Wnt Signaling Mediates Pathological Vascular Growth in Proliferative Retinopathy

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Background—Ischemic proliferative retinopathy, characterized by pathological 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 pathological vessels in retinopathy, we investigated the potential role of Wnt signaling in pathological retinal vascular growth in proliferative retinopathy.

Methods and Results—In this study, we show that Wnt receptors (Frizzled4 and low-density lipoprotein receptor–related protein5 [Lrp5]) and activity are significantly increased in pathological neovascularization in a mouse model of oxygen-induced proliferative retinopathy. Loss of Wnt coreceptor Lrp5 and downstream signaling molecule disheveled2 significantly decreases the formation of pathological 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 downregulation of tight junction protein claudin5 in Lrp5−/− vessels. Blocking claudin5 significantly suppresses Wnt pathway–driven endothelial cell sprouting in vitro and developmental and pathological vascular growth in retinopathy in vivo.

Conclusions—These results demonstrate an important role of Wnt signaling in pathological vascular development in retinopathy and show a novel function of Cln5 in promoting angiogenesis. (Circulation. 2011;124:1871-1881.)

Key Words: angiogenesis ■ retinopathy ■ vasculopathy

Pathological 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 in infants and proliferative diabetic retinopathy in working-age adults, are characterized by abnormal proliferation of pathological vessels. These vessels are distinctly different from normal vasculature morphologically, exhibit increased vascular leakage, and are associated with retinal detachment and blindness.1,2 Specifically targeting pathological neovessels while sparing normal vessels would be a major advancement in the treatment of proliferative retinopathy and other vascular diseases with pathological 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 pathological retinal blood vessel growth.

Clinical Perspective on p 1881

Wnt signaling may be involved in pathological vascular growth. Mutations in the Wnt pathway cause several hereditary vascular eye disorders, with defective retinal vascular development sharing some characteristics of proliferative retinopathy.3-9 The Wnt signaling pathway is fundamentally important in embryonic development and in cancer and cardiovascular diseases.10-14 It is essential for cardiac development and differentiation,15,16 and is linked to cardiac hypertrophy, cardiac failure, and vascular aging.17,18 However, the contribution of Wnt to pathological angiogenesis has not been determined.

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1871
Canonical Wnt pathway signaling starts with the binding of a Wnt ligand to the Wnt receptor Frizzled (Fzd) and recruitment of coreceptor low-density lipoprotein receptor–related protein (Lrp5/6), activating and stabilizing β-catenin. After translocation to the nucleus, β-catenin binds the transcription factor Lef/TCF and activates target gene transcription. Canonical Wnt signaling is implicated in neuronal development, cancer, regression of embryonic hyaloid vessels in the eye, and blood-brain barrier formation. Mutations in the Wnt receptors Fzd4 and Lrp5 and in the Wnt ligand Norrin are linked to hereditary vascular eye diseases in human and in mouse models. Mice deficient in the Wnt coreceptor Lrp5 have persistent embryonic hyaloid vessels in the eye (and low bone density), recapitulating human autosomal-recessive osteoporosis-pseudoglioma syndrome, a form of familial exudative vitreoretinopathy. Depletion or loss of Lrp5 results in abnormal vascular growing tips and dishevelled2 (Dvl2) results in significantly decreased levels of pathological neovascularization during other postnatally 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), we investigated the contribution of the Wnt signaling pathway in pathological vascular growth in retinopathy. We found that components of Wnt signaling are significantly upregulated in pathological neovessels with induced retinopathy. Loss of Lrp5 and Wnt signaling protein dishevelled2 (Dvl2) results in significantly decreased levels of pathological 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 downregulation of the tight junction protein claudin5 (Cln5) in retinal vessels. Importantly, inhibition of Cln5 suppresses endothelial sprouting in vitro and retinal vascular growth in vivo both during development and in retinopathy. These results indicate a crucial role of Wnt signaling in promoting pathological 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.

Methods

Retinal Vascular Phenotype Analysis and OIR
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 postnatal day (P) 7 to P12, followed by room air. At P17, when the neovascular response is greatest, pathological retinal neovascularization was evaluated. To examine the level of gene transcripts, cDNA was prepared from isolated retina at various ages, followed by quantitative reverse-transcription polymerase chain reaction analysis.

Endothelial Cell Spheroid Sprouting Assay
Human retinal microvascular endothelial cells were cultured and prepared for spheroids as described previously. Sprouting was photographed after treatment of Wnt3a, Wnt7a, vascular endothelial growth factor (VEGF), and Cln5 antibodies for 24 hours, and images were assessed with results expressed as mean±SEM (n is the number of spheroids quantified).

Intravitreal Injection
Intravitreal injections with Cln5 antibody or Cln5 small interference RNA (siRNA) were performed at specific ages following published protocols. Retinas were isolated for RNA analysis or flat mounted for analysis of vascular phenotype during development and in OIR.

