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The formation of an angiogenic astrocyte template is regulated by the neuroretina in a HIF-1-dependent manner

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ABSTRACT

The vascular and nervous systems display a high degree of cross-talk and depend on each other functionally. In the vascularization of the central nervous system, astrocytes have been thought to sense tissue oxygen levels in hypoxia-inducible factors (HIFs)-dependent manner and control the vascular growth into the hypoxic area by secreting VEGF. However, recent genetic evidences demonstrate that not only astrocyte HIFs but also astrocyte VEGF expression is dispensable for developmental angiogenesis of the retina. This study demonstrates that hypoxia-inducible factor 1 alpha subunit (HIF-1α), a key transcription factor involved in cellular responses to hypoxia, is most abundantly expressed in the neuroretina, especially retinal progenitor cells (RPCs). A neuroretina-specific knockout of HIF-1α ($\alpha Cre^{+}Hif1\alpha^{flox/flox}$) showed impaired vascular development characterized by decreased tip cell filopodia and reduced vessel branching. The astrocyte network was hypoplastic in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. Mechanistically, platelet-derived growth factor A (PDGF-A), a mitogen for astrocytes, was downregulated in the neuroretina of $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. Supplementing PDGF-A restored reduced astrocytic and vascular density in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. Our data demonstrates that the neuroretina but not astrocytes acts as a primary oxygen sensor which ultimately controls the retinal vascular development by regulating an angiogenic astrocyte template.

Introduction

Accumulating evidence shows that neuronal lineage cells contribute significantly to the formation of the vascular network (Segura et al., 2009). In the developing skin, for example, local signals such as vascular endothelial growth factor (VEGF), provided by organized sensory nerve fibers, define the pattern of blood vessel branching and arterial differentiation (Mukouyama et al., 2002). In retinal vascular development, specialized endothelial tip cells lead the outgrowth of blood vessels (Gerhardt et al., 2003). The retinal astrocyte network in the hypoxic retina provides guidance cues such as VEGF for endothelial tip cells (Dorrell et al., 2002; Ruhrberg et al., 2002; Stone et al., 1995; West et al., 2005), thereby astrocytes have been believed to play a pivotal role in the oxygen sensing mechanism during retinal vascular development (Gariano and Gardner, 2005; West et al., 2005). However, recent studies suggested that not only astrocyte hypoxic response but also astrocyte VEGF expression is dispensable for developmental angiogenesis of the retina (Scott et al., 2010; Weidemann et al., 2010), which implies a modification in the current understanding of oxygen-sensing mechanisms during the interaction between astrocytes and endothelial cells.

Retinal progenitor/stem cells (RPCs), counterparts of neural stem/progenitor cells in the brain, are multipotent and remain undifferentiated (Tropepe et al., 2000). RPCs are found in the early embryonic retina and gradually disappear during retinal development (Marquardt et al., 2001). Cell fate tracing experiments revealed that RPCs generate multiple cell types in the neuroretina including ganglion cells, photoreceptor cells, and bipolar cells but not astrocytes (Marquardt et al., 2001). In the mouse and xenopus retina, RPCs coexpress the transcription factors Pax6, Rx1 (rax), and Six3, prior to the onset of retinogenesis (Perron et al., 1999; Walther and Gruss, 1991). Transgenic mice expressing Cre recombinase under the control of the *Pax6* retina-specific regulatory element, α-promoter (*α-Cre*) are used to delete genes in

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the cells of the peripheral neuroretina including RPCs (Marquardt et al., 2001). Previously, we reported that α-Cre-specific conditional knockout mice for the von Hippel-Lindau tumor suppressor gene (*vhl*) exhibited persistent hyaloid vessels due to the increased activity of hypoxia-inducible factor 1 alpha subunit (HIF-1α), a key transcription factor in cellular responses to hypoxia (Kurihara et al., 2010). Retinal vascularization starts from the optic nerve head, and the vasculature radially grows towards the periphery along the retinal astrocyte scaffold. The primary plexus in the ganglion cell layer covers almost the entire retinal surface before P9. After P9, vascular growth subsequently occurs into deeper layers where again branching and fusion lead to formation of two additional plexuses: the intermediate plexus and the deep plexus (Gerhardt et al., 2003). Recently, Caprara et al. (2011) reported that HIF-1α knockout in the neuroretina resulted in impaired intermediate plexus. Although these results suggest that the neuroretina contributes to the retinal oxygen-sensing mechanism, the function of the neuroretina in the formation of primary vascular plexus and in the interaction of astrocytes and endothelial cell is unclear.

