Wnt Signaling Mediates Pathological Vascular Growth in Proliferative Retinopathy Clinical Perspective

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Accessibility
Wnt signaling mediates pathological vascular growth in proliferative retinopathy

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Abstract

Background—Ischemic proliferative retinopathy, characterized by pathologic retinal neovascularization, is a major cause of blindness in working age adults and children. Defining the molecular pathways distinguishing pathological neovascularization from normal vessels is critical to controlling these blinding diseases with targeted therapy. Because mutations in Wnt signaling cause defective retinal vasculature in humans with some characteristics of the pathologic vessels in retinopathy, we investigated the potential role of Wnt signaling in pathologic retinal vascular growth in proliferative retinopathy.

Methods and Results—In this study we show that Wnt receptors (Frizzled4 and Lrp5) and activity are significantly increased in pathologic neovascularization in a mouse model of oxygen-induced proliferative retinopathy. Loss of Wnt co-receptor Lrp5 and downstream signaling molecule disheveled2 significantly decreases the formation of pathologic retinal neovascularization in retinopathy. Loss of Lrp5 also affects retinal angiogenesis during development and formation of the blood retinal barrier, which is linked to significant down-regulation of tight junction protein claudin5 (Cln5) in Lrp5−/− vessels. Blocking Cln5 significantly suppresses Wnt-pathway driven endothelial cell sprouting in vitro and developmental and pathologic vascular growth in retinopathy in vivo.

Conclusions—These results demonstrate an important role of Wnt signaling in pathologic vascular development in retinopathy and show a novel function of Cln5 in promoting angiogenesis.
Keywords
angiogenesis; vessels; retinopathy; Wnt

Introduction

Pathologic retinal neovascularization precipitated by vascular loss and hypoxia is the most common cause of blindness in all age groups. Two prominent blinding neovascular eye diseases, retinopathy of prematurity (ROP) in infants and proliferative diabetic retinopathy in working age adults, are both characterized by abnormal proliferation of pathologic vessels. These vessels are distinctly different from normal vasculature morphologically, exhibit increased vascular leakage and are associated with retinal detachment and blindness\textsuperscript{1, 2}. Specifically targeting pathologic neovessels, while sparing normal vessels, would be a major advancement in treating proliferative retinopathy, as well as other vascular diseases with pathologic vessel proliferation, such as cancer. Development of such treatment strategies with selective molecular targeting requires a better understanding of the pathways involved in the regulation of pathologic retinal blood vessel growth.

Wnt signaling may be involved in pathologic vessel growth. Mutations in the Wnt pathway cause several hereditary vascular eye disorders with defective retinal vascular development sharing some characteristics of proliferative retinopathy\textsuperscript{3-5}. The Wnt signaling pathway is fundamentally important in embryonic development and in cancer and cardiovascular diseases\textsuperscript{10-14}. It is essential for cardiac development and differentiation\textsuperscript{15, 16} and linked to cardiac hypertrophy, cardiac failure and vascular aging\textsuperscript{17, 18}. However, the contribution of Wnt to pathological angiogenesis has not been determined.

Canonical Wnt pathway signaling starts with the binding of a Wnt ligand to the Wnt receptor Frizzled (Fzd) and recruitment of co-receptor low-density lipoprotein receptor-related protein (Lrp5/6), activating and stabilizing β-catenin\textsuperscript{14}. After translocation to the nucleus, β-catenin binds the transcription factor Lef/TCF and activates target gene transcription\textsuperscript{10, 14}. Canonical Wnt signaling is implicated in neuronal development\textsuperscript{19}, cancer\textsuperscript{20, 21}, regression of embryonic hyaloid vessels in the eye\textsuperscript{22}, and blood brain barrier formation\textsuperscript{23-25}. Mutations in the Wnt receptors Frizzled4 and Lrp5 as well as the Wnt ligand, Norrin, are all linked to hereditary vascular eye diseases in human and in mouse models\textsuperscript{26-29}. Mice deficient in Wnt co-receptor Lrp5 have persistent embryonic hyaloid vessels in the eye\textsuperscript{9, 28}, (as well as low bone density) recapitulating human autosomal-recessive osteoporosis-pseudoglioma syndrome (OPPG), a form of familial exudative vitreoretinopathy (FEVR). Depletion or mutations in Lrp5 result in lack of deeper retinal vessels\textsuperscript{30, 31}, similar to mice lacking Wnt receptor Frizzled4 or Wnt ligand Norrin\textsuperscript{26, 27, 29}. However, the potential role of Wnt signaling in pathologic neovascularization during other post-natally occurring retinal vascular diseases, such as retinopathy of prematurity and diabetic proliferative retinopathy, is not defined and is the focus of this study.

