Attenuation of EphrinB2 Reverse Signaling Decreases Vascularized Area and Preretinal Vascular Tuft Formation in the Murine Model of Oxygen-Induced Retinopathy

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PURPOSE. EphB4 and ephrinB2 are known key regulators of retinal vascular development, but due to their capacity for bidirectional signaling, delineation of their individual roles in this process remains unclear. To better dissect out individual contributions, a model of proliferative retinopathy in mice with attenuated ephrinB2 reverse signaling was studied. It was hypothesized that endothelial ephrinB2 reverse signaling regulates hypoxia-induced capillary sprouting, as well as the pathologic formation of neovascular tufts in postnatal retinal microvascular networks.

METHODS. Genetically manipulated mice with attenuated ephrinB2 reverse signaling (ephrinB2^{lacZ/+}), along with wild-type (WT) controls, were exposed to oxygen-induced retinopathy (OIR), a postnatal model of proliferative retinopathy. At peak disease (postnatal day 18), microvascular networks were analyzed to examine intraretinal revascularization, capillary sprouting, and pathologic neovascularization responses. EphB4 and phosphorylated ephrinB protein expression patterns along retinal microvessels were also assessed.

RESULTS. EphrinB2^{lacZ/+} mice exhibited reduced hypoxia-induced revascularization ($P \leq 0.04$) and reduced formation of neovascular tufts ($P < 0.001$), as compared with WT controls. Corresponding to the observed inhibition of retinal angiogenesis, ephrinB2^{lacZ/+} retinas displayed an increased number of blind-ended capillary sprout tips ($P < 0.02$) and endothelial filopodial processes ($P = 0.001$). In WT and ephrinB2^{lacZ/+} OIR-exposed retinas, ephrinB was confined to endothelial cells, with expression detected along angiogenic vascular processes including neovascular tufts and blind-ended capillary sprouts.

CONCLUSIONS. EphrinB2 reverse signaling is a regulator of key processes during retinal vascularization and controls pathologic retinal angiogenesis through direct effects on capillary sprouting and endothelial filopodia formation. (*Invest Ophthalmol Vis Sci*. 2012;53:5462–5470) DOI:10.1167/iovs.11-8599

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Supported in part by National Institutes of Health Grants R01 HL082838-02 (SMP), K08 EY019533-02 (PAY), and T32 GM08715 (TAM).

Submitted for publication September 15, 2011; revised June 11, 2012; accepted July 8, 2012.

Disclosure: A.C. Taylor, None; T.A. Mendel, None; K.E. Mason, None; K.E. Degen, None; P.A. Yates, None; S.M. Peirce, None

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Eph receptors constitute the largest family of receptor tyrosine kinases in the human genome. Ephs and their ephrin ligands are transmembrane proteins, with signaling occurring bidirectionally, through Eph receptors (forward signaling) as well as ephrin ligands (reverse signaling).^{1,2} EphB4 was found to mark venous endothelial cells, and ephrinB2 was exclusively expressed on arterial endothelial cells.³ Genetic knockouts of ephrinB2 and EphB4 display a multitude of vascular defects and are embryonically lethal.^{3,4} During normal postnatal development of the retinal vasculature, downregulation of ephrinB2 expression in endothelial cells reduced the size and complexity of the growing retinal vascular network.⁵ Similarly, impairment of the PDZ-dependent aspect of ephrinB2 reverse signaling resulted in a reduced number of blind-ended capillary tips in the mouse retina.⁶

In addition to normal vascular development, EphB4 and ephrinB2 are also implicated in processes of pathologic angiogenesis. In human patients with either diabetic retinopathy or retinopathy of prematurity (ROP), prevalent expression of ephrinB2 and EphB was detected within harvested retinal fibroproliferative membranes.⁷ Previous work utilizing the oxygen-induced retinopathy (OIR) mouse model of ROP has demonstrated the presence of activated ephrinB along retinal neovessels and activated Eph receptors along retinal blood vessels and neovascular tufts.^{8,9}

Targeting ephrinB2/EphB4 signaling can alter pathologic retinal neovascularization.^{9,10} In the mouse OIR model, intraocular injection of either soluble ephrinB2/Fc and EphB4/Fc chimeric proteins, capable of phosphorylating and activating the complementary protein, significantly reduced pathologic neovascularization.⁹ However, other studies have demonstrated a proangioproliferative effect of injections of either EphB4/Fc or ephrinB2/Fc for the OIR model.¹¹ Intravitreal injection of soluble monomeric EphB4, which binds but does not activate EphrinB2, was shown to reduce overall proliferative retinopathy, including vascular tuft formation, in the OIR model.¹⁰ Although it is clear that EphB4/ephrinB2 signaling regulates the angiogenic response of the OIR model, the specific contributions of EphB4 forward signaling and ephrinB2 reverse signaling to intraretinal revascularization or preretinal pathologic neovascularization remain unknown.

