Differential Expression of Claudins in Retinas during Normal Development and the Angiogenesis of Oxygen-Induced Retinopathy

Yan Luo,*1,2 Wei Xiao,1,2 Xiaobo Zhu,1 Yani Mao,5 Xialin Liu,1 Xiaoyun Chen,1 Juan Huang,1 Shibo Tang,*1, and Lawrence J. Rizzolo4

PURPOSE. Angiogenesis accompanies several retinal pathologies that impair the inner blood–retinal barrier. Claudins are key structural and functional proteins of the barrier. This study compared the expression of claudins during the normal angiogenesis of development with that of oxygen-induced retinopathy.

METHODS. Real-time PCR was used to monitor mRNA from postnatal day 8 (P8) to P21 in normal mice and oxygen-induced retinopathy (OIR) mice. Protein expression was monitored by immunoblotting and immunofluorescence. Isoclin B4 was used to identify blood vessels and occludin was used to identify tight junctions. Neovascularization and permeability were monitored using FITC–dextran and Evans blue.

RESULTS. The mRNA of claudin-1, -2, -3, -4, -5, -12, -22, and -23 was developmentally regulated, but only claudin-1, -2, and -5 were found in the tight junctions of retinal vessels. OIR induced the formation of leaky neovascular vessels. The mRNA and protein of claudin-2 and -5 were overexpressed, whereas claudin-1 and occludin were unaffected. Despite their overexpression, each claudin was distributed throughout the cell, especially in the neovascular tufts. Occludin was retained at the lateral membranes but exhibited a punctate distribution.

CONCLUSIONS. Claudin-1, -2, and -5 are the most prominent claudins of the inner blood-retinal barrier. The pathologic angiogenesis induced by oxygen formed a leaky barrier due to the mislocalization of these claudins. Studies of the mechanisms that regulate the intracellular distribution of claudins may lead to new therapeutic approaches for retinal vascular disease. (Invest Ophthalmol Vis Sci. 2011;52:7556–7564) DOI: 10.1167/iovs.11-7185

The microenvironment of the retina is separated from the circulation by the blood–retinal barrier. This barrier includes the outer blood–retinal barrier formed by the retinal pigment epithelium and the inner blood–retinal barrier (iBRB) formed by retinal vessel endothelial cells. The iBRB controls cellular and molecular trafficking between the blood and interstitium of the neural retina. Disruption of the iBRB occurs in a number of ocular fundus diseases, such as diabetic retinopathy3 and retinopathy of prematurity.2

The barrier function of the iBRB depends on the integrity of the tight junctions that seal the space between adjacent endothelial cells. This semiselective seal retards the diffusion of solutes across these paracellular spaces. Tight junctions also divide the plasma membrane into apical and basolateral membrane domains and maintain cell polarity.3–5 The numerous tight junction proteins include transmembrane and scaffold proteins. Transmembrane proteins include claudins, junctional adhesion molecules, occludin, and tricellulin.6

Claudins form the tight junctional strands that are observed by electron microscopy and determine the permeability and selectivity of the strands.7–9 Claudins are 20- to 27-kDa tetraspan proteins with a short cytoplasmic N-terminus, two extracellular loops, and a C-terminal cytoplasmic domain.10 At least 24 family members have been identified.11 Each tissue expresses its own subset of claudins, according to the physiologic role of that tissue.12–16 In the eye, tissue-specific expression has been reported for the lens, cornea, and conjunctiva.17,18

The expression profile and function of the claudins have not been reported for the iBRB. This study examines the mRNA and protein expression levels of claudin family members during the normal formation of the iBRB and the hyperstimulated retinal angiogenesis of oxygen-induced retinopathy (OIR). With the retinas of normal development and OIR, we could begin to address the questions of the expression and distribution of claudins during retinal angiogenesis.

METHODS

Animals

All the studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Ethics Committee in Animal and Human Experimentation of Sun Yat-sen University, Guangzhou, China. C57BL/6J mice were purchased from Southern Medical University, Guangzhou, China. Mouse pups and their
DNA was prevented using a commercial kit (DNase I Kit; Sigma-Aldrich, St. Louis, MO). DNase I–treated RNA (1 μg) was then converted to cDNA using reverse transcriptase (Takara, Siga, Japan). cDNA samples were aliquoted and stored at −80°C.

