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The Wnt7's Tale: A story of an orphan who finds her tie to a famous family

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Angiogenesis, Gpr124, Reck, Wnt7a/b, zebrafish

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The transformation suppressor gene *RECK* was isolated by cDNA expression cloning (1998), and *GPR124/TEM5* was detected as a tumor endothelial marker by differential screening (2000). The importance of *Wnt7a/b* and *Gpr124* in brain angiogenesis was demonstrated by reverse genetics in mice (2008–2010). A series of recent studies using genetically engineered mice and zebrafish as well as luciferase reporter assays in cultured cells led to the discovery of functional interactions among *Reck*, *Gpr124*, and *Wnt7a/b* in triggering canonical *Wnt* signaling with relevance to embryonic brain angiogenesis and blood–brain barrier formation.

Prologue

In Shakespeare's comedy "The Winter's Tale", the infant princess Perdita (lost one) was abandoned and her mother dies due to the king's outrageous jealousy of his rival king. After 16 years, the grown-up orphan travels to her homeland with her boyfriend Florizel (flower; the rival king's son trying to establish himself) and unexpectedly learns about her origin in a magical happy ending.

A series of recent studies has set the stage for a dramatic encounter of an orphan G protein-coupled receptor, *Gpr124*, accompanied by the *Reck* tumor suppressor, with a particular pair of the *Wnt* family, *Wnt7a/b*, which together trigger canonical *Wnt* signaling to induce brain angiogenesis and blood–brain barrier (BBB) formation in vertebrates.⁽¹⁾

Gpr124: An orphan with multiple names

The story begins with the provocative paper published in 2000 by St. Croix *et al.* describing the isolation of vascular endothelial cells through an elaborate series of negative and positive selections from colon tumor and normal mucosa and the comparison of gene expression profiles between these cells using the then-current technique called serial analysis of gene expression (SAGE). This screen allowed the researchers to identify nine previously unknown genes that showed more than 16-fold higher

expression in tumor endothelial cells than the normal counterpart and were therefore termed tumor endothelial markers (TEMs).⁽²⁾ One of these genes, *TEM5*, was found to encode an orphan G protein-coupled receptor (GPCR)⁽³⁾ and later termed *GPR124*⁽⁴⁾ which was eventually classified into the adhesion G protein-coupled receptor (ADGR) family, the second largest subfamily of GPCRs consisting of 33 members, and hence conferred yet another name, *ADGRA2*.⁽⁵⁾ Most ADGRs are characterized by a long NH₂-terminal ectodomain followed by the seven transmembrane domain and the COOH-terminal cytoplasmic tail (Fig. 1, left side).⁽⁵⁾ While membrane distal extracellular regions of different ADGRs can vary greatly, homology and family affiliation are confined and determined, respectively, by a highly conserved membrane proximal domain termed the GPCR autoproteolysis-inducing domain and the highly conserved seven-pass transmembrane domain.

An important function of *Gpr124* (*Tem5*) was revealed when this gene was inactivated in mice.⁽⁶⁾ *Gpr124*-null mice show lethality from embryonic day 15.5 (E15.5) onward with hemorrhage in the forebrain and neural tube. In these embryos, angiogenic sprouting from the perineural vascular plexus into the forebrain and ventral neural tube is severely impaired and formation of hemorrhagic glomeruloid vascular malformations at the periphery of the neuroepithelium is observed. Furthermore, filopodia are misoriented in tip-like cells belonging to invading angiogenic sprouts.⁽⁶⁾ In wild-type mice, although *Gpr124* (*Tem5*) is highly expressed in the central nervous

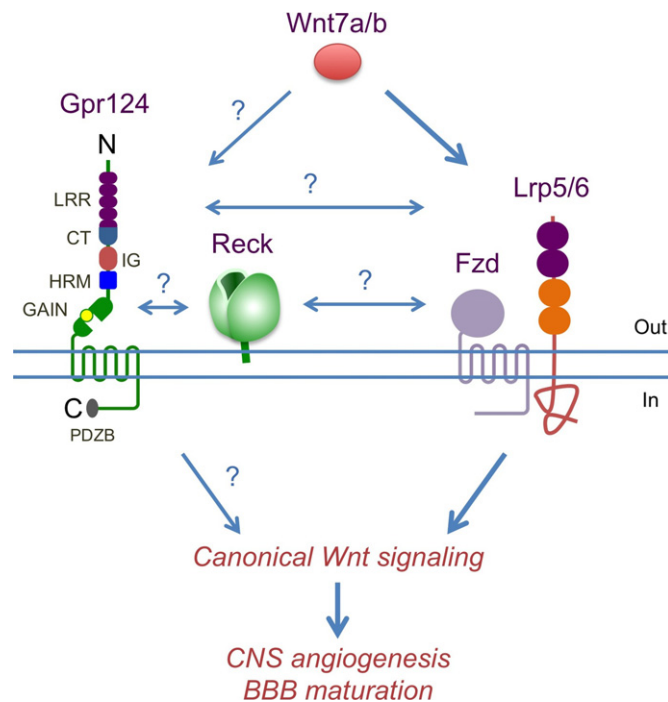


Fig. 1. Functional interactions among Gpr124, Reck, and Wnt7a/b-triggered canonical Wnt signaling which promotes central nervous system (CNS) angiogenesis and blood–brain barrier (BBB) formation. New genetic studies in mouse and zebrafish indicate functions of Gpr124 and Reck to enhance canonical Wnt signaling induced by Wnt7a/b. Question marks denote possible molecular interactions. C, COOH-terminus; CT, C-terminal domain; GAIN, G protein-coupled receptor autoproteolysis-inducing; HRM, hormone receptor motif; IG, immunoglobulin domain; LRR, leucine-rich repeat; N, NH₂-terminus; PDZB, PDZ domain-binding motif.^(3,5,14)