To this end, we sought to determine the specific role of ephrinB2 reverse signaling in pathologic postnatal retinal neovascularization of OIR, through the use of genetically manipulated mice possessing ephrinB2 cytoplasmic domain mutations (ephrinB2^{lacZ/+}) that replace the tyrosine kinase signaling domain with a *lacZ* reporter.¹² These transgenic mice allow for specific attenuation of ephrinB2 reverse signaling without disrupting EphB4 forward signaling.¹² Because ephrinB2 reverse signaling initiated by the EphB4 receptor is generally thought to promote angiogenesis by increasing endothelial cell proliferation and migration,^{13,14} we hypothe-

sized that decreased retinal angiogenesis would be observed in ephrinB2^{lacZ/+} mice in the OIR disease model.

MATERIALS AND METHODS

EphrinB2^{lacZ/+} Mice

EphrinB2^{lacZ/+} transgenic mice, developed on a CD-1/129 background and previously characterized elsewhere,¹² were obtained from Dr. Mark Henkemeyer (University of Texas Southwestern). These transgenic mice have defective ephrinB2 reverse signaling due to a mutation in the cytoplasmic domain of ephrinB2 but undisrupted forward signaling through the EphB4 receptor.¹² Specifically, *lacZ* is inserted into the fifth exon of the wild-type (WT) ephrinB2 gene and results in the translation of a fusion protein consisting of altered ephrinB2 and β -galactosidase, joined by a short peptide chain in the cytoplasmic domain. A colony was established at our facility and mice were genotyped using PCR on tail clippings to separate ephrinB2^{lacZ/+} mice from WT littermates using primer sequences previously described.¹² Because ephrinB2^{lacZ/+} females are poor breeders,¹² WT CD-1/129 mice from this strain were used for breeding so each litter possessed both ephrinB2^{lacZ/+} and WT pups.

Oxygen-Induced Retinopathy Model

All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Virginia's Institutional Animal Care and Use Committee (IACUC). The standard oxygen-induced retinopathy (OIR) model was implemented using a protocol previously established.¹⁵ EphrinB2^{lacZ/+} or WT mouse pups (postnatal day 7 [P7]) along with their nursing dam, were placed in a hyperoxia chamber, a clear, airtight chamber (Plexiglas) where the atmospheric oxygen concentration can be regulated. Using an oxygen tank and oxygen controller (ProOx model 110; Biospherix, Laconia, NY), the oxygen levels were raised to 75% O₂ inside the chamber. Carbon dioxide and moisture were removed by commercial anhydrous calcium sulfate filters (Drierite and activated carbon; Fisher Scientific, Pittsburgh, PA). The mice were checked twice daily through the transparent chamber, and temperature and humidity were monitored to ensure they stayed within the required ranges of 68 to 72°F and 30 to 60% relative humidity. Food and water levels were checked daily, although enough food was provided so that the chamber would not need to be opened during hyperoxia exposure. Animals were exposed to hyperoxia for 5 days and subsequently returned to normoxia (room air, 21% O₂) at P12. At the relevant time point (P12, P18, or P21), animals were euthanized and retinas were harvested and processed for analysis. Additional retinas from ephrinB2^{lacZ/+} and WT mice were harvested at P5, prior to hyperoxic exposure, to examine normal physiologic development of the retinal vasculature.

Retinal Whole-Mount Procedure and Immunohistochemistry

Mice were euthanized with CO₂ inhalation. Eyes were enucleated and fixed using 4% paraformaldehyde (PFA) (Alfa Aesar, Ward Hill, MA) in phosphate-buffered saline (PBS) for 30 minutes at 4°C. Following preliminary fixation, the cornea and lens were separated from the posterior eyecup using a stereomicroscope (Nikon SMZ1500; Nikon Instruments, Melville, NY). The posterior eyecup was fixed for an additional 90 minutes in 4% PFA at 4°C, and the retina was removed from the posterior eyecup and flat-mounted on a gelatin-coated slide.

To visualize microvascular networks, retinal tissue was stained with *Griffonia simplicifolia* isolectin B4 (GS-IB4) pre-conjugated to either a commercial dye (Alexa Fluor 488, 568, or 647; Molecular Probes/Invitrogen, Carlsbad, CA) or biotin (Sigma-Aldrich, St. Louis, MO), which labels endothelial cells^{16,17} and monoclonal antismooth muscle α -actin (SMA) antibody pre-conjugated to a commercial dye (Cy3; Sigma-Aldrich), which labels smooth muscle cells (SMCs).^{18–20} Retinas were