In this study, we show that the HIF-1α is most abundantly expressed in RPCs in the developing retina. Conditional deletion of *Hif1α* expression ($\alpha Cre^{+}Hif1\alpha^{flox/flox}$) revealed impaired vessel growth and a hypoplastic astrocyte network. Our data reveals a novel oxygen-sensing mechanism required for proper vascular patterning in the retina; the neuroretina acts as a primary oxygen sensor which controls the vascular growth by forming an angiogenic astrocyte template in a HIF-1-dependent manner.

Materials and methods

Mice

Animal care was performed in accordance with the Guidelines of Keio University for Animal and Recombinant DNA experiments. *α-Cre* transgenic mice (Marquardt et al., 2001) were mated with $Hif1\alpha^{flox/flox}$ mice (Ryan et al., 2000), and generated *α-Cre*-specific *Hif1α* knockout ($\alpha Cre^{+}Hif1\alpha^{flox/flox}$) mice. As control littermates for these knockout animals, $Hif1\alpha^{flox/flox}$ without the α-Cre transgene were used. *Flox-CAT-EGFP* mice (Kawamoto et al., 2000) were mated with *α-Cre* mice to obtain *α-Cre/Flox-CAT-EGFP* mice.

Whole-mount immunostaining and in situ hybridization

Enucleated eyes were fixed for 20 min in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) and then dissected. Retinal cups were post-fixed and stored in methanol at −20 °C. For the BrdU incorporation assay, 100 μg BrdU (BD Pharmingen, Franklin Lakes, NJ) per gram of body weight, dissolved in sterile PBS, was injected intraperitoneally 2 h before sacrifice. Isolated retinas were stained using a BrdU immunohistochemistry system (Calbiochem, Darmstadt, Germany). For in situ hybridization (ISH), retinas were briefly digested with proteinase K and hybridized with DIG-labeled antisense RNA probes. When ISH was combined with immunohistochemistry (IHC), IHC was performed after ISH procedures were completed. The following primary monoclonal antibodies were used: PDGFRα (APA5; eBioscience; San Diego, CA), α-smooth muscle actin (1A4; FITC- or Cy3-conjugated; Sigma-Aldrich, St. Louis, MO), neurofilament (2H3; Developmental Studies Hybridoma Bank, Iowa, IA), F4/80 (CI:A3-1; Serotech; Oxford, UK), GFAP (G-A-5; Cy3-conjugated; Sigma-Aldrich, St. Louis, MO), and desmin (DAKO, Glostrup, Denmark). The following primary polyclonal antibodies were used: rabbit anti-GFP (Molecular Probes; Alexa 488-conjugated; Eugene, OR), goat anti-GFP (Rockland; Gilbertsville, PA), Pax-2 (Covance, Berkeley, CA), collagen IV (Cosmo Bio, Tokyo, Japan), fibronectin (DAKO), Pax-6 (PAX6; Developmental Studies Hybridoma Bank), and HIF-1α (originally established by immunizing guinea pigs with a purified fusion protein, encompassing amino acids 416 to 785 of mouse HIF-1α) (Kurihara et al., 2010). The following secondary antibodies were used: Alexa 488 fluorescence-conjugated IgGs (Molecular Probes) or Cy3/Cy5-conjugated IgGs (Jackson ImmunoResearch, West Grove, PA). For nuclear staining, specimens were treated with DAPI (Molecular Probes). In some experiments, blood vessels and monocyte-lineage cells were simultaneously visualized using biotinylated isolectin B4 (iB4; Sigma) followed by fluorescent streptavidin conjugates (Molecular Probes).