### RNA Isolation and cDNA Preparation

After mice were anesthetized by an intraperitoneal (IP) injection of 10% chloral hydrate (2.5 mL/kg), retinas of postnatal day 8 (P8), P11, P13, P15, P18, and P21 mice, together with kidney and brain of 6-week-old mice, were collected. Total RNA was extracted using a commercial reagent (TRIzol; Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Potential contamination by genomic DNA was prevented using a commercial kit (DNase I Kit; Sigma-Aldrich, St. Louis, MO). DNase I–treated RNA (1 μg) was then converted to cDNA using reverse transcriptase (Takara, Siga, Japan). cDNA samples were aliquoted and stored at −80°C.

### Nonquantitative PCR and Quantitative Real-Time PCR

Primers for claudins, occludin, and 18S rRNA (Table 1) were designed using commercial software (Primer Premier 5.0; PREMIER Biosoft International, Palo Alto, CA). Specificity of the primer sequences was examined using the National Center for Biotechnology Information Basic Local Alignment Search Tool module. To reveal the mRNA expression of claudins, 30 cycles of PCR were used to amplify the claudin

### Table 1. Real-Time PCR Primers Used in This Study

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All primers were designed using commercial software (Primer Premier 5.0; PREMIER Biosoft International).

† Primers were used for real-time PCR.

‡ Primers were used for RT-PCR.
cDNA produced from normal P18 mouse retinas. The expression of claudins in the kidney and brain of 6-week postnatal mice was also amplified as a positive control. PCR products were separated by 2% agarose (Biowest, Seville, Spain) and visualized by double-stranded DNA binding fluorescent stain (GoldView dye; SBGene, Shanghai, China). For quantitative analysis of mRNA expression, an RT-PCR kit (SYBR PrimeScript; Takara, Siga, Japan) was used to amplify the target genes according to the manufacturer’s standard protocol. Real-time PCR reactions were performed with a sequence detection system (ABI Prism 7000; Applied Biosystems, Foster City, CA). Relative expression of mRNA was calculated using the established $2^{-\Delta\Delta CT}$ method. Briefly, the mRNA level of each target gene was normalized first to that of 18S rRNA and second to its mRNA level at P8.

**Immunofluorescent Staining of Frozen Section**

Eyes of normal P18 mice were enucleated and immediately embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA) at $-80^\circ$C. Sections (7 µm) were cut on a cryostat, fixed in cold acetone on ice for 8 minutes, then incubated with penetrating solution (0.05% Triton X-100 in PBS) at room temperature (RT) for 5 minutes, and then blocked with 5% BSA in PBS at RT for 1 hour. Sections were then incubated with primary antibodies (claudin-1, -2, -3, -5, and -12 from Zymed Laboratories/Invitrogen; claudin-4 from Santa Cruz Biotechnology, Santa Cruz, CA; claudin-23 from Abcam, Cambridge, UK) at 4°C overnight in a humidity chamber, then incubated with secondary antibodies conjugated with a fluorescent dye (Alexa Fluor 555; Molecular Probes/Invitrogen) and with isolectin B4, also conjugated with a fluorescent dye (Alexa Fluor 488; Molecular Probes/Invitrogen) for 1 hour. Finally, sections were incubated with 4',6-diamidino-2-phenylindole (Sigma-Aldrich) for 5 minutes, and then mounted with antifade mounting medium (Applygen Technologies, Beijing, China). Images were captured under a confocal microscope (LSM 510; Carl Zeiss Meditec, Oberkochen, Germany).

**Mouse Model of Oxygen-Induced Retinopathy**

Oxygen-induced retinopathy mice were generated as described previously. Briefly, at P7, mouse pups were exposed to 75% oxygen with their nursing mothers in an airtight chamber. Oxygen concentration was measured and controlled by a compact oxygen controller (ProOx, Model 110; BioSpherix, New York, NY). Temperature was maintained at 21 ± 1°C and humidity was between 40% and 70%. At P12, pups were returned to room air, resulting in retinal neovascularization.

**Measurement of Retinal Neovascularature**

The perfusion of the retinal vessels with FITC-conjugated dextran was performed as described previously. Briefly, P18 mice were perfused via the left ventricle with 2 mL of 25 mg/mL FITC-conjugated dextran (Mw = 2 × 10^5 Da; Sigma-Aldrich) in 0.15 M PBS. The retinas were dissected, flatmounted, and viewed under a fluorescence microscope (Axioplan 2 imaging; Carl Zeiss Meditec). Quantitative analysis of the vasculature was carefully delineated based on pixel intensities, as previously described. The total area of neovascularization was calculated using commercial software (Image-Pro Plus 5.1; Media Cybernetics, Silver Spring, MD).

