Endothelial *microRNA-150* is an intrinsic suppressor of pathologic ocular neovascularization

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Pathologic ocular neovascularization commonly causes blindness. It is critical to identify the factors altered in pathologically proliferating versus normally quiescent vessels to develop effective targeted therapeutics. MicroRNAs regulate both physiological and pathological angiogenesis through modulating expression of gene targets at the posttranscriptional level. However, it is not completely understood if specific microRNAs are altered in pathologic ocular blood vessels, influencing vascular eye diseases. Here we investigated the potential role of a specific microRNA, *miR-150*, in regulating ocular neovascularization. We found that *miR-150* was highly expressed in normal quiescent retinal blood vessels and significantly suppressed in pathologic neovessels in a mouse model of oxygen-induced proliferative retinopathy. *MiR-150* substantially decreased endothelial cell function including cell proliferation, migration, and tubular formation and specifically suppressed the expression of multiple angiogenic regulators, *CXCR4*, *DLL4*, and *FZD4*, in endothelial cells. Intravitreal injection of *miR-150* mimic significantly decreased pathologic retinal neovascularization in vivo in both wild-type and *miR-150* knockout mice. Loss of *miR-150* significantly promoted angiogenesis in aortic rings and choroidal explants ex vivo and laser-induced choroidal neovascularization in vivo. In conclusion, *miR-150* is specifically enriched in quiescent normal vessels and functions as an endothelium-specific endogenous inhibitor of pathologic ocular neovascularization.

neovascularization | endothelial cells | microRNA | *miR-150* | retinopathy

Angiogenesis plays important roles in both physiological development and pathological events. Dysregulated angiogenesis is associated with many diseases including cardiovascular diseases, tumorigenesis, proliferative retinopathies, and neurodegeneration (1). In the eye, pathologic retinal neovascularization is characterized by abnormally proliferating tuft-liked structures, which may lead to vision loss (2). During angiogenesis, endothelial cells (ECs) proliferate, sprout, and form new vessels following dual guidance cues from both angiogenic stimulators and inhibitors (3). Vascular endothelial growth factor (VEGF) is targeted in current antiangiogenic therapies for cancers and neovascular eye diseases (4). However, anti-VEGF therapies target the end neovascular stage of vascular eye diseases and therefore do not address the incipient cause of proliferation and ischemia and in fact may affect normal vessel homeostasis (5, 6). It is therefore critical to identify additional intrinsic factors that maintain normal vessel quiescence and control the switch to proliferative neovessels to design improved targeted therapies.

MicroRNAs (miRNAs) are a group of small endogenous noncoding RNA molecules that function by base paring to the complementary sequence in the 3′ untranslated region (3′ UTR) of target mRNAs, inducing their cleavage and translational repression (7). MiRNAs are recognized as key fine-tuning mediators of posttranscriptional regulation. MiRNAs play critical roles in many biological processes, such as cell proliferation, cell death, neuronal differentiation and development, and angiogenesis (8–11). Moreover, dysregulation of miRNA is associated with many diseases, such as cancer and heart diseases, and are being developed as important biomarkers (12, 13). In neovascular diseases, both pro- and antiangiogenic miRNAs were identified in vascular ECs and perivascular cells (8, 13, 14). However, it is not well understood whether miRNAs are intrinsically regulated in proliferative blood vessels to influence ocular vascular diseases.

In this study, we used mouse retinal and choroidal neovascularization (CNV) models to study the role of endogenous miRNAs in regulating pathologic ocular neovascularization, which may also be relevant for angiogenesis in other organs. We demonstrated that expression of *miR-150* was highly enriched in quiescent blood vessels isolated from normal retinas and significantly suppressed in pathologic neovessels isolated from retinas under an oxygen-induced proliferative retinopathy model. In addition, we found *miR-150* decreases the proliferative function and tube formation of ECs in vitro and decreased the expression of multiple angiogenic target genes: C-X-C chemokine receptor type 4 (*CXCR4*), Delta like ligand 4 (*DLL4*), and Frizzled-4 (*FZD4*). Treatment with *miR-150* mimics significantly suppressed neovascularization in vivo in retinopathy. On the other hand, loss of *miR-150* in mice promoted angiogenesis in aortic and choroidal explants ex vivo as well as CNV in vivo in a laser-induced model. Together our findings suggest that the vascular-enriched *miR-150* is an intrinsic suppressor of EC proliferation and pathologic ocular neovascularization. *MiR-150* may represent a potential therapeutic target for diseases with abnormal angiogenesis.

Results

Vascular-Enriched *miR-150* Was Significantly Suppressed in Pathologic Neovessels in OIR. To identify miRNAs specifically affected in pathological vessel growth, we analyzed retinal RNAs isolated

Significance

Pathologic vascular growth causes vision impairment in several vascular eye diseases. Specifically targeting molecular signatures distinguishing pathologic neovascularization from normal vessels will allow targeted treatment options. This study demonstrates that *miR-150* was specifically enriched in normal retinal vessels and down-regulated in pathologic neovessels in a mouse model of proliferative retinopathy. *MiR-150* suppressed pathologic ocular neovascularization in mice with decreased expression of angiogenic target genes and inhibited endothelial cell function in vitro. Loss of *miR-150* also promoted vascular sprouting in ex vivo aortic and choroidal assays and laser-induced choroidal neovascularization in mice. These data suggest that endothelial *miR-150* is an endogenous suppressor of ocular neovascularization and a drug target for vascular eye diseases.

