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MicroRNA-184 modulates canonical Wnt signaling through the regulation of frizzled-7 expression in the retina with ischemia-induced neovascularization

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Abstract

Aberrant activation of Wnt signaling contributes to ischemia-induced retinal neovascularization in oxygen-induced retinopathy (OIR), although the underlying mechanism is so far unclear. Here, we show that microRNA-184 (miR-184) is significantly down-regulated in the retina of OIR mice, and miR-184 negatively modulates Wnt signaling both *in vivo* and *in vitro*. Furthermore, we show that the Wnt receptor, frizzled-7, is a downstream target of miR-184, and delivery of miR-184 mimic inhibits Wnt signaling in the OIR retina. These results suggest that decreased levels of miR-184 are responsible, at least in part, for the aberrant activation of Wnt signaling in ischemia-induced retinal neovascularization.

Keywords

microRNA; neovascularization; oxygen-induced retinopathy; Wnt signaling pathway; gene delivery

1. Introduction

MicroRNAs (miRNAs) are single-stranded, small (19~24 nucleotides) non-coding RNA molecules [1] and serve as negative regulators in gene expression at the posttranscriptional level by partially complementary binding to the 3' un-translated region (3'UTR) of target

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mRNAs [2-8]. It is known that miRNAs are widely present in the body and regulate a variety of developmental and physiological processes [3]. Although it is reported that expression changes of some miRNAs are implicated in the development and progression of various diseases including diabetes [9,10], the pathogenic roles of miRNAs in diabetes and its complications remain unclear.

Diabetic retinopathy (DR), one of the major complications of diabetes, is the most common diabetic eye disease and a leading cause of blindness among the working age population in the US [11]. Accumulating evidence suggests that chronic inflammation and oxidative stress in the retina play important pathogenic roles in DR [12-14]. Oxygen-induced retinopathy (OIR) is a well-established animal model of ischemia-induced retinal inflammation and neovascularization (NV) which recapitulates pathologies of proliferative diabetic retinopathy (PDR). Thus, OIR is commonly used as a model of PDR [15].

Wnt signaling is a conserved intracellular signaling pathway consisting of Wnt ligands, their co-receptors and an intracellular signaling cascade and regulates multiple cellular processes, such as cell differentiation, inflammation, carcinogenesis and angiogenesis [16-19]. It has been reported that aberrant-activation of the Wnt signaling in the retina is a major pathogenic mechanism for retinal inflammation and NV in DR and in the OIR model [20-22]. However, the cause of the Wnt signaling activation in OIR is poorly understood.

It was reported that microRNA-184 (miR-184) is abundantly expressed in the cornea and lens [23], and single-base substitution in the miR-184 seed region is associated with corneal diseases and cataract [24-26]. In addition, an earlier study reported that several miRNAs including miR-184 are implicated in ocular NV in the OIR model and the non-ischemia retinopathy (laser-induced choroidal NV) model [27].

Here, we verified that miR-184 was down-regulated in the retina of OIR mice and identified that miR-184 negatively modulates canonical Wnt signaling through the regulation of expression of the Wnt receptor frizzled-7 (Fzd7).

2. Materials and Methods

2.1. Animals

C57BL/6J and *Axin2^{LacZ}* mice [28] were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in 12 hr light and 12 hr dark cycles. The *Axin2* gene is a known target gene of canonical Wnt signaling, and *Axin2^{LacZ}* mice were generated in-frame insertion of a nuclear-localized β -galactosidase (NLS-*LacZ*) to visualize the locations of Wnt signal activation [28,29]. All of the experiments involving mice were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Oklahoma Health Sciences Center, and performed following the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) statement for the "Use of Animals in Ophthalmic and Vision Research".

2.2. Oxygen-induced retinopathy (OIR) model

C57BL/6J or *Axin2^{LacZ}* mice were exposed to 75% O₂ from postnatal day 7 (P7) to P12. Age-matched mice with the same genetic background were maintained in constant room air (normoxia control) as the negative control. The retinas were isolated at P16 for RNA or protein analyses.

