Therapeutic Effect of Novel Single-Stranded RNAi Agent Targeting Periostin in Eyes with Retinal Neovascularization

Takahito Nakama,1 Shigeo Yoshida,1 Keijiros Ishikawa,1 Yuki Kubo,1 Yoshiyuki Kobayashi,1 Yedi Zhou,1 Shintaro Nakao,1 Toshibo Hisatomi,1 Yasuhiro Ikeda,1 Kazunasa Takao,2 Kazunori Yoshikawa,2 Akira Matsuda,3 Junya Ono,4 Shoichihiro Ohta,5 Kenji Izuhara,5 Akira Kudo,6 Koh-hei Sonoda,1 and Tatsuro Ishibashi1

1Department of Ophthalmology, Kyushu University Graduate School of Medical Sciences, Fukuoka 812-8582, Japan; 2AQUA Therapeutics Co., Ltd., Kobe 650-0035, Japan; 3Department of Ophthalmology, Juntendo University School of Medicine, Tokyo 113-8421, Japan; 4Shino-test Corporation, Sagamihara 252-0331, Japan; 5Division of Medical Biochemistry, Department of Biomolecular Sciences, Saga Medical School, Saga 849-8501, Japan; 6Department of Biological Information, Tokyo Institute of Technology, Yokohama 226-8501, Japan

Retinal neovascularization (NV) due to retinal ischemia remains one of the principal causes of vision impairment in patients with ischemic retinal diseases. We recently reported that periostin (POSTN) may play a role in the development of preretinal fibrovascular membranes, but its role in retinal NV has not been determined. The purpose of this study was to examine the expression of POSTN in the ischemic retinas of a mouse model of oxygen-induced retinal NV. We also studied the function of POSTN on retinal NV using Postn KO mice and human retinal endothelial cells (HRECs) in culture. In addition, we used a novel RNAi agent, NK0144, which targets POSTN to determine its effect on the development of retinal NV. Our results showed that the expression of POSTN was increased in the vascular endothelial cells, pericytes, and M2 macrophages in ischemic retinas. POSTN promoted the ischemia-induced retinal NV by Akt phosphorylation through integrin αvβ3. NK0144 had a greater inhibitory effect than canonical double-stranded siRNA on preretinal pathological NV in vivo and in vitro. These findings suggest a causal relationship between POSTN and retinal NV and indicate a potential therapeutic role of intravitreal injection of NK0144 for retinal neovascular diseases.

INTRODUCTION

Retinal neovascularization (NV) due to retinal ischemia is one of the principal causes of vision impairment in patients with ischemic retinal diseases such as proliferative diabetic retinopathy (PDR), retinal vein occlusion, and retinopathy of prematurity. Retinal NV can result in macular edema, vitreous hemorrhage, and traction retinal detachment, which are the causes of the vision reduction. Despite recent progress in pharmacological therapies and surgical techniques, there are still cases of treatment-resistant retinal NV.

Retinal NV is an example of excessive angiogenesis that is characterized by proliferation, migration, and tube formation of the retinal vascular endothelial cells. In this process, the extracellular matrix (ECM) components play a critical role in regulating retinal NV. Previous studies have shown that several growth factors, including the vascular endothelial growth factors (VEGFs), platelet-derived growth factors, transforming growth factor βs, and placenta growth factor, are involved in this process. Among these factors, the VEGFs have been well characterized and are known to play a causal role in retinal NV. Although anti-VEGF therapies have been shown to inhibit the progression of retinal NV to some degree, it was recently reported that the intravitreal injection of anti-VEGF antibodies can cause retinal damage and might promote retinal fibrosis. Therefore, retinal NV still remains a vision-threatening pathological condition.

Several laboratories including ours have demonstrated a higher expression of periostin (POSTN) in the fibrovascular membranes (FVMs) of patients with PDR than in those of patients with normal retinas by comprehensive gene and protein expression profiling. We have also detected increased levels of POSTN protein in the vitreous of patients with PDR. POSTN is a 90-kDa matricellular protein belonging to the fasciclin family that interacts not only with other ECMs but also with the integrins, such as αvβ3 and αvβ5, as a ligand. POSTN is associated with tissue development and remodeling through these interactions. POSTN has been reported to promote angiogenesis in an ischemic limb, in the choroid, in cancerous tissues, in keloids, and in cardiac valve degeneration. These findings suggest that POSTN may play a role in the development of pathological retinal NV. However, whether POSTN is involved in retinal NV has not been examined.