Genomic PCR and RT-PCR analysis

Quantitative RT-PCR assays were performed on an ABI 7500 Fast Real-Time PCR System using TaqMan Fast Universal PCR master mix (Applied Biosystems, Foster City, CA) and TaqMan® Gene Expression

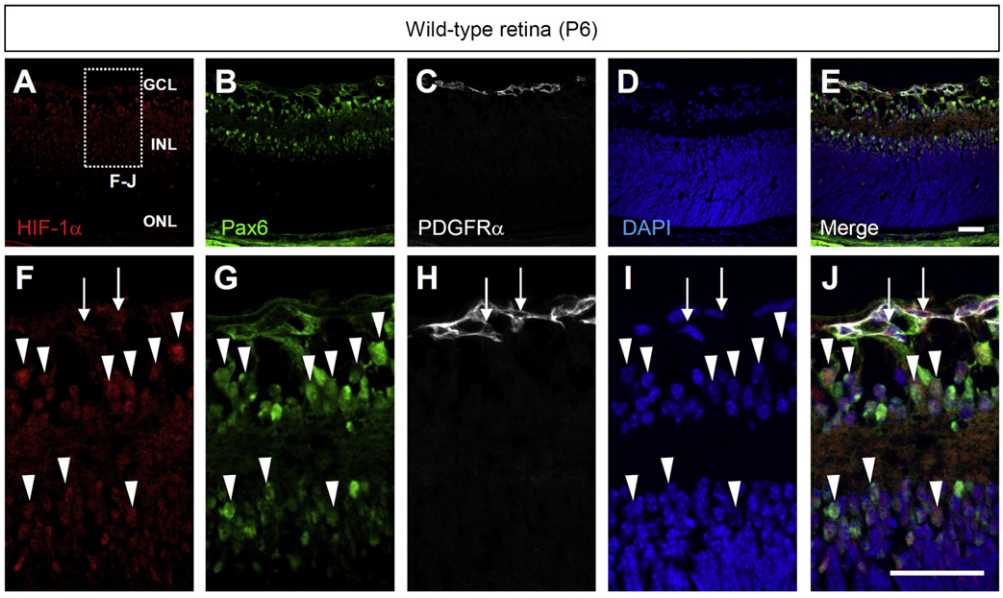

Fig. 1. HIF-1α is most abundantly expressed in RPCs in the developing retina. (A–J) Immunofluorescence for P6 retinas. HIF-1α is abundantly expressed in the ganglion cell layer (GCL) and the inner nuclear layer (INL) (A). In the high magnification view (F–J), HIF-1α is most abundantly detected in $Pax6^{+}$ RPCs (arrowheads), whereas a weaker HIF-1α expression is detected in astrocytes (arrows) and the cells in the outer nuclear layer (ONL). Scale bar: 50 μm.

Assay Mix of *Vegf-a* (Mm00437304_m1), *Pdgfa* (Mm01205760_m1), *Csfr1* (Mm00432689_m1), *Ang-1* (Mm00456503_ml), *Ang-2* (Mm00545822_m1), *Flk-1* (Mm00440099_m1), *Epo* (Mm01202755_m1), *Edn2* (Mm00432983_m1), or *Flt-1* (Mm00438980_ml). A mouse *β-actin* (Mm00607939_s1) assay mix served as an endogenous control. Data were analyzed by 7500 Fast System SDS Software 1.3.1. Each experiment was performed with four replicates from each sample, and the results were averaged.

Intra-ocular injections

Injections into the vitreous body were performed using 33 gauge needles, as described previously (Kubota et al., 2009). Approximately 0.2 μl of sterile PBS with or without 0.1 mg/ml of PDGF-A was injected at P3 or P5 and mice were sacrificed at P6.

Confocal microscopy

Fluorescent images were obtained using a confocal laser scanning microscope (FV1000; Olympus). Quantification of cells or substances of interest was usually done on a 500 μm × 500 μm field of view per sample in scanned images. Specifically, we set the area in the vascular front between arterial and venous area. Quantification of relative immunofluorescence was performed using the Scion image software Version 4.0.3.2 (Scion Corporation).

Statistical analysis

All results are expressed as the mean ± SD. The averaged variables were compared using the 2-tailed Student's *t*-test. P-values of less than 0.05 were considered statistically significant.

