

Wnt/Frizzled signaling in angiogenesis

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Abstract The roles of growth factors such as angiopoietin (Ang) and vascular endothelial growth factor (VEGF) in angiogenesis have been known for some time, yet we have just an incipient appreciation for the contribution of Wnts to this process. Cellular proliferation and polarity, apoptosis, branching morphogenesis, inductive processes, and the maintenance of stem cells in an undifferentiated, proliferative state are all regulated by Wnt signaling. The development and maintenance of vascular structures are dependent on all these processes, and their orchestration has, to some extent, been revealed in studies of VEGF and Ang receptors. Recent evidence links the Wnt/Frizzled signaling pathway to proper vascular growth in mammals but our knowledge of Wnt function in the vasculature is rudimentary. Further insight into vascular development and the process of angiogenesis depends on evaluating the function of novel endothelial regulatory pathways such as Wnt/Frizzled signaling.

Keywords Wnt · Frizzled · Signaling · Norrin · Retinal vasculature

Cellular proliferation and polarity, apoptosis, branching morphogenesis, inductive processes, and maintenance of stem cells in an undifferentiated state are all regulated by Wnt signaling [1–3]. The development of vascular structures involves these cellular processes and is dependent in large part by the action of vascular endothelial growth factor (VEGF) in concert with a variety of other signaling

pathways [4–9]. This review will discuss evidence that the Wnt/Frizzled signaling pathway, in concert with VEGF, is required for proper vascular growth in mammals.

Wnt signal transduction

Wnts are a family of 19 secreted glycoproteins that accumulate in the extracellular matrix to activate pathways in adjacent cells. Wnt ligands trigger these pathways by binding an appropriate receptor that belongs to a family of seven-pass transmembrane proteins, the frizzleds, of which there are 10 members [10]. A co-receptor, LRP5/6 [11–13], is required to activate some Wnt signaling pathways, and these can be blocked when LRP5/6 is bound to a secreted inhibitory protein, Dickkopf-1 [14–16]. Frizzleds are G-protein-coupled receptors [17, 18] and Wnt binding to frizzleds can activate more than one distinct branch of the Wnt signaling cascade. These distinct cascades are often referred to as the *canonical* or Wnt/ β -catenin pathway and the *non-canonical*, which includes Wnt/calcium signaling and the planar cell polarity (PCP) pathways. These are described in the ensuing sections and a cartoon representation of the common component proteins in the different branches of the Wnt signaling is provided in Fig. 1.

Canonical pathway—Wnt/ β -catenin

The accumulation of β -catenin in the cytoplasm of normal cells is prevented by gene products that promote its destruction by phosphorylation and ubiquitin-mediated proteosomal degradation [19, 20]. This is a cellular default mechanism that is interrupted, normally, by Wnt signaling. The tumor suppressor, adenomatous polyposis coli (APC),

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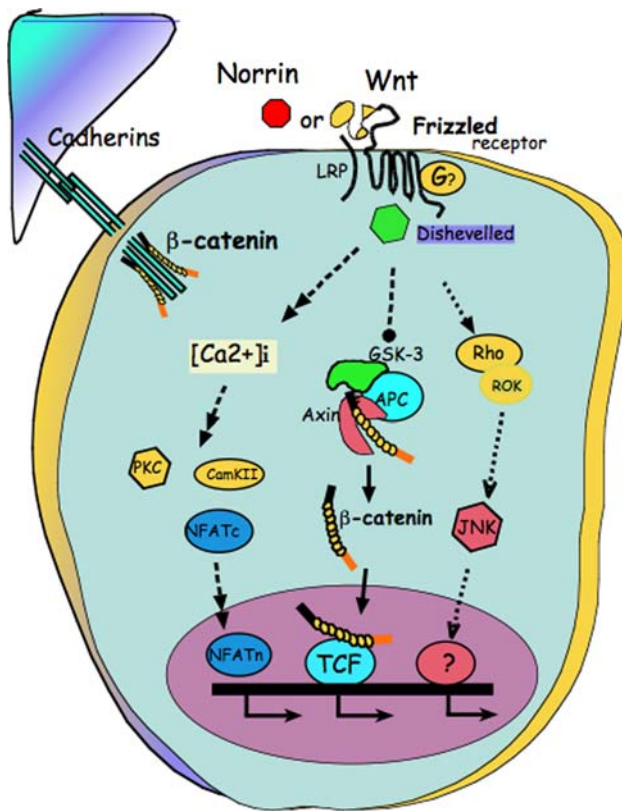