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from oxygen-induced retinopathy (OIR) and age-matched normoxic mice at postnatal day (P) 17 by miRNA array (Fig. 1*A*). *MiR-150* levels were suppressed about fivefold in OIR retinas compared with normoxic controls as validated by quantitative RT-PCR (qPCR) (Fig. 1*B*). We next explored the retinal localization of *miR-150* in retinal vascular and neuronal layers using laser capture microdissection (LCM) from P17 OIR and control normoxic retinas. The expression level of an EC marker vascular endothelial (VE)-cadherin was comparable between pathologic OIR vessels and normal vessels (*SI Appendix*, Fig. S1), confirming similar amounts of ECs in LCM isolated vessel samples. *MiR-150* was highly enriched in normal blood vessels compared with retinal neurons in normoxic retinas (Fig. 1*C*), suggesting a potential role of *miR-150* in regulating EC function. Moreover, *miR-150* expression was drastically reduced (>95%) in pathological neovessels from OIR retinas compared with normal retinal vessels (Fig. 1*C*), indicating a potential pathogenic role of *miR-150* deficiency in regulating ocular neovascularization.

***MiR-150* Regulated EC Migration, Proliferation, and Tubular Formation.** We next examined the angiogenic effects of *miR-150* in human retinal microvascular endothelial cells (HRMECs). Cells treated with *miR-150* mimic showed ~42% reduction in migration (Fig. 2 *A* and *B*) and ~22% reduction in proliferation (Fig. 2*C*) compared with nontargeting miRNA mimic control. In addition, *miR-150* substantially suppressed tube formation of HRMECs, resulting in reductions of ~48% in tubule length, ~60% in number of junctions, ~77% in mesh numbers, and ~78% in mesh area (Fig. 2*D*). In addition, in the presence of cotreatment with VEGF, *miR-150* mimic showed vascular-inhibitory effects independent of VEGF-induced HRMEC proliferation, migration, and tube formation

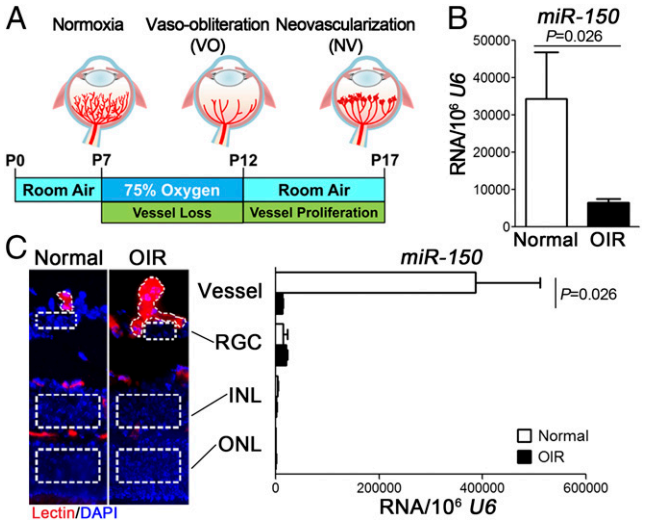

Fig. 1. *MiR-150* was enriched in normal retinal blood vessels and specifically suppressed in pathologic neovessels isolated from OIR retinas. (*A*) Schematic diagram of OIR. Neonatal mice were exposed to 75% oxygen from P7 to P12 to induce vessel loss and returned to room air from P12 to P17 to induce maximum pathologic neovascularization at P17. (*B*) Expression levels of *miR-150* were significantly suppressed in OIR whole retinas compared with normoxia controls, analyzed by qPCR normalized to *U6* snRNA as control ($n = 6$ per group). (*C*) Images on the left show representative retinal cross-sections from normoxic and OIR retinas stained with isolectin B_4 (red) for ECs and DAPI (blue) for nucleus, with dotted lines highlighting the area for LCM. *MiR-150* was found almost exclusively in LCM-isolated normal retinal blood vessels with minimal expression in retinal neurons. In addition, *miR-150* levels were significantly suppressed in pathologic vessels isolated from OIR retinas compared with normal vessels ($n = 4$–6 per group). INL, inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cells.