Laser-Capture Microdissection of Retinal Vessels
Retinal vessels were microdissected with laser capture in retinal cross sections from P8 Lrp5−/− and wild-type mice, as described previously. Enrichment of endothelial cells was confirmed. RNA was extracted and cDNA synthesized for quantitative reverse-transcription polymerase chain reaction analysis.

Statistics
Results are presented as mean±SEM for animal studies and mean±SD for nonanimal studies. For all statistical analysis, the F test (for 2 samples; for >2 samples, the Levene test) was performed first to assess whether the variances were homogeneous. If the variances were homogeneous, a 2-sample t test (or ANOVA for >2 groups of samples) assuming equal variance was performed. If not, a 2-sample t test (or ANOVA) assuming unequal variance was performed.

Results

Delayed and Incomplete Retinal Vasculature of Lrp5−/− Mice
To study the role of Wnt coreceptor 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 P8. The development of the deeper secondary and tertiary network of capillaries follows, and is complete ≈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 midperiphery of the primary vascular plexus (Figure 1A). Although Lrp5−/− retinas still display vertical vessels extending from the primary vascular plexus toward the deeper vascular layers, these vertical vessel sprouts terminate without forming interconnections, thus failing to create the 2 deeper layers of capillary networks in the inner and outer plexiform layers (Figure 1B and 1C). Additionally, gial fibrillary acidic protein levels are increased in astrocytes and Muller cells of Lrp5−/− eyes, indicating increased retinal stress associated with defective retinal vasculature (Figure 1C). The retinal vasculature of heterozygous Lrp5+/− mice appears normal (Figure 1A through 1C).

In addition to analyzing the mature retinal phenotype in adult retinas, we assessed retinal vascular development in Lrp5−/− retinas compared with the phenotypically normal Lrp5+/− mice (Figure 1D). At P5, the area of vascularized retina is ≈25% less in Lrp5−/− mice compared with heterozygous littermate controls (27±1.9% versus 36±1.3% of total retinal area; P=0.001; Figure 1D and 1E). 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 with littermate controls (12.6±1.1 versus 18.1±1.4 sprouts per view field; P=0.001; Figure 1D and 1E). 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 coreceptor 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 (at P6) (Figure 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 (Figure 1F), indicating that Lrp5 is expressed preferentially in newly formed vessels compared with 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 (Figure IA in the online-only Data Supplement). As expected, Lrp5−/− eyes do not stain for Lrp5 (Figure IB in the online-only Data Supplement).

Localization and Expression Patterns of Wnt Receptors and Activity in Pathological 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 pathological retinal neovascularization in OIR. In OIR, mouse pups are exposed to 75% oxygen from P7 to P12, followed by room air with a maximum neovascular response at P17 (Figure 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 Dvl and β-catenin stabilization; Figure 2B).

During OIR, the Wnt receptor Fzd4 is found specifically in pathological neovascular tufts (Figure 2C). Wnt activity was localized with the Wnt reporter TOP-Gal mice, which express the lacZ gene under the control of Tcf promoters and hence synthesize lacZ only in cells with active canonical Wnt/β-catenin signaling. Similar to Fzd4, activated Wnt signaling, as evidenced by anti-lacZ staining, is observed specifi-
Mice Lacking Wnt Signaling Show Decreased Levels of Pathological Neovascularization in Retinopathy

To assess whether Wnt signaling contributes to the formation of pathological neovascularization, retinopathy (OIR) was induced in Lrp5−/− mice. With loss of Lrp5, there was significantly less pathological neovascularization compared with wild-type controls (5.9±0.3% versus 8.3±0.6% of total retina area; P=0.001; Figure 3A). Because 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 Dvl2. Dvl2 is a cytoplasmic phosphoprotein that acts directly downstream of Wnt receptors and is required for transmitting Wnt receptor activation signals42 (Figure 2B). We found normal retinal vascular development in Dvl2−/− mice (Figure IIA and IIB in the online-only Data Supplement), potentially reflecting redundant roles of Dvl1, Dvl2, and Dvl3 in development.43 However, with OIR, Dvl2−/− retinas develop significantly less pathological neovascularization than littermate controls (6.5±1% versus 9.1±0.5%; P<0.01; Figure 3B). These results suggest that Wnt signaling through Lrp5 and Dvl2 is important for the formation of pathological neovascularization in the OIR model independently of abnormalities in normal retinal development, and that blocking the Wnt pathway by genetic depletion of either Lrp5 or Dvl2 suppresses pathological neovascularization formation.