**Assessment of Inner Blood–Retinal Barrier Function**

Evans blue dye (EB) (Sigma-Aldrich) was used to assess the permeability of retinal vessels. EB was dissolved in normal saline (30 mg/mL) and injected IP into the P18 mice. By 2 hours after EB injection, mice were anesthetized by an IP injection of 10% chloral hydrate. For morphologic studies, the eyes were enucleated and fixed by 4% paraformaldehyde (PFA) at RT for 1 hour. Retinal flatmounts were prepared to view the dye distribution in the retina under a fluorescent microscope with a 546-nm excitation filter. For quantitative analysis of iBRB function, vascular permeability was quantified by measuring EB leakage from blood vessels into the retina, as described. The mice were perfused via the left ventricle with 1% PFA in citrate buffer (pH 3.5) and the retinas were carefully dissected under an operating microscope. After retinas were fully dried at 4°C, EB was extracted by incubating each sample in 60 µL of formamide at 70°C for 18 hours. The extract was centrifuged at 10,000g (Heraeus Freco 17 Centrifuge; Thermo Scientific, Waltham, MA) at 4°C for 20 minutes. Absorbance was measured using 50 µL of the supernatant at 620 nm. The concentration of EB (µg of EB per µg of total protein) in the extract was calculated from a standard curve and normalized by the total protein concentration in each sample.

**Immunoblot Analysis**

Retinas were isolated, immersed in cell lysis buffer (BD Biosciences, San Jose, CA) containing a protease inhibitor cocktail (Calbiochem, EMD Biosciences, La Jolla, CA), and homogenized on ice for 5 minutes using an ultrasonic tissue destructor. Lysate was centrifuged at 12,000 rpm at 4°C for 30 minutes, after which supernatant was collected. Total protein concentration was determined by the bicinchoninic acid method protein assay kit (Shenneng, Shanghai, China). The samples were separated by standard RT-PCR (n = 3). Representative pictures for mRNA expression of claudins detected by standard RT-PCR are shown. In the normal P18 retina, claudin-5, -12, and -23 showed strong signals followed by claudin-1 and -20, but claudin-6, -8, -15, and -18 were not detected. Expressions of claudins in brain and kidney of 6-week-old mice were used as positive controls.

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933248/ on 11/21/2018)
were boiled for 10 minutes after 4× SDS sample buffer was added, and then stored at −80°C. Protein samples (20 μg) were resolved by 10% SDS-PAGE, and then transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The membranes were blocked in 5% nonfat milk at RT for 1 hour, and then incubated with primary antibodies at 4°C overnight. After being washed with PBS containing 0.1% Tween-20 (PBST) three times, the membranes were incubated with horseradish peroxidase–conjugated secondary antibody (Dako, Tokyo, Japan) for 1 hour. Visualization was performed using enhanced chemiluminescence (Cell Signaling Technology, Beverly, MA).

**Immunofluorescent Staining of Whole-Mount Retina**

For whole-mounted retinas, the eyes of anesthetized pups were enucleated and fixed with 4% PFA at RT for 15 minutes. The retinas were isolated under dissecting microscope, then fixed in ice-cold methanol for 10 minutes. After being blocked with 20% (v/v) fetal bovine serum (Invitrogen) in PBS at RT for 2 hours, retinas were incubated with primary antibodies raised against occludin and claudin-1, -2 or -5, at 4°C for 18 hours, washed with PBST, and then incubated with second-

**Figure 2.** Dynamic expression of claudins in normal retinas from P8 to P21. Data represent the fold increases of each gene, with the expression at P8 retinas taken as 1.0; n = 6 for each data point. Error bars, SD. The mRNA levels of eight claudins, claudin-1, -2, -3, -4, -5, -12, -22, and -23, varied >2× over this time course. The expression of claudin-1, -5, -12, and -23 displayed a transient peak around P15–P18. Claudin-22 mRNA increased during this period and reached a plateau around P18. Compared with those at P8, mRNA expression levels were significantly higher (*P < 0.05) from P13 to P18 for claudin-1 and -3; from P13 to P21 for claudin-2, -22, and -23; from P15 to P18 for claudin-4; from P13 to P15 for claudin-5; and from P11 to P18 for claudin-12. The mRNA expression levels of claudin-1 and claudin-5 at P21 were significantly lower than those at P8 (#P < 0.05).
ary antibodies conjugated with fluorescent dye (Alexa Fluor 555, for claudins, or Alexa Fluor 488, for occludin). After being washed with PBST, retinas were mounted with antifade mounting medium and images were collected by confocal microscope.