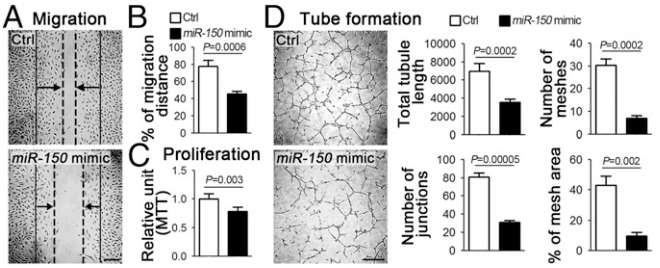

Fig. 2. *MiR-150* suppressed EC proliferation, migration, and tube formation in vitro. (*A* and *B*) Representative images and quantitative analysis of HRMEC wound healing assay. The solid lines indicate the start of cell migration, the dash lines indicate the front of the migration, and the arrows present the direction of the migration. HRMECs treated with *miR-150* mimic had significantly lower migration ability compared with cells treated with nontargeting mimic control (Ctrl) ($n = 4$–5 per group). (*C*) MTT assay results showed that *miR-150* mimic-treated HRMECs had lower levels of proliferative activity ($n = 3$ per condition). (*D*) Representative images and quantitative analysis of tube formation assay characterizing tubule length, junction numbers, mesh numbers, and percentage of mesh area. *MiR-150* mimic treatment significantly reduced the capacity of HRMECs to form tubes when cultured on Matrigel ($n = 3$–5 per group). [Scale bars, 250 μm (*A*) and 500 μm (*D*).]

(*SI Appendix*, Fig. S2). Together these results indicate that *miR-150* is a potent suppressor of EC function that may act through VEGF-independent pathways.

***MiR-150* Targeted Multiple Angiogenic Genes *CXCR4*, *DLL4*, and *FZD4* in EC.** To identify potential target genes of *miR-150*, we analyzed the seed sequence of *miR-150* (CUCCCAA), conserved in both human and murine for complementarity with predicted target mRNAs. Three candidates were identified, which are associated with angiogenesis and retinopathy (15–17): *CXCR4*, *DLL4*, and *FZD4*. All contained potential *miR-150* seed targeting sequences in their 3′ UTRs (Fig. 3*A*). In LCM-isolated pathologic OIR vessels, all three putative targets were significantly up-regulated by ~threefold (*Cxcr4*), 2.5-fold (*Dll4*), or fivefold (*Fzd4*), respectively, compared with the normoxic control vessels (Fig. 3*B*), consistent with decreased *miR-150* levels in OIR vessels (Fig. 1*C*). Moreover, *miR-150* significantly inhibited expression of *CXCR4*, *DLL4*, and *FZD4* in HRMECs, compared with nontargeting mimic control (Fig. 3*C*). Expression levels of other angiogenic factors, *VEGF*, fibroblast growth factor 2 (*FGF2*), and angiogenic receptors including vascular endothelial growth factor receptor 1 (*VEGFR1*), *VEGFR2*, *NOTCH1*, low-density lipoprotein receptor-related protein 5 (*LRP5*), and *LRP6*, were not significantly affected by *miR-150* (*SI Appendix*, Fig. S3). The direct regulation of *miR-150* on its angiogenic targets was further supported by reporter assays. When cotransfected with *miR-150* mimic in HEK293T cells, reporter constructs containing target sequences of *CXCR4*, *DLL4*, or *FZD4* demonstrated significant repression of their luciferase activities in a dose-dependent manner (Fig. 3*D*). Together these results illustrate that *miR-150* is a direct regulator of these angiogenic genes in ECs through targeting their 3′ UTR seed sequences.

Deficiency of *miR-150* Increased Angiogenesis in Aortic Rings and Choroidal Explants ex Vivo. We next investigated the effects of *miR-150* on angiogenesis in ex vivo tissue explants isolated from *miR-150*$^{-/-}$ mice. In an aortic ring angiogenic assay (18), from day 4–8 after aortic ring explanting, there was a significantly larger sprouting area in *miR-150*$^{-/-}$ aortic rings compared with WT controls (Fig. 4*A*, ~threefold at day 8). In the choroid sprouting assay (19), *miR-150*$^{-/-}$ choroid explants showed extensive outgrowth starting from 3 d after culturing and significantly and consistently larger sprouting areas (1.5-fold at day 7) compared with WT controls (Fig. 4*B*). In the presence of VEGF treatment,