Increased Expression of Wnt Ligands in Retinopathy

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

**Downregulation of Cln5 and Disruption of Blood-Retina Barrier in Lrp5<sup>−/−</sup> Retina**

To unravel the molecular mechanisms by which Wnt signaling regulates retinal angiogenesis, we analyzed mRNA expression in wild-type and Lrp5<sup>−/−</sup> 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 reverse-transcription polymerase chain reaction (Figure 4A). Protein levels of Cln5 are also markedly reduced in Lrp5<sup>−/−</sup> retinas (Figure 4B). Cln5 protein is localized predominantly by immunohistochemistry to retinal vessels in both wild-type and Lrp5<sup>−/−</sup> retina (with lower staining intensity in Lrp5<sup>−/−</sup> vessels; Figure 4C). Because immunohistochemistry is not quantitative, we used laser-capture microdissection to isolate retinal vessels from Lrp5<sup>−/−</sup> and wild-type mice, followed by reverse-transcription polymerase chain reaction to analyze gene expression specifically in vessels. We confirmed that Cln5 mRNA is significantly decreased in retinal vessels of Lrp5<sup>−/−</sup> mice, whereas Plvap, a marker of vascular permeability, is increased compared with vessels isolated from wild-type retinas (Figure 4D). Expression of the endothelial cell marker VE-Cadherin is the same (Figure 4D). The transcription factors Sox17 and Sox18 are also significantly downregulated in Lrp5<sup>−/−</sup> vessels compared with wild-type vessels by ∼2- and 4-fold, respectively (Figure 4D). The observation that Sox17 mRNA expression is decreased in Lrp5<sup>−/−</sup> vessels is in agreement with a previous study reporting decreased Sox17 transcripts in Wnt receptor Fzd4<sup>−/−</sup> endothelial cells. The finding that Sox18 is regulated by Wnt signaling, however, was not reported previously. Because Sox18 is known to control expression of Cln5, we assessed expression of Sox18 and Cln5 in human retinal microvascular endothelial cells treated with siRNA targeting Lrp5 and Sox18. Compared with control siRNA, Lrp5 siRNA significantly inhibited Lrp5 expression by ∼3 fold (Figure 4E). Lrp5 siRNA treatment significantly inhibited mRNA expression of both Sox18 and

![Figure 3.](http://circ.ahajournals.org/)

**Figure 3.** Loss of Wnt signaling significantly decreases formation of pathological neovascularization in oxygen-induced retinopathy (OIR). A, Quantification of pathological neovascularization in postnatal day (P) 17 retinas from Lrp5<sup>−/−</sup> mice exposed to OIR compared with wild-type controls. Areas of pathological neovascularization are quantified as percentage of total retinal area. n=16 to 29 per group; ***P≤0.001. B, Quantification of pathological neovascularization in P17 retinas from Dishevelled2<sup>−/−</sup> (Dvl2) mice exposed to OIR compared with heterozygous littermate controls. n=8 to 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, 1000 μm.
Sox18 siRNA treatment successfully inhibited Sox18 expression and, importantly, inhibited Cln5 mRNA expression by 3-fold (Figure 4E). Together, these in vitro data show that the transcription factor Sox18 mediates downregulation 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 function in Lrp5−/− mice and found increased blood-retinal barrier permeability when subjected to fluorescent

Figure 4. Downregulation of tight junction protein claudin5 (Cln5) and disruption of blood-retina barrier in Lrp5−/− retina. A, mRNA expression of Cln5 in whole retina isolated from postnatal day (P) 5 and P8 Lrp5−/− and wild-type (WT) mice. B, Protein levels of Cln5 in retinal extracts of P8 WT and Lrp5−/− retinas with Western blot. C, Localization of Cln5 in retinal vessels in cross section of P8 WT and Lrp5−/− eyes stained with Cln5 antibody (green), isolectin B4 (red, vessels), and DAPI (blue, nucleus). D, Quantification of Cln5, Plvap, Sox17, Sox18, and VE-Cadherin mRNA from retinal blood vessels isolated with laser-capture microdissection from P8 Lrp5−/− and WT retinas. n=3. E, Quantification of Lrp5, Sox18, and Cln5 mRNA from human retinal microvascular endothelial cells treated with siRNA targeting Lrp5 and Sox18 for 48 hours. Data were analyzed with 1-way ANOVA with the Dunnett multiple comparison tests against control siRNA group. F, Integrity of the blood-retinal barrier in Lrp5−/− and WT retinas examined with FITC-dextran perfusion (green) or by staining for mouse IgG (green), which is normally confined within blood vessels. Scale bars: C and F, 5 μm; G, 100 μm. *P<0.05, **P<0.01, ***P<0.001.
angiopathy with FITC-dextran (Figure 4F). In a complementary assay, mouse plasma IgG, which normally is confined to vessels in wild-type mice with intact blood-retinal barrier, extravasates into the neuronal tissue of the retina in Lrp5−/− mice, also indicating blood-retinal barrier breakdown (Figure 4G). Together, these results suggest that the vasculature of Lrp5−/− retinas is more permeable than that of wild-type retinas.

### 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 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 wild-type controls (Figure 5A). This observation suggests that the delay and lack of vascular growth in Lrp5−/− mice are not caused by VEGF deficiency. Other pathways that are critical for angiogenesis, such as the angioptoinet-Tie2 pathway50–52 and Notch pathway53,54 are also not significantly affected in Lrp5−/− retinas (Figure III in the online-only Data Supplement).