Results

HIF-1α is most abundantly expressed in RPCs in the developing retina

The initial step in our study was to examine the distribution of the HIF-1α protein in the developing retina. Immunofluorescence of retinal sections revealed that the nuclear translocation of HIF-1α was abundantly detected in the cells of the neuroretina located in the inner nuclear layer (INL), especially Pax6$^+$ RPCs (Figs. 1A–J). A weaker HIF-1α expression was detected in astrocytes (Figs. 1A–J). Transgenic mice expressing Cre recombinase under the control of the *Pax6* retina-specific regulatory element, α-promoter (*α-Cre*) are

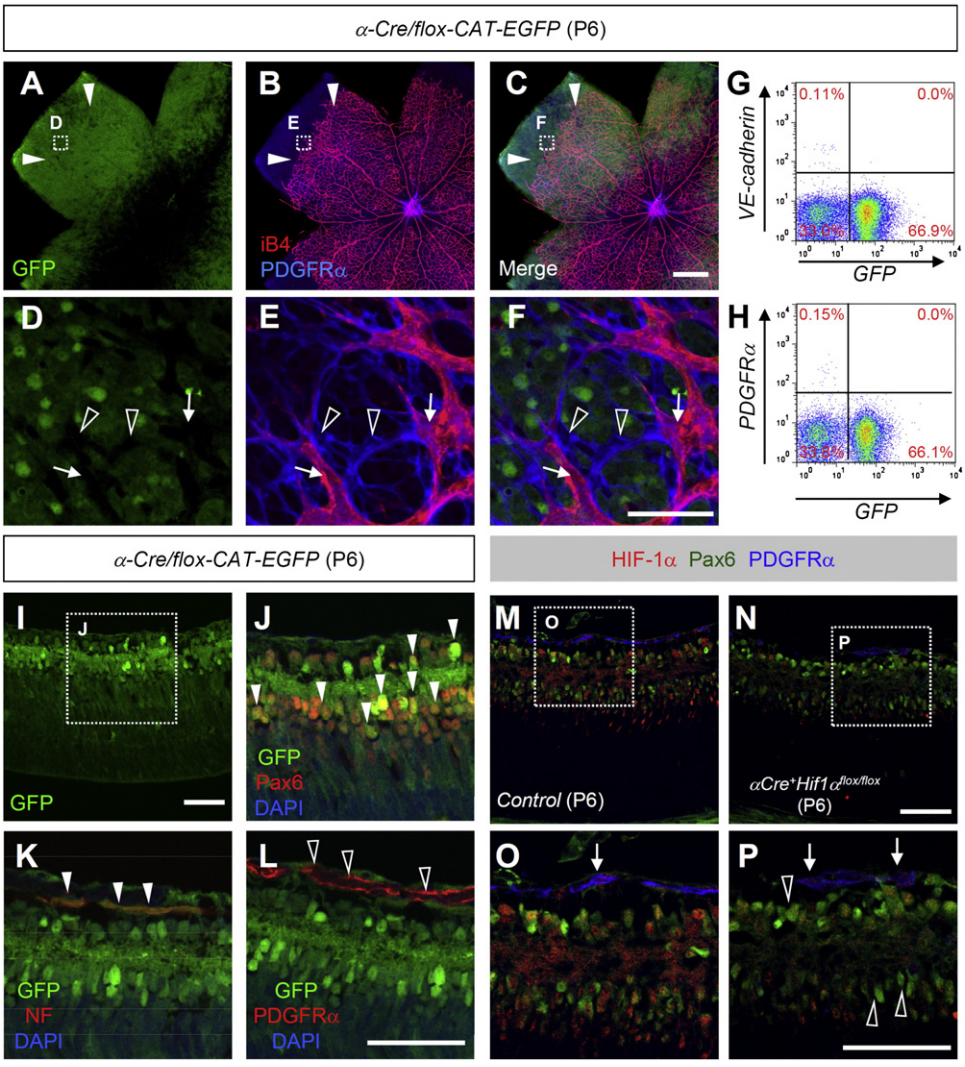

Fig. 2. *α-Cre*-mediated excision of Hif-1α in neuroretinal cells. (A–L) Whole-mount immunofluorescence (A–F), FACS study (G, H), and section immunofluorescence (I–L) for the P6 retinas of *α-Cre* mice crossed with *flox-CAT-EGFP* mice. GFP was detected in Pax6$^+$ RPCs (solid arrowheads in J), Neurofilament (NF)$^+$ ganglion cell axons (solid arrowheads in K), but not in astrocytes (open arrowheads in D–F, L) and Isolectin B4 (iB4)-positive endothelial cells (arrows in D–F). GFP was more abundantly expressed in the peripheral neuroretina (solid arrowheads in A–C) compared to proximal neuroretina. (M–P) Immunofluorescence of P6 retinas. HIF-1α expression was diminished in most of Pax6$^+$ RPCs of *αCre$^+$Hif1α$^{flox/flox}$* mice (arrowheads in P), but was detected in astrocytes from *αCre$^+$Hif1α$^{flox/flox}$* mice (arrows in O and P). Scale bars: 500 μm in A–C; 50 μm in D–F, I–P.

used to delete genes in the cells of the neuroretina including RPCs. It was also reported that Cre recombination does not occur in astrocytes and endothelial cells of *α-Cre* mice during embryogenesis and late developmental stages (Marquardt et al., 2001). We confirmed this expression pattern at P6, the developmental time at the focus of this study, when the vascular plexus was expected not to reach the recombination zone previous to this stage. We crossed α-Cre mice with a transgenic reporter line, *flox-CAT-EGFP*, and examined the GFP expression (Figs. 2A–L). Whole-mount immunofluorescence and FACS study revealed that GFP was not expressed in astrocytes