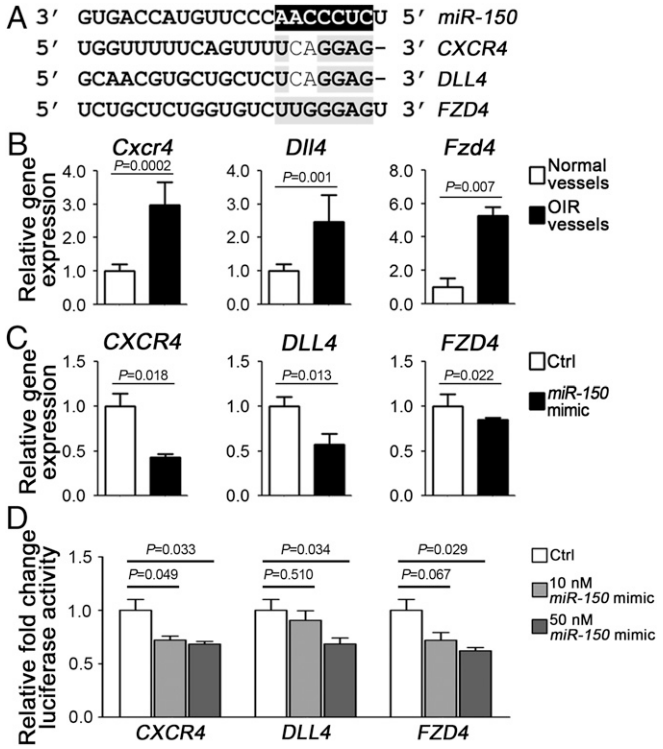

Fig. 3. *MiR-150* directly regulated expression levels of angiogenic target genes *CXCR4*, *DLL4*, and *FZD4* in ECs. (*A*) Alignment of seed sequences from 3′ UTR regions of candidate target genes (*CXCR4*, *DLL4*, and *FZD4*) with mature *miR-150*. (*B*) Expression levels of *Cxcr4*, *Dll4*, and *Fzd4* were significantly up-regulated in pathologic vessels from OIR retinas compared with normal blood vessels isolated from normoxia control retinas ($n = 6$ per group). Expression levels were normalized to cyclophlin A (*CypA*) and relative to normal vessel groups. (*C*) *MiR-150* mimic treatment significantly suppressed expression of *CXCR4*, *DLL4*, and *FZD4* in HRMECs ($n = 3$ per group). Expression levels were normalized to β-actin (*ACTB*) and relative to mimic control (Ctrl). (*D*) Luciferase reporter assays of potential *miR-150* target genes (*CXCR4*, *DLL4*, and *FZD4*) were performed in HEK293T cells. Cells were cotransfected with *miR-150* mimic (or mimic control) and constructs of luciferase reporter containing the *miR-150* target sequence of *CXCR4*, *DLL4*, or *FZD4* (as shown in *A*). Transfection with *miR-150* mimic resulted in significant repression of luciferase activities reflecting complementary binding of seed sequence and suppression of target genes expression ($n = 3$–6 per group). Firefly luciferase activities were normalized to the *Renilla* luciferase and to the levels in the mimic control (Ctrl).

both *miR-150*$^{-/-}$ aortic ring and choroid explants showed exacerbated vascular outgrowth in an additive manner (*SI Appendix*, Fig. S4). These data suggest that lack of endothelial *miR-150* intrinsically promotes angiogenesis ex vivo and the vascular effects of endothelial *miR-150* are additive to external VEGF stimulus.

***MiR-150* Suppressed Pathologic Retinal Angiogenesis in OIR.** Next we examined retinal vasculature in *miR-150*$^{-/-}$ and WT mice. At P7, there is no detectable developmental difference in retinal vascular growth areas between *miR-150*$^{-/-}$ and WT retinas (*SI Appendix*, Fig. S5), suggesting that *miR-150* is likely dispensable for developmental retinal angiogenesis. Because the levels of endogenous *miR-150* in WT OIR retinas were already drastically suppressed (Fig. 1 *B* and *C*), *miR-150*$^{-/-}$ mice showed no significant changes in vaso-obliteration and retinal neovascularization compared with WT mice at P17 (Fig. 5*A*). To determine whether modulation of *miR-150* may suppress pathologic retinal vessel growth, OIR mice were treated with intravitreal injection of *miR-150* mimic at P12 (Fig. 5*B*). At P17, *miR-150* suppressed retinal neovascularization in OIR by ∼50% in WT retinas and by ∼40% in *miR-150*$^{-/-}$ retinas compared with their relative contralateral eyes injected with mimic control (Fig. 5 *C* and *D*). Moreover, expression levels of *Cxcr4*, *Dll4*, and *Fzd4* were significantly suppressed in *miR-150* mimic-treated P17 WT retinas compared with contralateral retinas treated with mimic control (Fig. 5*E*), yet retinal *Vegfa*, *Vegfr1*, and *Vegfr2* levels were not significantly altered (*SI Appendix*, Fig. S6). Together these data indicate that *miR-150* suppressed pathologic neovascularization and the expression of target angiogenic factors in experimental retinopathy.

Deficiency of *miR-150* Increased Laser-Induced CNV. We next evaluated *miR-150*$^{-/-}$ mice in another angiogenic model of laser-induced CNV (20). At day 7 after laser photocoagulation, *miR-150*$^{-/-}$ mice had ∼1.4-fold larger CNV lesions than WT mice (Fig. 6*A*), without significantly impacting the levels of vascular leakage evaluated by fluorescent fundus angiography (Fig. 6*B*). Together, these findings suggested that *miR-150* deficiency resulted in increased pathologic CNV, further supporting an endogenous vaso-inhibitory role of *miR-150*.