Fig. 1 Schematic representation of the branches of the Wnt signaling cascade. The canonical pathway is represented in the center where β -catenin occurs in a complex, destined for degradation unless Wnt (yellow) or Norrin (red) transduces a signal through frizzled and its co-receptor, LRP, resulting in its transport to the nucleus as a co-transcription factor of TCF. Non-canonical pathways activate calcium ion influx and NFAT (on the left) or elements of planar cell polarity (on the right). LRP, LDL receptor related protein 5/6; G 2 , unspecified GTP binding protein; [Ca $^{2+}$] $_i$, calcium ion influx; GSK-3, glycogen synthase kinase-3; APC, adenomatous polyposis coli; PKC, protein kinase C; CamKII, Ca $^{2+}$ -calmodulin kinase II; NFATc and NFATn, nuclear factor of activated T cells in cytoplasm (c) and nucleus (n), respectively; TCF, T cell factor; Rho and Rok, Rho A family of small GTPases; JNK, c-jun N-terminal kinase

and axin function as a scaffold to bring the Ser/Thr kinase, glycogen synthase kinase (GSK)-3 into proximity with its target, β -catenin [21]. Phosphorylation of the β -catenin NH $_2$ -terminus by GSK-3 is a recognition signal for the E3-ubiquitin ligase β -TrCP [22]. Canonical Wnt signaling elevates the level of cytosolic β -catenin, transiently, by activating and recruiting Dishevelled (Dvl) to the membrane [23] thus blocking the phosphorylation of β -catenin by GSK-3 [24]. Dishevelled is juxtaposed to GSK-3 in the complex via an amino-terminal domain [25]. Wnt-induced β -catenin accumulation in the cytoplasm promotes its entry into the nucleus where it binds the co-factor Tcf/LEF-1 [26, 27] to stimulate the transcription of genes that are implicated in cell growth regulation [28–30].

Non-canonical pathway—Wnt/Ca $^{2+}$

This pathway has been identified as a distinct branch of Wnt signaling [31]. The release of intracellular Ca $^{2+}$ [17] and the activation of Ca $^{2+}$ -sensitive enzymes such as protein kinase C [32] and Ca $^{2+}$ -calmodulin kinase II (CaMKII) [33] are features that are characteristic of the Wnt/Ca $^{2+}$ pathway. It is believed that the non-canonical pathway turns off the Wnt/ β -catenin pathway upstream of TCF/LEF-1 by activating a kinase cascade that, ultimately, prevents TCF/LEF-1 binding DNA by NEMO-linked kinase (NLK). Thus, activated CaMKII phosphorylates TAK1 that, in turn, phosphorylates NLK [34, 35].

The Wnt/Ca $^{2+}$ pathway also activates calcineurin, a Ca $^{2+}$ -dependent phosphatase that activates the transcription factor NFAT [36]. One key NFAT target gene [37] is *DSCR1*, which encodes a calcineurin inhibitor. *DSCR1* is expressed in developing endocardium and endothelial cells [38]. *DSCR1* and NFAT are thought to exist in a feedback loop where *DSCR1* inhibits calcineurin-mediated activation of NFAT. Activated (dephosphorylated) NFAT, on the other hand, can stimulate the transcription of *DSCR1* due to the presence of NFAT binding sites within the *DSCR1* promoter. There is no known direct physiological relationship between non-canonical Wnt signaling and *DSCR1* at this time. Furthermore, it is not clear how these different Ca $^{2+}$ signaling effectors become activated by frizzleds.

Non-canonical pathway—planar cell polarity (PCP)

Manifestations of PCP, as originally described in *Drosophila*, include the parallel alignment of abdominal sensory bristles and cytoplasmic extensions in wing cells (or cellular hairs) and the orderly arrangement of ommatidia in the eye [39]. The organization of inner ear sensory hair cell bundles and the migration and intercalation of cells along the midline during gastrulation are manifestations of PCP in vertebrates [40, 41]. These morphogenetic processes require coordinated changes to the cytoskeleton and gene expression. Frizzleds, Dishevelled (Dvl), small GTPases RhoA and Rac1, and c-jun N-terminal kinase (JNK) regulate PCP. The relevance of PCP to the development and function of the vasculature is obscure.