washed four times for 10 minutes each with 0.1% saponin/PBS at 4°C and then permeabilized in 10% Triton-X 100 (Bio-Rad, Hercules, CA) for 2 hours at room temperature. Blocking of nonspecific binding was performed using 5% normal donkey serum (NDS) or normal goat serum (NGS) (Sigma-Aldrich) in 0.1% saponin/PBS solution for 1 hour at room temperature. Retinas were incubated overnight at 4°C with GS-IB4 primary (1:200) diluted in antibody buffer consisting of PBS, 0.1% saponin, 2% bovine serum albumin (Jackson ImmunoResearch, West Grove, PA), and 5% NDS or NGS. Retinas were washed on the following day eight times for 5 minutes each with 0.1% saponin/PBS. Some retinas were stained for EphB4 using goat polyclonal anti-EphB4 antibody (1:50) (R&D Systems, Minneapolis, MN) followed by dye-conjugated donkey anti-goat IgG (1:200, Alexa Fluor 647; Molecular Probes/Invitrogen). Activated ephrinB2 ligands were detected through the use of an anti-phospho-ephrinB antibody (Cell Signaling Technology, Danvers, MA) followed by dye-conjugated goat anti-rabbit IgG (Cy2; Jackson ImmunoResearch) as previously described.⁸ Positive antibody labeling was confirmed through the use of rabbit IgG (for anti-phospho-ephrinB antibody) and goat IgG (for anti-EphB4 antibody) (Jackson ImmunoResearch) plus secondary antibody controls. Some retinas were incubated with dye-conjugated rat anti-SMA antibody (1:200, Cy3; Sigma-Aldrich) during the secondary incubation. After washing, retinas were mounted (VectorShield; Vector Labs, Burlingame, CA) and stored at 4°C.

Whole-Eye Sections

Sagittal cross-sections were obtained from eyes ($n = 6$) of WT and ephrinB2^{lacZ/+} mice exposed to the OIR model. Following enucleation, eyes were fixed in 4% PFA at 4°C for 24 hours. Eyes were paraffin-embedded and six to eight representative 5- μ m-thick sagittal cross-sections were taken from the central region of each eye, approximately 40 μ m apart. Samples were stained with hematoxylin and eosin (H&E), to allow for the identification of preretinal neovascular tuft nuclei.

Image Acquisition

A microscope (Nikon TE 2000-E2, equipped with a Melles Griot Argon Ion Laser System and Nikon D-Eclipse C1 accessories; Nikon Instruments) was used to image whole-mounted retinal tissue. Digital confocal images of the retinal microvasculature were acquired using a $\times 10/0.30$ differential interference contrast (DIC) L/N1 dry objective, a $\times 20/0.75$ numerical aperture (NA) oil-immersion objective (Nikon Instruments), a $\times 60/1.45$ NA oil-immersion objective (Nikon Instruments), and commercial software (Nikon EZ-C1; Nikon Instruments). All images were acquired using consistent gain and aperture size settings on the confocal microscope. During the acquisition of $\times 10$ images, the confocal focal plane was standardized among images by focusing on the main radially oriented arterioles of the superficial retina. Whole-eye sections were imaged using an epifluorescence microscope (Nikon Eclipse 80i) with a $\times 20/0.50$ DIC M/N2 dry objective (Plan Fluor; Nikon Instruments) and a digital camera (Olympus Color Microfire Digital Camera; Olympus, Center Valley, PA).

Analysis of Microvascular Remodeling

Quantification of Retinal Developmental Vascularization and Hypoxia-Induced Revascularization. To quantify the amount of developmental vascularization, $\times 10$ images of lectin-labeled retinal whole-mounts (P5; $n = 5$ WT, $n = 6$ ephrinB2^{lacZ/+}) were montaged and using ImageJ image-analysis software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsbweb.nih.gov/ij/index.html>), the total retinal tissue area (μm^2) was measured. The area of retinal tissue containing retinal vessels (vascular area) was also measured. The total amount of vascularized retinal area (μm^2) was divided by the total area of the retina (μm^2) to yield the vascular area/total area ratio. To quantify the amount of revascularization of the central ischemic retina after hyperoxia-induced vaso-

obliteration, $\times 10$ images of lectin-labeled retinal whole-mounts (P12: $n = 19$ WT, $n = 6$ ephrinB2^{lacZ/+}; P18: $n = 11$ WT, $n = 10$ ephrinB2^{lacZ/+}; P21: $n = 8$ WT, $n = 2$ ephrinB2^{lacZ/+}) were montaged and the total retinal tissue area (μm^2) was measured. The area of any retinal tissue lacking retinal vessels (avascular area) was also measured. The total amount of avascular retinal area (μm^2) was divided by the total area of the retina (μm^2) to yield the avascular area/total area ratio. All data analysis was conducted in a blinded manner.

Quantification of Pathologic Neovascular Tuft Formation. To quantify the amount of preretinal neovascularization, $\times 20$ images of H&E-labeled whole eye sections were examined for the presence of preretinal neovascular tufts. For each whole-eye section, the total number of preretinal neovascular tuft nuclei penetrating the inner limiting membrane (ILM) of the retina was counted. As described previously,²¹ values for the WT in each litter were normalized to 1. All data analysis was conducted in a blinded manner.

Quantification of Blind-Ended Capillary Tips. Representative fields of view (FOVs) (two to six FOVs per retina) from lectin-labeled retinas ($n = 11$ for WT; $n = 9$ for ephrinB2^{lacZ/+}) containing a border between the avascularized and vascularized retinal tissue were acquired using confocal microscopy and a $\times 10$ objective. The number of endothelial blind-ended capillary tips along a vascular border length was counted and divided by the measured vascular border length (μm). All data analysis was conducted in a blinded manner.