Discussion

In this study, we identified an endothelium-specific function of *miR-150* as a key endogenous suppressor of pathological ocular neovascularization, which is supported by observations in several models of angiogenesis. First, in a mouse OIR model, *miR-150* transcript was specifically enriched in normal retinal blood vessels and decreased in pathologic neovessels. Administration of exogenous *miR-150* suppressed pathological neovascularization and its target gene expression in OIR, highlighting a vaso-inhibitory role of vascular-enriched *miR-150*. Second, ex vivo tissue explants from *miR-150*$^{-/-}$ mice showed notably increased sprouting ability compared with WT explants. In the laser-induced CNV model, *miR-150*$^{-/-}$ mice showed significantly larger neovascular lesion sizes. All of these findings support a potent

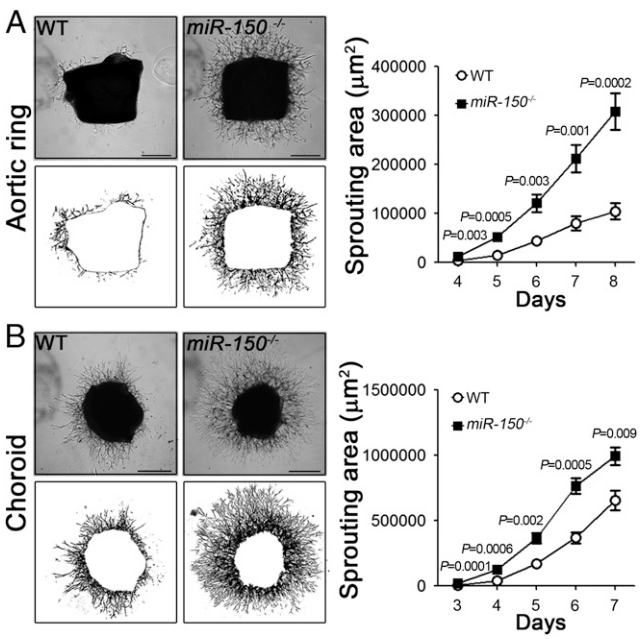

Fig. 4. *MiR-150* deficiency increased vascular growth in aortic ring and choroidal explant ex vivo. (*A*) Aortic rings isolated from 4-wk-old *miR-150*$^{-/-}$ mice showed significantly increased sprouting ability ex vivo compared with those from wild-type (WT) mice ($n = 3$ per group). (*Left* panels) Representative images from day 5 of explants (*Top*) and areas of vascular sprouts quantified (*Bottom*). (*B*) Representative images (*Top* panels) and quantification of microvascular sprouting area (*Bottom* panels) from *miR-150*$^{-/-}$ and WT adult mouse choroid explants. Knockout of *miR-150* results in significantly increased sprouting area ($n = 3$ per group). (Scale bar, 500 μm.)

