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

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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.1–3

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).2–5

One of these genes, TEM5, was found to encode an orphan G protein-coupled receptor (GPCR)3 and later termed GPR1244 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
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) indicating that the mutation not only causes hemorrhage and vascular defects in the brain by E15.5 and embryogenesis. 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; Slc2a1, has been used as a marker for the BBB. (1) Indeed, Glut1 expression was absent in Gpr124-deficient endothelium 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 Matsuuzaki, 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 (25) and precocious neuronal differentiation. (20)** 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 recomp
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 α,β3 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 BBF 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 arterioles (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, (26) 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 CTA 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 \textit{Reck} gene (tamoxifen-induced, \textit{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 \textit{Reck}-deficient mice show dilated blood vessels. It was unclear, however, why the phenotype of endothelial \textit{Reck} knockout mice was confined to the brain. A feasible answer to this question was provided by the new findings in zebrafish\(^{(15)}\) (see above).

Also in this system, vascular cells lacking \textit{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,\(^{(32)}\) 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 \textit{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 \textit{Gpr124}-deficient and \textit{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 \textit{Gpr124}-deficient and \textit{Reck}-deficient animals is the timing of death: global \textit{Reck} mutants die earlier (embryonic or postnatal) than \textit{Gpr124} mutants (some zebrafish mutants even survive to adulthood), although endothelial-specific \textit{Reck} mutants and \textit{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 \textit{Gpr124}-deficient mice show glomeruloid malformations, whereas the vessels in \textit{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 \textit{Gpr124} or \textit{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 \textit{Gpr124} and \textit{Reck} on the tip cell surface enhance Wnt7a/b signaling in tip cells, which instruct the behavior of stalk cells that do not require \textit{Gpr124} and/or \textit{Reck}. It should be noted, however, that the misbehaviors of tip cells could be caused by several different events: failures in proper 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 \textit{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 \textit{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 \textit{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 \textit{Gpr124} expression in mouse mutants; however, Wnt signaling has been implicated in DRG development.\(^{(47,48)}\) Third, the importance of mural \textit{Reck} for vascular development and survival has

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**Fig. 3.** Effects of \textit{Reck} in sprouting angiogenesis.\(^{(31)}\) (a) Summary of findings by aortic ring assay. When \textit{Reck} is present (\textit{+}; 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 \textit{Reck} is absent or reduced (\textit{−}; 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 \textit{Reck} promotes vascular stabilization. \textit{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.\(^{(15,39)}\) \textit{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.
|                      | Mouse                  | Zebrafish              | HEK-293 cells          |
|----------------------|------------------------|------------------------|------------------------|
|                      | **Phenotype**          | **Phenotype**          | **TOP-flash assay**    |
| Gpr124 KO            | Reck cKO (Tie2) cKO   | gpr124 KO              | reck MO+/KO            |
|                      | (Reck-CreER\textsuperscript{2}) |                       | Gpr124, Reck, Fzd, Lrp, Wnt7a/b |
| Brain vasculature    | - CNS hemorrhage       | Avascular brain        | - Avascular brain      |
|                      | - Glomeruloid          |                        |                        |
|                      | malformation           |                        |                        |
|                      | - PNVP thickening      |                        |                        |
|                      | - Avascular telencephalon |                      |                        |
|                      | - Low Glut-1           | - Cranial hemorrhage   | - Avascular brain      |
|                      | expression             | - Dilated vessels      |                        |
| BBB                  | ?                      | ?                      | ?                      |
| DRG                  | ?                      | Absent                | Absent, cell-autonomous\textsuperscript{†} (Prendargast) |
| Endothelial cells    | Failure in chemotactic migration and sprouting in vitro (Cdc42-dependent, VEGF-independent, cell-autonomous) | Tip cells mislocalized in aortic ring assay (Fig. 3) * | - Lack of vascular sprouting into the hindbrain tissue (non cell-autonomous) - PHBP hyperplasia (Ulrich) \textsuperscript{†} |
| Mural cells          | ?                      | Mislocalized in vivo and in aortic ring assay (Fig. 3) * | 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\textsuperscript{†} |
| Other findings       | Tg (Tie2) mice: brain hypervascularity | - Defects in cortical development - Reck KO: lethal around E10.5 with abdominal hemorrhage | - 50% healthy and fertile - Smaller body size - Disrupted skin pigmentation pattern |
| References           | Kuhner, Anderson, Cullen, Zhou, Posokhova | Almeida*, Oh, Chandana | Vanhollebeke, Ulrich\textsuperscript{†}, Prendargast\textsuperscript{†} |
|                      |                       |                       | Vanhollebeke, Zhou, Posokhova |

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 \textit{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.

\textbf{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 characteristics of Reck-null microvessels in culture (Fig. 3a) are due to the abortive canonical Wnt signaling.

Gpr124 might exhibit redundancy via its closest parologue 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 \textit{in vitro}.\(^{9,10}\) Impotence 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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