Using a mouse model of oxygen-induced proliferative retinopathy (OIR)\textsuperscript{32}, we investigated the contribution of the Wnt signaling pathway in pathologic vascular growth in retinopathy. We find that components of Wnt signaling are significantly upregulated in pathologic neovessels with induced retinopathy. Loss of Lrp5 and Wnt signaling protein dishevelled2 results in significantly decreased levels of pathologic neovascularization in retinopathy. In addition loss of Lrp5 results in abnormal vascular growing tips and breakdown of the blood retina barrier, which is linked to down-regulation of the tight junction protein claudin5 (Cln5) in retinal vessels. Importantly, inhibition of Cln5 suppresses endothelial sprouting \textit{in vitro} and retinal vascular growth \textit{in vivo} during both development and in retinopathy. These
results indicate a crucial role of Wnt signaling in promoting pathologic retinal neovascularization in retinopathy beyond its known role in developmental retinal angiogenesis, suggesting that specific targeting of this pathway may lead to treatment options for proliferative retinopathy.

**Results**

**Delayed and incomplete retinal vasculature of Lrp5−/− mice**

To study the role of Wnt co-receptor Lrp5 in retinal angiogenesis, we first characterized vascular development of the retina in the Lrp5−/− mouse. Murine retinal vascular development starts at birth. The superficial vascular plexus grows radially from the optic disc reaching the periphery by postnatal day (P) 8. The development of the deeper secondary and tertiary network of capillaries follows and is complete approximately 3 weeks after birth. This stereotyped development is perturbed in adult Lrp5−/− retinas, where vessels are large, dilated and tortuous, with abnormal aggregations of endothelial cells in the mid-periphery of the primary vascular plexus (Fig.1A). Although Lrp5−/− retinas still display vertical vessels extending from the primary vessel plexus toward the deeper vascular layers, these vertical vessel sprouts terminate without forming interconnections, thus failing to create the two deeper layers of capillary networks in the inner and outer plexiform layers (Fig.1B, C). Additionally, glial fibrillary acidic protein (GFAP) levels are increased in astrocytes and Müller cells of Lrp5−/− eyes, indicating increased retinal stress associated with defective retinal vasculature (Fig.1C). The retinal vasculature of heterozygous Lrp5+/− mice appears normal (Fig.1A, B, C).

In addition to analyzing the mature retinal phenotype in adult retinas, we assessed retinal vascular development in Lrp5−/− retinas compared to the phenotypically normal Lrp5+/− mice (Fig.1D). At P5, the area of vascularized retina is ~25% less in Lrp5−/− mice compared with heterozygous littermate controls (27±1.9% vs. 36±1.3% of total retinal area; p≤0.001; Fig.1D, E). At the leading edge of the developing vasculature in Lrp5−/− retinas, the vessels appear thickened and the number of vascular sprouting tips is significantly reduced (by ~30%) compared to littermate controls (12.6±1.1 vs. 18.1±1.4 sprouts per view field; p≤0.001; Fig.1D, E). At P8, when the superficial layer of control retinas is almost 100% vascularized, Lrp5−/− retinas are only 70% vascularized. These results suggest that loss of the Wnt co-receptor Lrp5 directly contributes to delayed retinal vessel development.

**Localization and expression of Lrp5 in neovessels in developing retina**

We next localized Lrp5 protein in the retina to the developing primary vessel plexus (P6) (Fig.1F). At P13 and P17, when deeper layers of retinal vessels are forming and the superficial vascular layer is complete, Lrp5 staining shifts predominantly to these newly developing vascular layers (Fig. 1F), indicating that Lrp5 is expressed preferentially in newly formed vessels compared to mature blood vessels. This notion is supported by the robust expression of Lrp5 mRNA in whole retina during the first week after birth when rapid vascular growth occurs in the primary vascular plexus. When the retina matures and vascular growth slows, Lrp5 expression gradually declines (Supp. Fig.1A). As expected, Lrp5−/− eyes do not stain for Lrp5 (Supp. Fig.1B).

**Localization and expression patterns of Wnt receptors and activity in pathologic retinal neovessels**

Having established the importance of Wnt signaling and Lrp5 in normal retinal vessel development, we next asked whether the Wnt pathway contributes to the formation of pathologic retinal neovascularization (NV) in oxygen-induced retinopathy (OIR)32. In OIR mouse pups are exposed to 75% oxygen from P7 to P12 followed by room air with a
maximum neovascular response at P17 (Fig. 2A). We assessed the effects on the pathological neovascular response of modulating the Wnt pathway (ligands and receptors, as well as the downstream signaling protein dishevelled and β-catenin stabilization) (Fig. 2B).

