Data represent more than four independent experiments with total of 65 rings prepared from 17 animals. (c) Effects of medium supplements. Freshly prepared aortic rings were embedded in the gel containing collagen type 1A and 4, fed with medium supplemented with FBS (2), fetal horse serum (FHS) (3), or VEGFa (4) at indicate concentration(s) and incubated in medium containing the same supplement with medium change at days 5 and 10. Phase-contrast images of typical rings (panels 1-4) and the number of microvessels at day 7 (bar graph, n=24 rings from 6 animals) are shown. (d) Effects of age of mice and position in aorta. Aortic rings from rostral quarter (superior), caudal quarter (inferior), and the remaining area (middle) of aorta (for instance, see the bottom photograph of a sample at 8 weeks of age) obtained from mice at indicated age were subjected to ARA, and the number of microvessels were counted at day 7. n=3 aortas per group (total 243 rings). Scale bar: 1 mm (a,c,d),. Data in bar graphs represent mean±s.e.m. Data with significant difference as detected by Student's t-Test are indicated as follows: ** $P < 0.01$, *** $P < 0.001$.

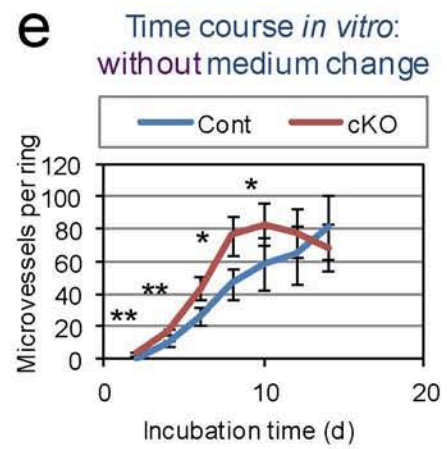
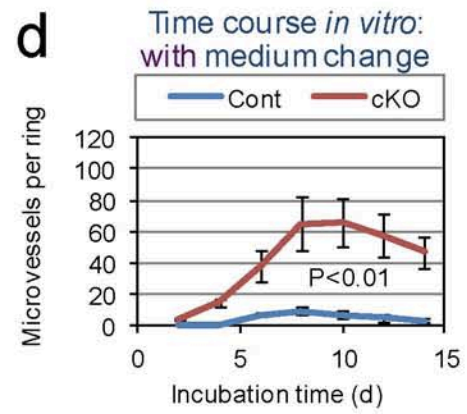
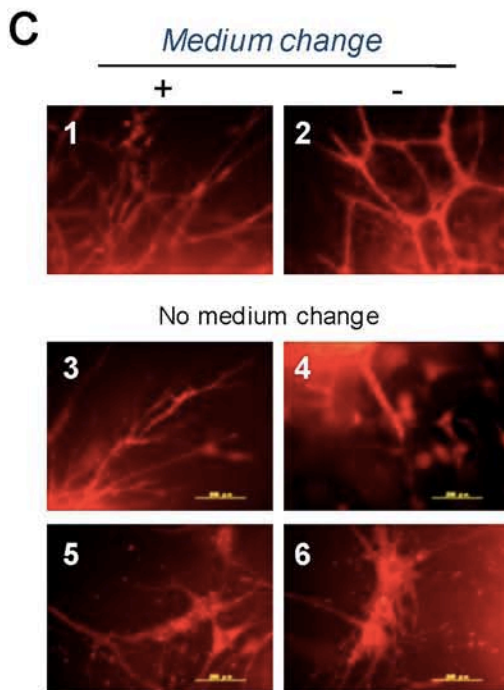
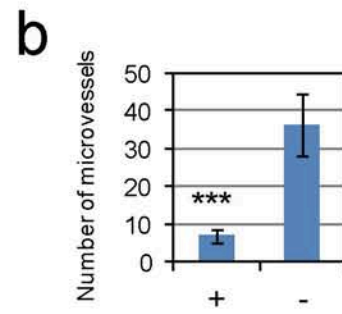
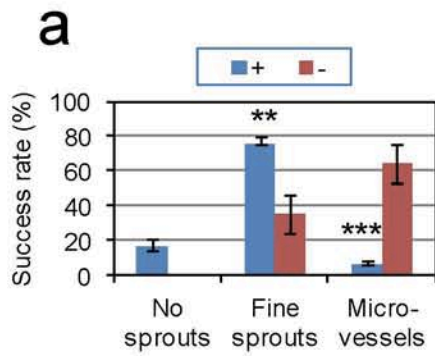
3.3 Effects of Reck on sprouting angiogenesis in ARA

While optimizing ARA for ICR mice, I also noticed that frequent changes of medium reduced its success rate. As I compared between the rings incubated for 14 days with two medium changes (day 5 and 10) and those incubated without medium changes (kept the dishes sealed to avoid evaporation), I obtained better results under the latter conditions. Namely, not only the frequency of rings per aorta (Figure 7a) but also the number of microvessels (Figure 7b) and morphological diversity of the cells (Figure 7c) were increased when medium was kept unchanged.

Then I compared the time courses of microvessel formation between the control

rings (Reck level=50% of wild type) and the Reck cKO rings in two different conditions: with (Figure 7d) and without (Figure 7e) medium changes. To my surprise, Reck cKO rings could form microvessel even when medium was changed (Figure 7d, red line). Without medium changes, Reck cKO rings formed microvessels earlier and larger in number in the early phase, but the curve was in bell-shaped (Figure 7e, red line), which is in contrast to the steady increase in the case of control rings (Figure 7e, blue line).

I tried to confirm this finding using different combination of *Reck* alleles expressing different levels of Reck protein: wild type mice (+/+) and Reck hypomorphic mice (Low/ Δ) that express Reck at about 25% of the wild type level. Indeed, Reck hypomorphic rings formed microvessels earlier and larger in number (Figure 7f, red line) than the wild type rings (Figure 7f, blue line) but the number of microvessels declined in the later phase in contrast to the wild type case. The decrease in microvessel number found in *Reck* mutant rings was accompanied by extensive aggregation and coalescence of microvessels (Figure 7f-2 arrowheads; Figure 7g).



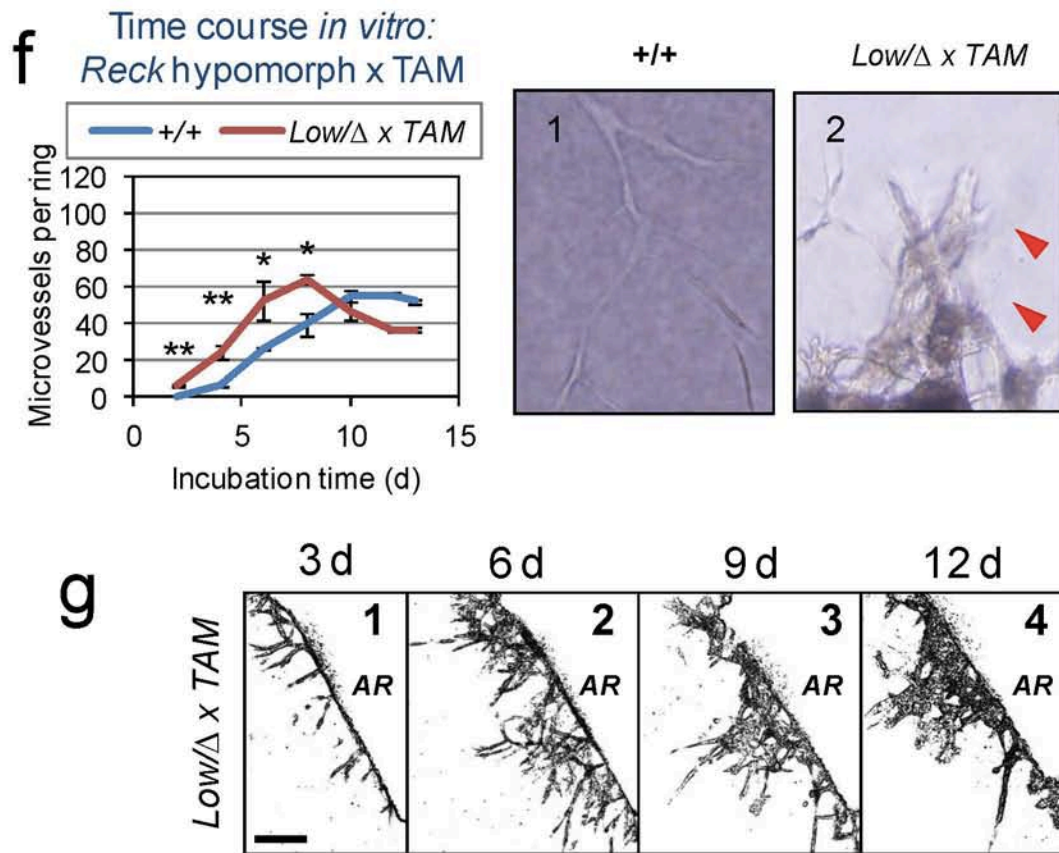


Figure 7. Effects of Medium changes. (a) Frequency of rings that gave rise to no sprouts, fine sprouts (cord-like structure Figure c-1) or more robust protrusions (Figure c-2 term “microvessels”). Data for the rings incubated with (+, n=12) or without (-, n=7) medium changes are compared. (b) Number of microvessels generated in Reck-CreRT2 aortic rings with and without medium changes. (c) *mT*-expressing aortic rings, pre-incubated overnight in serum-free medium, were subjected to ARA under the optimal gel components (collagens-1A and 4) and medium supplements (10% FHS); medium was either changed at days 5 and 10 (panel 1) or kept unchanged (panels 2-6). Micrographs were taken at day 7 focusing on morphology of sprouts (panels 1, 2) and various types of cells (panels 3-6). (d) Time course of microvessel formation (number per ring) when medium was refreshed at days 5 and 10. Aortic rings from 5-weeks-old control (*Reck*^{+/*Cre*}; *mTmG*, blue line, n=5) or cKO (*Reck*^{*E1f*/*Cre*}; *mTmG*, red line, n=3) mice were used. Note that cKO aortic rings could rapidly extend abundant microvessels under these conditions, while control aortic rings failed to do so. The reason for this difference has yet to be clarified. (e) Time course without medium change. Aortic ring from 5-weeks-old (*Reck*^{+/*Cre*}; *mTmG*, blue line, n=9) or cKO (*Reck*^{*E1f*/*Cre*}; *mTmG*, red line, n=4) mice were used the number of microvessels was counted every other day from day 2 to 14.. (f) Effects of *Reck*-deficiency on microvessel formation in ARA as examined using hypomorphic *Reck* mutant mice. Time course of microvessel formation (without

medium changes) by aortic rings derived from the wild type (+/+) or *Reck*^{Low/Δ} (Low/Δ) mice pretreated with tamoxifen. n=4. Phase contrast images of typical microvessels in wild type (1) and *Reck*^{Low/Δ} (2) cultures at day 10. Red arrowhead indicates the aggregation of microvessels. (g) Binary images of a typical region of a ring derived from tamoxifen-treated *Reck*^{Low/Δ} mice after indicated period of incubation in ARA (without medium changes). Phase-contrast images were converted to binary images using ImageJ software. Scale bar: 0.5 mm. In this mutant, basal *Reck* expression is about one fourth of the wild type level, and tamoxifen induces acute inactivation of residual *Reck* expression. Note that the initial increase and subsequent decline in microvessel number seem to be accelerated (b, red line) as compared to the cKO samples (a, red line). Data with significant difference as detected by Student's t-Test are indicated as follows: **P* < 0.05, ***P* < 0.01, *** *P* < 0.001.