system (CNS) vasculature, it is also detectable in other organs and in multiple cell types. It is therefore remarkable that the mutant phenotype (vascular malformation) appears to be confined to the CNS. These mice also failed to establish an effective BBB. These findings were underscored by two papers reporting similar phenotypes of Gpr124-null mice established independently.^(7,8)

The BBB is a special property of CNS endothelium, induced and maintained by a multicellular neurovascular unit. It separates the CNS from the peripheral blood circulation and maintains an environment that allows neurons to function properly. Endothelial cells of the neurovascular unit have special characteristics, such as continuous tight junctions, lack of fenestrations, and low rates of transcytosis, which greatly limits the movement of molecules through the endothelial layer.⁽⁹⁾ Instead, passage of molecules through the BBB is regulated by a series of specific transporters that allow delivery of nutrients to the brain and extrusion of unwanted molecules. One such transporter, Glut1/Slc2a1, has been used as a marker for the BBB.⁽¹⁾ Indeed, Glut1 expression was absent in Gpr124-deficient endothelium in the forebrain,^(6–8) indicating that the mutation not only causes anatomical vascular defects, as evidenced by massive brain hemorrhage, but also affects endothelial differentiation leading to the formation of a functional BBB.

Wnt7a/b: The royal couple

In E12.5 mouse embryos, canonical Wnt signaling (as detected by the TOP-GAL reporter transgene) co-localized with

endothelium in CNS but not in other areas. Wnt7a and Wnt7b were the canonical Wnt ligands expressed in the forebrain and on the ventral neural tube where CNS angiogenesis initiates.⁽¹⁰⁾ The phenotype of Wnt7a/Wnt7b double-knockout mice^(10,11) was reminiscent of that of Gpr124-null mice.^(6–8)

An actual link between Wnt7a/b and Gpr124 was reported by Zhou and Nathans⁽¹²⁾ and by Posokhova *et al.*⁽¹³⁾ in mice and by Vanhollebeke *et al.*⁽¹⁴⁾ in zebrafish using reporter assays *in vitro* and genetic studies *in vivo*. These studies established the role of Gpr124 as a ligand (Wnt7a/b)-specific coactivator of canonical Wnt/ β -catenin signaling.

Reck: The prince likened to a flower

Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) was first isolated as a cDNA inducing morphological reversion when expressed in a mouse embryo fibroblast cell line transformed by a RAS oncogene.⁽¹⁵⁾ Despite the *in vitro* screen used for isolation, the gene soon attracted the attention of many clinicians, as its expression tends to be downregulated in various cancer tissues, including those of the lung, colon, breast, pancreas, and testis.^(16–18) It was found that RECK expression was suppressed by various stimuli and signals, such as growth factors, low cell density, hypoxia, and oncogenes.^(15,19–21) Restored RECK expression in cancer cells has been reported to suppress cell proliferation, tumor angiogenesis, invasion, and metastasis, depending on the cell lines and assay systems used.^(21–23) Frequency of spontaneous tumors increases in mice with reduced *Reck* expression (Sachiyo Yamaguchi, Tomoko Matsuzaki, Makoto Noda, *et al.*, unpublished data), demonstrating the function of *Reck* as a bona fide tumor suppressor.

RECK encodes a glycosylphosphatidylinositol-anchored glycoprotein of approximately 125 kDa that forms a tulip-shaped dimer (Fig. 2)⁽²⁴⁾ and can negatively regulate multiple extracellular proteases, such as several members of the MMP family, Adam10, and CD13.^(15,22,25,26) At the cellular level, RECK can regulate directional cell migration^(27,28) and cell cycle progression.⁽²³⁾

RECK is not found in *Caenorhabditis elegans* but is conserved from fruit fly to human as a single gene. *Reck*-deficient mice die at approximately E10.5 with reduced tissue integrity, abdominal hemorrhage,⁽²²⁾ and precocious neuronal differentiation.⁽²⁶⁾ In wild-type embryos at this stage, Reck is abundantly expressed in blood vessels (both endothelial and mural components) and the neural precursor cells.^(22,26,29–31) Experiments using tamoxifen-inducible *Reck* knockout mice indicated that global inactivation of *Reck* starting from E11 resulted in hemorrhage and vascular defects in the brain by E15.5 and embryonic death before birth.⁽²⁹⁾ Hence, *Reck* is essential for mouse embryogenesis.