2.3. Quantitative real-time RT-PCR (qRT-PCR) for microRNA and mRNA expression analyses

RNA extraction and qRT-PCR for miRNA (TaqMan miRNA assay, Lifetech, Carlsbad, CA: miR-184, U6-snRNA) and mRNAs (*Vegf-a*, *Gfap*, *Hprt1*: primer sequences were shown in Table 1) were performed as described previously [30].

2.4. Western blot analysis

Briefly, equal amounts of total cellular proteins (20 µg) were resolved on 10% SDS-PAGE gel and electro-transferred onto a nitrocellulose membrane. Western blot analysis was conducted as described previously [30]. Rabbit polyclonal antibodies for phosphorylated-LRP6 and non-phosphorylated-β-catenin were purchased from Cell Signaling (Danvers, MA); a rabbit polyclonal antibody for VEGF-A was purchased from Santa Cruz (Dallas, TX); a mouse monoclonal antibody for β-actin was purchased from Sigma Aldrich (Saint Louis, IL) and an anti-Fzd7 mouse monoclonal antibody was purchased from LifeSpan Biosciences (Seattle, WA).

2.5. In vivo and in vitro Wnt signaling activity assays

Wnt signaling activation in the retina of Wnt signaling reporter (*Axin2^{LacZ}*) mice was evaluated by the Wnt reporter gene, β-galactosidase activity assay using Senescence Cells Histochemical staining kit (Sigma Aldrich). A rat Müller cell line, rMC-1 [31], with stably expressing a firefly luciferase controlled by Wnt/β-catenin system, was generated by lentivirus infection and antibiotic (puromycin, Sigma Aldrich) resistance selection [32]. The equal amounts (12.5 pmol/0.5 mL (25 nM) each) of negative control miRNA mimic or miR-184 mimic (Invitrogen, Carlsbad, CA), and 2.5 pmol/0.5 mL (5 nM) of miR-184 mimic plus 25 pmol/0.5 mL (50 nM) of negative control miRNA inhibitor or miR-184 inhibitor (Invitrogen, Carlsbad, CA) using Lipofectamine RNAiMAX (Invitrogen), and culture media were replaced at 6 hr post-transfection. The cells were treated with 10% Wnt3A-conditioned media (W3A) at 32 hr post-transfection, and a luciferase-based Wnt signaling activity assay (TOP flash assay) was conducted at 48 hr post-transfection following the manufacturer's protocol (Promega Corporation, Madison, WI).

2.6. Verification of downstream target of miR-184

The downstream target of miR-184 was validated with luciferase-based assay as described previously [30]. Briefly, approximately 700 base-pairs of the human Fzd7 3'UTR was cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). In addition, a scrambled sequence of miR-184 was cloned into the same vector as a negative control and a reverse complementary sequence of miR-184 was cloned into the same vector as a positive control. Further, we generated a point-mutant and a deletion mutant of the

miR-184-binding site in Fzd7 3'UTR to verify the impact of the binding site of miR-184 on Fzd7 expression. For *in vitro* analysis, the equal amount of assay vectors (0.2 µg each: scrambled, MIR-184, Fzd7-wt, Fzd7-Mut, and Fzd7-Del) were separately transfected into 293A cells (Qbiogen, Montreal, Canada) with 12.5 pmol/0.5 mL (25 nM) of either pre-miRNA negative control or miR-184 mimic (Invitrogen) using Lipofectamine2000 (Invitrogen). At 48 hr post-transfection, dual-luciferase assay (Promega) was conducted following the manufacturer's protocol.

2.7. Nanoparticle formulation and delivery into the eye

A pre-miRNA negative control and a miR-184 mimic (miRIDIAN Mimic, Thermo Fisher Scientific, Chicago, IL) were separately packed in liposome-based nanoparticles as described by Rajala et al [33]. One-µL of the prepared nanoparticle (approximately 5.5 pmol of pre-miRNA) was injected into the vitreous space of OIR mice at P12, and Wnt signaling activity in the retina was evaluated with X-gal staining at P16.

2.8. Statistical analysis

All of quantitative data were expressed as Mean ± SEM and represented as fold of control. At least three individual measurements were conducted and Student's t-test in Microsoft Excel (Microsoft, Redmond, WA) was performed to determine the statistical significance for comparison of two data sets. Significance was denoted as * p<0.05, ** p<0.01 and *** p<0.001, respectively.