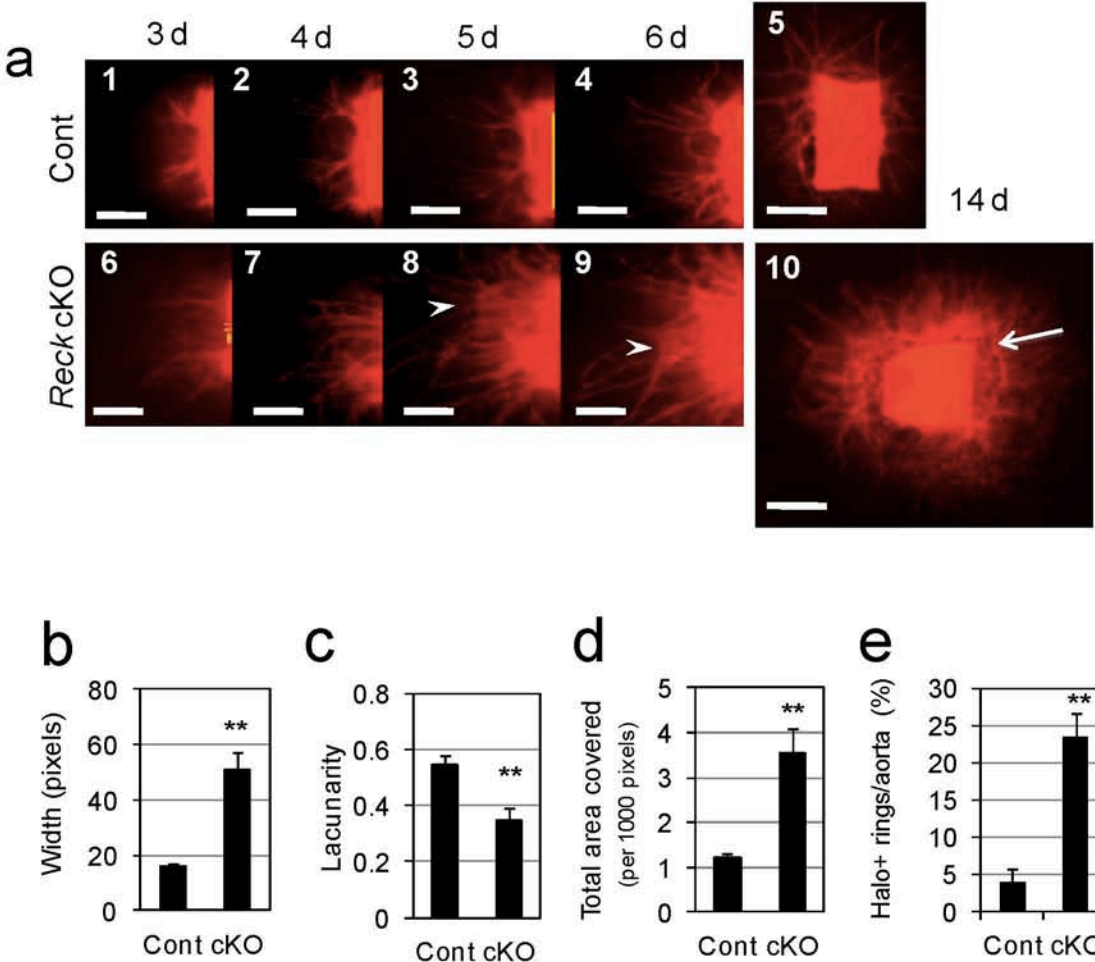
3.4 *Reck* affects sprouting and stability of microvessels

To better understand how *Reck* affects microvessels formation, I tried to quantify some of the morphological features with the aid multiple image analysis tools, including ImageJ and AngioTool ⁵⁸. For this purpose, I harvested control and mutant aortae from 5-weeks old mice, performed ARA without medium changes under the optimized conditions, and recorded the microvessel images from day 2 to 14 (Fig. 8a, f and data not shown).

Reck cKO microvessels acquired abnormal patterns around day 5 (Figure 8a-8 arrowhead) with aggregation and coalescence (Figure 8a-9 arrowhead) giving rise to the microvessels with greater width (Figure 8b) and fewer lacunae (empty spaces) among the microvessels (Figure 8c). Microvessels from *Reck* cKO rings grew faster (Fig. 8g) and covered larger area (Figure 8d) than the control. *Reck* cKO rings were also frequently accompanied by peri-aortic halos, the areas of excessive collagen digestion (Figure 8e and figure 8a-10 arrow).

Little difference was observed in the number of junctions and end points between *Reck* cKO and the control (Figure 8h, i) under these conditions. Taken together, these

data suggest that Reck regulates the frequency and timing of microvessel sprouting and the stability of individual microvessels.



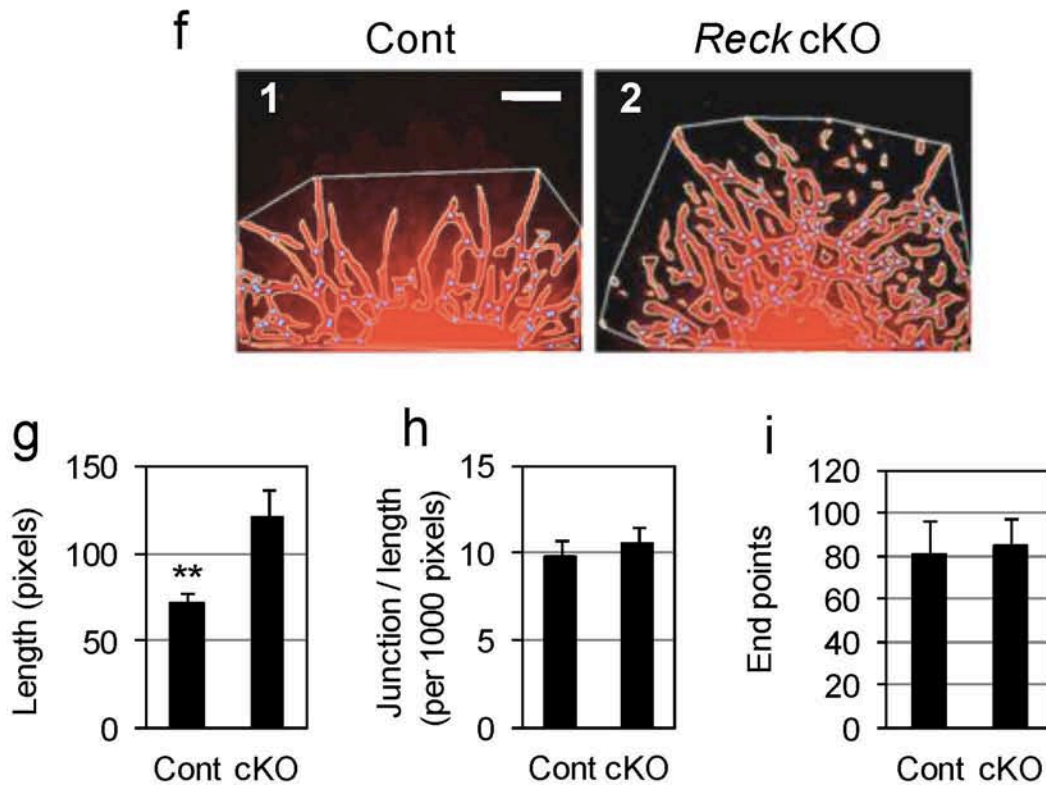


Figure 8. Effects of *Reck*-deficiency on microvessel formation in ARA. (a) Fluorescent micrographs of a control (panels 1-5) or *Reck* cKO (panels 6-10) ring after incubation for indicated period of time in ARA without medium changes. Arrow indicates peri-aortic halo, and arrowheads highlight aggregating microvessels. Scale bar: 1 mm (5, 10), 0.5 mm (other panels). (b) Relative width of microvessels measured using ImageJ on high magnification images. Summary of 6 independent experiments (n = 36 rings for Cont and 30 rings for *Reck* cKO in total). (c, d, h, i) Parameters measured using AngioTool¹. Summary of 8 independent experiments for Cont (46 rings) and 7 experiments for *Reck* cKO (43 rings). (e) Frequency of rings exhibiting peri-aortic halo. (f) Image treated with angiTool, emphasize length and expanded angiogenic area. (g) Relative length of microvessels measured using ImageJ on 100 μ m magnification images. Summary of 6 independent experiments for Cont (78 rings) and 3 independent experiments for *Reck Reck* cKO (30 rings).

3.5 *Reck* affects the number and localization of vascular cells

Toward understanding how *Reck*-deficiency brings about the abnormal microvessels in ARA, I tried to determine in which cells *Reck* is expressed in ARA and how these cells are affected by *Reck*-deficiency. First, I performed ARA with

aortae from mTmG mice carrying either the Reck-CreER^{T2}, Sm22-Cre, or Tie2-Cre transgene to compare the morphology of Reck-expressed (Reck-mG) cells with those of mural (Sm22-mG) and endothelial (Tie2-mG) cells. Reck-mG cells (Fig. 9a-1) were found in both tip (arrowhead) and stalk (arrow) regions of microvessels; the former resembled Sm22-mG cells (Figure 9a-2) while the latter Tie2-mG cells (Figure 9a-3).