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Correspondence: Shigeo Yoshida, Department of Ophthalmology, Kyushu University Graduate School of Medical Sciences, Fukuoka 812-8582, Japan.
E-mail: yoshida@eye.med.kyushu-u.ac.jp
The silencing of post-transcriptional genes by RNAi is an excellent method of inhibiting gene expressions because of its high selectivity and potency, which are advantages over conventional therapies using antibodies and small molecules.21 Moreover, RNAi agents have other advantages due to their easy synthesis and rapid identification and optimization. However, previous investigations have shown that canonical double-stranded small interfering RNAs (siRNAs) have several problems for their use, including the need of a drug delivery system (DDS), low biological stability, off-target gene silencing, and immunostimulatory effects through the activation of Toll-like receptor 3 (TLR3).22–25 We used a novel single-stranded RNAi agent that can overcome these obstacles, and we found that the naked single-stranded RNAi agent had an inhibitory effect on choroidal FVM formation with good stability, no sequence-independent choroidal neovascularization (CNV) suppression through TLR3, and no serious toxicity.17 Based on these findings, we hypothesized that POSTN is involved in retinal NV and can be used as a therapeutic target. To test this hypothesis, we investigated the role played by POSTN in retinal NV using a mouse model of oxygen-induced retinopathy (OIR) in vivo and human retinal microvascular endothelial cells (HRECs) in vitro. We also studied the therapeutic effect of the single-stranded RNAi agent targeting POSTN in retinal NV.

RESULTS
Expression and Localization of POSTN in OIR Retina
To determine whether POSTN is involved in retinal NV, the expression of the mRNA of Postn in OIR retinas was determined by real-time RT-PCR at several time points. The expression of the mRNA of Postn in the OIR retinas was significantly upregulated compared to that in control mice retinas and reached a peak on postnatal day 17 (P17) when retinal NV reaches its peak (p < 0.05, n = 4; Figure 1A).

To confirm the results of real-time RT-PCR, we performed ELISA and determined the protein concentration of POSTN. ELISA showed that the level of POSTN protein in the OIR retinas was significantly higher at P17 than that in the control group (p < 0.001; n = 4; Figure 1B).

Next, we stained retinal sections and retinal flat-mounts with antibodies to determine the location of the POSTN in the OIR retinas. Immunohistochemical analyses of retinal sections showed that POSTN-positive cells were co-stained with both CD31 and α-smooth muscle actin (αSMA) in the preretinal pathological NVs. In the retinal flat-mounts, POSTN co-stained with F4/80. In addition, POSTN co-stained the preretinal pathological NVs with CD206.
KO mice than in WT mice (p < 0.01, n = 6; Figures 2A and 2B). The mean avascular area was significantly larger in the OIR retinas of POSTN KO mice than that in WT mice (p < 0.01, n = 6; Figures 2A and 2B). These results indicated that POSTN promotes both preretinal pathological NV and physiological revascularization in OIR retinas.

**Attenuation of Ischemia-Induced Retinal Angiogenesis in OIR Retinas of Postn Knockout Mice**

To investigate whether POSTN alters the ischemia-induced retinal NV, we quantified the size of the neovascular tufts and avascular areas in the OIR retinas of wild-type (WT) mice and Postn knockout (KO) mice stained with isolecic B4 at P17. In the OIR retinas, the neovascular tufts indicate preretinal pathological NV, whereas the avascular areas represent the physiological revascularization.26 The size of the neovascular tufts was significantly smaller in the OIR retinas of Postn KO mice than in WT mice (p < 0.01, n = 6; Figures 2A and 2B). The mean avascular area was significantly larger in Postn KO mice than that in WT mice (p < 0.01, n = 6; Figures 2A and 2B). These results indicated that POSTN promotes both preretinal pathological NV and physiological revascularization in OIR retinas.