Quantification of Filopodial Processes on Blind-Ended Capillary Sprouts. Representative FOV (15–25 per retina) from lectin-labeled retinas ($n = 4$ for WT; $n = 5$ for ephrinB2^{lacZ/+}) containing a border between the avascularized and vascularized retinal tissue were acquired using confocal microscopy and a $\times 60$ objective. The number of endothelial filopodial processes along a vascular border length was counted, and divided by the measured vascular border length (μm). All data analysis was conducted in a blinded manner.

X-Gal Staining and Immunolabeling

EphrinB2^{lacZ/+} transgenic mice used in this study have defective ephrinB2 reverse signaling due to the insertion of a β -galactosidase domain (*lacZ* gene) into the cytoplasmic tail of ephrinB2.¹² The common technique of X-gal staining was used to detect the presence of the *lacZ* gene insert through a detection of β -galactosidase activity as previously described.¹¹ After exposure to the OIR model, whole-eyes ($n = 7$ for WT; $n = 3$ for ephrinB2^{lacZ/+}) were enucleated and fixed in 4% PFA for 30 minutes. The cornea and lens were then removed, and the posterior eyecup was fixed in PFA for an additional 45 minutes. Eyecups were transferred to X-gal staining solution containing 50 mM potassium ferrocyanide, 50 mM potassium ferricyanide, 1 M MgCl₂, X-gal (20 mg/mL) (Sigma-Aldrich), Nonidet-P40, and 10% sodium deoxycholate in PBS. Eyecups were incubated in the X-gal staining solution overnight with protection from light and gentle shaking at room temperature. After incubation, eyes were rinsed eight times in PBS and whole-mounted. Mounted tissue was imaged using a stereomicroscope (Nikon SMZ1500, equipped with a DS-2M Series Camera; Nikon Instruments). Whole-mounted retinas were subsequently processed for immunolabeling using conjugated isolectin (Alexa Fluor 568; Molecular Probes/Invitrogen) as described earlier.

To image both X-gal staining and immunofluorescent lectin labeling in the same retina, a microscope (Nikon TE 2000-E2), equipped with both halogen and xenon light sources, was used to image whole-mounted retinal tissue. Digital images of the X-gal and lectin-stained retinal microvasculature were acquired using a $\times 10/0.30$ DIC L/N1 dry objective and a digital camera (Olympus Color Microfire Digital Camera; Olympus). PCR genotyping was used to verify the specificity of X-gal staining.

Statistical Analysis

To test for statistically significant differences between the means of two groups, an unpaired standard Student's *t*-test was used (SigmaStat,

Aspire Software, Ashburn, VA). Data not normally distributed were analyzed using a Mann-Whitney rank-sum test. Statistical significance was asserted at a value of $P \leq 0.05$. All results compared using a *t*-test are displayed graphically as the mean $\pm$ SEM. Data analyzed using a Mann-Whitney rank-sum test are displayed as a box plot in which the ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles. The fifth and 95th percentile values are shown with circles.

RESULTS

Attenuation of EphrinB2 Reverse Signaling Results in Delayed Development of Retinal Vasculature

To determine the impact of ephrinB2 reverse signaling on normal physiologic vascularization, retinas from ephrinB2^{lacZ/+} mice were examined prior to hyperoxic exposure at P5. Attenuated ephrinB2 reverse signaling resulted in significantly decreased developmental vascularization of the retina at P5, as compared with wild-type (WT) controls, with radial outgrowth reduced by 54% ($P < 0.001$) (Fig. 1).

Attenuation of EphrinB2 Reverse Signaling Results in Reduced Retinal Revascularization following Hyperoxic Insult

The OIR model results in characteristic loss of central retinal vessels by P12, followed by hypoxia-induced regeneration of the central vascular plexus and development of preretinal neovascularization.¹⁵ EphrinB2^{lacZ/+} mice were exposed to the OIR to test the hypothesis that ephrinB2 reverse signaling contributes to hypoxia-induced revascularization of the central retina. To investigate the vasoregressive response in the OIR model, we measured the avascular central retinal zones at P12 in ephrinB2^{lacZ/+} and WT mice, immediately after the hyperoxic period when the mice had been returned to normoxia. Importantly, despite the ephrinB2^{lacZ/+} mice retinal vasculature developing more slowly than WT as measured at P5, the avascular area to total area ratio at P12 for the retinas of ephrinB2^{lacZ/+} and WT mice were comparable (0.166 for ephrinB2^{lacZ/+}; 0.164 for WT, $P = 0.84$). Attenuated ephrinB2 reverse signaling resulted in decreased central revascularization at P18 following exposure to OIR, as indicated by the larger area of avascular retinal tissue space remaining at P18 as compared with WT controls (Fig. 2A). A substantial central avascular area remains in the ephrinB2^{lacZ/+} mice, whereas in WT retinas much of the avascular area has already been invaded by new vessels (Fig. 2A). Quantification of avascular retinal area at P18 and P21 indicated that attenuated ephrinB2 reverse signaling resulted in a significant decrease in the amount of intraretinal revascularization in ephrinB2^{lacZ/+} mice as compared with WT controls ($P = 0.003$) (Fig. 2B). A significant reduction in intraretinal avascular area (60%) occurred in WT retinas between P18 and P21 (Fig. 2B). However, in ephrinB2^{lacZ/+} mice, there was no statistically significant change in intraretinal avascular area between P12 and P18, and only a moderate trend (38%) toward reduced avascular area between P18 and P21.