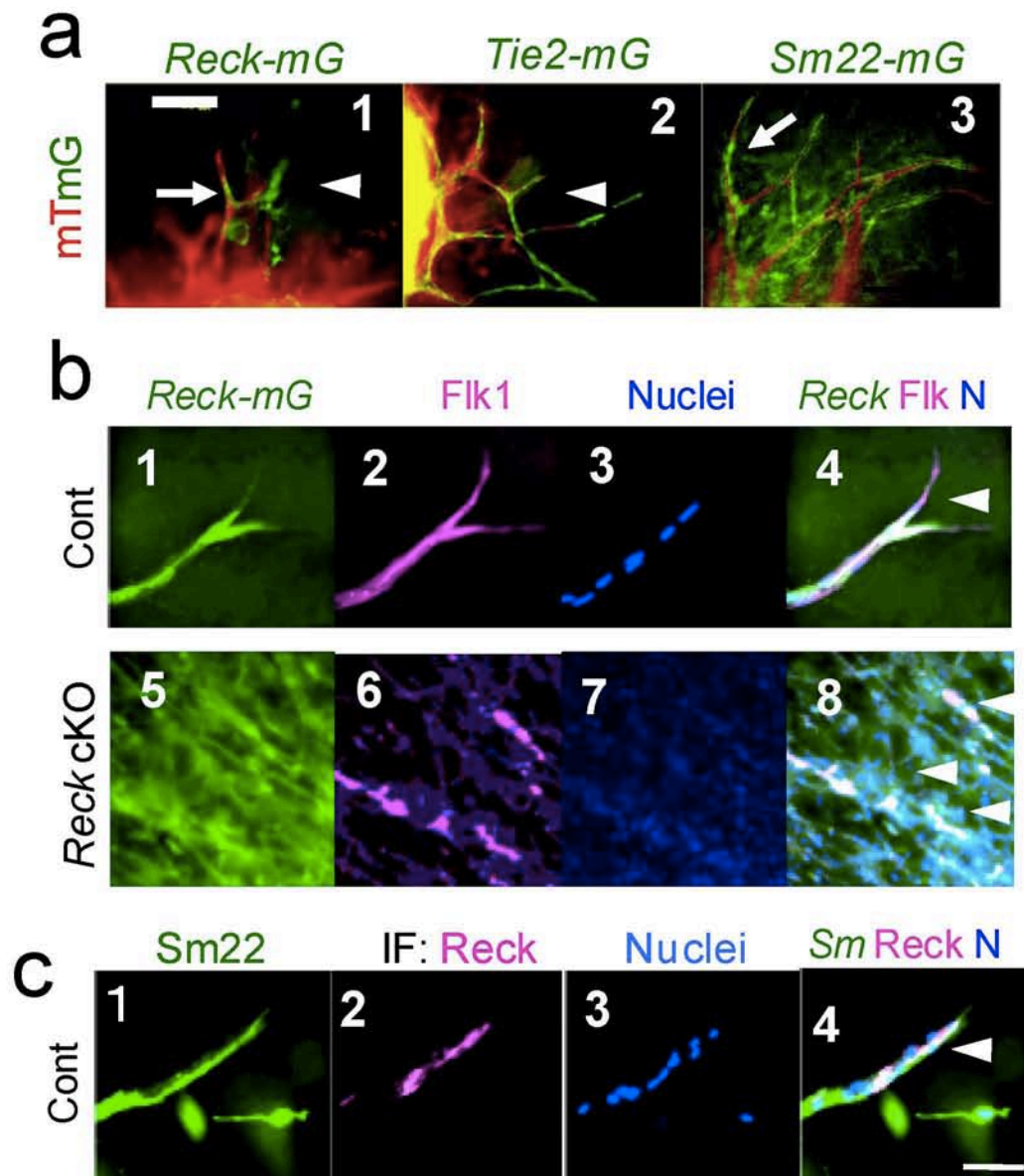
Double labeling experiments with control rings indicated that both endothelial tip cells and mural stalk cells can express Reck. Namely, the Reck-mG cells at the tip regions were positive for the “tip cell” marker Flk-1 (Figure 9 b1-4) as well as the endothelial marker CD31 (Figure 9 d-8, arrowhead); some of the Sm22-mG cells at the stalk regions were positive for Reck (Figure 9c) and conversely, the Reck-mG cells at the stalk regions were positive for the mural cell marker α Sma (Figure 9 d and g, panel 3, arrow).

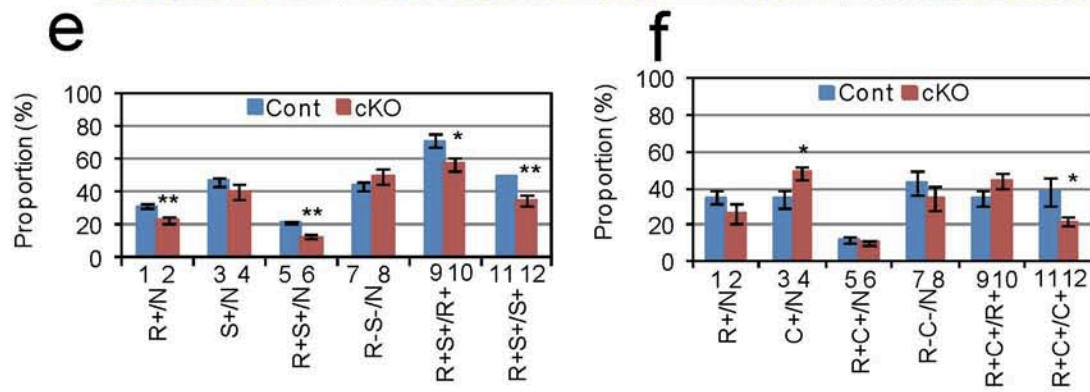
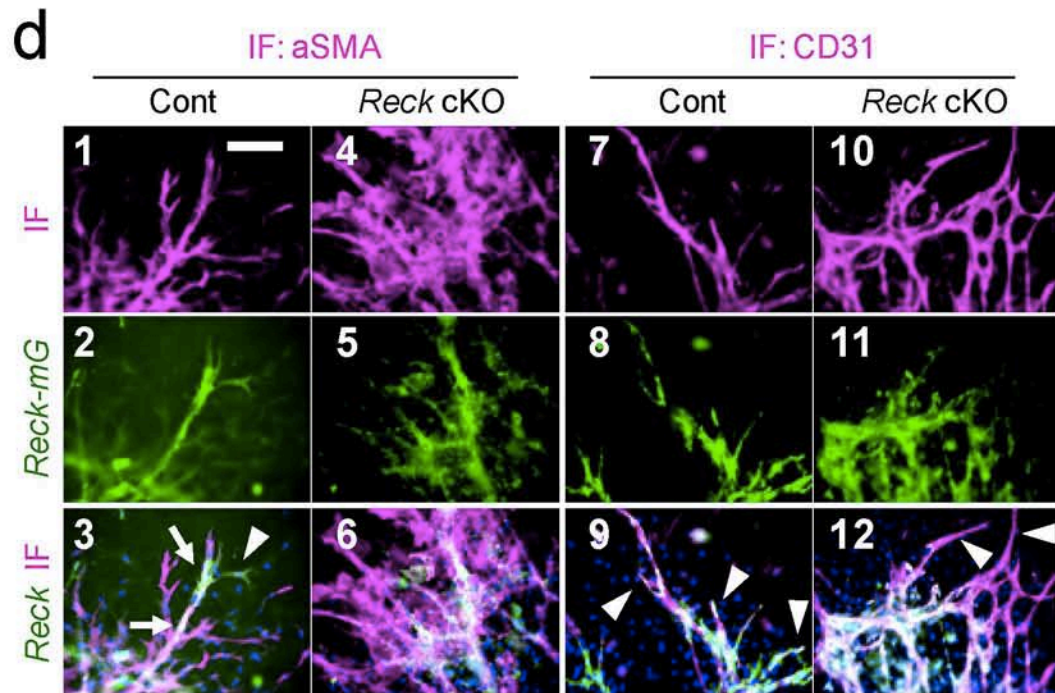
Double labeling experiments with Reck cKO rings, on the other hand, indicated that Flk-1/CD31 positive cells were mislocalized at the stalk regions (Figure 9 b5-8; Figure 9 d and g, panel 12) and many α Sma-positive mural cells were not associated with microvessels (Figure 9d and g, panel 6). I also counted the number of total nuclei as well as single-positive and double positive cells in the experiments as shown in Figure 9 d (Figure 9e, f). In control cultures ~70% of Reck-mG cells were of mural type (Figure 9e, bar 9) and ~30% were of endothelial type (Figure 9f, bar 9).