3. Results

3.1. Activation of canonical Wnt signaling in the retina of OIR mice

Firstly, we evaluated Wnt signaling activation in the retina of OIR mice. The *Axin2^{LacZ}* Wnt signal reporter mice [28,29] were placed in the oxygen chamber (75% Oxygen) at P7 for 5 days (OIR model). Wnt signaling activation was evaluated by X-gal staining of retinal sections of normoxia control (Fig. 1A) and OIR (Fig. 1B) *Axin2^{LacZ}* mice at P16. Substantial blue color staining was observed in the retina of OIR *Axin2^{LacZ}* mice, but not in the normoxia control, indicating Wnt signaling activation in the OIR retina. In addition, Western blot analyses showed up-regulation of phosphorylated-LRP6 [34] (Fig. 1C, D) and non-phosphorylated-β-catenin [35] in the retinas of OIR C57BL/6J mice (Fig. 1C, E), further verifying activation of Wnt signaling in the OIR retina. Further, mRNA expression of *Vegf-a* (Fig. 1F), a major angiogenic factor and target gene of Wnt signaling, and *Gfap* (Fig. 1G), a commonly used stress marker in the retina [36,37], were significantly up-regulated in the retina of OIR mice. These results suggested that canonical Wnt signaling was activated in the retina of OIR mice, consistent with previous reports [20].

3.2. Expression level of miR-184 in the retina of OIR mice and Wnt signaling modulation by miR-184

Following the OIR procedure, the retinas were isolated at P16 for RNA extraction. As shown by qRT-PCR, the level of miR-184 was significantly down-regulated in the retina of OIR mice compared to that in the normoxia control (Fig. 2A). Furthermore, an rMC1-TOP cells were generated using rMC1 cells, a rat Müller cell line [31], infected with lentivirus

expressing luciferase reporter gene under a promoter containing TCF/ β -catenin-binding sites [32] to evaluate Wnt signaling activity in retinal cells. The rMC1-TOP cells were separately transfected with the negative control miRNA, miR-184 mimic or miR-184 inhibitor. *In vitro* Wnt signaling activation assay (TOP-Flash assay) was conducted at 48 hr post-transfection. Transfection of miR-184 mimic significantly inhibited Wnt signaling activity, whereas the miR-184 inhibitor significantly enhanced Wnt activity (Fig. 2B). It should be noted that the Wnt signaling activity in the cells treated with W3A-conditioned media increased approximately 5.2-fold over that of negative control L-cell conditioned media (data not shown).

3.3. Identification of downstream targets of miR-184

Bioinformatics analysis predicted that the mRNA of frizzled-7 (Fzd7), which is a receptor of canonical Wnt signaling, contains a binding site of miR-184 in the 3' UTR (Fig. 3A, C). Therefore, the Fzd7 cDNA 3'UTR including the predicted binding site was subcloned into the pmirGLO vector (Fzd7 Wt) (Fig. 3A). The interaction of miR-184 with this binding site in Fzd7 3'UTR was evaluated using luciferase-based assays. The miR-184 mimic transfection significantly down-regulated the expression of firefly luciferase in the cells cotransfected with the positive control (MIR-184) and Wt Fzd7 assay vectors, whereas no effect was observed in the negative control miRNA transfection (Fig. 3D). It should be noted that other predicted downstream target genes of miR-184 related to Wnt signaling did not regulate the expression of firefly luciferase (Suppl. Fig. 1 and 2). To further verify the miR-184-binding site in the 3'UTR of the Fzd7 mRNA, we have mutated and deleted the predicted miR-184-binding site in the assay vectors. Luciferase activity assays showed that miR-184 failed to regulate the reporter gene expression after the binding site was mutated (Fzd7 Mut) or deleted (Fzd7 Del). These results suggest that *Fzd7* is a downstream target of miR-184. Furthermore, the protein level of Fzd7 in the retina of OIR mice was evaluated by Western blot analysis, which showed that Fzd7 protein was significantly up-regulated in the retina of OIR mice (Fig. 3E, F).