During OIR, Wnt receptor Frizzled 4 (Fzd4) is found specifically in pathologic neovascular tufts (Fig. 2C). Wnt activity was localized using the Wnt reporter TOPGAL mice, which express lacZ gene under the control of Tcf promoters and, hence, synthesize lacZ only in cells with active canonical Wnt/β-catenin signaling. Similar to Frizzled 4, activated Wnt signaling, as evidenced by anti-lacZ staining, is observed specifically in neovascular tufts in OIR retinas (Fig. 2C). Moreover, we also localized the Wnt ligand Norrin to a subset of macrophages associated with retinal vessels as seen by co-localization with CSF1-R (Fig. 2C). Because in situ hybridization is not quantitative, we used laser capture microdissection to isolate pathologic NV tufts from OIR mice and normal vessels from control mice to measure specific mRNA expression of Wnt receptors in retinal vasculature. Wnt receptor Fzd4 and Lrp5 mRNA levels are ~3 to 5 fold higher in neovessels compared to normal vessels (Fig. 2D).

Mice lacking Wnt signaling show decreased levels of pathologic neovascularization in retinopathy

To assess whether Wnt signaling contributes to the formation of pathologic NV, retinopathy (OIR) was induced in Lrp5−/− mice. With loss of Lrp5 there was significantly less pathologic NV compared to wild type controls (5.9±0.3% vs. 8.3±0.6% of total retina area; p≤0.001, Fig. 3A). Since Lrp5−/− mice have delayed vascular growth during development which may affect the neovascular response of Lrp5−/− mice in OIR, we examined OIR in another transgenic mouse line Dvl2−/− lacking the Wnt signaling component Dishevelled2 (Dvl2). Dvl2 is a cytoplasmatic phospho-protein that acts directly downstream of Wnt receptors and is required for transmitting Wnt receptor activation signals. We found normal retinal vascular development in Dvl2−/− mice (Supp. Fig. 2A, B), potentially reflecting redundant roles of Dvl1, Dvl2 and Dvl3 in development. However, with OIR, Dvl2−/− retinas develop significantly less pathological NV than littermate controls (6.5±1% vs. 9.1±0.5%; p≤0.01; Fig. 3B). These results suggest that Wnt signaling through Lrp5 and Dvl2 is important for formation of pathologic neovascularization in the OIR model independent of abnormalities in normal retinal development, and that blocking the Wnt pathway by genetic depletion of either Lrp5 or Dvl2 suppresses pathologic NV formation.

Increased expression of Wnt ligands in retinopathy

Having established that loss of Wnt signaling affects pathologic NV in retinopathy, we next asked which Wnt ligands are regulated during pathological neovascularization. Wnt3a, Wnt7a and Wnt10a mRNA expression is significantly up-regulated in OIR retinas at P17 compared with room air controls, with Wnt7a and Wnt10a mRNA increasing up to 7-10 fold (Fig. 3C). Expression of the Wnt ligand Norrin is not significantly altered during OIR (Fig. 3C). These data suggest that Wnt3a, Wnt7a and Wnt10a are likely Wnt ligands contributing to pathologic neovessel formation in the OIR retina.

Down-regulation of Cln5 and disruption of blood retina barrier in Lrp5−/− retina

To unravel the molecular mechanisms by which Wnt signaling regulates retinal angiogenesis, we analyzed mRNA expression in wild type and Lrp5−/− retinas at P8 with a microarray. With loss of Lrp5 we identified a significant ~8 fold reduction in expression of the tight junction protein Cln5, confirmed by quantitative RT-PCR (Fig. 4A). Protein levels of Cln5 are also markedly reduced in Lrp5−/− retinas (Fig. 4B). Cln5 protein is predominantly localized by immunohistochemistry to retinal vessels in both WT and Lrp5−/− retina (with lower staining intensity in Lrp5−/− vessels) (Fig. 4C). Since...
immunohistochemistry is not quantitative we used laser-captured microdissection to isolate retinal vessels from Lrp5−/− and WT mice followed by RT-PCR to analyze gene expression specifically in vessels. We confirmed that Cln5 mRNA is significantly decreased in retinal vessels of Lrp5−/− mice; while Plvap, a marker of vascular permeability, is increased compared to vessels isolated from wild type retinas (Fig.4D). Expression of the endothelial cell marker VE-Cadherin is the same (Fig.4D). Transcription factors Sox17 and Sox18 are also significantly down-regulated in Lrp5−/− vessels compared with wild type vessels by ~2 and 4 fold respectively (Fig.4D). The observation that Sox17 mRNA expression is decreased in Lrp5−/− vessels is in agreement with a previous study reporting decreased Sox17 transcripts in Wnt receptor Frizzled4−/− endothelial cells. The finding that Sox18 is regulated by Wnt signaling, however, was not reported previously. Since Sox18 is known to control expression of Cln5, we assessed expression of Sox18 and Cln5 in human retinal microvascular endothelial cells (HRMEC) treated with small interference RNA (siRNA) targeting Lrp5 and Sox18. Compared with control siRNA, Lrp5 siRNA significantly inhibited Lrp5 expression by >3 fold (Fig,4E). Lrp5 siRNA treatment significantly inhibited mRNA expression of both Sox18 and Cln5. Sox18 siRNA treatment successfully inhibited Sox18 expression, and importantly inhibited Cln5 mRNA expression by >3 fold (Fig.4E). Together these in vitro data shows that the transcription factor Sox18 mediates down-regulation of Cln5 in Lrp5 deficient endothelial cells.