Mice expressing Reck at a level <25% of the normal level are viable but show a defect in anterior–posterior limb patterning most similar to that found in Wnt7a-null mice,⁽³²⁾ albeit with peculiar asymmetry (right-dominant, forelimb-specific).⁽³³⁾

News from the aquarium

In 2012, Prendergast *et al.*⁽³⁴⁾ announced an important breakthrough in better understanding the physiological functions of RECK. They carried out a forward genetic screen in zebrafish for mutations causing deficiencies in dorsal root ganglia (DRG). They isolated four mutants lacking all DRG and found that these mutants carry independent mutations in the *reck*

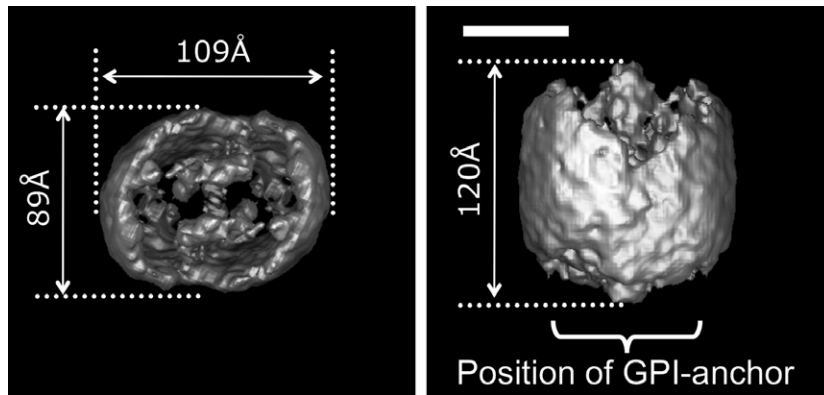


Fig. 2. Surface representation of the RECK protein. Three-dimensional reconstruction of the RECK-His dimer viewed from the top (a) and the side (b). GPI, glycosylphosphatidylinositol. Scale bar = 50 Å.⁽²⁴⁾

gene. Morpholino-mediated knockdown of *reck* phenocopied the DRG deficiency, supporting its involvement and the recessive (loss-of-function type) nature of the mutations. Transplantation experiments indicated that *reck* was required in a cell-autonomous fashion in DRG neurons. The *reck* deficiency led to slower migration and mislocalization of the neural crest-derived DRG precursor cells.⁽³⁴⁾ Interestingly, this migration requires Mmp17, a glycosylphosphatidylinositol-anchored member of the MMP family, co-expressed with Reck in DRG precursor cells.⁽³⁵⁾

More surprise from the aquarium

Vanhollebeke and coworkers introduced mutations in the zebrafish *gpr124* gene using transcription activator-like effector nucleases (TALEN).⁽¹⁴⁾ These mutant fish recapitulate the brain vascular defects found in mice, although the defects seem to be less life-threatening, for half of the mutants reached adulthood and some were even fertile. In addition, they found a lack of DRG in these mutant fish. To test the possibility that common molecules underlie these two events, they knocked down genes known to be involved in DRG formation, including *erbb3*, *sorbs3*, and *reck*, and found that *reck* morpholino could induce brain vascular defects reminiscent of that induced by *gpr124* mutations.

Furthermore, the TOP-flash Wnt reporter assay showed that Wnt7a/b-triggered canonical Wnt signaling *in vitro* was markedly enhanced when Gpr124 and Reck co-exist with the Wnt receptor, for example, Fzd4/Lrp6. They also used double immunofluorescence staining and a proximity ligation assay to show co-localization of overexpressed Gpr124 and Reck on the surface of HEK293T cells. However, although the proximity ligation assay technique demonstrates proximity, it does not unequivocally reveal direct physical interaction between Gpr124 and Reck, which yet remains to be determined.

Through elegant experiments *in vivo* with zebrafish, Vanhollebeke and colleagues further demonstrated the following points: (i) Wnt signaling is required for brain angiogenesis and DRG neurogenesis (through pharmacological and genetic approaches); (ii) both *gpr124* and *reck* are required for endothelial-specific Wnt signaling; and (iii) Gpr124/Reck-mediated Wnt signaling is required in a tip cell-specific manner during angiogenic sprouting in the brain (through cell transplantations, mosaic vessel quantification, and live imaging).

Based on these findings, Vanhollebeke *et al.* proposed three alternative models of how Gpr124 and Reck contribute to Wnt signaling: (i) Reck protects an Fzd/Lrp/Gpr124 complex from

proteolysis; (ii) Fzd/Lrp/Gpr124/Reck form a high affinity Wnt7a/b receptor; or (iii) Gpr124/Reck constitute an independent Wnt7a/b receptor (Fig. 1).⁽¹⁴⁾

The first model seems to be relevant to the previous findings that the GPR124 (TEM5) ectodomain can be shed by MMP9 and mediates endothelial cell survival by linking integrin $\alpha_v\beta_3$ to glycosaminoglycans⁽³⁶⁾ and that RECK regulates MMP9.^(15,37) Vanhollebeke *et al.*, however, argued against this model, based on the difficulty to rescue *reck* morphant phenotypes by treating embryos with various MMP/ADAM inhibitors. They instead suggested the involvement of other activities of Reck, for instance, regulation of endocytic trafficking,⁽²⁵⁾ focal adhesions, and cell polarity⁽²⁷⁾ that could influence canonical Wnt signaling.^(38,39)