Wnt/Frizzled function in the vasculature

Genetic analysis of Wnt/Frizzled in vasculature

Compelling evidence for a role of Wnt signaling in the vasculature comes from analyses of mice that have targeted disruptions of Wnt/frizzled genes and from analyses of

several human vascular disorders linked to Frizzled-4 (Fz-4). These studies show that canonical and non-canonical Wnt signaling function in the vasculature.

Wnt-2

Targeted disruption of the *Wnt-2* gene in mice leads to defects in the placental vasculature that is marked by reduced capillaries of fetal origin [42]. This defect arises from either increased apoptosis or decreased proliferation of endothelial cells. Other manifestations of the phenotype include pooling of maternal blood, edema, and disruption of the labyrinth zone, possibly due to reduced blood flow to the placenta. The expression of *Wnt-2* in fetal vessels of the placenta is consistent with a placental vasculature function [42]. *Wnt-2* is a ligand that activates the *Wnt/β*-catenin signaling cascade [43].

Wnt-4

Vascular formation in the mammalian gonad occurs in a sex-specific manner, during which endothelial cells migrate from the mesonephros into the gonad to form a coelomic blood vessel. Analysis of *Wnt-4* knock-out mice shows that *WNT4* represses mesonephric endothelium in the XX gonad, preventing the formation of male-specific coelomic blood vessels [44].

Wnt-7b

Disruption of the *Wnt-7b* gene in mice leads to perinatal death due to respiratory failure that is presumably from a vascular defect [8, 45]. Newborn mutant mice exhibit severe defects in the smooth muscle component of the major pulmonary vessels causing rupture of major vessels and hemorrhage in the lungs after birth. *Wnt-7b* gene expression in airway epithelia may facilitate survival of pulmonary smooth muscle cells. It is unclear which branch of Wnt signaling is activated by *Wnt-7b* ligand.

Frizzled-5

Angiogenesis of the yolk sac and placenta is regulated by *Fz-5* gene expression [46]. Disruption of the murine *Fz-5* gene leads to embryonic lethality by E11.5 due to improper yolk sac and placental angiogenesis. The phenotype is characterized by defects in large vitelline vessels and disorganization of the capillary plexus. It is thought that these defects arise during angiogenic remodeling because the

primary vessel structure seems to form normally. There is a reduction in the proliferation of yolk sac endothelial cells. It is unclear which branch of the Wnt signaling cascade is regulated by *Fz-5* in this context because it is capable of responding to *Wnt-2*, known to stimulate *Wnt/β*-catenin signaling and *Wnt-5a*, which is thought to activate the non-canonical pathways [47].

Frizzled-4

The human *Fz-4* gene is linked to familial exudative vitreoretinopathy (FEVR), a hereditary ocular disorder characterized by a failure of peripheral retinal vascularization [48]. Mutations in the *Fz-4* gene that are linked to FEVR are predicted to result in expression of a truncated *Fz-4* protein that lacks the cytoplasmic domain. FEVR is an autosomal dominant disorder where mutant *Fz-4* protein may interfere with the activity of the normal gene product. Biochemical signaling assays reveal that mutant *Fz-4* protein lacks signaling activity [48]. Expression of normal *Fz-4* causes activation of CAMKII and protein kinase C, both components of the *Wnt/Ca²⁺* pathway. *Frizzled-4* does not appear to activate the *Wnt/β*-catenin pathway [48]. Thus, defective retinal angiogenesis that is associated with FEVR may be linked to improper *Fz-4*-mediated *Wnt/Ca²⁺* signaling. *Frizzleds* can homo- and hetero-oligomerize in the endoplasmic reticulum (ER). A recent report shows that *Fz-4* mutants that are associated with FEVR form defective hetero-oligomers with wild-type that accumulate in the ER of cultured cells [49]. This explains the genetic dominance of mutant *Fz-4* in FEVR. The authors also report that *Fz-4* can activate the *Wnt/β*-catenin pathway [49]. Thus, *Fz-4* may transduce signals through *Wnt/β*-catenin and *Wnt/Ca²⁺* pathways. Defects in one or both pathways may contribute to the FEVR phenotype.