Statistical Analysis
All the experiments were performed at least three times with similar results, and representative figures are shown. Data were collected and expressed as mean ± SD. Student’s t-test was used to statistically compare the total area of neovascularization and EB leakage between normal and OIR mice. Variation of mRNA and protein expression from P8 to P21 between groups was evaluated by one-way ANOVA and post hoc test. A value of P < 0.05 was considered significant.

RESULTS
Claudin Expression in the Normal Retinas
To identify the claudins of the iBRB, we examined the mRNA expression profiles of claudins in the retinas of normal mice. Because claudin-21 and -24 have been identified only from sequence analysis of the human genome, we focused on claudin-1 through -20, -22, and -23. Kidney and brain of 6-week-old mice were used as positive controls because they express all the claudins, except claudin-6, -11, -13, -17, and -18 (Fig. 1). Although undetected in kidney or brain, claudin-11, -13, and -17 mRNA were detected in normal

![Claudin Expression](https://example.com/claudin-expression.png)

**Figure 3.** Localization of claudins in the retinas of normal mice. Frozen sections of normal P18 retinas were immunolabeled with primary antibodies against claudin-1, -2, -3, -4, -5, -12, and -23, followed by fluorescent dye-conjugated (Alexa Fluor 555 dye) secondary antibodies and fluorescent dye-conjugated (Alexa Fluor 488) isolectin B4. Representative pictures for each protein are shown (n = 6). Note the signals for claudin-1, -2, and -5 (red) colocalized with those of isolectin B4 (green), yielding a yellow fluorescent signal in the merged image, in the retinal ganglion cell (RGC), inner plexiform (IPL), and outer plexiform (OPL) layers. Claudin-3, -4, -12, and -23 showed no colocalization with isolectin B4. Staining of claudin-3 was localized in the RGC layer and claudin-12 was distributed in the OPL. Claudin-4 was distributed in both the RGC layer and the OPL whereas staining of claudin-23 was localized in the RGC layer and INL. INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars, 50 μm.
retina. Claudin-6, -8, -15, and -18 mRNA were undetected in normal retina (Fig. 1).

To determine which Claudins were most critical for forming and maintaining the tight junctions of the iBRB, we examined how the expression of Claudins changed between P8 and P21 by real-time PCR analysis. Twofold changes were considered biologically significant. The expression of Claudin-8 and -13 to -18 mRNA was always low, and the expression of Claudin-7, -9, -10, -11, -19, and -20 mRNA was unchanged (data not shown). In contrast, the expression of Claudin-1, -5, -12, and -23 displayed a transient peak at approximately P15–P18. Claudin-22 mRNA increased during this period and reached a plateau at approximately P18. Compared with those at P8, mRNA expression levels were significantly higher (Fig. 2; *P < 0.05) from P13 to P18 for Claudin-1 and -5; from P13 to P21 for Claudin-2, -22, and -23; from P15 to P18 for Claudin-4; from P13 to P15 for Claudin-5; and from P11 to P18 for Claudin-12. The mRNA expression levels of Claudin-1 and Claudin-5 at P21 were significantly lower than those at P8 (Fig. 2; *P < 0.05).

The presence of mRNA does not indicate that Claudins localized to retinal blood vessels. Indirect immunofluorescence was used to localize the Claudins that were developmentally regulated. Because the commercial antibody for Claudin-22 was unavailable, Claudin-22 was not included. Isolectin B4 was used to identify the retinal vasculature. In P18 retinas, Immunofluorescent signals for Claudin-1, -2, and -5 colocalized with that of Isolectin B4 in the retinal ganglion cell (RGC) layer, and the inner (IPL) and outer (OPL) plexiform layers. Claudin-3, -4, -12, and -23 did not colocalize with Isolectin B4. Claudin-3 immunofluorescence was evident in the RGC layer and Claudin-12 was evident in the OPL. Claudin-4 immunofluorescence was found in both the RGC layer and OPL, whereas Claudin-23 immunofluorescence was found in the RGC layer and inner nuclear layer (INL) (Fig. 3).