Attenuation of EphrinB2 Reverse Signaling Reduces Formation of Pathologic Neovascular Tufts following OIR Exposure

In addition to revascularization of the central retina, the OIR model induces pathologic formation of neovascular tufts that penetrate the internal limiting membrane of the retina.^{9,15} An examination of H&E whole-eye sections harvested at P21, the

FIGURE 1. Attenuated ephrinB2 reverse signaling resulted in decreased developmental vascularization of the ephrinB2^{lacZ/+} retina, as compared with WT controls. Whole-mounted lectin-stained retina from wild-type control (**A**) demonstrated more advanced vascular outgrowth when compared with ephrinB2^{lacZ/+} mouse (**B**) (scale bar: 1 mm). Quantification of the vascular area/total area ratio at P5 demonstrated that attenuated reverse ephrinB2 signaling significantly reduced the amount of vascularized retinal tissue (* $P < 0.001$) (**C**).

peak of this pathologic response,¹⁵ revealed that the formation of preretinal neovascular tufts was significantly reduced in ephrinB2^{lacZ/+} mice as compared with WT controls ($P < 0.001$) (Fig. 3).

Attenuation of EphrinB2 Reverse Signaling Results in Increased Formation of Blind-Ended Capillary Tips and Endothelial Filopodial Processes

In the OIR model, capillary sprouting occurs at the transition zone between vascularized peripheral retina and vasoobliterated central retina in response to local tissue hypoxia following the return of the mice to room air (i.e., after P12).¹⁵ Since a significant difference in intraretinal revascularization following OIR exposure was detected between ephrinB2^{lacZ/+} and WT mice, retinal whole-mounts were examined to determine any morphologic differences in capillary sprouting, a key component in the revascularization process. An investigation of lectin-labeled capillary sprouts at avascular/vascular area transition zones in P18 retinas demonstrated an increased formation of blind-ended capillary sprout tips in ephrinB2^{lacZ/+} retinas than that in WT controls ($P < 0.02$) (Fig. 4).

To determine whether attenuation of ephrinB2 reverse signaling influenced the formation of filopodia along the capillary sprouts of ephrinB2^{lacZ/+} mice, high-magnification ($\times 60$) images of lectin-labeled capillary sprouts at avascular/vascular area transition zones in whole-mounted retinas were

obtained (Fig. 5A). Quantification of filopodia per vascular length in these images indicated that the formation of filopodial processes was significantly greater in P18 ephrinB2^{lacZ/+} retinas as compared with that in WT controls (Fig. 5B). Interestingly, we found filopodia per vascular length varied significantly, depending on the time point and stage of vascular development examined. At P5 during normal vascular development, there was a decrease in filopodia counts in ephrinB2^{lacZ/+} versus WT mice, trending toward significance ($P = 0.067$), although both mutant and WT counts were considerably higher at P5 than those at P18 (Fig. 5B). At P12 following OIR exposure, there were comparable filopodial counts in ephrinB2^{lacZ/+} and WT mice ($P = 0.349$) (Fig. 5B).

EphB4 and EphrinB Expression along Retinal Microvascular Networks

After exposure to OIR, P21 retinas were harvested and immunolabeled with anti-EphB4 antibody and lectin to examine EphB4 protein expression between WT and ephrinB2^{lacZ/+} mice. We found EphB4 expression localization along the microvasculature was comparable between these mice, with protein detected along venules, neovascular tufts, and capillaries (Fig. 6A). Positive EphB4 staining was verified by incubating retinal tissue with goat IgG plus secondary antibody (Supplemental Fig. S1A; link to supplemental material: <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8599/-/DCSupplemental>). Analysis at P18 also showed EphB4 protein

FIGURE 2. Attenuated ephrinB2 reverse signaling decreased revascularization of ephrinB2^{lacZ/+} retinas as compared with WT controls. (A) Whole-mounted immunostained P18 retinas from ephrinB2^{lacZ/+} mice (*right*) exhibited larger areas of avascular retinal tissue space (*) compared with WT retinas (*left*) (scale bar: 100 μ m). (B) The ratio of avascular to total area in ephrinB2^{lacZ/+} mice was larger compared with WT control retinas at P18 (* P = 0.003) and P21 (** P = 0.04). As expected with the OIR model, which involves progressive revascularization of the avascular retinal tissue space, the avascular area decreased in both WT and ephrinB2^{lacZ/+} retinas from P18 to P21. However, this decrease was statistically significant only for WT retinas (* P = 0.003).