3.4. Regulation of Wnt signaling by miR-184 in the retina of OIR mice

To elucidate the functional role of miR-184 *in vivo*, the negative control miRNA mimic and miR-184 mimic were separately packed in nanoparticles and injected into the vitreous space of OIR mice at P12. Its impact on Wnt signaling was examined at P16. A retinal section of OIR *Axin2^{Lacz}* mice injected with the negative control nanoparticle showed intense X-gal staining (Fig. 4A) indicating activation of Wnt signaling. This was consistent with the result in the retinal section of OIR mice without miRNA injection as shown in Figure 1B. On the other hand, the retinal section of OIR mice injected with miR-184 nanoparticles showed substantial reduction of X-gal staining (Fig. 4B) compared to that injected with the negative control miRNA (Fig. 4A), indicating that Wnt signaling was attenuated by miR-184 delivery. In addition, Western blot analyses showed significant reductions of phosphorylated-LRP6 (Fig. 4C, D) and non-phosphorylated- β -catenin (Fig. 4C, E) in the retina of OIR mice injected with miR-184, suggesting attenuation of Wnt signaling. Furthermore, expression levels of Fzd7, which is identified as a downstream target of miR-184 in this study, and Vegf-a, a target gene of Wnt signaling, were reduced in the retina of OIR mice with miR-184 injection (Fig. 4C, F).

4. Discussion

Our earlier studies suggested that Wnt signaling is activated in the diabetic retina, which plays a major pathogenic role in retinal inflammation and NV in DR [20,38,39]. However, the role of miRNAs in the Wnt signaling regulation in DR is not well- understood. In the present study, we identified the functional role of miR-184 in ischemia-induced retinal inflammation and NV (oxygen-induced retinopathy, OIR), a mouse model of PDR. We firstly verified that Wnt signaling was activated (Fig. 1), while the expression of miR-184 was significantly down-regulated in the retina of OIR mice (Fig. 2A). These results were consistent with earlier studies [20,27,38,39].

Then, we generated a rat Müller cell line stably expressing a Wnt signaling reporter gene, a firefly luciferase controlled by the Wnt/ β -catenin system (rMC1-TOP) to examine whether Wnt signaling in Müller cells is regulated by miR-184, since Wnt signaling activation was predominantly observed in the cells in the inner retina (Fig. 1A). Wnt signaling in the rMC1-TOP cells was significantly inhibited by transfection of the miR-184 mimic, while enhanced by the miR-184 inhibitor (Fig. 2B). These results strongly suggest that there is a link between the reduction of miR-184 expression and activation of Wnt signaling in the retina of OIR mice.

Next, we investigated a new downstream target of miR-184 implicated in canonical Wnt signaling. Through the bioinformatics analysis, mutagenesis of the predicted miR-184-binding site and luciferase-based assays, for the first time we identified that *Fzd7* is a direct target gene of miR-184 among a number of candidate target genes in Wnt signaling (Fig. 3 and Suppl. Fig. 1 and 2). In addition, Western blot analysis showed that *Fzd7* protein was increased in the OIR mouse retina (Fig. 3E). This provides a further support that *Fzd7* is the target gene of miR-184. For comparison, we also examined a luciferase assay vector containing another putative binding site of miR-184 in the 3'UTR of *Fzd4* (accession number NM_008055.4: nucleotide number, 3592-3612), and it did not reduce the luciferase expression by miR-184 mimic transfection (Suppl. Fig. 1 and 2). This result is consistent in-line with a previous observation that *Fzd4* protein was not reduced in the OIR mouse retina by pre-miR-184 (miR-184 mimic) injection [27].

In addition to miR-184, other miRNAs (miR-1, 23b, 27a) were recently identified as miRNAs regulating *Fzd7* in stem cells and cancer cells [40-42], suggesting that these miRNAs may modulate Wnt signaling in the same fashion as miR-184. It was also reported that *Fzd7* is involved in both canonical and non-canonical Wnt signaling in cancer cells [43], and *Wnt3A* acts as a ligand of *Fzd7* in canonical Wnt signaling [44]. Moreover, it was suggested that *Fzd7* is a target gene of canonical Wnt signaling, since there is a TCF/ β -catenin-binding site in the promoter region of the *Fzd7* gene [45]. This suggests that *Fzd7* may create a positive feedback loop in canonical Wnt signaling, and down-regulation of *Fzd7* expression by miR-184 may be ascribed to the modulation of the canonical Wnt signaling.