**In Vitro Effects of POSTN on Proliferation, Migration, and Tube Formation of HRECs**

To test the functional role of POSTN in retinal NV, we examined the effect of POSTN on proliferation, migration, and tube formation of HRECs, which are key steps of angiogenesis. POSTN promotes the proliferation of HRECs in a dose-dependent manner as determined by bromodeoxyuridine (BrdU) incorporation, and the difference of proliferation between the 100 ng/mL POSTN group and the control group was significant (p < 0.05, n = 6; Figure 3A).

The migration of HRECs examined in Boyden chambers was also significantly promoted by POSTN (p < 0.0001, n = 12; Figure 3B). Furthermore, POSTN significantly increased the tube formation of HRECs in a dose-dependent manner (p < 0.0001, n = 12; Figure 3C). These results indicated a causal role of POSTN in retinal NV.

**Signaling Pathway for Retinal NV Promoted by POSTN**

We next investigated the signaling pathway for the retinal NV regulated by POSTN. Because POSTN can bind to integrin 33 or vβ3 as a ligand, and because FAK/Akt phosphorylation is involved in cell proliferation, migration, and tube formation through integrin 33 or vβ3, we examined the effect of POSTN on FAK/Akt phosphorylation in HRECs. FAK-Tyr397 and Akt-Ser473 phosphorylation was increased at 15 min after POSTN stimulation (Figures 4A and S1A). In addition, we observed the integrin-linked kinase 1 (ILK1) phosphorylation, which is a key mediator between ECMs and integrins. ILK1 phosphorylation was also increased 15 min after POSTN stimulation (Figure 4A). To confirm that the FAK/Akt phosphorylation induced by POSTN was mediated through integrin 33 or vβ3, we used antibodies against integrin 33 or vβ3 in HRECs 30 min before the POSTN stimulation. Inhibition of integrin 33 reduced the FAK/Akt phosphorylation, whereas inhibition of integrin vβ3 had no effect on the Akt phosphorylation (Figures 4B and S1B). These results indicated that POSTN stimulated FAK/Akt phosphorylation through integrin vβ3 and ILK1 in HRECs.

To confirm that the FAK/Akt phosphorylation enhanced by POSTN through integrin vβ3 is involved in key steps of angiogenesis, we inhibited integrin vβ3 and Akt by the antibody (Ab) and the inhibitor (LY294002). The inhibition of integrin vβ3 and Akt significantly suppressed the POSTN-induced migration (p < 0.0001, n = 12; Figure 4C) and the tube formation of HRECs (p < 0.01 or 0.0001, n = 4; Figure 4D). These results demonstrated that POSTN stimulated Akt phosphorylation through integrin vβ3, which then resulted in the promotion of migration and tube formation of HRECs.

**In Vitro NK0144-Mediated Inhibition of POSTN Expression in HRECs**

A novel single-stranded RNAi agent targeting POSTN (NK0144) was used as described in Materials and Methods. The difference in the
structure of canonical double-stranded siRNA (NI0079) and NK0144 is shown in Figure 5A.

First, we tested the effects of inflammatory cytokines related to PDR on the synthesis of POSTN by HRECs. Interleukin-6 (IL-6), IL-8, IL-13, MCP-1, and VEGF were used for stimulation.27,28 The expression of POSTN in HRECs was significantly increased in a dose-dependent manner only by IL-13 (p < 0.01 or 0.0001, n = 4; Figure 5B). IL-13 was also expressed by CD4-positive cells in OIR retinas (Figure 5C). HRECs exposed to the other cytokines did not show a significant induction in the expression of POSTN (data not shown).

Next, the knockdown effect of NK0144 on the expression of the mRNA of POSTN in HRECs was examined. Real-time RT-PCR showed that the expression of POSTN mRNA in HRECs induced by IL-13 was significantly decreased following transfection with 10 nM NK0144 (p < 0.01 or 0.0001, n = 4; Figure 5D). Transfection with 10 nM of single-stranded scramble RNAi agent (NK0000) as a negative control RNAi agent had no significant inhibitory effect on the expression of POSTN mRNA.