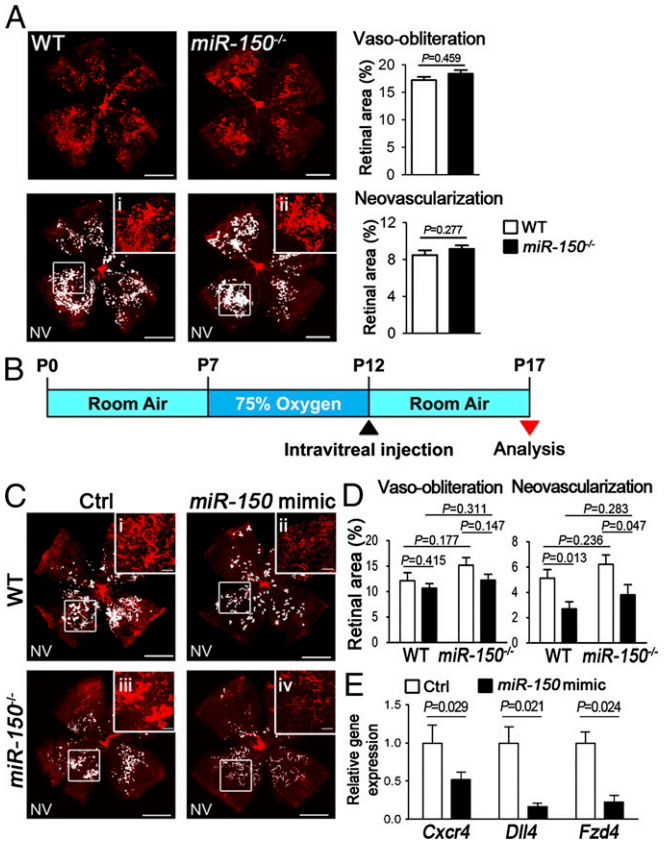

Fig. 5. *MiR-150* suppressed pathologic neovascularization in OIR. (*A*) Retinal vaso-obliteration and neovascularization of WT and *miR-150*$^{-/-}$ retinas in OIR. Retinal flat mounts from P17 WT and *miR-150*$^{-/-}$ mice were stained with isolectin B_4 (red). Pathologic neovascular tufts (NV) were labeled (white highlight), and selected areas were enlarged at high magnification (*i* and *ii*). Quantification of vaso-obliteration and neovascularization showed no significant difference between WT and *miR-150*$^{-/-}$ OIR retinas at P17 (n = 10–18 per group). (*B*) Schematic diagram of intravitreal injection of *miR-150* mimic or mimic control in WT and *miR-150*$^{-/-}$ OIR eyes at P12, with *miR-150* mimic injected in one eye and negative mimic control in the contralateral eye. At P17 in OIR, retinal vasculature was analyzed. (*C*) Representative images of P17 WT and *miR-150*$^{-/-}$ OIR retinas with injection of *miR-150* mimic or mimic control. Pathologic NVs were labeled as white highlight, and *Insets* were the selected areas at high magnification (*i–iv*). (*D*) Quantitative analysis of vaso-obliteration and pathologic neovascularization at P17 in OIR. *MiR-150* mimic treatment significantly reduced pathologic neovascularization in both WT and *miR-150*$^{-/-}$ mice compared with contralateral retinas treated with mimic control, with no significant difference in vaso-obliteration (n = 12 or 13 per group). (*E*) Substantial decrease of *Cxcr4*, *Dll4*, and *Fzd4* expression levels was observed in *miR-150* mimic-treated P17 WT OIR retinas (n = 3 per group). Expression levels were normalized to *CypA* and relative to contralateral eyes injected with negative mimic control. [Scale bars, 1 mm (*A* and *C*) and 200 μm (*C*, *i–iv*).]

antiangiogenic role of endogenous *miR-150*. Finally, exogenous *miR-150* treatment inhibited angiogenic functions specifically in HRMECs and down-regulated multiple angiogenic target genes through direct posttranscriptional control. Collectively, our data suggest that in normal retinal vessels, expression of *miR-150* is abundant and may maintain vascular quiescence through repressing its downstream angiogenic target genes—*CXCR4*, *DLL4*, and *FZD4*. In pathological neovessels, however, substantial down-regulation of the angiogenic suppressor *miR-150* in ECs may lead to up-regulation of downstream angiogenic targets, contributing to formation of pathologic neovascularization. *MiR-150* mimics may compensate for the loss of endogenous *miR-150* in pathologic neovessels and thereby alleviate abnormal angiogenesis in neovascular ocular diseases (Fig. 7). This antiangiogenic role of *miR-150* is potentially relevant for other vascular diseases as well.

Our data localizing *miR-150* in normal blood vessels identified a direct cell-specific role of *miR-150* in suppressing EC functions and expanded the current knowledge of its cellular localization, previously reported in monocytes, and mature lymphocytes (21, 22). Our findings of *miR-150* suppression of vascular EC function is consistent with previous reports of *miR-150* in regulating B-cell proliferation, development, and differentiation (22–24) and EC differentiation and vasculogenesis by targeting *ZEB1* in human embryonic stem cells (25). A previous study identified multiple miRNAs substantially regulated in ischemic mouse retinas (26), yet the cell-specific roles of these miRNAs in the pathogenesis of neovascular eye diseases were not clear. In addition, several highly expressed miRNAs were identified in human ECs, including *miR-126*, *miR-221/222*, *miR-21*, the *let-7* family, the *miR-17~92* cluster, and the *miR-23~24* cluster (27, 28). Our work suggests that *miR-150* is an additional EC-enriched miRNA functioning as a molecular regulatory switch controlling EC quiescence and pathologic proliferation.

The intrinsic vaso-inhibitory role of *miR-150* was highlighted in two in vivo models of pathologic ocular angiogenesis. Although no significant difference was found in neovascularization between WT and *miR-150*$^{-/-}$ OIR mice, this observation is likely attributable to very low levels of *miR-150* levels in WT OIR retinas, resulting in lack of a significant difference between WT and *miR-150*$^{-/-}$ OIR retinas. Importantly, intravitreal injection of *miR-150* significantly suppressed OIR, suggesting a protective role of exogenously applied *miR-150* in inhibiting pathologic retinal angiogenesis. In addition, lack of *miR-150* significantly exacerbated CNV lesions in the laser-induced CNV model and increased sprouting in aortic ring and choroidal explants, further supporting an antiangiogenic effect of *miR-150*. These findings are consistent with another study showing increased lung angiogenesis in *miR-150*$^{-/-}$ mice in a model of oxygen-induced neonatal lung injury (29). Although these data strongly support a primarily direct effect of vascular-enriched *miR-150* on endothelial function and ocular angiogenesis, we cannot rule out potential contributions from circulating *miR-150*, which was found in monocyte-derived microvesicles to regulate target genes in the recipient ECs and affect pathological angiogenesis in tumor and diabetic models (30, 31). In addition, genetic variation in research mouse strains may also contribute to their observed eye phenotype, as was discovered for *rd8* mutation (32), and potentially vascular phenotype. The *miR-150*$^{-/-}$ mice we used do not harbor *rd8* mutation, as validated by