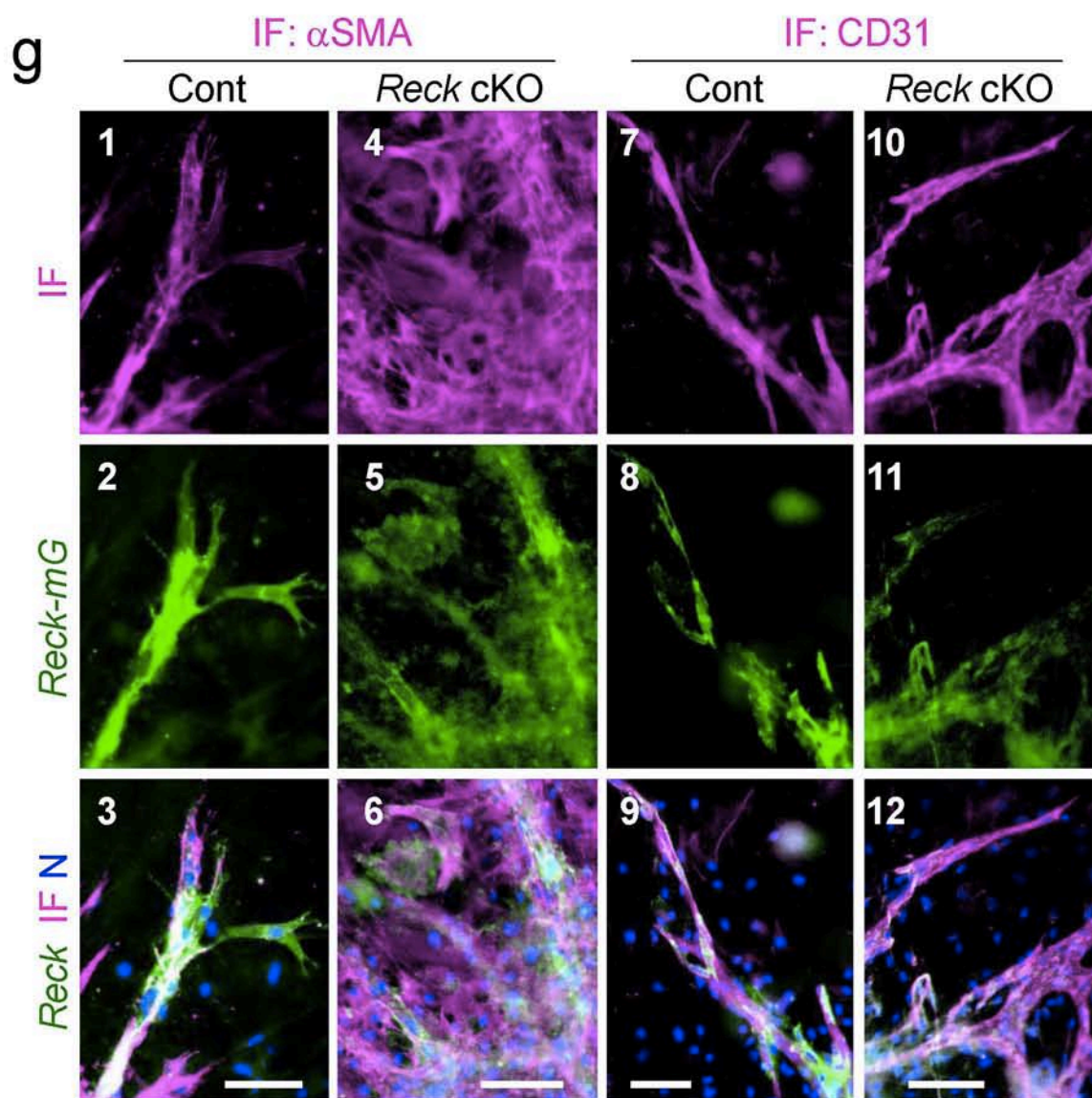
In Reck cKO culture, frequencies of both Reck-mG cells and Reck-mG/ α Sma-double positive cells decreased (Figure 9e, bar 1, 6, 10, 12), while the frequency of CD31-positive cells increased (Figure 9 f, bar 9) and the proportion of Reck-mG cells among these CD31-positive cells decreased (Figure 9 f, bar 12).

Hence, while Reck-deficiency leads to the mis-localization of both endothelial and

mural *Reck*-mG cells (Figure 9 h), it may also affect the number of certain populations:
 e.g., negatively on *Reck*-mG cells and positively on endothelial cells.







h

Category	Genotype	Alleles	Reck expression after TAX (%)	Phenotype
Wild type	+/+		100	
Reck Control	+/ <i>Reck-CreER</i> ^{T2}		50	
Reck mutant	<i>R1/Reck-CreER</i> ^{T2}		0	
VSMCs and others	Sm22-cre		100	
ECs and others	Tie2-cre		100	

Figure 9. Identity and behavior of *Reck-mG* cells in ARA (a) Morphology and microvessel-association of *Reck-mG* (1), *Tie2-mG* (2), and *Sm22-mG* (3) cells (green) in ARA at day 8. (b) Control and mutant microvessels were immunostained for a vascular tip-cell marker, Flk1/Vegfr2 (magenta in panel 2 and 4 for control and 6 and 8 for mutant). (c) Sm22-Cre microvessel was immunostained for Reck at day 10. (d) Effects of *Reck*-deficiency on the nature of, and relationship between, *Reck-mG*, endothelial, and mural cells. Aortic rings from the control (*Reck*^{+/*CreER*}; *mTmG*) or *Reck* cKO (*Reck*^{*E1fc/CreER*}; *mTmG*) mice were subjected to ARA and immunostained for αSMA (magenta in panels 1, 3, 4, 6) or CD31 (magenta in panels 7, 9, 10, 12) at day 11. Green signals represent *Reck-mG* cells. *Reck-mG*/αSMA double positive staining could be observed at microvessel stalks from control mice (white stain, arrow, panel 3). In control cultures, *Reck-mG*/CD31 double positive staining could be observed at microvessel tips (white stain, arrowheads, panel 9) and *Reck-mG* single positive staining found at microvessel stalks (green stain, panel 9). This was lost in *Reck* cKO mice as observed by the lack of *Reck-mG*/CD31 double positive at the microvessel tips (arrowheads, panel 12). (e) Frequency of *Reck-mG*/αSMA double labeling as shown in (b), panels 1-6, and (f) frequency of *Reck-mG*/CD31 double labeling as shown in (f), panels 7-12. N=total nuclei, R=*Reck-mG*, S=αSMA, C=CD31, “+” means “-positive”. (g) Aortic rings from the control (*Reck*^{+/*CreER*}; *mTmG*) or *Reck* cKO (*Reck*^{*E1fc/CreER*}; *mTmG*) mice were subjected to ARA and immunostained for αSMA (magenta in panels 1, 3, 4, 6) or CD31 (magenta in panels 7, 9, 10, 12) at day 11. Green signals represent *Reck-mG* cells. Scale bar: 50 μm. Arrows indicate *Reck-mG*/αSMA double positive cells

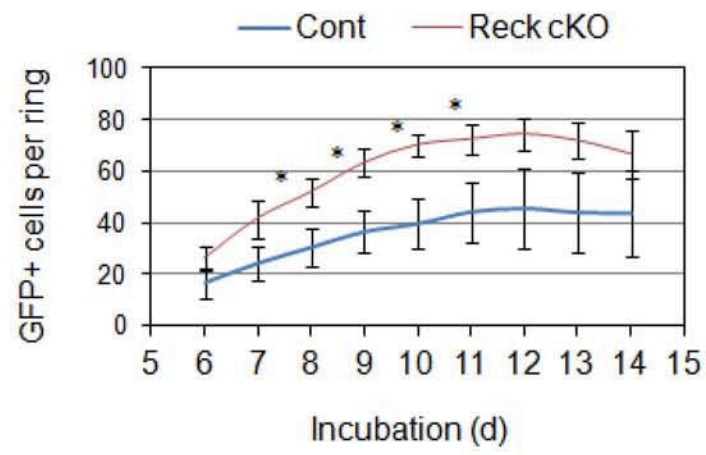
surrounding the stalk. Arrowhead show cells near the tip of a sprout. **(h)** Summary of findings. Number of images analyzed: (d) Cont (n=17), *Reck* cKO (n=12). (e) Cont (n=6), *Reck* cKO (n=9). Bars 1-8 represent proportion among all cells. Bars 9 and 10 represent proportion among *Reck-mG* cells Bars 11 and 12 represent proportion among α SMA-positive cells (d) or CD31-positive cells (e). Aortic rings were prepared from at least 3 animals per group. Scale bar: (a) 50 μ m, (b) 100 μ m, (c) 20 μ m.

3.6 *Reck* regulates cell proliferation and cell-cell interactions

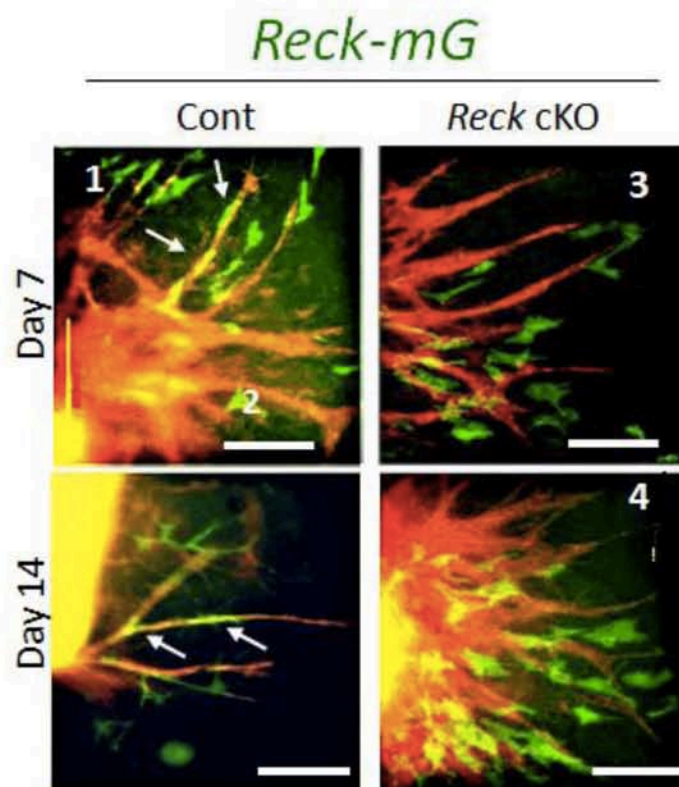
I also quantified the number of *Reck-mG* cells association with microvessels in the control and *Reck* cKO cultures (Figure 10). First, I found that the total number of *Reck-mG* cells was higher in *Reck* cKO cultures (Figure 10a), but many of these cells were not associated with microvessels (Figure 10 b-e). Second, *Reck* cKO cultures showed higher number of proliferative cells as assessed by Ki67-immunoreactivity (Figure 10f, g), suggesting that the increased number of *Reck-mG* cells in *Reck* cKO cultures may be explained, at least in part, by increased proliferation of these cells.