To confirm the results of real-time RT-PCR, we performed ELISA to examine whether the protein level of POSTN was also decreased in the supernatant after transfection with NK0144. ELISA showed that the level of POSTN protein in the supernatant from HRECs transfected with NK0144 was significantly decreased (222.4 ± 14.0 pg/mL, p < 0.01; n = 4) compared with that in the control group (554.0 ± 77.9 pg/mL; n = 4; Figure 5D). These results indicated that NK0144 can inhibit the expression of POSTN from vascular endothelial cells induced by IL-13.

In Vitro Inhibitory Effect of NK0144 on Migration and Tube Formation of HRECs

We next investigated whether NK0144 affected the migration and tube formation of HRECs facilitated by IL-13. The migration of HRECs induced by 50 ng/mL IL-13 was significantly inhibited by 10 nM NK0144 transfection (p < 0.0001, n = 18; Figure 6A) compared to that with 10 nM NK0000 transfection. The tube formation of HRECs was also significantly reduced by 10 nM NK0144 transfection (p < 0.01, n = 6; Figure 6B). These results indicated that NK0144 can inhibit the angiogenesis of HREC in vitro.

In Vivo NK0144-Mediated Inhibition of Ischemia-Induced Retinal Angiogenesis in OIR Retinas

To determine whether NK0144 also inhibits the ischemia-induced retinal NV in vivo, NK0144 was injected intravitreally in OIR mice.
Figure 4. Signaling Pathway in Retinal NV Promoted by POSTN

HRECs were stimulated with POSTN and treated with or without control IgG, integrin αvβ3 Ab, αvβ5 Ab, or LY294002 (Akt inhibitor). (A) The phosphorylation of FAK-Tyr397, Akt-Ser473, ILK1 was increased at 15 min after POSTN stimulation. (B) Inhibition of integrin αvβ3 effectively reduced the FAK/Akt phosphorylation promoted by POSTN, whereas inhibition of integrin αvβ5 had no influence on the Akt phosphorylation. (C) The inhibition of integrin αvβ3 and Akt significantly suppressed the POSTN-induced migration (n = 12/group). *p < 0.0001. (D) Tube formation of HRECs was significantly inhibited by integrin αvβ3 Ab and Akt inhibitor (n = 4/group). *p < 0.0001, **p < 0.01, ***p < 0.05. Error bars are SEM.
at P12. At P17, the eyes that received an intravitreal injection of 1 μL of 10 μM NK0144 had smaller preretinal pathological NV area than eyes after 1 μL of 10 μM NK0000 injections (p < 0.001, n = 4; Figures 7A and 7B). In addition, the inhibitory effect of NK0144 on preretinal pathological NV was significantly greater than that of the canonical double-stranded siRNA (p < 0.05, n = 4; Figures 7A and 7B).

We further investigated the effect of NK0144 on the size of the avascular area. At P17, there was no significant difference in the avascular area between the NK0000 treatment group and the NK0144 treatment group, whereas the difference between the novel single-stranded RNAi agents (NK0000 and NK0144) treatment groups and canonical double-stranded siRNAs (NI0079 and NI0000) treatment groups was significant (p < 0.05, n = 4; Figures 7A and 7B). These results indicated that the single-stranded RNAi agent (NK0144) had a greater inhibitory effect on preretinal pathological NV than the canonical double-stranded siRNA, whereas the inhibitory effect of NK0144 on physiological revascularization was less than that of canonical double-stranded siRNA.

**DISCUSSION**

The results of this study showed the functional role of POSTN in the pathogenesis of ischemia-induced retinal NV, and the possible therapeutic effect of a novel single-stranded RNAi agent targeting POSTN in retinal NV. We showed that POSTN promoted Akt phosphorylation through integrin αvβ3, which can promote retinal NV. We also demonstrated that a novel single-stranded RNAi agent targeting POSTN (NK0144) can inhibit retinal NV more than a canonical double-stranded siRNA targeting POSTN (NI0079) both in vivo and in vitro. Our studies thus raise the possibility that this single-stranded RNAi agent targeting POSTN can be considered as a potential therapeutic agent for pathological retinal NV.