Because Cln5 is a known tight junction protein essential for maintaining vessel integrity and Plvap is an indicator of vessel permeability, we assessed the blood-retinal barrier (BRB) function in Lrp5−/− mice and found increased BRB permeability when subjected to fluorescent angiography with FITC-dextran (Fig.4F). In a complementary assay, mouse plasma IgG, which normally is confined to vessels in wild type mice with intact BRB, extravasates into the neuronal tissue of the retina in Lrp5−/− mice, also indicating BRB breakdown (Fig.4G). Together these results suggest that the vasculature of Lrp5−/− retinas is more permeable than WT.

**Blocking Cln5 suppresses Wnt-stimulated endothelial spheroid sprouting in vitro and developmental retinal angiogenesis in vivo**

To further investigate the molecular mechanisms that cause impaired angiogenesis in Lrp5−/− mice, we examined vascular endothelial growth factor (VEGF) in Lrp5−/− retinas. Lrp5−/− retinas display similar levels of VEGF mRNA expression at P5 and P8 during vascular development of the superficial vascular layer. After the initial vascular growth there is a breakdown of vascular development after P8 likely leading to inadequate perfusion of the developing retina. Without normal vascular development there is a significantly increased level of VEGF at P12 and P17 compared with WT controls (Fig.5A). This observation suggests that the delay and lack of vascular growth in Lrp5−/− mice is not caused by VEGF deficiency. Other pathways that are critical for angiogenesis such as the angiopoietin-Tie2 pathway and Notch pathway are also not significantly affected in Lrp5−/− retinas (Supp. Fig.3).

Given that Cln5 is significantly down-regulated in Lrp5−/− retinas, we asked whether deficiency in Cln5 might contribute directly to the lack of vessel growth-by affecting endothelial cell adhesion and migration for further vascular development. Using an in vitro spheroid sprouting assay, we found that both Wnt ligands Wnt3a and Wnt7a significantly stimulated HRMEC sprouting, as potently as VEGF (Fig.5B,C). Inhibition of Cln5 with an anti-Cln5 antibody significantly suppressed HRMEC sprouting to basal control levels in Wnt3a and Wnt7a stimulated groups, as well as in VEGF stimulated group (Fig.5B,C), suggesting that Cln5 is essential for proper endothelial cell (EC) sprout formation. These results were confirmed using a second anti-Cln5 antibody (Supp. Fig.4). Toxic effects of the antibody solution were ruled out using sodium azide-containing vehicle control groups.
Moreover, inhibition of Cln5 with intraocular injection of Cln5 antibody at P2 during normal retinal development results in a significant delay of retinal vascular growth in the superficial retinal layer at P7 compared with fellow eyes injected with pre-immune rabbit IgG (superficial retinal area without vessels: 16.1±1.4% vs. 11.1±1.0% of total retina area; \( p \leq 0.02 \) Fig.5D). In addition, to assess the effect of Cln5 on deep vessel layer formation, we injected Cln5 antibody intravitreally at P4 and found that inhibition of Cln5 at P9 also significantly suppresses retinal vascular growth in the deep retinal layer compared with control IgG (deep retinal area without vessels: 88.1±1.8% vs. 66.0±7.9% of total retina area; \( p \leq 0.01 \) Fig.5E).