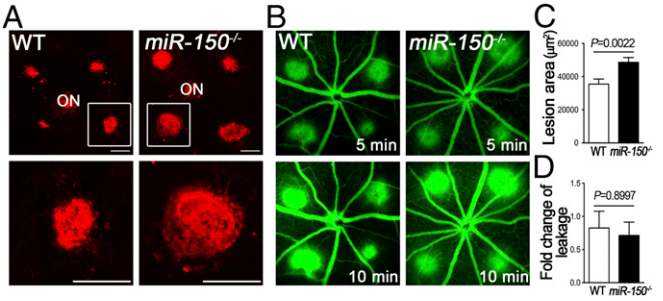

Fig. 6. *MiR-150* deficiency promoted laser-induced CNV. (*A*) Choroidal flat mounts at 7 d after photocoagulation were stained with isolectin B_4 (red) to visualize CNV lesions. (*B*) Fundus fluorescein angiography images from WT and *miR-150*$^{-/-}$ mice taken at 1 wk after laser photocoagulation showed the subretinal CNV lesions and associated vascular leakage at 5 and 10 min. (*C*) Quantitative analysis of lesion size showed that *miR-150* deficiency resulted in significantly increased CNV lesion size 7 d after laser exposure (n = 8–14 per group). (*D*) Quantification analysis revealed no significant difference of the vascular leakage between WT and *miR-150*$^{-/-}$ mice (n = 7–9 per group). ON, optic nerve. (Scale bar, 500 μm.)

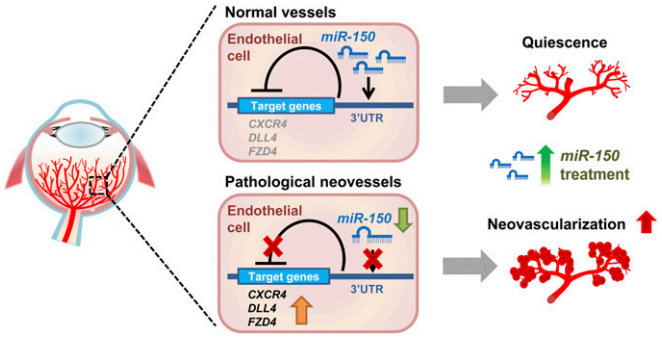

Fig. 7. Schematic illustration of an endothelium-specific inhibitory role of *miR-150* in pathological ocular neovascularization. In normal retinal vessels, endothelial-enriched *miR-150* represses the expression of its downstream angiogenic target genes by binding to their 3′ UTRs to maintain quiescence of retinal vessels. In pathological neovessels, expression of endothelial *miR-150* is decreased, releasing the repression of downstream angiogenic targets (*CXCR4*, *DLL4*, and *FZD4*) and leading to their up-regulation, thereby contributing to formation of pathologic retinal neovascularization. Supplementing *miR-150* may compensate for the loss of *miR-150* in pathologic neovessels and alleviate pathologic neovascularization in retinopathy.

genetic testing using PCR and sequencing (*SI Appendix*, Fig. S7), yet minor unknown background strain differences may exist between the WT (C57BL/6J) and *miR-150*$^{-/-}$ colonies we used in this study.

The deficiency of *miR-150* in pathological OIR neovessels is associated with reciprocal up-regulation of multiple target angiogenic genes that are usually suppressed in quiescent mature vasculature. Indeed, blood vessels isolated from normoxic retinas had high levels of *miR-150* and low expression levels of target angiogenic genes, including *Cxcr4*, *Dll4*, and *Fzd4*, that may act separately through stromal cell-derived factor 1 (SDF-1)/CXCR4, Dll4/Notch, or a Wnt signaling pathway to regulate sprouting angiogenesis. The antiangiogenic effect of *miR-150* in the eye may be explained by its direct suppression of *CXCR4*, *DLL4*, or *FZD4*, as confirmed in EC culture and in OIR retinas. All of these genes are potent proangiogenic genes associated with ocular angiogenesis. FZD4 is a receptor for Wnt signaling, which is a highly conserved angiogenic pathway activated in both OIR and laser-induced CNV models (16, 33, 34). On the other hand, Dll4/Notch signaling controls vessel sprouting and branching, and inhibition of DLL4 showed reduced pathological neovessels in OIR (35). Moreover, CXCR4, a receptor for SDF-1 signaling, showed increased expression in diabetic epiretinal membranes (36), and inhibition of CXCR4 reduces the laser-induced CNV lesion sizes and leakage in rats (37). Together these findings are consistent with our data of *miR-150*, a suppressor of *Fzd4*, *Dll4*, and *Cxcr4*, protecting against OIR. In addition, VEGF, a prominent angiogenic growth factor, was reported as a downstream gene regulated by *miR-150* (38), yet in our study we did not find significant regulation of VEGF and its receptors' (VEGFR1 and VEGFR2) expression in *miR-150* mimic-treated HRMECs (*SI Appendix*, Fig. S3) or *miR-150* mimic-treated OIR retinas (*SI Appendix*, Fig. S6). *Vegfa* expression from isolated retinal pigment epithelium (RPE) was also not significantly affected in *miR-150*$^{-/-}$ mice compared with WT (*SI Appendix*, Fig. S8). Moreover, the vascular effects of *miR-150* appear to be additive to the effects of VEGF treatment on HRMEC proliferation, migration, tube formation (*SI Appendix*, Fig. S2), as well as aortic ring and choroidal explant sprouting (*SI Appendix*, Fig. S4). Together these data suggest that endothelial *miR-150* acts in a VEGF-independent manner in our study, although *miR-150* may suppress *Vegfa* expression in other VEGF-enriched cell types in a tissue- and cell-specific context. Although the upstream regulators of *miR-150* deficiency in OIR have yet to be identified, one possible regulator is local tissue hypoxia, which may regulate a subset of miRNAs—namely, hypoxamiRs—to influence the posttranscriptional control in hypoxia-controlled cell proliferation, apoptosis, differentiation, and angiogenesis (39–41). Negative regulation of *miR-150* by hypoxia-inducible factor (HIF) was reported during hypoxia in liver regeneration (42).