Disruption of the mouse *Fz-4* gene leads to progressive cerebellar, auditory, and esophageal dysfunction [50]; however, further analysis of these mice reveals a retinal vascular defect [51]. This defect is reminiscent of the human FEVR phenotype.

Norrin/Frizzled-4

Incomplete retinal neovascularization occurs in both Norrie disease (ND) [52] and FEVR. Norrie disease is a sex-linked, congenital, progressive oculo-acoustico-cerebral degenerative condition [52]. ND is characterized by a spectrum of fibrous and vascular changes of the retina at birth through childhood that causes varying degrees of visual impairment. The phenotype of the human disorder mimics the spectrum of phenotypes in the *Fz-4* knockout

mouse. Norrin, the protein product of the ND gene (also referred to as Norrie disease protein, NDP), is a cysteine-rich secreted protein having no structural similarity to Wnts [53]. The Norrin protein is linked to several eye disorders that are manifested by abnormal retinal vascular development, including ND, FEVR, retinopathy of prematurity (ROP) [54], and Coats' disease [55]. Coats' disease is characterized by abnormal retinal vascular development, and Coats' telangiectasis has been linked to somatic mutations in the NDP gene that affects Norrin within the developing retina [56]. Norrin and Fz-4 function as a ligand-receptor pair [51], a finding that is remarkable, in itself, as proteins that are unrelated to Wnts can function as Frizzled ligands. The evidence that Norrin signals through Fz-4 during retinal vascular development includes: (1) the similarity in vascular phenotypes of Norrin and Fz-4 mutations in humans and mice, (2) the specificity of Norrin–Fz-4 binding, (3) Norrin induces Fz-4-dependent activation of the Wnt/ β -catenin pathway, and (4) the signaling defects displayed by disease-associated variants of Norrin and Fz-4. Norrin–Fz-4 signaling thus plays a central role in vascular development in the eye. Differing inheritance patterns of FEVR and ND suggest separate etiologies. However, the discovery of a functional and physical relationship between Norrin and Fz-4 demonstrates that these two retinopathies are manifestations of the same physiological pathway. This is true for osteoporosis pseudoglioma as well, where mutation in the LRP5 gene contributes to detached retina and bone density disorders [57, 58]. Four FEVR-linked loci have been mapped and characterized, EVR-1, 2, 3, and 4. EVR1, 2, and 4 encode Fz-4, Norrin and LRP5, respectively. Norrie Disease and FEVR have different inheritance patterns because Norrin is X-linked recessive whereas Fz-4 exhibits dominant and recessive inheritance patterns, as does LRP-5. Patients with FEVR can present with a range of symptoms including complete blindness due to retinal detachment, retinal vascular defects, and persistence of fetal vasculature, digital malformation, and bone density abnormalities. Thus, multi-gene control of FEVR suggests a wide range of abnormalities.

Wnt/Frizzled expression and activity in endothelial cells

The Wnt/ β -catenin pathway is thought to promote the survival and/or proliferation of primary endothelial cells. Microvascular endothelial cells cultured *in vitro* express Wnt-5a, Wnt-7a, Wnt-10b and vascular smooth muscle cells express Wnt-5a [59]. Wnts are paracrine factors that could, conceivably, be expressed from a variety of cellular sources (including endothelial, mural, or epithelial

cells) to stimulate frizzled-bearing endothelial cells. Our data demonstrates that Fz-4, Fz-5, Fz-6 expression occurs in cultured human umbilical vein endothelial cells (HUVEC) [60] as are Fz-1, 3, 5 and 7 genes [59, 61]. These Frizzleds are also expressed *in vivo* in a variety of mammalian tissues [62–64]. Thus, Frizzled genes are expressed by endothelial cells *in vitro* but their expression is not restricted to endothelial cells *in vivo*.

Endothelial cells respond to the activation of the Wnt/ β -catenin pathway *in vitro* [60, 65, 66]. One study reports that expression of Wnt-1, but not Wnt-5a, in HUVEC increases cell proliferation, induces β -catenin stabilization, and activates a TCF-responsive transcriptional reporter [59]. Increased degradation of β -catenin correlates with endothelial cell survival [67]. We find that ectopic expression of a stabilized β -catenin, Wnt-1, or Wnt-5a gene stimulates HUVEC proliferation [60, 68]. β -catenin activity is implicated downstream of several distinct signaling pathways that function in endothelial cells [69, 70]. In addition, PECAM, a key endothelial surface protein, may signal to induce β -catenin levels [71]. Changes in localization of β -catenin from the membrane to the cytosol, an indicator of Wnt/ β -catenin signaling, occur in endothelial cells during neovascularization after experimentally induced myocardial infarction [72].