Inhibition of Cln5 with siRNA suppresses pathologic retinal neovascularization in retinopathy

To corroborate these in vivo results obtained with Cln5 antibodies, we assessed the effect of Cln5 on vascular growth in vivo with siRNA targeting Cln5. The specificity and effectiveness of the Cln5 siRNA were previously validated\(^ {49} \). We confirmed that intravitreal injection of Cln5 siRNA significantly suppresses retinal Cln5 mRNA expression by more than 3 fold (Fig.6A), and in development significantly inhibits retinal vascular growth compared with contralateral eyes injected with control siRNA (avascular area: 12.8±1.1% vs. 8.2±1.0% of total retinal area, \( p \leq 0.01 \), Fig.6B). To assess whether suppression of Cln5 also suppresses pathologic retinal neovessels, we injected intravitreally Cln5 siRNA in OIR mice at P14. At P17, Cln5 siRNA injected eyes had significantly less pathologic neovascularization (9.2±0.7% of total retinal area) compared with contralateral eye injected with control siRNA (11.4±0.7% of total retinal area, \( p \leq 0.01 \), Fig.6C). Together these data suggest that inhibition of Cln5 significantly suppresses angiogenesis both in vitro and in vivo, indicating a novel function of Cln5 in promoting angiogenesis not only during development but also during pathologic neovessel formation.

Discussion

In this study we present novel evidence showing that Wnt pathway signaling is important for formation of pathologic neovascularization in retinopathy. It is important to distinguish biochemical pathways differentially involved in pathological neovascularization to consider specific control of pathologic angiogenesis (characterized by abnormal proliferation and increased vascular leakage) versus normal mature vessels. Mutations in Wnt ligand Norrin and receptors Frizzled4 and Lrp5 have been linked previously to several rare human eye diseases such as Norrie disease\(^ {26} \) and FEVR\(^ {5, 8} \), which both have features of abnormal retinal vessel development and vascular leakage. Recent studies have identified a new binding protein of Wnt receptors, tetraspannin (TSPAN12), which enhances Wnt signaling\(^ {50} \), and a transcription factor Sox17 which is upregulated by Wnt signaling\(^ {38} \). However, the role of Wnt signaling in pathological proliferative neovascularization remained undefined.

Here we find that Wnt receptors and activity are selectively upregulated in pathologic neovessels in the retina in a mouse model of oxygen-induced proliferative retinopathy (OIR). Reduction of Wnt signaling in Lrp5\(^ {−/−} \) mice, or dishevelled2\(^ {−/−} \) mice, significantly suppresses pathologic neovessel formation in OIR. These results are in line with a previous study showing ectopic expression of Wnt ligand Norrin promotes vascular regrowth in OIR\(^ {51} \). Together these findings suggest that modulation of Wnt signaling has significant implications not only for congenital hereditary eye diseases with mutations in Wnt pathway, but also for the much more prevalent postnatally-occurring retinal vascular diseases like diabetic retinopathy, ROP and macular degeneration all of which are characterized by pathologic vascular growth. Interestingly in this context, ROP in some severe cases has been
linked to mutations of the Wnt ligand Norrin\textsuperscript{52}, and age-related macular degeneration has been associated with gene polymorphisms in the Wnt co-receptor Lrp6\textsuperscript{53}.

In addition to defining the role of Wnt signaling in proliferative retinopathy, in this paper we also expanded the characterization of Lrp5 in developmental retinal angiogenesis. Decreased numbers of retinal vascular sprouts and an incomplete deep layer of blood vessels in \textit{Lrp5}\textsuperscript{−/−} retinas is similar to retinal vascular development abnormalities seen in \textit{Norrin}\textsuperscript{−/−} and \textit{Frizzled4}\textsuperscript{−/−} mice\textsuperscript{26, 27, 30, 38}. \textit{Frizzled4} expression is same in \textit{Lrp5}\textsuperscript{−/−} retina compare to WT, however a few other Frizzled receptors (\textit{Fzd2}, \textit{Fzd3}, \textit{Fzd7}) are down-regulated reflecting potential compensatory regulation (Supp. Fig.5). Lrp5 protein is expressed preferentially in newly formed versus mature retinal vessels during development and in pathologic neo vessels during OIR. Although expression in other retinal cell types such as neurons and Muller glia cells cannot be excluded\textsuperscript{31}, it is likely that vascular Wnt receptor Fzd4 and Lrp5 are direct regulators of vascular growth in retinopathy, consistent with a previous report showing that conditional loss of Frizzled4 in vascular endothelial cells results in similar retinal vascular defects as systemic Frizzled4 null mice\textsuperscript{38}.