Interestingly, Dismuke et al. recently showed that miR-184 abundantly exists in exosomes of the intraocular fluid [46]. As described earlier, it was reported that the major sources of

miR-184 in the eye are the cornea and lens [23,47]. It is well-established that exosomes containing unique miRNA species are implicated in cell-to-cell communication [48,49]. This exosome-mediated delivery of miR-184 from the cornea and lens might be an additional mechanism responsible for the elevated level of miR-184 in the retina, in addition to up-regulation in retinal cells.

To further establish the causative role of miR-184 in Wnt signaling activation in the retina, we showed that delivery of the miR-184 mimic formulated in nanoparticles substantially inhibited Wnt signaling in the retina of OIR mice (Fig. 4B), compared to the negative control nanoparticle (Fig. 4A). Although the present study did not conduct the functional assays on inflammatory responses, vascular leakage and neovascularization in the retina, our previous studies have demonstrated that inhibition of Wnt signaling in the OIR model ameliorates these pathogenic features [20,38,39].

Another recent study also showed that the level of miR-184 was down-regulated in a mouse model of spinal cord of diabetic neuropathic pain [50]. It was reported that Wnt signaling regulates neuroinflammation to promote chronic pain, and the miR-184 reduction-mediated activation of Wnt signaling causes the development of neuropathic pain [51]. The present study may reveal the therapeutic potential using miR-184 nanoparticles as a new class of drugs for diseases associated with dysregulation of Wnt signaling.

In summary, the present study for the first time showed that delivery (or over-expression) of miR-184 in the retina with ischemia-induced retinal NV inhibits Wnt signaling as well as vegf-a (a major proinflammatory and angiogenic factor) expression. This may offer a new therapeutic strategy to prevent the inflammatory responses, vascular leakage and NV through canonical Wnt signaling in DR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

OIR	oxygen-induced retinopathy
NV	neovascularization
miRNA	microRNA
DR	Diabetic retinopathy
3'UTR	3' un-translated region

Fzd7	frizzled-7
Vegf-a	vascular endothelial growth factor-a
Gfap	glial fibrillary acidic protein
Hprt1	hypoxanthine phosphoribosyltransferase 1
TCF/LEF	T-cell factor/lymphoid enhancing factor

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Highlights

- MiR-184 is down-regulated in the retina of OIR mice.
- Canonical Wnt signaling is regulated by miR-184.
- Fzd7 is a downstream target of miR-184.
- Delivery of precursor of miR-184 inhibits Wnt signaling in the retina of OIR mice.

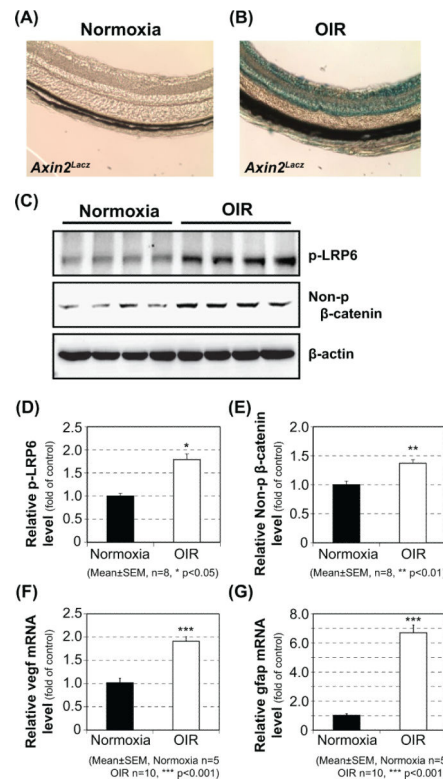


Figure 1. Activation of canonical Wnt signaling in the retina of OIR mice

Wnt signaling activation was evaluated by X-gal staining of retinal sections of normoxia control (A) or OIR (B) *Axin2^{LacZ}* mice at P16. Western blot analysis of phosphorylated-LRP6 (C, D) and non-phosphorylated-β-catenin (C, E) was performed using the retinas from the OIR C57BL/6J mice and normoxia control. The expression of *Vegf-a* (F), a major inflammatory factor, and *Gfap* (G), a commonly used stress marker, at the mRNA level in the retina of OIR mice was analyzed by qRT-PCR.