No food for thought

Another forward genetics study using zebrafish has recently provided evidence *in vivo* for the involvement of Reck in Wnt signaling and BBB formation. Shaw *et al.*⁽⁴⁰⁾ previously mutagenized zebrafish expressing GFP in endothelial cells and screened mutants with defects in vascular patterning. In one such line, *no food for thought* (*nft*), a missense (C254Y) mutation in *reck* was found to be responsible for its phenotypes including loss of intracerebral central arteries (CtAs) and downregulation of Glut1 as well as Wnt signaling in cerebral vasculature.⁽⁴¹⁾ The mutant phenotype was phenocopied by inactivating Wnt signaling (by overexpression of a dominant negative form of TCF3).

The loss of CtAs in the *nft* mutant is accompanied by hyperplasia of the primordial hindbrain channels, which is reminiscent of the disorganized perineural vascular plexus found in *Reck* KO mice,⁽²⁹⁾ suggesting inefficient vascular sprouting from primordial hindbrain channels. Transplantation of wild-type endothelial cells into *nft* mutant zebrafish embryos resulted in phenotypic suppression and chimeric CtAs formation, suggesting a non-cell autonomous contribution of Reck to intracerebral vascularization.

The heart bleeds, but here is more matter for a hot brain

Another recent study using tissue-selective *Reck* knockout mice indicated that the lack of *Reck* expression in mural cells mediated by Sm22-Cre largely recapitulates the phenotypes of global *Reck* knockout mice, that is, death around E10.5 accompanied by abdominal hemorrhage and loss of the pericardial membrane.⁽³¹⁾ In contrast, the lack of *Reck* expression in endothelial cells mediated by Tie2-Cre leads to embryonic

death at much later stages (between E15.5 and P0). Of note, the most prominent phenotype in these mice is found in the brain: dilated blood vessels accompanied by massive hemorrhage and impaired cortical structure.⁽³¹⁾

Aortic tissue explants from young mice with conditionally inactivated *Reck* gene (tamoxifen-induced, *Reck*⁺ cell-specific knockout) showed (in addition to the increased collagenolytic activity) excessive sprouting of microvessels, which tended to aggregate and form thicker vessels than the control;⁽³¹⁾ this vessel instability may explain why *Reck*-deficient mice show dilated blood vessels. It was unclear, however, why the phenotype of endothelial *Reck* knockout mice was confined to the brain. A feasible answer to this question was provided by the new findings in zebrafish⁽¹⁴⁾ (see above).

Also in this system, vascular cells lacking *Reck* expression failed to associate properly with microvessels: namely, the endothelial tip cells were mislocalized while the mural cells failed to tightly associate with microvessels⁽³¹⁾ (Fig. 3a). According to the model proposed by Senger and Davis,⁽⁴²⁾ the association of mural cells to endothelial tubules triggers fibronectin (FN) deposition followed by vascular basement membrane deposition required for vascular stability (Fig. 3b). Interestingly, exogenous FN could ameliorate the instability of *Reck* conditional knockout (cKO) microvessels but not the poor association of mural cells with microvessels,⁽³¹⁾ an event upstream of FN deposition in this model (Fig. 3b).

What animals lack from head to heel

A common phenotype found in *Gpr124*-deficient and *Reck*-deficient animals (both mice and zebrafish) is the lack of blood vessels, at least in a certain part of the brain, often accompanied by brain hemorrhage. A major difference between *Gpr124*-deficient and *Reck*-deficient animals is the timing of death: global *Reck* mutants die earlier (embryonic or postnatal) than *Gpr124* mutants (some zebrafish mutants even survive to adulthood), although endothelial-specific *Reck* mutants and *Gpr124* mutants look similar in their survival curves. More subtle differences are found in the morphology of mouse

blood vessels: forebrain and neural tube vessels in *Gpr124*-deficient mice show glomeruloid malformations, whereas the vessels in *Reck*-deficient mice show larger, irregularly shaped, and often broken luminal space.

It is remarkable that independent observations by imaging (i.e., intravital imaging in zebrafish, and murine cells or tissue explants in culture) let us focus on the same phenomenon: misbehaviors of endothelial (tip) cells that lack *Gpr124* or *Reck* (Table 1). The activity of these molecules to make the tip cells function and/or localize properly seem to be cell-autonomous,⁽¹⁴⁾ whereas their activity to promote proper vascular sprouting seems to be non-cell-autonomous.^(14,41) These findings are consistent with the model that *Gpr124* and *Reck* on the tip cell surface enhance Wnt7a/b signaling in tip cells, which instruct the behavior of stalk cells that do not require *Gpr124* and/or *Reck*. It should be noted, however, that the misbehaviors of tip cells could be caused by several different events: failures in proper cell–cell or cell–matrix association,⁽³¹⁾ process extension,⁽¹⁴⁾ and directional migration.⁽⁶⁾ It was previously found that forebrain cortical cells secrete guidance cues that stimulate *Gpr124*-dependent migration of brain endothelial cells.⁽⁶⁾ Wnt7b, known as a forebrain marker,⁽⁴³⁾ is an obvious candidate for such a guidance cue. In contrast, a wide range of functions have been assigned to Wnt7a, based mainly on the neuronal phenotypes of Wnt7a knockout mice.^(44,45) In light of the recent findings discussed here, however, some of the previous interpretations may need to be re-evaluated whether or not the neuronal phenotypes represent a secondary manifestation of vascular defects.