expression along neovascular tufts, central arterioles, and blind-ended capillary sprouts (data not shown), consistent with results from our previous work in rat mesenteric microvascular networks.²⁰

Activated ephrinB2 ligands along the retinal microvascular networks were detected through the use of an anti-phospho-ephrinB antibody.⁸ Comparing activated ephrinB expression localization in WT and ephrinB2^{lacZ/+} retinas, p-ephrinB spatial expression patterns appeared similar in P21 WT and ephrinB2^{lacZ/+} mice exposed to the OIR model. In WT mice, p-ephrinB was detected along blind-ended capillary sprouts, neovascular tufts, and interconnected capillaries. In ephrinB2^{lacZ/+} mice, p-ephrinB was also detected along blind-ended capillary sprouts, neovascular tufts, and interconnected capillaries, as well as central arterioles (Fig. 6B).

High-magnification confocal images demonstrated endothelial cell-specific expression of activated ephrinB. Using $\times 60$ magnification, p-ephrinB expression in the remodeling retinal vasculature was detected along endothelial cells, with apparent localization to cell membranes. A representative high-magnification image taken of a venule from a P18 ephrinB2^{lacZ/+} retina

exposed to the OIR model demonstrates a similar staining pattern between the anti-p-ephrinB antibody and the endothelial cell marker lectin (Fig. 6C). Conversely, there is little apparent overlap in the expression of p-ephrinB and smooth muscle actin on perivascular cells that surround these endothelial cells (Fig. 6C). Immunolabeling of retinal vasculature, including neovascular tufts and capillaries, by the anti-phosphorylated-ephrinB antibody but not the rabbit IgG/secondary control, served to verify antibody specificity (Supplemental Fig. S1B).

X-gal staining was also used to identify the presence of β -galactosidase (β -gal) activity,¹¹ to determine the spatial expression pattern of defective ephrinB2 along the hierarchy of retinal microvascular networks of ephrinB2^{lacZ/+} mice. Low-magnification staining using X-gal in whole-mounted P20 OIR-exposed WT and ephrinB2^{lacZ/+} retinas revealed the presence of the ephrinB2-lacZ insert along the central radial vessels and downstream branches of the retinal vasculature of ephrinB2^{lacZ/+} pups, in addition to nonvascular background staining of the entire retina (Fig. 7A). No β -gal was detected in WT control retinas after X-gal staining (Fig. 7D). Higher-magnifica-

FIGURE 3. Formation of preretinal pathologic neovascular tufts was reduced in OIR-exposed ephrinB2^{lacZ/+} retinas harvested at P21, the peak of neovascular tuft formation.¹⁵ (A) H&E staining of retina cross-sections revealed neovascular tufts (short arrows) penetrating the ILM (long arrows) in WT (left) and ephrinB2^{lacZ/+} (right) retinas (scale bar: 50 μ m). (B) The number of neovascular nuclei per retina cross-section was found to be significantly less in ephrinB2^{lacZ/+} retinas than that in WT controls (* P < 0.001).

tion X-gal staining (Fig. 7B) followed by lectin (Fig. 7C) labeling in whole-mounted P20 ephrinB2^{lacZ/+} retinas revealed the presence of ephrinB2-lacZ along retinal arterioles and neovascular tufts. Although central arterioles displayed prominent β -gal activity, central venules radiating from the optic disk to the retinal periphery appeared to lack X-gal staining. Blind-ended capillary sprouts also appeared to exhibit some β -gal staining (Fig. 7B). The pattern of vascular expression of ephrinB2-lacZ in the retina is largely consistent with that of p-ephrinB in Figure 6.

DISCUSSION

The results of our study support a proangiogenic role of ephrinB2 reverse signaling in settings of postnatal pathologic retinal angiogenesis. Mice with defective ephrinB2 reverse signaling displayed attenuated revascularization of hypoxic retinal tissue, as well as decreased formation of pathologic preretinal neovascular tufts. Although inhibition of ephrinB2 reverse signaling decreases the amount of functional vascular network expansion, we observed an increase in blind-ended capillary tips and filopodial sprout formation following OIR compared with WT mice. Given that ephrinB2 is expressed in these blind-ended capillary tips, this suggests a direct role for ephrinB2 reverse signaling during pathologic retinal microvas-

FIGURE 4. EphrinB2^{lacZ/+} retinas possessed increased numbers of blind-ended capillary sprout tips as compared with WT controls at P18. (A) In whole-mounted retinas stained with lectin (white) from WT (left) and ephrinB2^{lacZ/+} (right) mice, blind-ended capillary tips (arrows) are visible at the border between the avascularized and vascularized retinal tissue (scale bar: 50 μ m). (B) EphrinB2^{lacZ/+} retinas exhibited a significantly increased number of capillary tips as compared with WT control retinas (* P < 0.02).

cular remodeling at the level of individual tip cells. To the best of our knowledge, this is the first study to elucidate the specific role of ephrinB2 reverse signaling in a disease model of postnatal retinal microvascular remodeling *in vivo*.