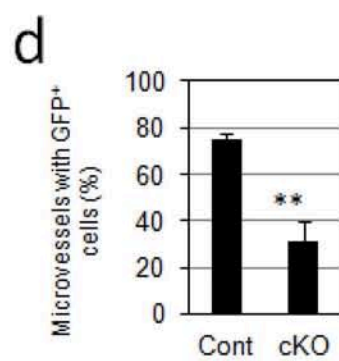
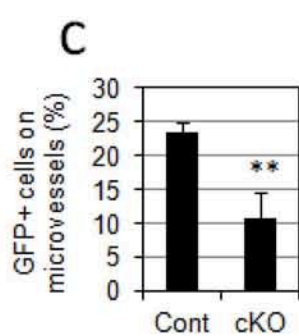
With these data in hand, we re-examined the histology of the *Reck* cKO (Sm) mutant mice and found interesting phenotypes *in vivo*. Namely, dorsal aortae of the mutant mice were unevenly surrounded by fewer number of α Sma-positive cells (Figure 10 h, I, panel 4) as compared to the control (Figure 10 h, I, panel 2), suggesting a cell-autonomous function of *Reck* in mural cells required for making intact aortae by promoting proper mural-endothelial interactions.

a

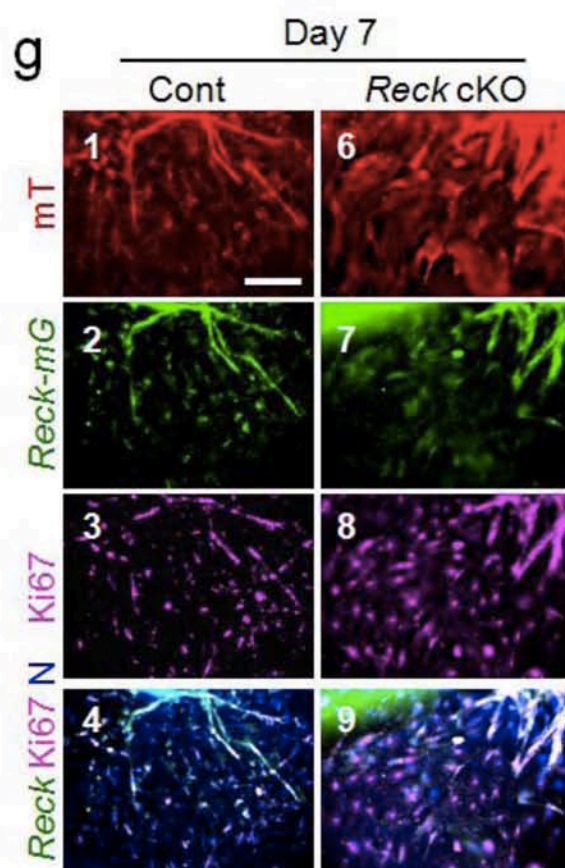
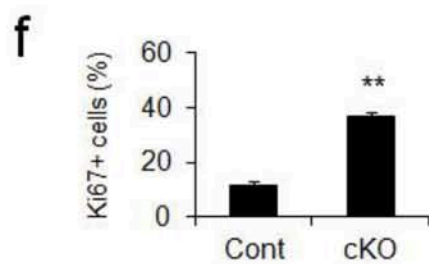
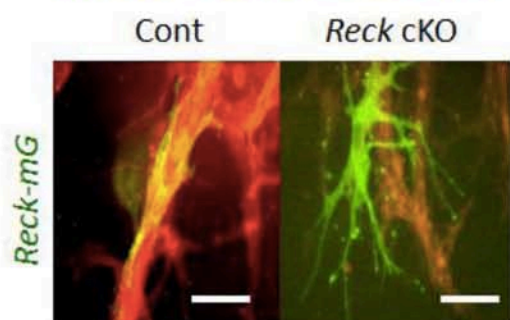


b





e Mural-ECs interactions



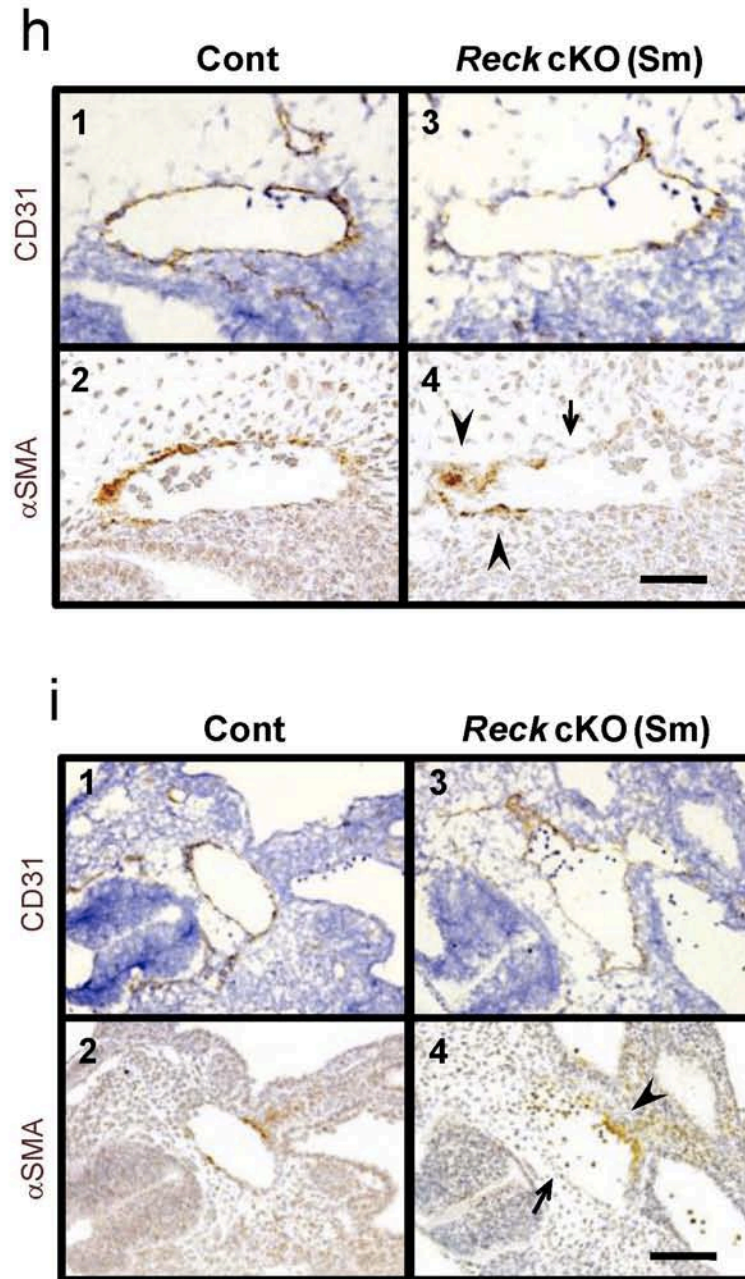


Figure 10. Effects of *Reck* on vessel-association of *Reck*-mG cells in ARA and mural cells *in vivo*. (a) Time course for the number of *Reck*-mG cells in ARA. Aortic rings from the 5-weeks-old control (n=10) or *Reck* cKO (n=7) mice were subjected to ARA and *Reck*-mG cells surrounding each ring were counted from day 6 to 14. (b) Distribution and morphology of *Reck*-mG cells (green) at day 7 and 14 in ARA. In control cultures, *Reck*-mG cells are tightly associated with microvessel stalks (arrows in panel 1 and 2) but this is impaired *Reck* cKO (panel 3 and 4). (c) Frequency of *Reck*-mG cells tightly associated with microvessels at day 8. n=3. (d) Frequency of microvessels with tightly associated *Reck*-mG cells at day 8.

n=3. (e-g) Effect of *Reck*-deficiency on the distribution of mural cells *in vivo*. (e) Typical images of Reck-mG cells found near microvessels. Control Reck-mG cells tightly wrap around microvessels (1), while cKO Reck-mG cells do not tightly attach the microvessels and show peculiar morphology (2). (f) Effects of *Reck* on cell proliferation in ARA. The proportion of Ki67-positive nuclei was estimated using ImageJ (3) and was found to be significantly increased in *Reck* cKO cells. **P=0.004. (g) Aortic rings from the control (*Reck*^{+/-CrER};mTmG) or cKO (*Reck*^{E1f6/CrER};mTmG) mice were subjected to ARA and stained for Ki67 at day 7 (magenta in panels 3, 8). Note the substantial increase in proliferating *Reck*-mG (white) as well as proliferating non-*Reck*-mG (magenta) signals in the cKO samples (panels 9). (h) Sagittal sections of *Reck*^{E1f6/Δ} mice (control; panels 1, 2) and *Reck* cKO (Sm) mice (panels 3, 4) at E10.5. Adjacent sections were immunostained for CD31 (with Hematoxylin counter-stain; panels 1, 3) and for αSMA (panels 2, 4), respectively. Micrographs focusing on large aortas in the middle (h) and caudal (i) part of the body are shown. In (h) - (i), arrowhead indicates an area of vessel wall abundant in mural cells, and arrow highlights an area where the mural cell layer is very thin or absent. Scale bar: b 100 μm, c 50 μm (Cont) 50 μm (cKO), d 50 μm, (g) 100 μm, (h) 50 μm, (i) 100 μm.

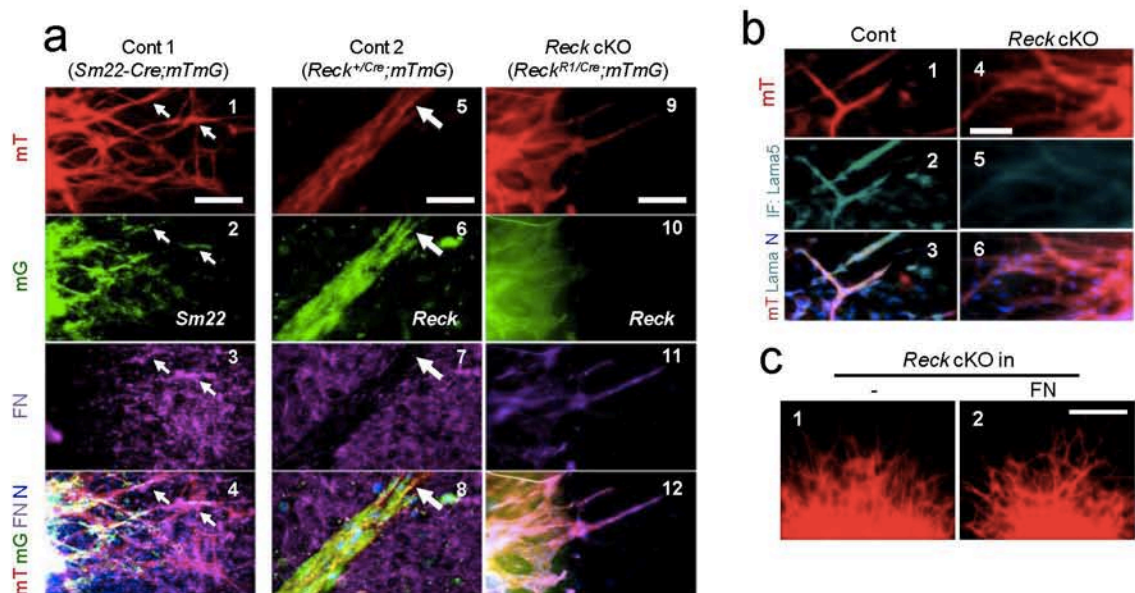
3.7 Functional relationship between Reck and FN in angiogenesis