Our results demonstrated that POSTN was expressed in vascular endothelial cells during ischemia-induced preretinal pathological NV. In addition, IL-13 stimulation enhanced the expression of POSTN in the vascular endothelial cells. These results are in line with earlier reports showing that IL-13 induced POSTN production by human microvascular endothelial cells from lung and skin blood vessels. Our study, IL-13 was increased in CD4-positive cells in OIR retinas compared with normal retinas. IL-13 is a Th2 cytokine and has angiogenic activities. A previous study has shown that Th2 cells are one of the cellular components in FVMs of PDR patients. We have also reported an increase of the IL-13 level in the vitreous of patients with PDR. Based on these findings, the IL-13 produced by CD4-positive Th2 cells may be the inducer of POSTN expression.
of POSTN secreted by the retinal vascular endothelial cells in ischemic retinas.

Our results showed that POSTN also co-stained with markers of pericytes and M2 macrophages in ischemic retinas. Earlier studies including ours have reported that zSMA-positive cells can secrete POSTN.13,31,32 In addition, our earlier studies have shown a strong positive correlation between M2 macrophage markers and POSTN in the vitreous of PDR patients.33 We have subsequently shown that macrophage polarization toward M2 phenotype by IL-13 resulted in the production of POSTN.28 Thus, zSMA-positive pericytes and M2 macrophages polarized by IL-13 may also produce POSTN in ischemic retinas.

Our data demonstrated that genetic ablation of POSTN resulted in a reduction of both preretinal pathological NV and physiological revascularization. Additionally, we observed the promotion effects of POSTN on the proliferation, migration, and tube formation of retinal vascular endothelial cells and Akt phosphorylation through integrin αvβ3. These findings are consistent with our previous reports showing that POSTN promotes CNV.17 In addition, several reports have shown that POSTN can promote NV through integrin αvβ3 in ischemic limbs, cancerous cells, and keloid cells.16,18,19 Furthermore, the expression of integrin αvβ3 on the vascular cells in tissues from patients with PDR has been reported.34 Previous papers including ours also showed the FAK/Akt phosphorylation through integrin αv by POSTN in endothelial cells and epithelial cells.31,35 Our laboratory has also shown that M2 macrophages enhance preretinal pathological NV in OIR retinas.36 Thus, an elevated expression of POSTN by retinal vascular endothelial cells, pericytes, and M2 macrophages may promote the development of ischemia-induced retinal NV mediated by Akt phosphorylation through integrin αvβ3 in both an autocrine and paracrine fashion.

Although recent studies have shown the attractive and promising aspects of canonical double-stranded siRNAs as a new therapy for retinal NV, some hurdles still remain to be overcome before their clinical application.37 The obstacles are the lack of a safe DDS, adverse off-target effects through TLR3 activation, and the lack of stability. We used a novel single-stranded RNAi agent that self-anneals into a unique structure containing a canonical double-stranded RNA to overcome these obstacles. Our results demonstrated that this single-stranded RNAi agent targeting POSTN can significantly inhibit the preretinal pathological NV following an intravitreal injection without any DDS. Moreover, the inhibitory effect of the single-stranded RNAi agent was greater than the canonical double-stranded siRNA, whereas treatment with the single-stranded RNAi agent led to a significant decrease in the avascular areas in OIR retinas compared to treatment with canonical double-stranded siRNAs. Furthermore, the sequence used for POSTN knockdown is present in not only human POSTN but also in mouse, rat, rabbit, and rhesus macaque POSTN.17 This indicates that NK0144 can be used for both in vitro and in vivo experiments and would also be suitable for future human clinical trials. Previous studies including ours have shown that, compared with canonical double-stranded siRNA, the single-stranded RNAi agent has a greater effect without target sequence-independent NV suppression through TLR3 activation.17,38–40 The mechanisms causing the differences of the effect on ischemia-induced retinal NV between the single-stranded RNAi agent and the canonical double-stranded siRNA were not completely determined. However, we suggest that these are because the single-stranded RNAi agent has better stability against nuclease, no off-target gene silencing, and no immunostimulatory effects through TLR3 activation.17,38–40 Thus, intravitreal injection of naked single-stranded RNAi agent targeting POSTN may be a safer and a more efficient therapeutic method of inhibiting preretinal pathological NV.
Although anti-VEGF therapy for PDR is now a mainstream therapy to prevent retinal NV, it was recently reported that anti-VEGF therapy might be associated with impairment of the function of normal retina and the maintenance of the choriocapillaris. This is partly because VEGF plays an important role in retinal homeostasis. Thus, therapies that block VEGF to inhibit pathological NV could result in unexpected complications of the normal retina and should be used cautiously. In contrast to VEGF, we have reported that POSTN was barely detectable in the normal retina. We also reported that the correlation between the vitreous concentration of POSTN and VEGF was weak in patients with PDR. In addition, previous studies have shown that the binding of VEGF with VEGFR2 promoted angiogenesis mainly through the PLCγ/PKC/MAPK pathway, whereas the binding of POSTN with integrin αvβ3 promotes angiogenesis mainly through the FAK/Akt pathway. This is good evidence of the concept that anti-POSTN therapy might have independent effect on retinal NV from anti-VEGF therapy. Together with the results of anti-POSTN therapy, POSTN might be an interesting therapeutic target to regulate “disease-specific” pathways involved in the development of retinal NV while minimizing the unfavorable side effects on the normal retina.