It would also be worthwhile to clarify which findings in one system have not been confirmed in the other (Table 1, indicated by question marks). First, BBB breakdown accompanied by *Glut1* downregulation has not been confirmed in *Reck*-deficient mice. Second, DRG deficiency has been found in zebrafish mutants but not in mouse mutants. A possible explanation for this difference might be the lack of *Gpr124* expression in mouse DRG.⁽⁴⁶⁾ Of note in this context, however, Wnt signaling has been implicated in DRG development.^(47,48) Third, the importance of mural *Reck* for vascular development and survival has

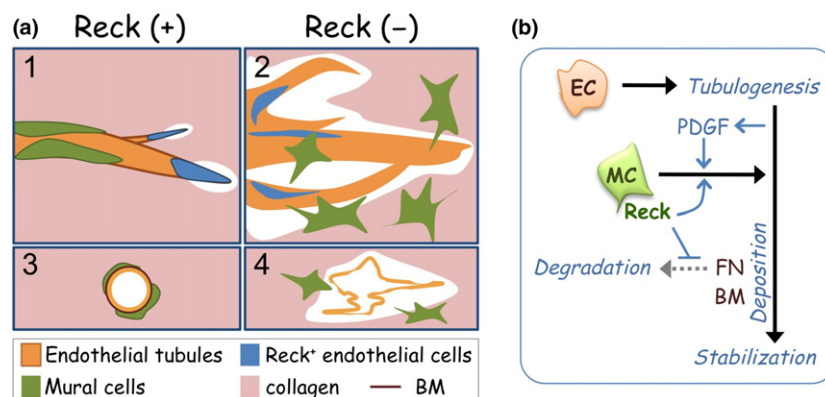


Fig. 3. Effects of *Reck* in sprouting angiogenesis.⁽³¹⁾ (a) Summary of findings by aortic ring assay. When *Reck* is present (+; panels 1 and 3), endothelial sprouting led by tip cells (blue) is appropriately regulated; tight association of mural cells (green) with endothelial tubules (orange) is promoted, and individual vessels are stabilized. When *Reck* is absent or reduced (-; panels 2 and 4), endothelial sprouting and collagenolysis are activated; tip cells and mural cells localize inappropriately, and microvessels are destabilized, permitting lateral fusion and ectopic anastomoses. (b) A model to explain how *Reck* promotes vascular stabilization. *Reck* promotes (blue arrow) tight association of mural cells (MC) to the tubule-forming endothelial cells (EC), an event known to trigger perivascular fibronectin (FN) deposition.^(2,3) *Reck* also protects FN from degradation, thereby promoting downstream events, including perivascular basement membrane (BM) deposition, which helps establish vascular stability. PDGF, platelet-derived growth factor.

Table 1. Comparison of findings on Gpr124, Reck, and their functional relationship in different experimental systems

	<i>In vivo</i>		<i>In vitro</i>
	Mouse	Zebrafish	
	Phenotype	Phenotype	TOP-flash assay
	Gpr124 KO	gpr124 KO	Gpr124, Reck, Fzd, Lrp, Wnt7a/b
	Reck cKO (Tie2) [*] cKO (Reck-CreER ^{T2})	reck MO/ [†] KO	
Brain vasculature	- CNS hemorrhage - Glomeruloid malformation - PNVP thickening - Avascular telencephalon - Low Glut-1 expression - Breakdown (tracer; Cullen) ?	- Cranial hemorrhage - Dilated vessels Avascular brain	- Avascular brain
BBB	?	?	?
DRG	?	Absent	Absent, cell-autonomous [†] (Prendargast)
Endothelial cells	Failure in chemotactic migration and sprouting <i>in vitro</i> (Cdc42-dependent, VEGF-independent, cell-autonomous) ?	Tip cells mislocalized in aortic ring assay (Fig. 3) [*] - Lack of vascular sprouting into the hindbrain tissue - Loss of tip-cell sprouts from PHBC (cell autonomous)	- Lack of vascular sprouting into the hindbrain tissue (non cell-autonomous) - PHBP hyperplasia (Ulrich) [†]
Mural cells	?	No change	No change
Wnt signaling	Reduced	Reduced	Reduced Ligand (Wnt7a/b)-specific enhancement of canonical Wnt signaling by Gpr124 plus Reck
Timing of death	E15-before adulthood	E15-P0	Before day 12 [†]
Other findings	Tg (Tie2) mice: brain hypervascularity	- Defects in cortical development - Reck KO: lethal around E10.5 with abdominal hemorrhage	- Co-localization of Gpr124 and Reck (PLA; Vanhollebeke) - Gpr124 interacts with DLG1 (Posokhova)
References	Kuhner, Anderson, Cullen, Zhou, Posokhova	Almeida [*] , Oh, Chandana	Vanhollebeke, Zhou, Posokhova Prendargast [†]