Recent advances in miRNA research have identified many miRNAs expressed in the eye and during retinal angiogenesis as well as regulated in pathologic neovascularization (43). Targeting multiple angiogenic genes using miRNAs may offer a complementary approach for vascular eye disease management and treatment, in addition to current ablative surgeries and pharmacologic interventions, and may be additive to anti-VEGF therapies. As current ongoing trials testing the miRNA approach in other diseases such as cancers are promising (44), the translational prospects of modulation miRNAs including *miR-150* in vascular eye diseases are encouraging.

In summary, our study presents direct evidence suggesting that *miR-150* is an intrinsic suppressor of pathologic ocular angiogenesis in ECs. *MiR-150* was expressed abundantly in quiescent ECs and can suppress abnormal endothelial activation through targeting multiple angiogenic signaling pathways specifically in the endothelium. On the other hand, suppression of endogenous *miR-150* in pathologic neovessels may induce endothelial activation to trigger pathological angiogenesis. Our findings of *miR-150* as an endothelium-specific intrinsic inhibitor of pathologic ocular angiogenesis suggest the potential of modulating *miR-150* as a therapeutic intervention for the treatment of neovascular eye diseases and potentially other vascular diseases.

Materials and Methods

Animals. Animal studies were approved by the Boston Children's Hospital Animal Care and Use Committee and adhered to the Association for Research in Vision and Ophthalmology *Statement for the Use of Animals in Ophthalmic and Vision Research* (45). *MiR-150* knockout mice [B6(C)-*Mir150*^{tm1Rsky}/J, stock no. 007750] and C57BL/6J mice (stock no. 000664) were obtained from Jackson Laboratory. Age-matched C57BL/6J mice were used as wild-type controls for comparison with the *miR-150* knockout mice according to vendor instruction.

OIR Model and Vessel Quantification. OIR was generated as previously described (46, 47). Neonatal mice with their nursing mother were exposed to 75% oxygen from P7–12 to induce retinopathy and killed at P17, followed by retinal dissection and staining overnight with fluorescent *Griffonia simplicifolia* isolectin B_4 (Invitrogen). Vaso-obliteration and neovascularization in OIR were quantified by using Adobe Photoshop and ImageJ (48). Quantification was performed with the identity of the samples masked, with n being the number of mice quantified.

LCM. LCM was performed on OIR or normoxia P17 wide-type frozen retinal sections that were dehydrated and stained with isolectin B_4 (33). Retinal layers were laser-capture microdissected with the Leica LMD 6000 system (Leica Microsystems) and collected directly into lysis buffer from the RNeasy Micro kit.

Intravitreal Injection. Intravitreal injections were performed following established protocols (49, 50). We injected 1 μg of *miR-150* mimics (Ambion) using a 33-gauge needle behind the limbus of the eye, and the contralateral eye of the same animal was injected with an equal amount of negative control mimics.

Laser-Induced CNV. Laser photocoagulation was carried out as described previously (18, 51) in 6–8-wk-old *miR-150*$^{-/-}$ and WT mice with laser-induced CNV analyzed 1 wk postlaser. Additional information regarding laser coagulation and lesion analysis is provided in *SI Appendix*.

Cell Culture and Assays. HRMECs and human embryonic kidney 293T cells (HEK293T; ATCC) were cultured for endothelial functional assays (migration, proliferation, and tube formation) and luciferase assays, respectively. FAM-labeled premiRNA (Ambion) was used to analyze transfection efficiency of miRNAs (52) and showed around 90% transfection efficiency in HRMECs (*SI Appendix*, Fig. S9). Detailed culture and assay conditions are detailed in *SI Appendix*.

Aortic Ring and Chroidal Sprouting Assays. Aortic ring and choroidal sprouting assays were performed as previously described (18). Fragments of aortae or RPE/choroid were isolated from 4-wk-old *miR-150*$^{-/-}$ and WT mice and cultured in Matrigel, imaged daily, and analyzed for vascular growth.

RNA Isolation and qPCR. Total RNA was extracted from mouse retinas or from HRMEC culture with RNeasy Kit (Qiagen). MiRNA was isolated from retinas or LCM isolated vessels with the miRNeasy Micro Kit (Qiagen). Synthesis of cDNA and qPCR were performed using established protocols as detailed in *SI Appendix*.

Statistical Analysis. Results were presented as mean ± SEM for animal studies and nonanimal studies. *P* values less than 0.05 were considered significant, according to two-tailed Student's *t* test (two groups) or ANOVA (more than two groups).

Additional detailed methods are available in *SI Appendix*.

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