Given that Cln5 is significantly downregulated 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 human retinal microvascular endothelial cell sprouting as potently as VEGF (Figure 5B and 5C). Inhibition of Cln5 with an anti-Cln5 antibody significantly suppressed human retinal microvascular endothelial cells sprouting to basal control levels in Wnt3a- and Wnt7a-stimulated groups and in the VEGF-stimulated group (Figure 5B and 5C), suggesting that Cln5 is essential for proper endothelial cell sprout formation. These results were confirmed with a second anti-Cln5 antibody (Figure IV in the online-only Data Supplement). Toxic effects of the antibody solution were ruled out with sodium azide–containing vehicle control groups (Figure IV in the online-only Data Supplement). 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 the other eyes injected with preimmune rabbit IgG (superficial retinal area without vessels, 16.1 ± 1.4% versus 11.1 ± 1.0% of total retina area; P = 0.02; Figure 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% versus 66.0 ± 7.9% of total retina area; P = 0.01; Figure 5E).
Inhibition of Cln5 With siRNA Suppresses Pathological 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.55 We confirmed that intravitreal injection of Cln5 siRNA significantly suppresses retinal Cln5 mRNA expression by >3-fold (Figure 6A) and in development significantly inhibits its retinal vascular growth compared with contralateral eyes injected with control siRNA (avascular area, 12.8±1.1% versus 8.2±1.0% of total retinal area; P<0.01; Figure 6B). To assess whether suppression of Cln5 also suppresses pathological retinal neovessels, we injected intravitreally Cln5 siRNA in OIR mice at P14. At P17, Cln5 siRNA-injected eyes had significantly less pathological neovascularization (9.2±0.7% of total retinal area) compared with contralateral eyes injected with control siRNA (11.4±0.7% of total retinal area; P<0.01; Figure 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 pathological neovessel formation.

Discussion

In this study, we present novel evidence showing that the Wnt pathway signaling is important for formation of pathological neovascularization in retinopathy. It is important to distinguish biochemical pathways differentially involved in pathological neovascularization to consider specific control of pathological angiogenesis (characterized by abnormal proliferation and increased vascular leakage) versus normal mature vessels. Mutations in the Wnt ligand Norrin and receptors Fzd4 and Lrp5 have previously been linked to several rare human eye diseases such as Norrie disease26 and familial exudative vitreoretinopathy,5,8 both of which have features of abnormal retinal vessel development and vascular leakage. Recent studies have identified a new binding protein of the Wnt receptor tetraspannin (TSPAN12), which enhances Wnt signaling,56 and transcription factor Sox17, which is upregulated by Wnt signaling.44 However, the role of Wnt signaling in pathological proliferative neovascularization remained undefined.

Here, we find that Wnt receptors and activity are selectively upregulated in pathological neovessels in the retina in a mouse model of OIR. Reduction of Wnt signaling in Lrp5−/− mice or Dvl2−/− mice significantly suppresses pathological neovessel formation in OIR. 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, retinopathy of prematurity, and macular degeneration, all of which are characterized by pathological vascular growth. Interestingly, in this context, retinopathy of prematurity in some severe cases has been linked to mutations of the Wnt ligand Norrin,58 and age-related macular degeneration has been associated with gene polymorphisms in the Wnt coreceptor Lrp6.59

In addition to defining the role of Wnt signaling in proliferative retinopathy, in this article, we expanded the characterization of Lrp5 in developmental retinal angiogenesis. Decreased numbers of retinal vascular sprouts and an incomplete deep layer of blood vessels in Lrp5−/− retinas are similar to the retinal vascular development abnormalities seen in Norrin−/− and Fzd4−/− mice.26,27,30,44 Fzd4 expression is the same in Lrp5−/− retina compared with wild-type retina; however, a few other Fzd receptors (Fzd2, Fzd3, Fzd7) are downregulated, reflecting potential compensatory regulation (Figure V in the online-only Data Supplement). Lrp5 protein is expressed preferentially in newly formed versus mature retinal vessels during development and in pathological neovessels during OIR. Although expression in other retinal cell types, such as neurons and Muller glia cells, cannot be excluded,11 it is likely that the vascular Wnt receptors Fzd4 and Lrp5 are direct regulators of vascular growth in retinopathy, consistent with a previous report showing that conditional loss of Fzd4 in vascular endothelial cells results in retinal vascular defects similar to systemic Fzd4-null mice.44