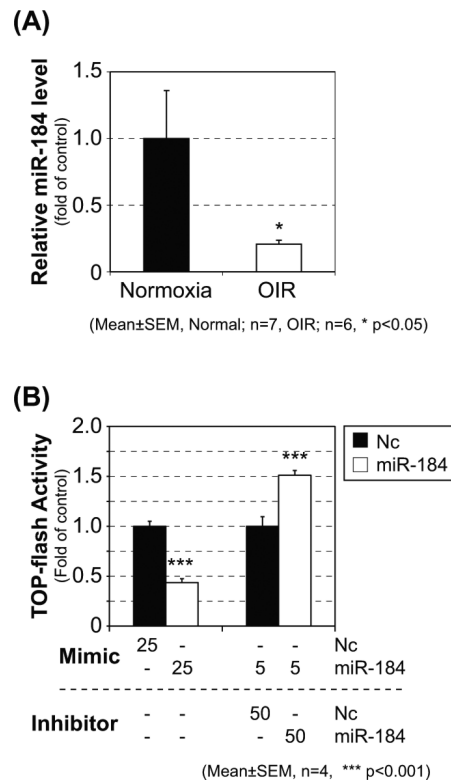


Figure 2. Expression of miR-184 in the retina of OIR mice and regulation of Wnt signaling by miR-184

C57BL/6J mice were under room air (normoxia control) or in the oxygen chamber from P7 to P12 (OIR model). Expression levels of miR-184 in the retinas at P16 were evaluated by qRT-PCR (A). MiR-184 mimic and its control (25 nM each) or miR-184 inhibitor and its control (50 nM each plus 5 nM miR-184 mimic) were separately transfected into rMC1-TOP cells stably expressing firefly luciferase under the control of the Wnt/ β -catenin system. The cells were treated with W3A-conditioned medium (10%) at 32 hr post-transfection and Wnt signaling activity assay (TOP flash assay) was conducted at 48 hr post-transfection (B).