KO, global knockout; cKO, conditional or cell type-specific knockout; MO, morpholino-mediated knockdown; Tie2, endothelial cell-specific promoter; PNVP, perineural vascular plexus; Tg, transgenic; PLA, proximity ligation assay. ^{*}Results with Reck cKO mice; [†]Results with reck KO fish.

been documented in mouse.⁽³¹⁾ What about mural Gpr124? In this respect, the glomeruloid malformations in global *Gpr124*-deficient mice are phenocopied by endothelial-specific deletion of Gpr124. Furthermore, the glomeruloid malformations are covered by pericytes,⁽⁶⁾ suggesting that Gpr124 is dispensable for pericyte function *in vivo*. Vanhollebeke *et al.*⁽¹⁴⁾ reported that vascular wall coverage by pericytes was unaffected in *gpr124*-deficient zebrafish. Fourth, is Wnt signaling also affected in *Reck*-deficient mice? Finally, adult zebrafish *gpr124* mutants and a mouse *Reck* hypomorphic mutant show smaller body sizes. What is the reason for this phenotype? Addressing these questions is not just filling the space in the table but may yield more clues to new discoveries.

Toward a happy ending

Several major questions are in sight at this stage. First, how do Reck and Gpr124 cooperate in Wnt signaling? To address this question, we need to better understand the nature of molecular interactions: which protein directly interacts with which through which domains? The role(s) of DLG1, identified as a Gpr124-interactor,⁽¹³⁾ in CNS angiogenesis and other events also needs to be investigated.

A second important question is how far we can extend our knowledge of the functions of Reck and Gpr124 along this line. In other words, is enhancement of Wnt7a/b signaling the only function of Reck and Gpr124? This question has to do with a more general question of what we could learn from phenotypes of knockout animals. Phenotypes of recessive mutants usually appear in the places where functional redundancy is poor or absent. In this regard, the earlier death of *Reck* mutants with broader spectra of deficiencies than the *Gpr124* mutants (Table 1) may indicate either a broader spectrum of *Reck*'s functions or the absence of redundancy for *Reck* in broader areas of the body. Likewise, the similarity in phenotype between endothelial *Reck* cKO and *Gpr124* KO mice and the earlier death of global *Reck* KO and mural *Reck* cKO mice may indicate either the importance of *Reck* in both cell types or the absence of redundancy for *Gpr124* in endothelial cells. Nevertheless, it seems unlikely that all char-

acteristics of *Reck*-null microvessels in culture (Fig. 3a) are due to the abortive canonical Wnt signaling.

Gpr124 might exhibit redundancy via its closest paralogue Gpr125,⁽⁵⁾ which has been characterized as a spermatogonial progenitor marker,⁽⁴⁹⁾ and is expressed in choroid plexus.⁽⁵⁰⁾ Gpr125, however, regulates non-canonical Wnt/PCP signaling in zebrafish⁽⁵¹⁾ and shows little effects on canonical Wnt signaling *in vitro*.^(9,10) Importance of the Gpr124 ectodomain in mediating canonical Wnt7 signaling has been reported.⁽¹⁰⁾ Some of the other ADGR family members share the same motifs with Gpr124 (e.g., hormone receptor motif) in their ectodomain.⁽⁵⁾ Comparison between precise expression patterns and knockout phenotypes of these genes during embryogenesis may provide some clues to this issue. Efforts to address the first question (e.g., elucidating the partners and domains involved in molecular interactions) may also provide information useful for designing further genetic experiments. A previous study with *Reck* hypomorphic mice suggested a functional link between Reck and Wnt7a in limb patterning.^(32,33) Whether this reflects an extra-CNS and Gpr124-independent role of Reck in Wnt7a signaling or a totally different mechanism is another interesting question to be addressed in future studies. Additionally, it will be interesting to explore the role of Gpr124 and Reck in adult pathologic states.

But now, let us celebrate the happy reunion of the orphan (Gpr124) and her tulip-shaped partner (Reck) with the royal couple (Wnt7a/b)!

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Disclosure Statement

The authors have no conflict of interest.