Our study identifies the tight junction protein Cln5 as a direct mediator of retinal angiogenesis regulated by Lrp5.
through the transcription factor Sox18. Cln5 is a key component of endothelial cell tight junctions essential in maintaining cell-cell adhesion. Blocking Cln5 significantly suppresses retinal blood vessel development in both superficial and deep retinal layers and suppresses pathological neovessel formation in proliferative retinopathy in mice. This effect is corroborated in vitro in that 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.60 Inhibition of the endothelial cell adhesion protein VE-cadherin also suppresses neovessel sprouting and angiogenesis.61–64 Similarly, Cln5 may be necessary for cell-cell adhesion, endothelial cell migration, and tubule formation, and thus would play an essential role in 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. Downregulation 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 Lrp5−/− retinas. It is important to note that Cln5 is also suppressed in Norrin−/− retinas (seen in a gene expression microarray study65), 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 familial exudative vitreoretinopathy and retinopathy of prematurity are characterized by not only inadequate retinal vascularization, but also peripheral retinal vascular leakage,2,3 which might be attributable in part to a lack of Cln5. In line with our observations that loss of Wnt signaling in Lrp5−/− retinas results in decreased blood-retinal barrier integrity, loss of Wnt ligands Wnt7a and Wnt7b is associated with disruption of tight junctions and loss of blood-brain barrier properties in the central nervous system.23–25 In the brain, deficiency of Cln5 has been linked to blood-brain barrier breakdown,47 and Cln5-null mice die soon after birth as a result of defective blood-brain barrier formation.46 Similarly, in line with our findings that Cln5 is a downstream target of Lrp5 via Sox18, Cln5 has been found to be downregulated in vascular endothelial cells in the absence of β-catenin.24,53 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.

**Conclusions**

This article provides direct evidence for an important role of Wnt signaling mediating pathological 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 in which pathological angiogenesis and loss of vascular integrity play a significant role.

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**Disclosures**

None.

**References**


**CLINICAL PERSPECTIVE**

Pathological neovascularization in ischemic proliferative retinopathies such as retinopathy of prematurity and diabetic retinopathy is a major cause of blindness in children and working-age adults. Although anti–vascular endothelial growth factor therapy has been proven partially successful in the suppression of neovascularization, identification of other signaling mechanisms involved in this disease process is essential for developing therapies specifically targeting pathological vessels while sparing normal vessels. Here, we found that the Wnt signaling pathway, a pathway important for cardiac development and differentiation, is a major component in regulating pathological neovascularization in retinopathy. Using a mouse model of oxygen-induced retinopathy, we found that Wnt ligands and receptors are highly upregulated in retinas with induced retinopathy and pathological neovessels, respectively. Mutant mice lacking Wnt coreceptor Lrp5 or downstream signaling molecule dishevelled2 have significantly decreased levels of neovascularization. Importantly, the proangiogenic effect of Wnt signaling is mediated through tight junction protein Claudin5, which is highly downregulated in Lrp5-null vessels. Suppression of claudin5 significantly inhibits Wnt-mediated vascular growth in vitro and pathological vessel growth in vivo. Our data suggest that Wnt signaling pathway plays a significant role in mediating pathological vascular growth in ischemic proliferative retinopathy, and selectively targeting this pathway might be a potentially useful strategy to develop future therapies for retinopathy.
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**Supplemental methods:**

*Animals:*

These studies adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Children’s Hospital Boston Animal Care and Use Committee. Unless otherwise indicated, C57Bl/6J mice (stock no. 000664; Jackson Laboratory) were used for the study. The Lrp5−/− mice (stock no. 005823; Jackson Laboratory) were developed by Deltagen Inc. TOPGAL transgenic Wnt activity reporter mice (stock no. 004623; Jackson Laboratory) contain the lacZ gene under the control of a regulatory sequence consisting of three consensus LEF/TCF-binding motifs upstream of a minimal c-fos promoter, as described previously 1, 2. Mice with macrophage marker Csf1r-driven GFP (stock no. 005070; JAX) were used to visualize microglia/macrophages in the retina. Dishevelled2−/− from Jackson Laboratory (stock no. 008001) were described previously 3.

*Retina dissection, vessel staining and flat mount:*

Mice at various ages during development were anesthetized with Avertin (Sigma-Aldrich) and sacrificed by cervical dislocation. Eyes were enucleated and fixed in 4% paraformaldehyde for 1 h at room temperature, followed by isolation and subsequent dissection of the retina. To visualize vessels, retinas were stained overnight at room temperature with fluoresceinated Griffonia Bandeiraea Simplicifolia Isolectin B4 (Alexa Fluor 594 conjugated; I21413; Invitrogen; 1:100 dilution) in 1 mM CaCl2 in 1x PBS. Following 2 h of washes in 1x PBS, retinas were whole-mounted onto Superfrost/Plus microscope slides (12-550-15; Fisher Scientific) with the photoreceptor side down and imbedded in SlowFade Antifade reagent (S2828; Invitrogen).

*Quantification of vascular development and neovascularization:*
Quantification of retinal vasculature was carried out as described previously. Images of whole-mounted retina were taken at ×5 magnification on a Zeiss AxioObserver.Z1 microscope and merged using AxioVision 4.6.3.0 software to produce an image of the entire retina. Vascular growth during development, and neovascular tuft formation in OIR were quantified in Adobe Photoshop or Image J. The number of pixels in vascular area during development is visualized with isolecin staining and outlined in Photoshop and compared to total number of pixels in the whole retina. Retinal areas with pathologic neovascular tufts structures were visually identified by their abnormal aggregated morphology that is distinctly different from the normal finely branched vascular network. The number of pixels in pathologic neovascular area is quantified and compared with the total number of pixels in the whole retina, either by manual selection based on morphology in Photoshop or by the SWIFT_NV method which consists of a set of macros on NIH’s free ImageJ platform to isolate the neovascular structures to stand out clearly against the background fluorescence of normal vessels. Percentages of neovascularization in total retina were compared between retinas from littermate heterozygous or age-matched wild type mice with identical oxygen conditions. For quantification of endothelial sprouts during development, images were taken at ×20 magnification at the center of each quadrant of the retina. The number of vascular sprouts with extending filopodia was quantified and compared in Lrp5−/− mice and their littermate controls. Evaluation was done blind to the identity of the sample. n is number of eyes quantified.