In support of a role for non-canonical Wnt signaling function in angiogenesis, studies using *in vitro* angiogenesis assays demonstrate that expression of Wnt-5a, which activates non-canonical Wnt signaling, affects endothelial cell behavior [68]. Ectopic expression of Wnt-5a in HUVEC promotes capillary-like endothelial network formation and proliferation, whereas experimental reduction of Wnt-5a expression inhibits capillary-like endothelial network formation [68]. Further, a pharmacological inhibitor, TNP-470, is known to inhibit endothelial cell growth *in vitro* [73, 74] and has also been reported to inhibit non-canonical Wnt signaling [75]. Studies will be needed to know if pharmacological inhibition of non-canonical Wnt signaling is relevant to strategies to block pathological angiogenesis.

These studies, taken together, implicate the Wnt/ β -catenin pathway and the non-canonical pathway as mediators of endothelial cell growth and survival. Few studies have been done to explore the activity of frizzled proteins in endothelial cells. FrzA, a frizzled related protein (also known as sFrp-1) and an inhibitor of Wnt signaling, can reduce the proliferation of cultured endothelial cells [76]. sFrp-1 binds Fz-4 and 7 and it appears to induce the re-organization of actin stress fibers in endothelial cells by activating GSK-3, a component of canonical Wnt signaling, and Rac-1, presumably acting on the non-canonical Wnt pathway [77]. Finally, a compelling case that activated β -catenin signaling occurs in

endothelial cells *in vivo* is made by observing TCF transcriptional activity in whole animals [78]. One such transgenic mouse strain, BAT, contains a galactosidase reporter under the control of β -catenin/TCF responsive elements. It responds in all tissues where canonical Wnt signaling occur and it identifies notochord, brain, and endothelial cells (co-stained with PECAM) as a site of β -catenin/TCF signaling. Thus, β -catenin is clearly activated in endothelial cells *in vivo* based on the analysis of the BAT mouse.

Wnt target genes encoding angiogenic regulators

The Wnt/ β -catenin pathway appears to regulate VEGF-A gene transcription [79]. Seven β -catenin/Tcf binding sites occur in the VEGF-A gene promoter [80]. VEGF-A is consistently up-regulated in colon cancer samples where deregulated β -catenin is a determinant in colon carcinogenesis. Furthermore, mice that carry a defect in the APC gene, where dysregulation of β -catenin is a consequence, also produce an abnormal amount of VEGF (Min/+ mice) [80]. Thus, the VEGF gene may be responsive to physiological and pathological Wnt/ β -catenin signaling.

Other Wnt target genes that are pertinent to this review include Interleukin-8, a cytokine that has angiogenic activity [81–83] and is transcriptionally activated by β -catenin/Tcf signaling in hepatocytes and HUVEC [84]. Matrix metalloproteinases (MMP) degrade components of the extracellular matrix, a function that is essential to the movement, proliferation, and assembly of endothelial cells into blood vessels [85]. We find that MMP-1 is induced by Wnt-5a via non-canonical signaling [68].

Summary

There is no doubt that the frizzled family of receptors transmits signals from its ligands, Wnt and Norrin, that are critical to the angiogenic processes. To be sure, the consequence of defective Wnt signaling in the eye has profound effects on retinal vasculature and can lead to blindness. Canonical and non-canonical Wnt signaling influence endothelial cell behavior by promoting key steps in angiogenesis, such as endothelial cell proliferation and migration. In the case of canonical Wnt/ β -catenin signaling, the evidence is compelling that retinal angiogenesis requires functional Wnt or Wnt-like signals in humans and mice. The combined analysis of Norrie and FEVR diseases show that Fz-4 is a key angiogenic receptor, and one wonders whether this characteristic can be extended to other frizzleds. We are also left to wonder

whether there are other Wnt ligand substitutes, besides Norrin. Non-canonical Wnt signaling in endothelial cells in whole animals is more obscure because, unlike Wnt/ β -catenin signaling, there are no known vascular pathologies that are linked to non-canonical signaling.

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