Comparison of Claudin Expression in Normal and OIR Retinas

To confirm that high oxygen tension induced angiogenesis, we assessed neovascularization using high molecular weight FITC-dextran and barrier property using EB. In P18 retinas, large avascular areas and neovascular tufts were observed in OIR retinas (Fig. 4A). Quantitatively, the area of neovascularization was significantly larger in OIR retinas (1.76 ± 0.09 mm²) than that in normal retinas (0.15 ± 0.01 mm²) (P < 0.05, Fig. 4B). Little, if any, EB leaked from the retinal vessels of normal mice, but EB leakage was observed in the retinas of OIR mice (Fig. 4C, arrow). Quantitatively, leakage from the retinal vessels of OIR mice was 12.7-fold higher than that of normal mice (P < 0.05, Fig. 4D).

Because OIR was associated with retinal neovascularization and iBRB leakiness, we examined the effects on the Claudins associated with blood vessels, Claudin-1, -2, and -5. Gene expression was examined by real-time PCR (Fig. 5A). Data represent the fold increase of each mRNA relative to the expression in normal P8 retinas. The mRNA expression levels of Claudin-2 and -5 in the OIR retinas were more than twofold higher than those in normal retinas at P18 (P < 0.05, Fig. 5A), and Claudin-2 mRNA in the OIR retinas rose to 4.2-fold higher than that in normal retinas at P21 (P < 0.05, Fig. 5A). For Claudin-1 and occludin, there was no statistical difference in mRNA expression levels between OIR and normal retinas.

Immunoblotting was used to examine whether the protein expression was regulated independently of mRNA expression (Figs. 5B, 5C). Like its mRNA, no significant difference was detected for occludin expression between normal and OIR retinas (P > 0.05). Claudin-1 was expressed at similar levels in OIR and normal retinas for each age. Similar to the mRNA levels, protein levels of Claudin-2 and -5 in OIR retinas were upregulated dramatically at P18 compared with that of the normal retinas (P < 0.05).

Distribution of Claudin-1, -2, and -5 in Normal and OIR Retinas

To investigate the apparent contradiction of Claudins being upregulated in a leakier iBRB, we examined the distribution
of claudin-1, -2, and -5 within the retinal blood vessels. Confocal micrographs demonstrated that claudin-1, -2, and -5 were expressed specifically in retinal vascular endothelial cells. In normal retinas, claudin-1, -2, and -5 were distributed in the plasma membranes of large and small vessels. The images of large vessels in the OIR mice (Figs. 6B, 6F, 6J) were taken adjacent to avascular retina and the images of small vessels (Figs. 6D, 6H, 6L) were taken within the peripheral vascularized portion. The claudins colocalized with occludin in tight junctions (Figs. 6A, 6C, 6E, 6G, 6I, 6K; arrowhead). In OIR retinas, claudins were found in a nonjunctional pool that might be intracellular or dispersed along the plasma membrane and occludin exhibited a punctate distribution (Figs. 6B, 6D, 6F, 6H; small arrow). Moreover, the intense staining of claudin-1, -2, and -5 often accompanied the weak staining of occludin in the retinal vessels of OIR mice, especially in the neovascular tufts (Figs. 6B, 6D, 6F, 6H, 6J, 6L; arrow).

**DISCUSSION**

The blood–brain barrier (BBB) requires a specialized microenvironment that is regulated in part by the tight junctions of capillaries. Previous investigators identified claudin-1, -3, -5, and -12 as the claudin family members present in the capillaries of this barrier. Because of their role in determining the permeability and selectivity of tight junctions, these findings potentially give some insight into the nature of the barrier. Claudin-5 was shown to be a major component of BBB tight junctions because it restricts the penetration of molecules with a molecular weight <800 Da.25 The role of the other claudins in this tissue remains to be clarified. In contrast to those reports, which studied whole brain, the present study focuses on one specific region of the BBB, the iBRB. By examining how the iBRB develops normally and during OIR, we found that claudin-1, -2, and -5 were the most prominent. This finding indicates regional variation in the BBB. In this case, the iBRB might be more permeable to ions in general, due to the lack of claudin-3,26 and more permeable still to Na⁺, due to the presence of claudin-2 that would increase the permeability to sodium.27