**Intravitreal injection:**

For analysis of superficial retinal vessel development, claudin5 antibody (0.25µg/µl in 0.5µl volume) was injected intravitreally at P2 with contra-lateral eyes injected with pre-immune IgG as control. Retinas were dissected, lectin stained and flatmounted at P7. Images were taken with Zeiss AxioObserver.Z1 microscope. The experiment was repeated in 3 litters. For analysis of deep retinal vessel development, claudin5 antibody (0.25µg/µl in 0.5µl volume) was injected intravitreally at P4 with contra-lateral eyes injected with pre-immune IgG as control. Retinas were dissected at P9, permeabilized, lectin stained and
flatmounted. Images were taken with a Leica confocal microscope at 10X magnification. For each image of deep layer of retinal vessels, the focus was adjusted to capture the maximum vascular area in the outer plexiform layer. Images were merged and quantified with Adobe Photoshop.

For the study with claudin5 siRNA during retinal development, claudin5 siRNA (Dharmacon: Sense sequence: 5’-CGU UGG AAA UUC UGG GUC UUU dTdT-3’, Antisense sequence: 5’-AGA CCC AGA AUU UCC AAC GUU dTdT-3’) were injected intravitreally at P4 with contra-lateral eyes injected with control siRNA (1027310, Qiagen). At P6 retinas were dissected and RNA isolated followed by quantitative real-time PCR analysis of claudin5 gene expression. At P7, retinas were dissected and flatmounted for analysis of vascular growth. For the study of claudin5 siRNA in retinopathy, retinopathy were induced in mice by exposure to 75% oxygen from P7 to P12. At P14 claudin5 siRNA were injected intravitreally with contra-lateral eyes injected with control siRNA. Retinas were dissected and flatmounted at P17 with lectin staining to analyze pathologic neovessels.

**RNA isolation and cDNA preparation:**

Total RNA was extracted from the retinas of 6 mice, each from a different litter; the RNA was pooled to reduce biologic variability (n=6). Retinas from each time point were lysed with a mortar and pestle and filtered through QiaShredder columns (Qiagen, Chatsworth, MD, USA). RNA was then extracted as per manufacturer’s instructions using the RNeasy Kit (Qiagen). To generate cDNA, 1 μg total RNA was treated with DNase I (Ambion Inc.) to remove any contaminating genomic DNA, and was then reverse transcribed using random hexamers, and SuperScript III reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA). All cDNA samples were aliquoted and stored at –80°C.

**Quantitative real-time PCR analysis of gene expression:**

PCR primers targeting Lrp5 (F: 5’-AAG GGT GCT GTG TAC TGG AC-3’, R: 5'-AGA AGA GAA CCT TAC GGG ACG-3’), Frizzled4 (F: 5’-TTC CTT TGT TCG GTT TAT GTG CC-3’, R: 5’-CTC TCA
GGA CTG GTT CAC AGC -3'), Norrin (F: 5'-CGC TGC ATG AGA CAC CAT TAT-3'; R: 5'-CTC AGA GCG TGA TGC CTG G-3'), Wnt3 (F: 5'-GGT TCG TGC GGA TGG GTG GG-3', R: 5'-GGG GCC AGA TGG GAG CTG GA-3'), Wnt7a (F: 5'- CAC TTG TGG TCT CAG GGG TT-3', R: 5'-GCA TCT GAG TTT CAC CAG CA-3'), Wnt10a (F: 5'-GCT CAA CGC CAA CAC AGT G-3', R: 5'-CGA AAA CCT CGG CTG AAG ATG-3'), Claudin5 (F: 5'-GCA AGG TGT ATG AAT CTG TGC T-3', R: 5'-GTC AAG GTA ACA AAG AGT GCC A-3'), Plvap (F: 5'-GCT GGT ACT ACC TGC GCT ATT-3', R: 5'-CCT GTG AGG CAG ATA GTC CA-3') and an unchanging control gene, Cyclophilin A (F: 5'-AGG TGG AGA GCA CCA AGA CAG A-3', R: 5'-TGC CGG AGT CGA CAA TGA T-3'), were designed using Primer Bank and NCBI Primer Blast Software. Express software (Applied BioSystems). We used three methods to analyze primer sequences for specificity of gene detection. First, NCBI Blast module was used to identify primer and probe sequences that specifically detected the sequence of choice. Second, amplicons generated during a PCR reaction were analyzed using the first derivative primer melting curve software supplied by Applied BioSystems. This analysis determined the presence of amplicons on the basis of their specific melting point temperatures. Third, amplicons generated were gel purified and sequenced by the Children's Hospital Boston Core Sequencing Facility. This further confirmed the selection of the desired sequence. Quantitative analysis of gene expression was determined using an ABI Prism 7700 Sequence Detection System (TaqMan) and the SYBR Green Master mix kit. Standard curves for each gene were plotted with quantified cDNA template during each real-time PCR reaction. Each target gene mRNA copy number was normalized to 10^6 copies of the house keeping gene, cyclophilin A.