In conclusion, our results show a causal link between POSTN and retinal NV, and the effects of a naked, unmodified single-stranded RNAi agent targeting POSTN. Although additional preclinical studies on the toxicity, stability, and effect on duration are underway, a POSTN-targeting RNAi agent may be a new therapeutic agent against preretinal pathological NV.

Figure 7. In Vivo NK0144-Mediated Inhibition of Ischemia-Induced Retinal NV in OIR Retinas
Each RNAi agent was intravitreally injected at P12 soon after returning to room air. (A) Representative images of the neovascular tufts and avascular areas at P17 in each treatment group. (B) The percentage of the neovascular tufts in NK0144 treatment group was significantly smaller than that of scramble RNAi agents. The inhibitory effect of NK0144 on preretinal pathological NV is significantly greater than that of N0000. The avascular area of novel single-stranded RNAi agents (NK0000 and NK0144) treatment group was significantly smaller than canonical double-stranded siRNAs (N0000 and N0079) treatment group (n = 4/group). *p < 0.001, **p < 0.05. Error bars are SEM.

MATERIALS AND METHODS

Animals
All animal experiments were 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. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Kyushu University.

WT C57BL/6J mice (CLEA) and Postn KO mice were used for the animal experiments. PCR was used to determine the genotype of the experimental mice as described in detail.

Mouse Model of OIR
The OIR mice were generated as described in detail. Briefly, pups were exposed to 75 ± 2% oxygen from P7 to P12 and then returned to room air. Mice in the control group were kept in room air continuously.

qRT-PCR
Total RNAs were extracted from homogenized retinas at the selected time points using a MagDEA RNA kit (Precision System Science) according to the manufacturer’s protocol. Total RNAs were also extracted from HRECs treated with RNAi agents. cDNAs were synthesized by reverse transcription with a First Strand cDNA Synthesis Kit (Roche) following the quantification of the RNAs concentration. qRT-PCR was performed and analyzed using a LightCycler 96 PCR system (Roche) and a SYBR Premix Ex Taq (Takara). The primer sequences were as follows: for mouse Postn, 5'-CTTTCGAGAAACTGCCACGAG-3' and 5'-CCTTCCATGGTCTCAAACG-3'; for mouse β-actin, 5'-GATGACCCAGATCATGTTTGA-3' and 5'-GGAGAGCATAGCCCTCGTAG-3'; and for human POSTN, 5'-TGCAGCTTTGCCTCAAACG-3' and 5'-CTTCTACAAATGTGCGTG-3'.
The quality and specificity of the PCR were determined by melting curves, and the relative expression levels by standard curves.

**ELISA**

Total protein was isolated from sonicated retinas using Tissue Protein Extraction Reagent with protease inhibitor (T-PER; Thermo). The concentrations of POSTN in the mouse retinas and the supernatants from the cultured HRECs were measured with a mouse POSTN immunoassay kit (R&D Systems) and human POSTN ELISA according to the manufacturer’s instructions.