Our study identifies the tight junction protein Cln5 as a direct mediator of retinal angiogenesis regulated by Lrp5 through the transcription factor \textit{Sox18}. Cln5 is a key component of endothelial cell tight junctions essential in maintaining cell-cell adhesion. Blocking Cln5 significantly suppresses retinal blood vessel during development in both superficial and deep retinal layers, as well as suppresses pathologic neo vessel formation in proliferative retinopathy in mice. This effect is corroborated \textit{in vitro} as blocking Cln5 significantly suppresses Wnt dependent endothelial cell spheroid sprouting. It has been shown previously that blocking a different adhesive protein, R-cadherin, inhibits retinal angiogenesis\textsuperscript{54}. Inhibition of the endothelial cell adhesion protein VE-cadherin also suppresses neo vessel sprouting and angiogenesis\textsuperscript{55-58}. Similarly, Cln5 may be necessary for cell-cell adhesion, endothelial cell migration and tube formation and thus would play an essential role for the proper growth of vascular sprouts and vessel growth. It is likely that Cln5 is finely regulated in the retina for optimal tight junction function. Down regulation of Cln5 may contribute, at least in part, to the delayed formation of the primary plexus, and to the complete lack of deeper capillaries in \textit{Lrp5}\textsuperscript{−/−} retinas. It is important to note that \textit{Cln5} is also suppressed in \textit{Norrin}\textsuperscript{−/−} retinas (seen in a gene expression microarray study\textsuperscript{59}), suggesting that Cln5 may mediate common effects of Wnt signaling in vascular growth.

This previously undiscovered function of Cln5 in retinal angiogenesis is in addition to its known role in blood brain (or blood retinal) barrier formation. Both FEVR and ROP are characterized by not only inadequate retinal vascularization but also peripheral retinal vascular leakage\textsuperscript{2, 3}, which might be attributable in part to lack of Cln5. In line with our observations that loss of Wnt signaling in \textit{Lrp5}\textsuperscript{−/−} retinas results in decreased blood-retinal barrier integrity, loss of Wnt ligands Wnt7a and Wnt7b are associated with disruption of tight junctions and loss of blood-brain barrier properties in the central nervous system\textsuperscript{23-25}. In the brain, deficiency of Cln5 has been linked to blood brain barrier breakdown\textsuperscript{41} and Cln5 null mice die soon after birth due to defective blood brain barrier formation\textsuperscript{40}. Similarly, in line with our findings that Cln5 is a downstream target of Lrp5 via Sox18, Cln5 has been found to be down-regulated in vascular endothelial cells in the absence of β-catenin\textsuperscript{24, 47}. Together, these results support the concept that canonical Wnt signaling through Lrp5, β-catenin and Sox18 regulates Cln5 expression in endothelial cells, which in turn mediates not only endothelial barrier function in the retina, but also retinal blood vessel growth in development and in pathology.

In summary, this paper provides direct evidence for an important role of Wnt signaling mediating pathologic neovascularization. Given the selectivity of Wnt signaling for
proliferating vessels, the therapeutic implications for modulating components of Wnt/β-catenin pathways in pathologic vessel proliferation are broad. Our identification of the Wnt-dependent angiogenic effects of Cln5 further adds to the potential therapeutic spectrum of Wnt-modulation in angioproliferative diseases. In this respect, modulation of canonical Wnt pathways could be potentially advantageous for treating not only retinopathy, but also other diseases such as tumors where pathological angiogenesis and loss of vascular integrity play a significant role.

**Experimental Procedures**

**Retinal vascular phenotype analysis and oxygen-induced retinopathy**

Retinal dissection vascular staining and image analysis were performed using standard published protocols. To induce pathological neovascularization in retina, mice with their nursing mother were exposed to 75% oxygen from P7 to P12 followed by room air. At P17 when the neovascular response is greatest, pathologic retinal neovascularization was evaluated. To examine the level of gene transcripts, cDNA was prepared from isolated retina at various age followed by RT-qPCR analysis.

**Endothelial cell spheroid sprouting assay**

Human retinal microvascular endothelial cells (HRMEC) were cultured and prepared for spheroids as described previously. Sprouting were photographed after treatment of Wnt 3a, Wnt7a, VEGF and Cln5 antibodies for 24 hrs and images were assessed with results expressed as mean ± SEM. n is number of spheroids quantified.

**Intravitreal injection**

Intravitreal injections with Cln5 antibody or Cln5 siRNA were performed at specific ages following published protocols. Retinas were isolated for RNA analysis or flatmount for analysis of vascular phenotype during development and in oxygen-induced retinopathy.

**Laser capture microdissection of retinal vessels**

Retinal vessels were micro-dissected with laser-capture in retinal cross-section from P8 Lrp5−/− and WT mice as described previously. Enrichment of endothelial cells was confirmed. RNA was extracted and cDNA synthesis for RT-qPCR analysis.