Here we present an examination of the spatial and cellular expression patterns of EphB4 and activated ephrinB proteins along the hierarchy of an entire retinal microvascular network. Detection of both EphB4 and activated ephrinB along the endothelial cells of neovascular tufts and blind-ended capillary sprouts indicates that juxtapositional interactions between the receptor/ligand pair EphB4/ephrinB2 may be important in regulating the hypoxia-induced angiogenic response of the OIR model. In support of this finding, ephrinB2 and EphB4 in the developing retinal vasculature were both recently found to be colocalized on capillaries and sprouts, further indicating that ephrinB2 and EphB4 can physically interact in angiogenic vessels.⁵

In support of our findings, ephrinB2 transcription has been detected along neovessels in the adult corneal micropocket assay and along small vessels in tissue undergoing wound healing.²² In additional adult settings of angiogenesis, such as in tumors of the female reproductive system, the endothelium of a subset of new vessels was found to strongly express ephrinB2.²³ p-EphrinB was detected along angiogenic blood vessels in postnatal settings of wound healing, tumor growth, and retinal development.⁸ Moreover, activated ephrinB ligands

FIGURE 5. The formation of filopodial processes was increased in ephrinB2^{lacZ/+} retinas as compared with WT controls at P18. (A) Endothelial filopodial extensions (green dots; inset) from blinded-ended capillary sprouts are visible at the border between the avascularized and vascularized retinal tissue in whole mount WT (left) and ephrinB2^{lacZ/+} (right) retinas stained with lectin (white) (scale bar: 50 μm). (B) EphrinB2^{lacZ/+} retinas exhibited a significantly greater number of filopodial extensions (green dots in A) per 100 μm of measured vascular length (red lines in A) as compared with WT control retinas at P18 (**P* = 0.001).

were detected along angiogenic retinal blood vessels in mouse pups exposed to the OIR model,⁸ although the precise spatial and cellular expression patterns of p-ephrinB remained unclear until our study.

We used ephrinB2^{lacZ/+} transgenic mice to investigate the role of ephrinB2 reverse signaling in postnatal retinal angiogenesis. In these mice, the cytoplasmic domain of ephrinB2 is specially replaced with β-gal, and the ephrinB2-β-gal protein will traffic to the membrane as normal and interact with Eph receptors on adjacent cells to activate forward signaling, but due to the β-gal insert will not be able to interact with intracellular SH2 and PDZ domain-containing proteins and will be unable to participate in reverse signaling.¹² Previous work has demonstrated phenotypic abnormalities of these mice in other systems and tissues,²⁴ and here we demonstrate a notable vascular phenotype in a model of retinal disease.

The functional importance of ephrinB2 reverse signaling in retinal angiogenesis is also supported by recent published studies. Inactivation of the ephrinB2 gene was previously found to reduce the size and complexity of the vascular network,^{5,6} which agrees with our data. However, we see a discrepancy between the filopodia and capillary sprout behaviors measured in our study, conducted in a murine model of pathologic retinal angiogenesis, as compared with these two other recent studies that were conducted in the setting of normal physiologic development. Whereas we

observed attenuated revascularization commensurate with increased filopodia extension and capillary sprouting, Sawami-phak et al.⁶ and Wang et al.⁵ both reported attenuated revascularization commensurate with decreased filopodia extension and capillary sprouting. We postulate a number of possible explanations for these discrepancies. Our studies were conducted in the setting of pathologic angiogenesis, where higher levels of vascular endothelial growth factor (VEGF) have been measured, as compared with normal retinal development.²⁵ Second, attenuation of ephrinB2 reverse signaling was accomplished by a mutation in the cytoplasmic domain of ephrinB2, as opposed to the approach taken by Wang et al.,⁵ which specifically reduced ephrinB2 PDZ-dependent signaling. The ability of ephrinB2 to still signal through other reverse signaling adaptors, such as Grb4,²⁶ in the mice of this study versus the ephrinB2^{lacZ/+} mice used in our study may also account for the opposing results. Nonetheless, our data taken in the context of these relevant prior studies suggest that the processes of filopodia extension and capillary sprout extension in the retina may be coupled to varying degrees to the ultimate vascular patterning outcome in the retina, and that this dependence may be specific to the local environmental cues (e.g., levels of hypoxia).