References

- Vallon M, Chang J, Zhang H, Kuo CJ. Developmental and pathological angiogenesis in the central nervous system. *Cell Mol Life Sci* 2014; **71**: 3489–506.
- St Croix B, Rago C, Velculescu V *et al*. Genes expressed in human tumor endothelium. *Science* 2000; **289**: 1197–202.
- Carson-Walter EB, Watkins DN, Nanda A, Vogelstein B, Kinzler KW, St Croix B. Cell surface tumor endothelial markers are conserved in mice and humans. *Cancer Res* 2001; **61**: 6649–55.
- Fredriksson R, Gloriam DE, Hoglund PJ, Lagerstrom MC, Schiöth HB. There exist at least 30 human G-protein-coupled receptors with long Ser/Thr-rich N-termini. *Biochem Biophys Res Commun* 2003; **301**: 725–34.
- Hamann J, Aust G, Arac D *et al*. International Union of Basic and Clinical Pharmacology. XCIV. Adhesion G protein-coupled receptors. *Pharmacol Rev* 2015; **67**: 338–67.
- Kuhnert F, Mancuso MR, Shamloo A *et al*. Essential regulation of CNS angiogenesis by the orphan G protein-coupled receptor GPR124. *Science* 2010; **330**: 985–9.
- Cullen M, Elzarrad MK, Seaman S *et al*. GPR124, an orphan G protein-coupled receptor, is required for CNS-specific vascularization and establishment of the blood-brain barrier. *Proc Natl Acad Sci USA* 2011; **108**: 5759–64.
- Anderson KD, Pan L, Yang XM *et al*. Angiogenic sprouting into neural tissue requires Gpr124, an orphan G protein-coupled receptor. *Proc Natl Acad Sci USA* 2011; **108**: 2807–12.
- Obermeier B, Daneman R, Ransohoff RM. Development, maintenance and disruption of the blood–brain barrier. *Nat Med* 2013; **19**: 1584–96.
- Daneman R, Agalliu D, Zhou L, Kuhnert F, Kuo CJ, Barres BA. Wnt/beta-catenin signaling is required for CNS, but not non-CNS, angiogenesis. *Proc Natl Acad Sci USA* 2009; **106**: 641–6.
- Stenman JM, Rajagopal J, Carroll TJ, Ishibashi M, McMahon J, McMahon AP. Canonical Wnt signaling regulates organ-specific assembly and differentiation of CNS vasculature. *Science* 2008; **322**: 1247–50.
- Zhou Y, Nathans J. Gpr124 controls CNS angiogenesis and blood-brain barrier integrity by promoting ligand-specific canonical wnt signaling. *Dev Cell* 2014; **31**: 248–56.
- Posokhova E, Shukla A, Seaman S *et al*. GPR124 functions as a WNT7-specific coactivator of canonical beta-catenin signaling. *Cell Rep* 2015; **10**: 123–30.
- Vanhollebeke B, Stone OA, Bostaille N *et al*. Tip cell-specific requirement for an atypical Gpr124- and Reck-dependent Wnt/beta-catenin pathway during brain angiogenesis. *Elife* 2015; **4**. doi: 10.7554/eLife.06489.
- Takahashi C, Sheng Z, Horan TP *et al*. Regulation of matrix metalloproteinase-9 and inhibition of tumor invasion by the membrane-anchored glycoprotein RECK. *Proc Natl Acad Sci USA* 1998; **95**: 13221–6.
- Clark JC, Thomas DM, Choong PF, Dass CR. RECK—a newly discovered inhibitor of metastasis with prognostic significance in multiple forms of cancer. *Cancer Metastasis Rev* 2007; **26**: 675–83.
- Noda M, Takahashi C. Recklessness as a hallmark of aggressive cancer. *Cancer Sci* 2007; **98**: 1659–65.