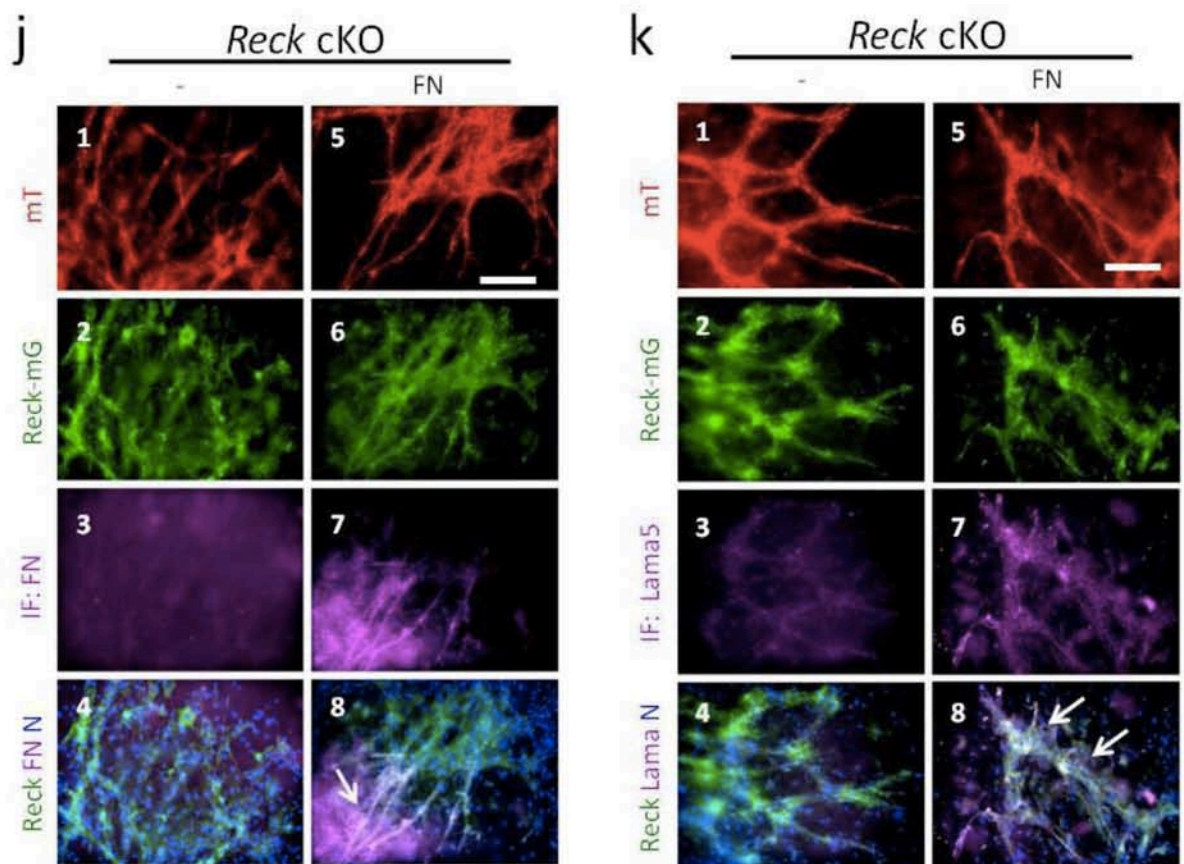
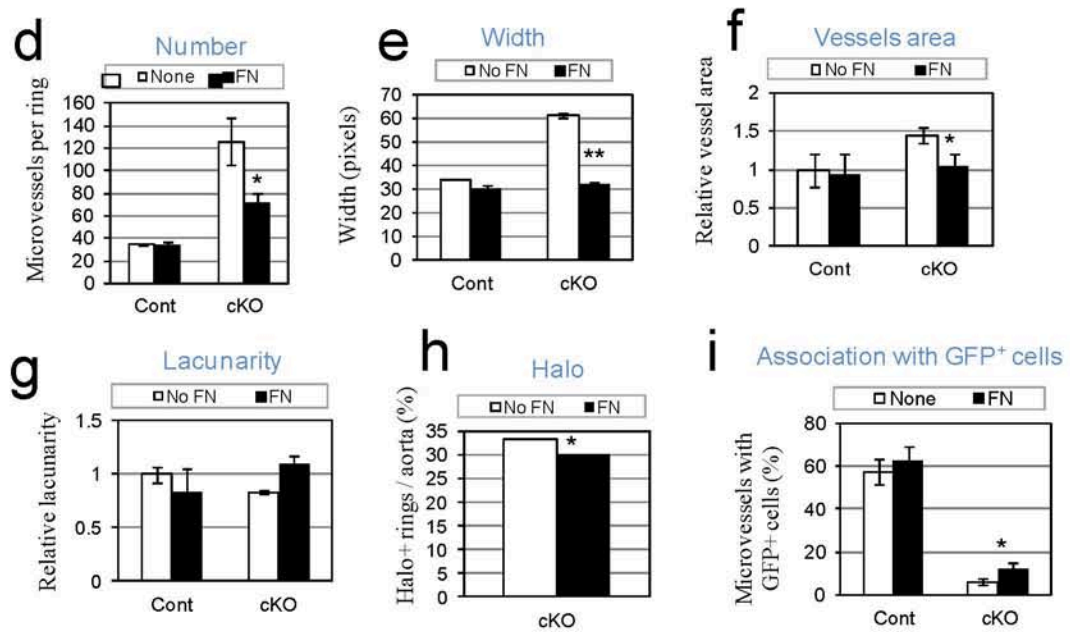
To find some clues to the question of how Reck affects the number and behaviors of Reck-mG cells and leads to vascular stabilization, I investigated the effects of Reck-deficiency on ECM components. Immunofluorescent staining indicated that fibronectin (FN), mainly derived from medium FHS, were abundant in control culture (Figure 11a, panel 3, 7), accumulated in the area where mural cells tightly associated with microvessels (Figure 11a, panels 1-4, arrows), and absent in the areas occupied by microvessels (Figure 11a, panel 7). In *Reck* cKO cultures, however, not only FN but also laminin α5 signals were greatly reduced (Figure 11a-11; Figure 11b-5).

FN is known to be protected by RECK from MMP-mediated degradation *in vitro*³⁴ and to rescue some of the phenotypes of RECK-deficient cells in culture^{43,44}. I therefore asked whether FN could rescue any of the *Reck* cKO phenotype in ARA. When the collagen gel was supplemented with purified FN, microvessels from *Reck* cKO rings showed altered morphology (Figure 11c). Morphometric analyses indicated significant

improvement in some of the phenotypes, including the reduction in microvessel number (Figure 11d), width (Figure 11e), vessels area (Figure 11f), and increase in lacunarity (Figure 11g), suggesting that these events require protection of FN by Reck. In contrast, FN showed little effects on the number of peri-aortic halo (Figure 11h) and the association of Reck-mG cells with microvessels (Figure 11i), suggesting that these events require FN-independent actions of RECK.

In this system, FN was found to associate with microvessels (Figure 11j) and to increase the amount of laminin a5 associated with microvessels (Figure 11k). These findings are consistent with the current model on the roles of mural cells in vascular stability, which proposes that FN-deposition on endothelial tubes, triggered by the association of mural cells, is prerequisite for basement membrane deposition and consequent vascular stabilization (Figure 11l) ⁷.





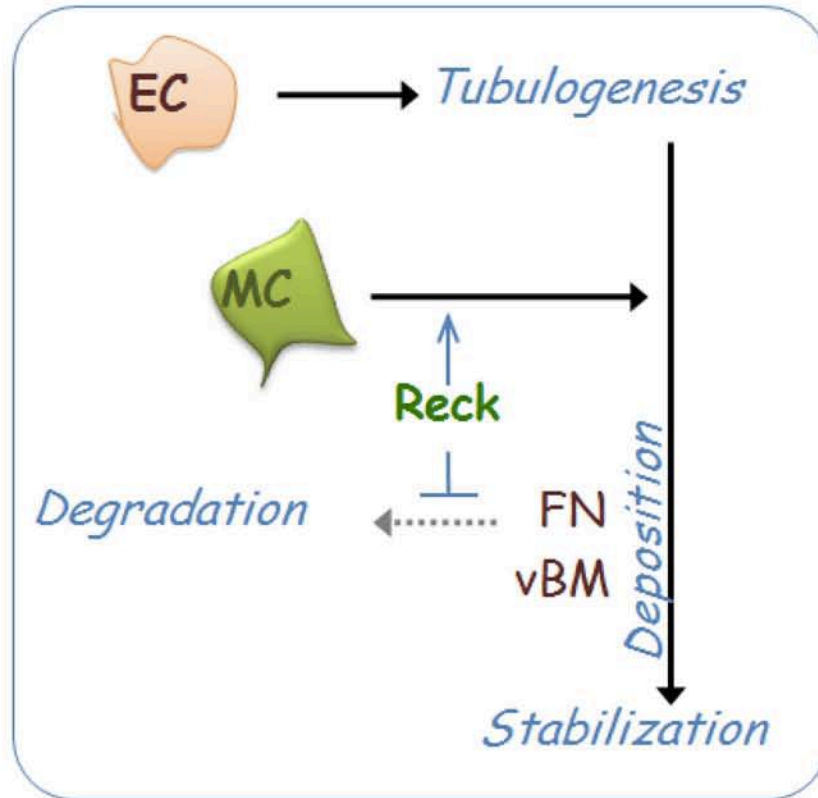
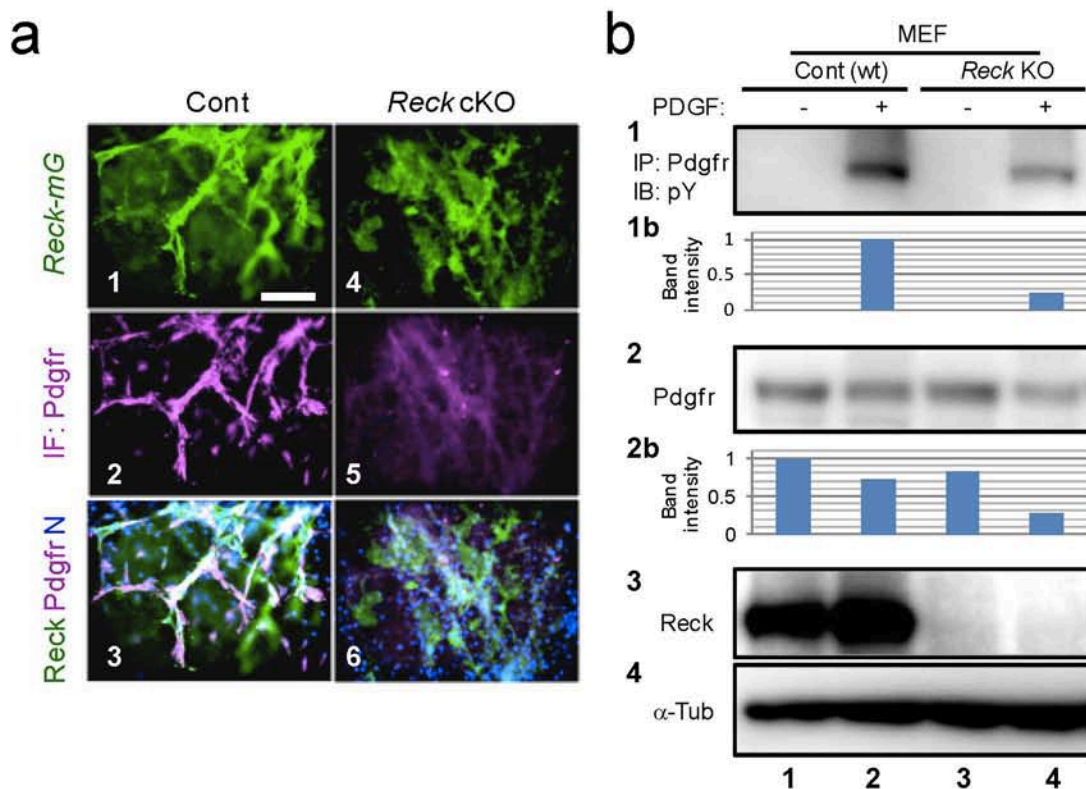