Although the molecular processes governing the assembly of filopodial extensions in endothelial cells remains relatively unclear, currently the main regulators of filopodia formation are considered to be Rho small GTPases, of which RhoA, Rac1, and Cdc42 have been most extensively studied.²⁷ Insights regarding this process in endothelial cells have been obtained from studies examining axon growth cones, in which the activation of these small GTPases in growth cones was found to occur in response to stimulation of many membrane receptor tyrosine kinases, including VEGFR2.^{27,28} Interestingly, other studies of axon guidance have established the link between Eph-mediated forward signaling and activation of small Rho GTPases.²⁹ Of relevance to our study, tyrosine phosphorylation of the ephrinB1 cytoplasmic domains in neuronal cells and fibroblasts was shown to regulate the activity of focal adhesion kinase and lead to the disassembly of focal adhesions and the loss of polymerized F-actin structures,²⁶ the key component of filopodia.²⁷ However, it remains unknown whether this actin remodeling response contributes to the regulation of filopodia formation in endothelial cells. Because the netrin guidance receptor Unc5B has been implicated in filopodia formation in the developing retina via activation of GTPases,^{27,30} it is reasonable to speculate that other classical axon guidance molecules, such as ephrins, may also help regulate endothelial tip cell navigation in this manner.

Classic axon guidance molecules, such as the semaphorins, Slits, netrins, and ephrins have all been implicated as regulators of vessel guidance.^{30–32} Although the focus of our study was ephrinB2 in a vascular setting, Ephs/ephrins were first identified in the context of axon guidance.³³ In the retina, a function for EphB1, B2, and B3 receptor extracellular domains in inhibiting axon extension through growth cone collapse has been demonstrated.³⁴ EphB receptor extracellular domains can function as guidance cues to alter axon behavior, possibly through downstream signaling through the cytoplasmic domains of their ephrinB ligands. Clear evidence exists that the EphB/ephrinB system functions in axon pathfinding and neuronal growth,^{29,34,35} and we propose that ephrinB2 reverse signaling may provide a similar modulation of capillary sprout guidance and elongation during postnatal angiogenesis.

We determined that attenuation of ephrinB2 signaling led to the increased formation of endothelial filopodial extensions along blind-ended capillary sprouts, and this was correlated with an overall reduction in the intraretinal revascularization response, perhaps a result of the impaired ability of the sprout

FIGURE 6. In P21 WT and ephrinB2^{lacZ/+} OIR-exposed retinas, EphB4 and ephrinB were confined to endothelial cells, with expression detected along angiogenic vascular processes including neovascular tufts and blind-ended capillary sprouts. (A) In both WT and ephrinB2^{lacZ/+} retinas, EphB4 protein expression was detected along venules (V), neovascular tufts (arrowheads), and capillaries (arrows) (scale bar: 50 μ m). (B) Phosphorylated ephrinB (p-ephrinB) expression (white or green) in WT and ephrinB2^{lacZ/+} retinas stained with lectin (red) was detected along blind-ended capillary sprouts (arrows) as well as interconnected capillaries. A central arteriole (A) exhibited active reverse ephrinB signaling (scale bar: 50 μ m). (C) A high-magnification FOV demonstrates the lack of p-ephrinB staining on SMA-positive (red) perivascular cells and the colocalization of endothelial cell marker lectin (blue) and p-ephrinB (green) staining. P-ephrinB-expressing (green) cells along both neovascular tufts and capillaries were enwrapped by SMA-expressing perivascular cells (scale bar: 25 μ m).

tip cell to sense and/or respond to chemotactic cues. Interestingly, in cortical neurons filopodia motility is reduced when EphB expression is knocked down.³⁶ This previous work provides evidence that the EphB/ephrinB signaling system actively modulates filopodial function in dendrites, which is

possibly recapitulated in the vascular system as supported by our data.

In this work, we implicate a role for ephrinB2 reverse signaling in endothelial filopodial process formation in a retinal disease model of hypoxia-induced capillary sprouting, and the results of this study contribute to our understanding of the role of ephrinB2 reverse signaling in postnatal microvascular remodeling *in vivo*. Considering the importance of ephrinB2 reverse signaling in both pathologic preretinal neovascular tuft formation and capillary sprouting angiogenesis demonstrated in this study, specific attenuation or induction of ephrinB2 reverse signaling may be useful in a variety of therapeutic applications, including antiangiogenic therapies as with proliferative retinopathies, or proangiogenic therapies as with tissue ischemia-induced hypoxia.

Acknowledgments

The authors thank Jessica Ungerleider for her help with the filopodia formation data analysis, the personnel of the Tissue Histology Core at the University of Virginia for providing the whole-eye cross-sections, and Mark Henkemeyer (University of Texas Southwestern) for supplying the ephrinB2^{lacZ/+} mice.

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FIGURE 7. X-gal staining in whole-mounted ephrinB2^{lacZ/+} retinas reveals the presence of ephrinB2-lacZ along the retinal microvasculature at P21. In ephrinB2^{lacZ/+} retinas, lacZ was detected along central radial vessels and their branches (arrows) (A), but no β -gal (the enzyme encoded by the lacZ gene) was detected in WT retinas (D) (scale bar: 200 μ m). Neovascular tufts (arrowheads) labeled with lectin (C) displayed β -gal activity (B). Central arterioles (A) displayed prominent β -gal activity, as compared with central venules (V) radiating from the optic disk (*) to the retinal periphery. Blind-ended capillary sprouts (arrows) also appeared to exhibit β -gal activity (scale bar: 100 μ m).

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