- 18 Noda M, Takahashi C, Matsuzaki T, Kitayama H. What we learn from transformation suppressor genes: lessons from RECK. *Future Oncol* 2010; **6**: 1105–16.
- 19 Sasahara RM, Takahashi C, Noda M. Involvement of the Sp1 site in ras-mediated downregulation of the RECK metastasis suppressor gene. *Biochem Biophys Res Commun* 1999; **264**: 668–75.
- 20 Hatta M, Matsuzaki T, Morioka Y, Yoshida Y, Noda M. Density- and serum-dependent regulation of the Reck tumor suppressor in mouse embryo fibroblasts. *Cell Signal* 2009; **21**: 1885–93.
- 21 Loayza-Puch F, Yoshida Y, Matsuzaki T, Takahashi C, Kitayama H, Noda M. Hypoxia and RAS-signaling pathways converge on, and cooperatively downregulate, the RECK tumor-suppressor protein through microRNAs. *Oncogene* 2010; **29**: 2638–48.
- 22 Oh J, Takahashi R, Kondo S *et al*. The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. *Cell* 2001; **107**: 789–800.
- 23 Yoshida Y, Ninomiya K, Hamada H, Noda M. Involvement of the SKP2-p27(KIP1) pathway in suppression of cancer cell proliferation by RECK. *Oncogene* 2012; **31**: 4128–38.
- 24 Omura A, Matsuzaki T, Mio K *et al*. RECK forms cowbell-shaped dimers with membrane type 1 matrix metalloproteinase-catalyzed cleavage of fibronectin. *J Biol Chem* 2009; **284**: 3461–9.
- 25 Miki T, Takegami Y, Okawa K, Muraguchi T, Noda M, Takahashi C. The reversion-inducing cysteine-rich protein with Kazal motifs (RECK) interacts with membrane type 1 matrix metalloproteinase and CD13/aminopeptidase N and modulates their endocytic pathways. *J Biol Chem* 2007; **282**: 12341–52.
- 26 Muraguchi T, Takegami Y, Ohtsuka T *et al*. RECK modulates Notch signaling during cortical neurogenesis by regulating ADAM10 activity. *Nat Neurosci* 2007; **10**: 838–45.
- 27 Morioka Y, Monypenny J, Matsuzaki T *et al*. The membrane-anchored metalloproteinase regulator RECK stabilizes focal adhesions and anterior-posterior polarity in fibroblasts. *Oncogene* 2009; **28**: 1454–64.
- 28 Yuki K, Yoshida Y, Inagaki R, Hiai H, Noda M. E-cadherin-downregulation and RECK-upregulation are coupled in the non-malignant epithelial cell line MCF10A but not in multiple carcinoma-derived cell lines. *Sci Rep* 2014; **4**: 4568.
- 29 Chandana EP, Maeda Y, Ueda A *et al*. Involvement of the Reck tumor suppressor protein in maternal and embryonic vascular remodeling in mice. *BMC Dev Biol* 2010; **10**: 84.
- 30 Wang H, Imamura Y, Ishibashi R, Chandana EP, Yamamoto M, Noda M. The Reck tumor suppressor protein alleviates tissue damage and promotes functional recovery after transient cerebral ischemia in mice. *J Neurochem* 2010; **115**: 385–98.
- 31 Almeida GM, Yamamoto M, Morioka Y, Ogawa S, Matsuzaki T, Noda M. Critical roles for murine Reck in the regulation of vascular patterning and stabilization. *Sci Rep* 2015; **5**: 17860.
- 32 Parr BA, McMahon AP. Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. *Nature* 1995; **374**: 350–3.
- 33 Yamamoto M, Matsuzaki T, Takahashi R *et al*. The transformation suppressor gene Reck is required for postaxial patterning in mouse forelimbs. *Biol Open* 2012; **1**: 458–66.
- 34 Prendergast A, Linbo TH, Swarts T *et al*. The metalloproteinase inhibitor Reck is essential for zebrafish DRG development. *Development* 2012; **139**: 1141–52.
- 35 Leigh NR, Schupp MO, Li K *et al*. Mmp17b is essential for proper neural crest cell migration in vivo. *PLoS ONE* 2013; **8**: e76484.
- 36 Vallon M, Essler M. Proteolytically processed soluble tumor endothelial marker (TEM) 5 mediates endothelial cell survival during angiogenesis by linking integrin alpha(v)beta3 to glycosaminoglycans. *J Biol Chem* 2006; **281**: 34179–88.
- 37 Takagi S, Simizu S, Osada H. RECK negatively regulates matrix metalloproteinase-9 transcription. *Cancer Res* 2009; **69**: 1502–8.
- 38 Fonar Y, Frank D. FAK and WNT signaling: the meeting of two pathways in cancer and development. *Anticancer Agents Med Chem* 2011; **11**: 600–6.
- 39 Feng Q, Gao N. Keeping Wnt signalosome in check by vesicular traffic. *J Cell Physiol* 2014; **230**: 1170–80.
- 40 Shaw KM, Castranova DA, Pham VN *et al*. fused-somites-like mutants exhibit defects in trunk vessel patterning. *Dev Dyn* 2006; **235**: 1753–60.
- 41 Ulrich F, Carretero-Ortega J, Menendez J *et al*. Reck enables cerebrovascular development by promoting canonical Wnt signaling. *Development* 2015; **143**: 147–59.
- 42 Senger DR, Davis GE. Angiogenesis. *Cold Spring Harb Perspect Biol* 2011; **3**: a005090.
- 43 Parr BA, Shea MJ, Vassileva G, McMahon AP. Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* 1993; **119**: 247–61.
- 44 Qu Q, Sun G, Murai K *et al*. Wnt7a regulates multiple steps of neurogenesis. *Mol Cell Biol* 2013; **33**: 2551–9.
- 45 Fernando CV, Kele J, Bye CR *et al*. Diverse roles for Wnt7a in ventral mid-brain neurogenesis and dopaminergic axon morphogenesis. *Stem Cells Dev* 2014; **23**: 1991–2003.
- 46 Homma S, Shimada T, Hikake T, Yaginuma H. Expression pattern of LRR and Ig domain-containing protein (LRRIG protein) in the early mouse embryo. *Gene Expr Patterns* 2009; **9**: 1–26.
- 47 Hari L, Brault V, Kleber M *et al*. Lineage-specific requirements of beta-catenin in neural crest development. *J Cell Biol* 2002; **159**: 867–80.
- 48 Lee HY, Kleber M, Hari L *et al*. Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. *Science* 2004; **303**: 1020–3.
- 49 Seandel M, Falciatori I, Shmelkov SV, Kim J, James D, Rafii S. Niche players: spermatogonial progenitors marked by GPR125. *Cell Cycle* 2008; **7**: 135–40.
- 50 Pickering C, Hagglund M, Szmydynger-Chodobska J *et al*. The adhesion GPCR GPR125 is specifically expressed in the choroid plexus and is upregulated following brain injury. *BMC Neurosci* 2008; **9**: 97.
- 51 Li X, Roszko I, Sepich DS *et al*. Gpr125 modulates Dishevelled distribution and planar cell polarity signaling. *Development* 2013; **140**: 3028–39.