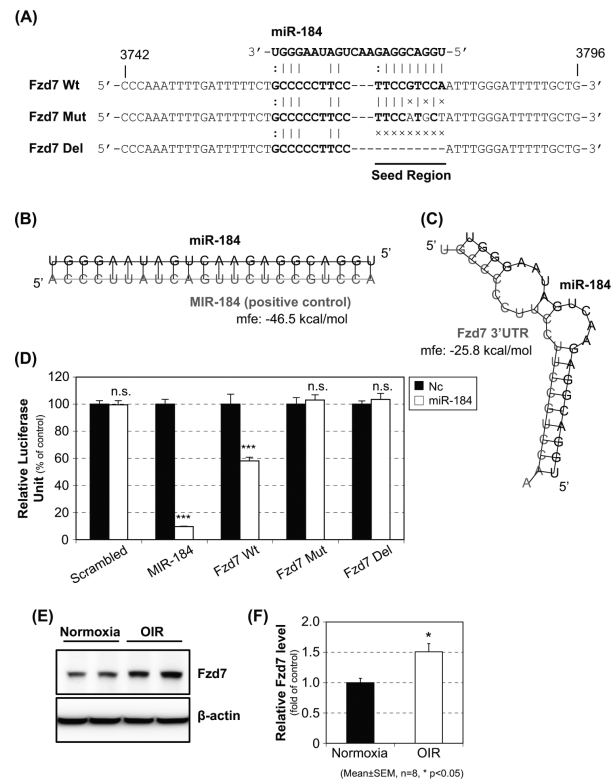


Figure 3. Verification of downstream target of miR-184

Approximately 700 bps of the 3'UTR of the Fzd7 cDNA including the predicted miR-184-binding site was subcloned into pmirGLO vector (A; **Fzd7 Wt**). Further, the predicted binding site of miR-184 (seed region) in the Fzd7 3'UTR was either mutated (A; **Fzd7 Mut**, 3 nucleotides in seed region were substituted) or deleted (A; **Fzd7 Del**). Generated pmirGLO vector containing a scrambled sequence of miR-184 was used as a negative control (**Scrambled**) and (B; **MIR-184**). The 3'UTR of **Fzd7 Wt**, **Mut**, and **Del** were co-transfected with negative control miRNA mimic (**Nc**, black bar) or miR-184 mimic (**miR-184**, white bar). Predicted secondary structure and minimum free energy (mfe) of the miR-184-binding site in the Fzd7 3'UTR were represented (C). At 48 hr post-transfection, luciferase assay was conducted (D). The retinas were collected from normoxia or OIR mice at P16, and Western blot analysis was conducted to evaluate the retinal level of Fzd7 protein (E, F).

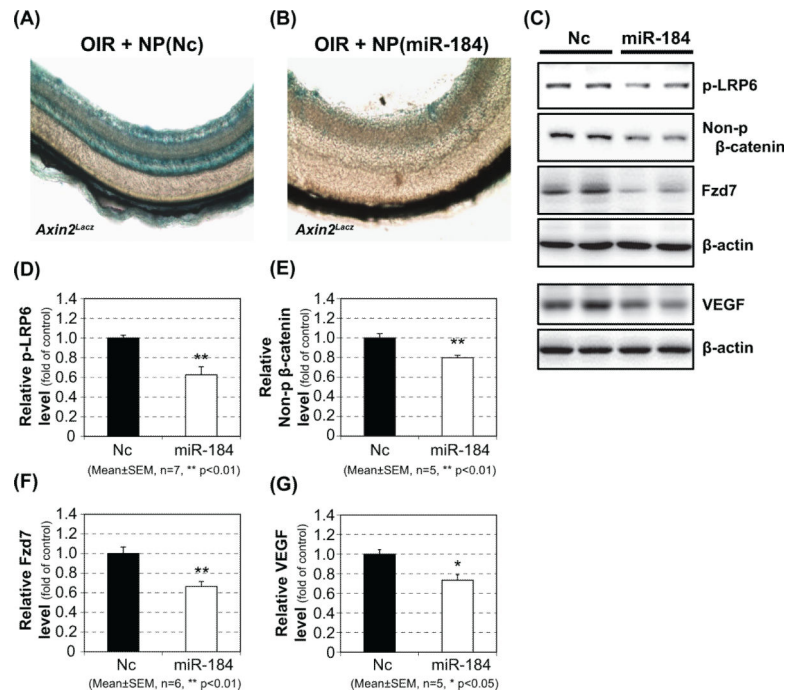


Figure 4. Delivery of miR-184 into the retina of OIR mice and its impact on Wn signaling
Wnt signaling activation was evaluated by X-gal staining of retinal sections on negative control nanoparticle-injected (A, OIR+NP(Nc)) or miR-184 nanoparticle-injected (B, OIR +NP(miR-184)) OIR-*Axin2* mice at P16. Western blot analyses of phosphorylated-LRP6 (C, D) and using negative control or miR-184 nanoparticle-injected OIR retina at P16. Further, expression of Fzd7 (C, F), a downstream target of miR-184, and Vegf-a (C, G), a target gene of Wnt signaling, in the retina of the miRNAs-injected OIR mice was evaluated by Western blot analysis.

Table 1

Primer sequences in this study.

Primer name	Primer Sequence (5' --- 3')	Product size
<i>Vegf-a</i> Fwd	GCCAGCACATAGAGAGAATGAGC	97
<i>Vegf-a</i> Rev	CAAGGCTCACAGTGATTTTCTGG	
<i>Gfap</i> Fwd	ATCGAGATCGCCACCTACAG	150
<i>Gfap</i> Rev	TACCACGATGTTCTCTTGA	
<i>Hprt1</i> Fwd	CAGGCCAGACTTTGTTGGAT	147
<i>Hprt1</i> Rev	TTGCGCTCATCTTAGGCTTT	

Vegf-a, vascular endothelial growth factor-a [40]; *Gfap*, glial fibrillary acidic protein; *Hprt1*, hypoxanthine phosphoribosyltransferase 1 [28].

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