Laser capture microdissection of retinal vessels:

Eyes were embedded in OCT and flash frozen immediately following enucleation. Eyes were cyrosectioned under RNase free conditions into 8-μm sections, and collected on RNase-free polyethylene naphthalate glass slides (11505189, Leica). Sections were dehydrated with 50%, 70%, and 100% ethanol washes and stained with isolectin (1:50 in 1mM CaCl₂) and counterstained with H&E. Retinal vessels
were microdissected with a Leica LMD 6000 system (Leica Microsystems) and collected directly into RNA stabilizing buffer from the RNeasy Micro kit (Qiagen, Chatsworth, CA). RNA was extracted from microdissected tissues using the RNeasy Kit as described above (Qiagen), and real-time PCR was performed with the generated cDNA.

*Permeability assay:*

For visualizing retinal vascular permeability, P8 *Lrp5*–/– mice or WT control were injected with fluoresceinated dextran 70kD (10mg/ml) with Isolectin B4 (Alexa Fluor 594 conjugated). Mice were sacrificed after 5 min, followed by eye fixation, retinal dissection and flatmounting. In addition, retinal cross-section from adult *Lrp5*–/– mice or WT control were stained immunohistochemically for mouse IgG.

*Endothelial cell culture and spheroid sprouting assay:*

Human retinal microvascular endothelial cells (HRMECs, Cell Systems, Kirkland, USA) were culture in Complete Medium and used from passage 3–7 for experiments. Small interference siRNA-mediated knockdown was performed using the RNA interference technique. siRNA duplexes used were as follows; human *Lrp5*: 5’-GCCCUACAUCAUUCGAGGAAU-3’, human *Sox18*: 5’-GGGUUACAUUUUUGAAGCA-3’ (Ambion, ID: 109098). Cells were transfected with 10nM of siRNA duplexes using Silentfect (BioRad, Hercules, CA) according to the manufacturer’s instructions and cells were harvested after incubation for 48 hours. As a control, siRNA duplex with irrelevant sequence (Ambion) was used. The silencing efficiency and gene expression were assessed with RT-qPCR. For spheroid sprouting assay, cells were cultured as monolayers at 37°C, 5% CO2 in a humidified atmosphere in Complete Media (Cell Systems, Kirkland, USA). The preparation of EC spheroids was performed as described previously 7, 8. Briefly, cells were harvested from subconfluent monolayers by trypsinization and suspended in Complete Media containing 10% FBS and 0.25% (w/v) carboxymethylcellulose (Sigma). 500 cells were seeded together in one hanging drop to assemble into a single spheroid within 24 h at 37°C, 5% CO2. After 24 h spheroids were harvested and used for sprouting analysis in a matrix of
type I collagen. Briefly, 30 EC spheroids per group were seeded into 0.5 ml collagen solution in non-adherent 24-well plates, with a final concentration of rat type I collagen of 1.5 mg/ml. Freshly prepared gels were transferred rapidly into a humidified incubator (37°C, 5% CO2) and after the gels had solidified, 0.1 ml Serum-Free Media (Cell Systems, Kirkland, USA) was added per well containing Wnt3a (100ng/ml), Wnt7a (100ng/ml), VEGF (25ng/ml), and Cln5 antibody (10µg/ml) as indicated. After 24 h, gels were photographed and spheroid sprouting was assessed quantitatively using Adobe Photoshop®. Results are expressed as mean ± SEM. n is number of spheroids quantified.

Immunohistochemical staining.

For retina cross-sections immunostaining, eyes were fixed in 4% paraformaldehyde for 1 h and incubated in 30% sucrose at 4°C, followed by embedding in OCT. 10-µm thick sections were blocked in PBS with 0.1% Triton X-100 and 5% goat serum. Sections were stained with Isolectin to visualize vessels and/or primary antibodies followed by secondary antibodies. For whole-mount immunohistochemical staining, retinas fixed in 4% paraformaldehyde for 1 h were rinsed in 1x PBS, permeabilized overnight at 4°C with 0.5% Triton X-100 in PBS, stained with Isolectin B4, followed by primary and secondary antibody staining. Retinal whole-mounts were prepared and imaged as described previously. Retinal microglia/macrophages were visualized in retinas from mice with Csf1r-driven GFP, with fluorescence enhanced with FITC labeled rabbit antibodies against GFP.