**Immunohistochemistry**

Eyes enucleated from OIR mice were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and cut at 3-μm thickness. After deparaffinization, rehydration, antigen retrieval by citric acid, and blocking with 5% skim milk, the sections were incubated with the primary antibodies overnight at 4°C, and the secondary antibodies were added for 1 hr at room temperature. Nuclei were counterstained with Hoechst 33342 (Molecular Probes). After washing with PBS, the slides were coverslipped with an aqueous mounting medium (Thermo). A fluorescent microscope (BZ-9000; Keyence) was used to examine and analyze the slides.

The primary antibodies were POSTN (MAB 3548: 5 μg/mL; R&D Systems), CD31 (550274: 1:50 dilution; BD Biosciences), αSMA (F3777: 1:250 dilution; Sigma-Aldrich), F4/80 (MCA497: 1:100 dilution; Biolegend), IL-13 (F3777: 1:250 dilution; Sigma-Aldrich), F4/80 (MCA497: 1:100 dilution; R&D Systems). The secondary antibodies were Alexa Fluor 488 and 647 (ab106732: 1:100 dilution; Abcam), and CD4 (sc-1140: 1:100 dilution; AbD Serotec), CD206 (1:100 dilution; Biolegend), IL-13 (F3777: 1:250 dilution; Sigma-Aldrich), F4/80 (MCA497: 1:100 dilution; R&D Systems) containing 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO₂. Cultured HRECs with normal morphology at the fifth to seventh passages were used for these experiments.

**Quantification of Neovascular Tufts and Avascular Areas**

Both preretinal pathologic NV and physiological revascularization in the OIR retinas were quantified at P17 as described in detail.26 Briefly, after fixation in 4% PFA, the neurosensory retinas were isolated from the eye cups. The retinas were washed with PBS and placed in 50% and 100% methanol for 10 min each at room temperature. The retinas were then blocked by PBS containing 1% BSA and 0.5% Triton X-100 for 1 hr at room temperature. This was followed by incubation with fluorescein-labeled isoelectin B4 (FL1201; 1:200 dilution; Vector Laboratories) overnight at 4°C. The retinal flat mounts were mounted with mounting medium and were examined with a fluorescent microscope (BZ-9000; Keyence), and each area was quantified with Adobe Photoshop CS6.

**Cell Proliferation Assay**

Starved HRECs were seeded in each well of 96-well plates at 1 × 10⁴ cells and incubated with recombinant POSTN. After 48 hr, the degree of proliferation was assessed using BrdU ELISA (Roche) according to the manufacturer’s instructions.

**Cell Migration Assay**

Migration of HRECs was determined using a modified Boyden chamber containing polycarbonate membranes (Transwell, 8-μm pore size; Corning) coated with 10 μg/mL of collagen for 1 hr at 37°C. Starved HRECs were seeded into the upper chamber at 2 × 10⁴ cells/insert in medium containing recombinant POSTN (R&D Systems) or recombinant IL-13 (50 ng/mL; R&D Systems). The lower chamber was filled with medium without serum and growth factors. After 16 hr of incubation at 37°C, non-migrated cells on the upper surface of the membrane were removed by gentle scraping with cotton swabs, and the migrated cells on the lower surface of the membrane were stained with Hoechst 33342 (Molecular Probes). Three photographs were taken of each insert at randomly selected sites with a fluorescence microscope (BZ-9000; Keyence). The number of migrated cells was counted using Adobe Photoshop CS6.

**Tube Formation Assay**

Tube formation assay was performed as described in detail.36 In brief, starved HRECs were suspended in 96-well plate coated with growth factor-reduced Matrigel matrix (BD Biosciences) at 2 × 10⁴ cells. The cells were cultured with recombinant POSTN or recombinant IL-13 (50 ng/mL). After culturing for 24 hr, the cells were photographed with a phase contrast microscope (Olympus CK2; Olympus). The images were analyzed using the Angiogenesis Analyzer toolset for NIH ImageJ software.