Figure 11. Functional relationship between Reck and FN in microvessel formation in ARA. (a) Effects of *Reck*-deficiency on FN fibrils in ARA. Aortic rings from control 1 (*Reck*^{+/+}; *Sm22-Cre*; *mTmG*), control 2 (*Reck*^{+/CreER}; *mTmG*), and *Reck* cKO (*Reck*^{EFf/crER}; *mTmG*) mice pretreated with tamoxifen were subjected to ARA and stained for FN (magenta, panels 3, 4, 7, 8, 11, 12) at day 10. Note that in the control samples, FN (magenta) signals are abundant around the extending sprouts (panels 3, 7), particularly accumulated in the areas where mT (red) and Sm22 (green) signals overlap (arrows in panels 1-4), but sparse near the tip of the sprouts (arrow in panels 5-8). Paucity of FN (blue) signals in *Reck* cKO sample (panels 9-12) could also be observed. **(b)** Similar cultures stained for laminin a5 (Lama5, turquoise). **(c-i)** Effects of exogenous FN on the phenotype of *Reck* cKO microvessels in ARA. ARAs were performed without or with addition of FN (1 µg/ml) in the gel. **(c)** Typical morphology of microvessels from *Reck* cKO aortic rings after incubation for 9 days in the absence (1) or presence (2) of exogenous FN. **(d-i)** Indicated parameters were measured as described in Fig 2c-f (d-h) and Fig. 4d (i) using micrographs (as shown in c) taken at day 10. Cont: n≥14 rings (3 aortae); *Reck* cKO: n≥43 rings (6 aortae). Scale bar: (a) 20 µm in panels 5-8, 100µm in others; (b) 50 µm; (c) 1 mm.

3.8. Other effects of Reck on microvessels

Dr. Morioka in the group previously found that Reck-deficient fibroblasts show defects in chemotactic movement towards PDGF⁴³. In investigating the reason for this behavioral defect, we found that PDGF receptor is decreased in these cells (Figure 12b). I also found that PDGF receptor immunoreactivity was markedly reduced on Reck cKO microvessels (Figure 12a), suggesting that Reck may protect endothelial PDGF receptor from degradation. I also noticed that the partial rescue of the association of Reck-mG cells with Reck cKO microvessels (see Figure 11i) largely represented the partially improved association of CD31-positive endothelial cells to the tip region of microvessels (Figure 12c-8). It is still true, however, that a large part of the failure in cell-cell interactions found in Reck cKO cultures cannot be explained by excessive FN degradation in the absence of Reck (see Figure 11a).



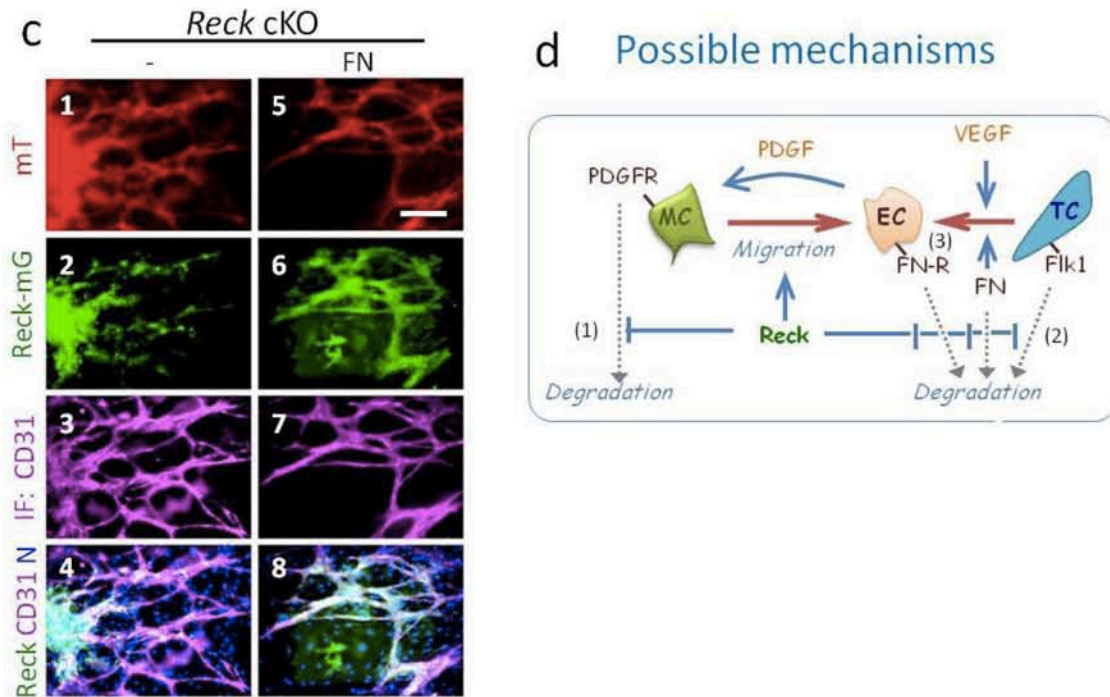


Figure 12. Possible mechanisms by which *Reck* promotes correct interactions between vascular cells. (a) Immunofluorescence for PDGF-receptor on *Reck-mG* cells. Control (1-3) and cKO (3-6) aortic rings were cultured as described in Figure 3b for 14 days and stained for PDGF-receptor (magenta) followed by nuclear-counter-staining (blue). Note that in control culture, PDGF-receptor-signals are abundant on *Reck-mG* cells (green) associated with microvessels (white signals in panel 3), whilst dampened signals are found in cKO samples (6). (b) Effects of *Reck* on abundance and activation of PDGF-receptor in fibroblasts. Sub-confluent cultures of fibroblasts prepared from *wt* or *Reck*^{-/-} (KO) mice were serum-starved for 24 h and then incubated for 10 min in the same medium (-) or medium containing 10 µg/ml PDGF-BB (R&D Systems). Cell lysates were either immunoprecipitated with anti-PDGF-receptor followed by immunoblot assay using anti-phospho-tyrosine antibody (Cell Signaling Technology #9411; panel 1) or directly subjected to immunoblot assay using antibodies against either PDGF-receptor (2), *Reck* (3), or α -tubulin (4). Intensity of bands determined using ImageJ is presented in panels 1b (ratio of absolute values) and 2b (ratio of PDGFR value divided by α -tubulin value). Note the reduced signals in KO samples, especially in the presence of PDGF (lane 4 vs. 2). (c) Effects of FN on localization of endothelial *Reck-mG* cells. Typical images of microvessels from cKO aortic rings after incubation for 13 days in the absence (1-4) or presence (5-8) of exogenous FN, followed by immunofluorescent staining for CD31 (magenta), and nuclear-counter-staining (blue). Note that white signals (i.e., endothelial *Reck-mG* cells) could be found at or near microvessel tips (arrowheads) in samples incubated in FN-supplemented gel. Scale bar: a, b 50 µm. (d) Possible mechanisms by which *Reck* facilitates mural-endothelial and tip-stalk interactions. *Reck* protects PDGF-receptors on mural cells (MC), thereby keeping MC sensitive

to the attractant (PDGF) produced by endothelial cells (EC). Reck also protects FN and Flk1 to stabilize the tip cell phenotype or to promote tip-stalk interaction directly².

Discussion

Several new findings, supporting the idea that Reck is critical for angiogenesis, were obtained in this study. First, tissue-selective Reck knockout in mural cells and endothelial cells resulted in lethality with distinct phenotypes and survival curves. In mural cells, Reck inactivation was lethal at E10.5 displaying abnormal vasculature and pericardial membrane that are reminiscent of the results of global Reck inactivation. In endothelial cells, Reck inactivation was lethal at around E18.5 showing defects mainly in brain vasculature. Second, medium changes have strong impacts on the efficiency of sprouting angiogenesis *in vitro*. Third, Reck has strong impacts on at least two steps in sprouting angiogenesis as revealed by ARA: frequency of sprouting and microvessel stability. I shall discuss these points and possible mechanisms of actions of Reck in vascular developments in this section.

4.1 Defects in dorsal aorta and heart in *Reck cKO (Sm)* mice

Chandana et al. previously proposed that dilated vessels found in *Reck*-deficient mice may result from impaired “non-sprouting” angiogenesis such as intussusception⁴⁶. The present data, however, support an alternative model that dilated vessels could result from abortive “sprouting” angiogenesis resulting from aggregation and lateral fusion of excessive, unstable microvessels (Figure 13).

Another striking phenotype shared by global Reck KO and Reck cKO (Sm) mice, the absence or breakage of pericardial (or pleuropericardial) membrane in sagittal tissue sections, was newly found in this study. Although more extensive anatomical studies

are needed to understand the exact nature of this defect, it is tempting to speculate that Reck is required for proper attachment or migration of precursor cells to this area which requires transient formation of ECM bridge ⁶⁴.