**Statistics**

Results are presented as mean±SEM for animal studies and mean±SD for non-animal studies. For all statistical analysis, F-Test (for two samples, or Levene’s test for more than two samples) was performed first to assess whether the variance are homogenous. If the variances were homogenous, two sample T-test (or ANOVA if more than two groups of samples) assuming equal variance was performed. If not, two sample T-test (or ANOVA) assuming unequal variance was performed.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Abnormal and delayed vascular development in \( \text{Lrp5}^{-/-} \) retina and localization of retinal Lrp5. Retinal vessels were visualized with Isolectin B\(_{4} \) staining (red). A. Retinal flat mounts of age-matched adult wild type, \( \text{Lrp5}^{+/+} \), and \( \text{Lrp5}^{-/-} \) mice (1 month old). B. Flat mounts illustrating the three layers of retinal vascular network in adult littermate \( \text{Lrp5}^{+/+} \) and \( \text{Lrp5}^{-/-} \) retina. NFL: nerve fiber layer, IPL: inner plexiform layer, OPL: outer plexiform layer. C. Cross section of adult littermate \( \text{Lrp5}^{+/+} \) and \( \text{Lrp5}^{-/-} \) retina stained with isolectin (red, vessels), GFAP (green, astrocytes and Muller cells) and DAPI (blue, nucleus). D. Retinal flat mounts of littermate \( \text{Lrp5}^{+/+} \) and \( \text{Lrp5}^{-/-} \) mice at postnatal day (P) 5. Growth fronts of vessels are enlarged in lower panels with vascular sprouts highlighted (*). E. Quantification of the vascularized retinal area and number of vascular sprouts in \( \text{Lrp5}^{+/+} \) and \( \text{Lrp5}^{-/-} \) retinas at P5. \( n=6-9 \) per group. *** \( p \leq 0.001 \). F: Immunohistochemical localization of Lrp5 protein in retinal cross-sections from P6, P13 and P17 wild type mice. Retinal sections are stained with Lrp5 antibody (green), Isolectin B\(_{4} \) (red, vessels) and DAPI (blue, nucleus). Scale bars: A: 1000\( \mu \)m. B-F: 100\( \mu \)m.
Figure 2.
Localization and expression patterns of Wnt receptors and activity in oxygen-induced retinopathy (OIR). A. Schematic illustration of oxygen-induced retinopathy. Neonatal mice with nursing mothers are exposed to oxygen from postnatal day (P) 7 to P12. Retinas are isolated at P17 and stained with Isolectin B4 to visualize vessels. B. Illustration of Wnt signaling components. Wnt signaling starts with binding of Wnt ligands to Wnt receptors, is mediated by dishevelled protein and results in β-catenin stabilization and target gene transcription. C. Localization of Wnt receptor Frizzled4, Wnt activity and Wnt ligand Norrin in the OIR retina. Retinopathy is induced in wild type mice or TOP-Gal Wnt reporter mice. P17 wild type retinas with OIR are stained with Frizzled4 (magenta) or norrin (magenta), Isolectin B4 (red, vessel) and CSF1-R (green, microglia). P17 retinas of TOP-Gal Wnt reporter mice with OIR are stained with β-Galactosidase (magenta, lacZ expression for Wnt activity) and Isolectin B4 (red, vessel). Both Frizzled4 and Wnt activity (lacZ) show co-localization with pathologic neovessels (red). Norrin (magenta) colocalizes with a subset of macrophages (green) associated with retinal vessels (red in merged image). D. mRNA expression of Wnt receptor Frizzled4 and Lrp5 in laser-capture microdissected pathologic neovessels (tufts) from OIR wild type mice compared to normal vessels from age-matched control mice raised in room air. Scale bars: A: 1000μm. C: 100μm. D: 50μm.
Figure 3.
Loss of Wnt signaling significantly decreases formation of pathologic neovascularization in OIR. A. Quantification of pathologic neovascularization in P17 retinas from Lrp5$^{-/-}$ mice exposed to OIR compared to wild type controls. Areas of pathologic neovascularization are quantified as percentage of total retinal area. n=16-29 per group; ***p≤0.001. B: Quantification of pathologic neovascularization in P17 retinas from Dishevelled2$^{+/+}$ (Dvl2) mice exposed to OIR compared with heterozygous littermates controls. n=8-24 per group; **p≤0.01. C. mRNA expression of Wnt ligands Wnt3a, Wnt7a, Wnt10a and Norrin in P17 wild type retinas exposed to OIR compared with age matched mice raised in room air. Scale bars: A and B: 1000μm.
Figure 4.
Down-regulation of tight junction protein claudin5 (Cln5) and disruption of blood retina barrier in \textit{Lrp5}^{-/-} retina. \textbf{A.} mRNA expression of Cln5 in whole retina isolated from P5 and P8 \textit{Lrp5}^{-/-} and wild type mice. \textbf{B.} Protein levels of Cln5 in retinal extracts of P8 wild type and \textit{Lrp5}^{-/-} retinas with Western blot. \textbf{C.} Localization of Cln5 in retinal vessels in cross-section of P8 wild type and \textit{Lrp5}^{-/-} eyes stained with Cln5 antibody (green), Isolectin B\textsubscript{4} (red, vessels) and DAPI (blue, nucleus). \textbf{D.} Quantification of \textit{Cln5, Plvap, Sox17, Sox18} and \textit{VE-Cadherin} mRNA from retinal blood vessels isolated with laser-capture micro-dissection from P8 \textit{Lrp5}^{-/-} and wild type retinas. n=3. \textbf{E.} Quantification of \textit{Lrp5, Sox18} and \textit{Cln5} mRNA from human retinal microvascular endothelial cells (HRMEC) treated with siRNA targeting \textit{Lrp5} and \textit{Sox18} for 48 hours. Data were analyzed with one-way ANOVA with Dunnett’s MCP against control siRNA group. \textbf{F.} Integrity of blood retinal barrier in \textit{Lrp5}^{-/-} and wild type retinas examined with FITC-dextran perfusion (green) or \textbf{G.} by staining for mouse IgG (green) which is normally confined within blood vessels. Scale bars: C and E: 50\mu m. F: 100\mu m. *\textit{p} \leq 0.05, **\textit{p} \leq 0.01, ***\textit{p} \leq 0.001.
Figure 5.