Western blot:

Retinas were dissected as described above. 25 µg protein of retinal lysate from Lrp5<sup>−/−</sup> or WT mice was loaded on an SDS-PAGE gel and transferred onto a PVDF membrane. After blocking, the membranes were incubated overnight with rabbit anti-mouse Cln5 antibody followed by anti-rabbit secondary antibody conjugated with horseradish peroxidase for one hour at room temperature. Chemiluminescence signals were generated with ECL plus substrate and captured with KODAK film.
**Antibodies:**

The following primary antibodies were used in this study: anti-Lrp5 (36-5400, Invitrogen), anti-Frizzled4 (MAB194, R&D systems), anti-Norrin (AF3014, R&D systems), rabbit antibody against GFP (A11122; Invitrogen); anti-β-GAL (ab616, Abcam), anti-Claudin5 (34-1600 and 352588, Invitrogen) and anti-Mouse IgG (A-21200, Invitrogen). Secondary antibodies: Chicken anti-rabbit Alexa 488 (A-21441, Invitrogen); Goat anti-Rabbit conjugated with horse-radish peroxidase (NA934V, Amersham Pharmacia).

**Supplemental References:**

Supplemental Figure 1. Time course of Lrp5 mRNA expression. A. Quantification of retinal Lrp5 mRNA with RT-qPCR during normal retinal development. B. Specificity of Lrp5 antibody is verified with absence of Lrp5 antibody staining in Lrp5<sup>−/−</sup> eyes. Scale bar: 100µm.

Supplemental Figure 2. Normal retinal vasculature in Dvl2<sup>−/−</sup> eyes. A. Retinal flat mount of Dvl2<sup>−/−</sup> mice (1 month old) shows normal retinal vascular structure. Retinal vessels are visualized with isolectin B<sub>4</sub> staining (red). B. Retinal cross section of Dvl2<sup>−/−</sup> mice (1 month old) stained with isolectin (red, vessels) and DAPI (blue, nucleus), showing normal retinal vessels in all three layers. Scale bars: A: 1000µm. B: 100µm.

Supplemental Figure 3. Pathways not regulated at mRNA level in Lrp5<sup>−/−</sup> eyes. A. Quantification of Angiopoietin (Ang) 1 and Ang2 in WT and Lrp5<sup>−/−</sup> retina at P5 and P8. B. Quantification of Dll4, Notch1 and Notch2 in WT and Lrp5<sup>−/−</sup> retina at P5 and P8.

Supplemental Figure 4. Blocking cln5 with a second antibody suppresses spheroid sprouting. A. Human retinal microvascular endothelial cells (HRMEC) were cultured as multicellular spheroids in the presence of Wnt 3a, Wnt7a and VEGF in combination with a second Cln5 antibody to confirm the results shown in Figure 4c. Quantification of HRMEC spheroid sprouting shows that blocking Cln5 with this second antibody also suppresses HRMEC spheroid sprouting. n=10-20 per group, n.s.: not significant, *p≤0.05, **p≤0.001. B. Quantification of HRMEC spheroid sprouting in the presence of VEGF in combination with NaN<sub>3</sub> shows lack of toxic effects of the antibody solution containing NaN<sub>3</sub>. n=10-20 per group, n.s.: not significant.

Supplemental Figure 5. Expression levels of Frizzled(Fzd) receptors in Lrp5<sup>−/−</sup> eyes. Quantification of Fzd2, Fzd3 Fzd4 Fzd7 and Fzd8 mRNA in WT and Lrp5<sup>−/−</sup> retina at P5 and P8.
Supplemental Figure 1

A

![Graph showing mRNA levels of Lrp5](graph.png)

B

![Immunofluorescence staining for Lrp5](image.png)
Supplemental Figure 3

A

Ang1

mRNA / 10^6 cyclophilin

WT  Lrp5^-/-

5  8

Postnatal Day

Ang2

mRNA / 10^6 cyclophilin

WT  Lrp5^-/-

5  8

Postnatal Day

B

Dll4

mRNA / 10^6 cyclophilin

WT  Lrp5^-/-

5  8

Postnatal Day

Notch1

mRNA / 10^6 cyclophilin

WT  Lrp5^-/-

5  8

Postnatal Day

Notch2

mRNA / 10^6 cyclophilin

WT  Lrp5^-/-

5  8

Postnatal Day
Supplemental Figure 4

A

Spheroids sprouting (relative units)

- Cln5 ab.
+ Cln5 ab.

Control
Wnt3a
Wnt7a
VEGF

***
***

B

Spheroids sprouting (relative units)

VEGF
VEGF + NaN₃

n.s.
Supplemental Figure 5

The figure shows mRNA levels of various Fzd receptors (Fzd2, Fzd3, Fzd4, Fzd7, Fzd8) normalized to cyclophilin expression. The data is presented for two genotypes: WT and Lrp5−/−, across postnatal days 5 and 8. The x-axis represents postnatal day (P), and the y-axis represents mRNA levels per 10^6 cyclophilin. Stars indicate significant differences between the genotypes for each day.