**Western Blot Analysis**

HRECs were seeded in collagen-coated six-well plates. After starvation with serum-free medium for 24 hr, the cells were cultured with POSTN following treatment with control IgG, integrin αβ3 Ab, integrin αβ5 Ab, or LY294020 for 30 min. Total cell lysates of HRECs were extracted using lysis buffer with protease inhibitor and phosphatase inhibitor (Thermo). The extracted cell lysates were added to 4%–12% SDS-NuPAGE, and the blots were incubated with antibodies against phosphorylated FAK (Tyr397, 3283: 1:1,000 dilution; Cell Signaling Technology), FAK (3285: 1:1,000 dilution; Cell Signaling Technology), phosphorylated Akt (Ser473, 4606: 1:2,000 dilution; Cell Signaling Technology), Akt (4691: 1:1,000 dilution; Cell Signaling Technology), or ILK1 (3862: 1:1,000 dilution; Cell Signaling Technology). The signals were made visible with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo) detection system. Differences in lane loading were determined by blotting the membranes with an Ab against β-actin (4970: 1:1,000; Cell Signaling Technology).

**RNAi Agent Targeting POSTN**

The novel single-stranded RNAi agent (NK) and the canonical double-stranded siRNA (NI) targeting POSTN that were used were as
follows: canonical double-stranded siRNA (N0079), sense 5'-GCA
CCAAAAAGAAAUACUUTT-3', antisense 5'-AAGUAUUUUCUU
UUUGUGGCTT-3'; canonical double-stranded scramble siRNA (N0000), sense 5'-UACAUUUUGCACGGAAGTT-3', antisense
5'-CUUCGGGUCUGAAUAUGATT-3'; novel single-stranded RNAi
agent (NK0144), 5'-AGCACACAAAAAGAAAUACUUUUCCCCAC
ACC GGAAAGAUAUUUCUUUUUGGCUGCUUUCCGGG-3', novel
single-stranded scramble RNAi agent (NK0000), 5'-AUACAUUUGC
ACCGGAAGUUCCCCACACCGGAACUUCGCGUGUCGAAUA
GUAUUUCCGG-3'.

The sequence for the POSTN knockdown was designed to target the
mRNA of both human and mouse POSTN. The novel single-stranded
RNAi agent was constructed by incorporating the sense and antisense
nucleotides of the canonical double-stranded siRNA into the scaffold
of a unique RNAi platform named nkRNA. The nkRNAs spontaneous-
ly anneal to form a helical structure containing a double-stranded
central stem and two loops within a molecule.

In Vitro Transfection
HRECs were cultured to a confluence of 50%–70%. RNAi agents were
mixed (10 nM, final concentration) with RNA transfection reagent
(Lipofectamine RNAiMAX; Invitrogen) according to the manufac-
turer’s protocol. The mixtures for transfection were replaced after
24 hr by medium containing IL-13 at a final concentration of
50 ng/mL as the inducer of POSTN followed by each assay.

In Vivo RNAi Treatment
Immediately after returning the pups to room air at P12, they were
given an intravitreal injection of 1 µL of PBS containing 10 µM
scramble RNAi agent in one eye and RNAi agent targeting POSTN
in the other eye. The intravitreal injections were performed 0.5 mm
away from the limbus using a 1-µL Hamilton syringe (Hamilton)
and a 33-gauge needle under a surgical microscope.

Statistical Analyses
All results were expressed as means ± SEMs. The statistical signifi-
cance of differences between groups was determined by two-tailed
t tests. For comparisons with the control group, Dunnett’s test
was used. Differences were considered significant with p < 0.05.
Statistical analyses were performed using JMP, version 11.0.0 (SAS
Institute).

SUPPLEMENTAL INFORMATION
Supplemental Information includes one figure and can be found with
this article online at http://dx.doi.org/10.1016/j.omtn.2017.01.004.

AUTHOR CONTRIBUTIONS
S.Y. designed the study, and T.N. wrote the initial draft of the manu-
script. T.N., S.Y., K. Ishikawa, and S.N. contributed to analysis and
interpretation of data, and assisted in the preparation of the manu-
script. All other authors have contributed to data collection and inter-
pretation, and critically reviewed the manuscript. The final version of
the manuscript was approved by all authors.

CONFLICTS OF INTEREST
The patent on periostin (WPO Patent WO/2013/147140) became
public, and in these patents, the names of T.N., S.Y., K. Ishikawa,
and T.I. are included. K.T. and K.Y. are employees of AQUA Thera-
peutics and hold equity. The other authors have no conflict of interest.

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