Blocking claudin5 (Cln5) with antibodies suppresses endothelial cell spheroid sprouting \textit{in vitro} and retinal angiogenesis \textit{in vivo}. \textbf{A.} Quantification of VEGF mRNA expression in \textit{Lrp5}−/− retinas at P5, 8, 12 and 17. \(n=6\) per group. \(*\ast\ast\ast p \leq 0.001\) between WT and \textit{Lrp5}−/− retinas. \textbf{B.} Human retinal microvascular endothelial cells (HRMEC) were cultured as multicellular spheroids in the presence of Wnt 3a, Wnt7a and VEGF in combination with Cln5 antibody. Images show spheroidal sprouting after culturing for 24h in collagen matrix. \textbf{C.} Quantification of HRMEC spheroid sprouting with treatment of Wnt3a, Wnt7a and VEGF, in combination with Cln5 antibody. \(n=10-20\) per group. n.s.: not significant, \(*\ast\ast\ast p \leq 0.001\) between groups with and without Cln5 antibody. \textbf{D.} Mice injected intravitreally with Cln5 antibody in one eye and rabbit IgG as control in the fellow eye at P2. Retinas are isolated at P7 and percent of avascular retinal area (retinal area without lectin-stained vessels in red) quantified. Cln5 ab. injected eyes show significantly less vascular growth in the superficial retinal layer. \(n=10\). \(*\ast\ast p \leq 0.01\). \textbf{E.} Mice injected intravitreally with Cln5 antibody in one eye and rabbit IgG as control in the fellow eye at P4. Retinas are isolated at P9 and growth of the deep vascular layer (red) is quantified as percent of avascular retinal area. Cln5 ab. significantly suppresses vascular growth in the deep retinal layer. \(n=6\). \(*\ast\ast p \leq 0.01\).

Scale bars: \textbf{B}: 100\(\mu\)m. \textbf{D} and \textbf{E}: 1000\(\mu\)m.
Figure 6. Claudin5 (Cln5) siRNA suppresses developmental retinal angiogenesis and formation of pathologic neovascularization in OIR. A. Cln5 siRNA significantly suppresses retinal expression of Cln5. Mice were injected intravitreally with Cln5 siRNA in one eye and control negative siRNA in the other eye at P4. Retinas were isolated at P6 and Cln5 mRNA expression quantified with RT-qPCR. n=3. *p ≤ 0.05. B. Quantification of P7 retinal vascular growth during development with Cln5 siRNA injected intravitreally at P4 and control siRNA injected in contralateral eye. n=10. **p ≤ 0.01. C. Quantification of P17 OIR retina with Cln5 siRNA and control siRNA injected intravitreally at P14 after mice were exposed to 75% oxygen from P7 to P12 to induce retinopathy. n=14. **p ≤ 0.01.