In our initial screen, we examined claudin family members and occludin by RT-PCR. We found that claudin family members show different expression levels in the mouse retina. At P18, claudin-6, -8, -15, and -18 were not expressed and the
Figure 6. Expression and distribution of claudin-1, -2, and -5 on the normal and OIR retinal vessels. Retinas from P18 normal or OIR mice were incubated with primary antibodies against occludin and claudin-1, -2 or -5, followed by fluorescent-dye (Alexa Fluor 488 and 555)-conjugated secondary antibodies. Representative pictures (n = 6) of large vessels (A, B, E, F, I, J) and small vessels (C, D, G, H, K, L) are shown. In the normal retinas, arrowbeads show the colocalization of claudin-1 (A, C), -2 (E, G), and -5 (I, K) (red) with occludin (green) at the junction of adjacent vascular endothelial cells. In OIR retinas, claudin-1 (B, D), -2 (F, H), and -5 (J, L) expressed in a nonjunctional pool that might be intracellular or dispersed along the plasma membrane in large and small vessels. Occludin exhibited a punctate distribution along cell–cell borders (B, D, F, H; small arrow). The intense staining of claudin-1, -2, and -5, with the weak staining of occludin, was frequently observed on retinal vessels of OIR mice, especially in the neovascular tufts (B, D, F, H, J, L, arrow). Scale bars, 50 μm.

others were expressed at different levels. Claudin-6 was not detected in postnatal retina, brain, or kidney, in agreement with an earlier report, which concluded claudin-6 is expressed in mouse embryonic epithelium.28 The relative amounts of claudin mRNA varying between P8 and P21 were determined by quantitative RT-PCR. The mRNA levels of claudin-1, -2, -3, -4, -5, -12, -22, and -23 varied more than twofold. Claudin-22 mRNA increased throughout this period, but the others exhibited transient peaks. Claudin-1, -3, -5, and -12 mRNA exhibited broad peaks centered about P13–P15. Claudin-2 and -23 peaked at P15, with claudin-4 peaking at P15–P18.

Of all the claudins expressed in the neural retina, only claudin-1, -2, and -5 were found in the blood vessels identified by isoelectin B4. Each of these three claudins was detected in inner, outer, and ganglion cell vascular layers. By contrast, claudin-3, -4, -12, and -23 localized to extravascular cells. Tight junctions do occur in other cell types, including Schwann cells29 and neurons,30 but the identities of these extravascular cells in the retina need to be investigated further. We focused our attention on the vascular claudins.

Besides mRNA, cells also regulated the steady state level and intracellular distribution of claudins. Immunoblots demonstrated that the steady state levels of occludin and claudin-1 and -5 remained high, even though the amount of their mRNA decreased at P21. The steady state level of claudin-2 paralleled the expression of its mRNA. Each claudin localized to the lateral membranes and colocalized with the tight junction marker, occludin.

Pathologic angiogenesis is often accompanied by vascular hyperpermeability, particularly in the blood–brain and blood–retinal barriers.31 We used the OIR mouse model to explore how pathologic angiogenesis affected claudin-1, -2, and -5. Hyperpermeable, neovascular tufts formed in the OIR retinas. Claudin-2 and -5 mRNA and protein were overexpressed in P15–P21 retinas, whereas the expressions of occludin and claudin-1 were unaffected. Conceivably, increased vascularity of the OIR retinas might explain these data. We believe this is unlikely because such an artifact would affect all the proteins tested the same way. Further, quantitative RT-PCR revealed no significant differences in the expression of the endothelial cell marker, CD31, between control and OIR animals (data not shown).

Although overexpression of claudins might be explained by neovascularization, immunofluorescence demonstrated that each of the claudins was also mislocalized to the cytosolic compartment or distributed to nonjunctional regions of the plasma membrane. This phenomenon was observed in both large and small vessels. Further, occludin exhibited a punctate distribution and was no longer distributed along the entire lateral membranes of the cells. Despite the overexpression of the claudins, these data suggest that the network of tight junctional strands no longer encircle each cell as a continuous network. This hypothesis was supported by the observation that injected EB was readily extracted from the harvested retinas. Because EB binds serum proteins, it could leak out of retinal vessels only if the tight junctional strands fail to form a continuous network. Rather than a complete dissociation of the junctional network, a rudimentary complex appears to remain that can retain very large tracers, such as the large FITC–dextran used in this study.

To our knowledge, this is the first study that has examined differential expression of claudins in retinas of normal development and the hyperstimulated angiogenesis of OIR. Further studies are required to find the exact roles and interactions of tight junction proteins essential for the proper formation of the iBRB. Knowledge of how cells regulate the expression and localization of claudin-1, -2, and -5 may lead to new therapeutic strategies for retinal vascular diseases associated with neovascularization and the breakdown of the iBRB.

References