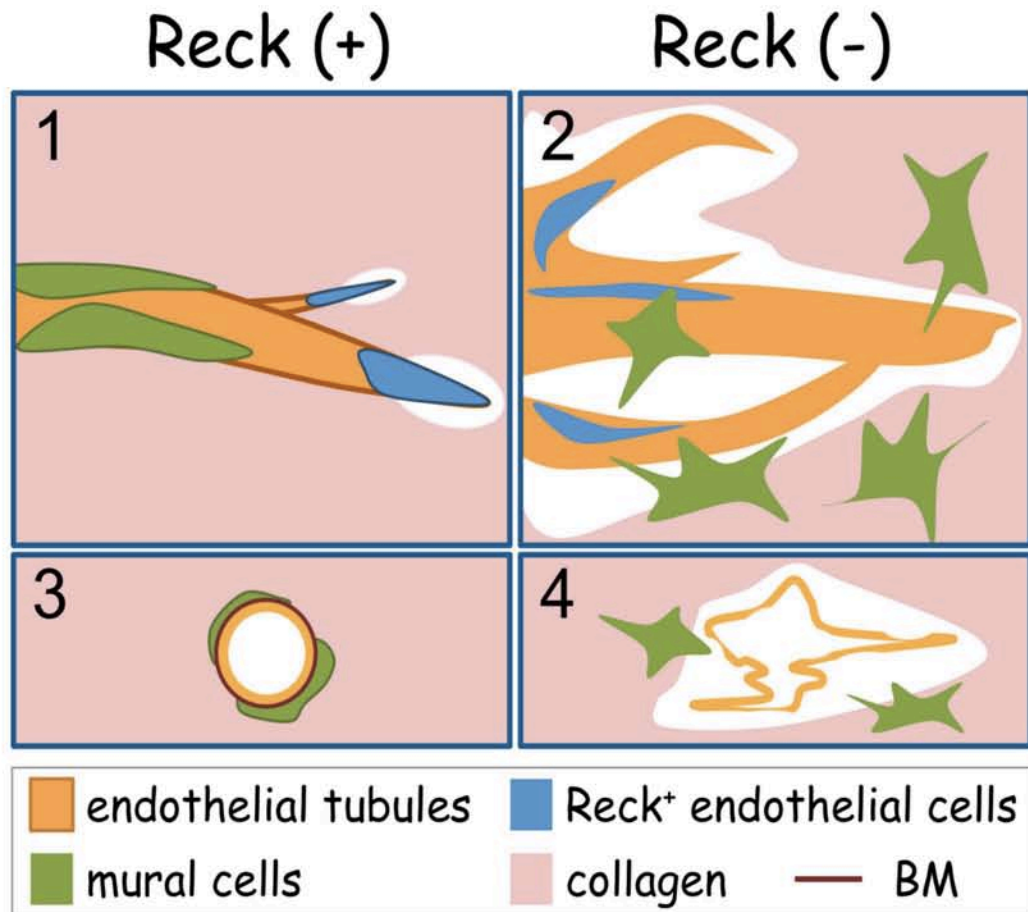


Figure 13. Reck is essential for angiogenesis. This model consistent with our findings *in vitro*. When Reck is present (panels 1, 3), endothelial sprouting led by tip cells (blue) is appropriately regulated; tight association of mural cells (green) with endothelial tubules (orange) is promoted; and individual vessels are stabilised. When Reck is absent or reduced (panels 2, 4), endothelial sprouting and collagenolysis are activated; tip cells and mural cells localise inappropriately; and microvessels destabilise, permitting lateral fusion and ectopic anastomoses.

4.2 Defects in brain vasculature in *Reck* cKO (Tie) mice

Inactivation of *Reck* in endothelial cells resulted in severe defects in brain vasculature without gross defects in other organs, such as the heart, liver, and lung, although more detailed histological analyses are required to exclude small defects. The reason for this organ-specific phenotype was unclear when we submitted our paper to the journal. A recent work by Vanhollebeke et al. has revealed, however, that *reck* and an orphan G protein-coupled receptor, *gpr124*, are required for brain angiogenesis as well as the formation of dorsal root ganglion (DRG) in zebrafish and that the mechanism involves enhancement of canonical Wnt signaling triggered specifically by *wnt7a/wnt7b* ⁴⁹. Although the reason for the organ-specific phenotype and the exact molecular mechanisms remain unclear, this new discovery will boost our understanding of how *Reck* works in angiogenesis. An advantage of zebrafish system is its transparency, and Vanhollebeke et al. found by live-imaging that *reck* and *gpr124* is required for tip cell formation in a cell-autonomous fashion ⁴⁹. This fits nicely with our finding in ARA that tip cells are mislocalized in *Reck* cKO cultures (Figure 11b-8, 11d-12, 11g-12).

4.3 Medium changes disturbs sprouting angiogenesis

I found in this study that medium changes disturb microvessel formation and that *Reck* cKO rings tolerate this effect (Figure 8). At the moment, it is unclear whether these two phenomena represent two sides of a coin or independent mechanisms. Possible mechanisms can be categorized into two groups: chemical and physical. Chemical factors include accumulation of inducers and decrease in inhibitors. Physical factors include movement of the dish, medium flow, pH, and oxygen.

Two obvious candidates of chemical factors are accumulation of

pro-angiogenic cytokines, such as VEGF, and wasting of nutrient and/or serum growth factors, thereby promoting vascular differentiation. Feasible physical factors, on the other hand, include hypoxia that triggers VEGF production, medium acidification due to glycolysis and lactate secretion ⁶⁵, and medium flow that may dilute the autocrine pro-angiogenic factors accumulated in the close vicinity of the cells. It has been hypothesized that endothelial cells use glycolysis to minimize oxygen consumption, supply efficiently avascular tissues, and synthesize quickly the block nutrients for its anabolic activities ⁶⁶, especially when they transient from quiescent to proliferative during angiogenesis, although glycolysis is also essential for endothelial homeostasis ⁶⁷.

Upon VEGF stimulation, quiescent ECs increase glycolysis by upregulating the expression of GLUT-1 (glucose transporter 1), LDH-A (lactate dehydrogenase-A), and PFKFB3 (6-phosphofructose-2 kinase/fructose-2,6-bisphosphatase-3) ⁶⁸⁻⁷¹. Intense glycolysis generates lactate that activates HIFa by inhibiting PHD2 leading to VEGF expression ^{66,72}. Lactate also inhibits IKBa, and stimulates autocrine pro-angiogenic factors, such as IL-8 ⁷³. On the other hand, low glucose makes cells highly sensitive to oxygen deprivation ⁷⁴. In this context, nutrient deprivation stimulates endothelial cells to express SIRT-1, which directly binds Notch ^{75,76} and affects tip-stalk specification. Thus, it would be interesting to test whether such molecular mechanisms may explain the effects of medium changes and/or Reck on the efficiency of microvessel formation.

4.4 Effects of Reck on vascular sprouting and stability

At least two steps of sprouting angiogenesis appear to be affected by Reck: negative effects on the rate of sprouting and positive effects on vessel stabilization. Again, it is unclear at the moment whether these two phenomena represent two sides of a coin or

independent mechanisms. Based on the known biochemical activities of Reck, a feasible model is that Reck protects ECM thereby inhibiting excessive sprouting (which would require ECM degradation) on the one hand and promoting vessel stability (which would require ECM deposition) on the other.

In light of the recent discovery that Reck enhances Wnt7a/Wnt7b signaling in tip cells⁴⁹ and our finding that tip cells are mislocalized in the absence of Reck (Figure 11), we speculate that the attachment of the non-proliferative, migratory cells (i.e., tip cells) at the tip of the sprouts may have negative effects on the growth of the proliferative stalk cells. How Reck helps keep the tip cells in place is an important question (see 4.2). Our rescue experiments suggested the involvement of FN in this process, but the extent of rescue was partial (Figure 13i). The roles of FN and the Wnt7 signaling and their relationship are interesting issues to be addressed in future studies.

4.5 Clinical implications

Our data raise the interesting possibility that Reck may serve as a key regulator of vascular morphogenesis by regulating the number and size of the vessels, the area covered by the vessels, as well as the site and timing of anastomoses (Figure 13). We speculate that RECK-dysfunction may underlie various conditions that give rise to fragile, leaky blood vessels, such as cancers⁷⁷ and RASopathies⁷⁸, since several activated oncogenes, including mutated RAS, strongly suppress RECK expression^{27,37}. Compounds capable of upregulating endogenous RECK⁷⁹ may be useful in ameliorating such conditions.

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Acknowledgements

This thesis is a result of excellent guided research under the supervision of Professor *Makoto Noda*, who I am very grateful for accepting me, and teaching me how to do high quality science.

I am very grateful to prof. *Dai Watanabe*, prof. *Naoki Watanabe*, prof. *Osamu Chisaka* and prof. *Yukinobu Nakaseko* for instructions and valueable discussion.

I sincerely thank Dr. *Tomoko Matsuzaki*, for her constant aiding during mice experiments, and for helping me to improve my technical skills and knowledge about mice. I am also very thankful to Dr *Mako Yamamoto*, Dr. *Yoko Morioka*, and *Shuichiro Ogawa* for technical training, their friendly conversations, and helping with many other matters of my life in Japan.

I would like to thank prof. *Hitoshi Kitayama*, prof. *Yoko Yoshida*, prof *Takao Miki*, Dr. *Kanako Yuki*, and all member from Molecular Oncology laboratory for their collaboration. I am also grateful to *Emi Nishimoto*, *Hai-Ou Gu*, *Mika Fujiwara*, and *Kumi Kawade* for technical assistance, and *Aki Miyazaki* for secretarial assistance. I am very thankful to MEXT for supporting my studies and for KAKENHI [Grants-in-Aid for Creative Scientific Research, Scientific Research on Priority Areas, and Scientific Research on Innovative Areas] for supporting this project. I also would like to thank my husband and my family for understanding and encouragement me during these years.

This thesis is based on the material contained in following scholar article

Glicia Maria de Almeida, Mako Yamamoto, Yoko Morioka, Shuichiro Ogawa, Tomoko Matsuzaki & Makoto Noda

Critical roles for murine Reck in the regulation of vascular patterning and stabilization. Scientific Reports 2015, 5:17860 DOI: 10.1038/srep17860