and endothelial cells (Figs. 2A–H). As reported, the GFP expression pattern varied according to area and was more abundantly expressed in the peripheral neuroretina compared to proximal neuroretina (Fig. 2A). Section immunohistochemistry showed that GFP expression was detected in all neuroretinal cells including Pax6$^+$ RPCs and Neurofilament (NF)$^+$ ganglion cells (Figs. 2I–K). In contrast, GFP expression was not detected in astrocytes (Fig. 2L). Next, to delete HIF-1α in the neuroretinal cells, we crossed *α-Cre* mice with *Hif1α$^{flox/flox}$* mice and generated an *α-Cre*-specific *Hif1α* knockout (*αCre$^+$Hif1α$^{flox/flox}$*). Consistent with the expression pattern of *α-Cre*, HIF-1α expression was mostly diminished in the neuroretinal cells including Pax6$^+$ RPCs from *αCre$^+$Hif1α$^{flox/flox}$* mice, but was detected in astrocytes of *αCre$^+$Hif1α$^{flox/flox}$* mice (Figs. 2M–P). The number of Pax6$^+$ RPCs was not affected in *αCre$^+$Hif1α$^{flox/flox}$* mice (Figs. 2M, N), suggesting that the loss of HIF-1α does not impair the proliferation and survival of these cells.

Decreased endothelial tip cell numbers and reduced vessel branching in αCre$^+$Hif1α$^{flox/flox}$ mice

Next, we examined retinal vascular structures in *αCre$^+$Hif1α$^{flox/flox}$* mice. At P6, decreased vessel branching and irregular growing edges were detected in the peripheral retina of *αCre$^+$Hif1α$^{flox/flox}$* mice (Figs. 3A, C, G), although the proximal retina did not show significant difference. A high magnification view of sprouting edges at P6 revealed a decrease in the number of endothelial tip cells and their filopodia in *αCre$^+$Hif1α$^{flox/flox}$* mice (Figs. 3E, F, I, J). At P9, irregularities were still detected in the peripheral vasculature of *αCre$^+$Hif1α$^{flox/flox}$* mice (Figs. 3B, D). Vascularized area at P9 is slightly but significantly decreased in *αCre$^+$Hif1α$^{flox/flox}$* mice at P9 (Fig. 3H).

αCre$^+$Hif1α$^{flox/flox}$ mice show moderately decreased endothelial proliferation and no alteration in perivascular cells

A short-term (2 h) BrdU incorporation assay showed a moderate decrease in endothelial proliferation in *αCre$^+$Hif1α$^{flox/flox}$* mice

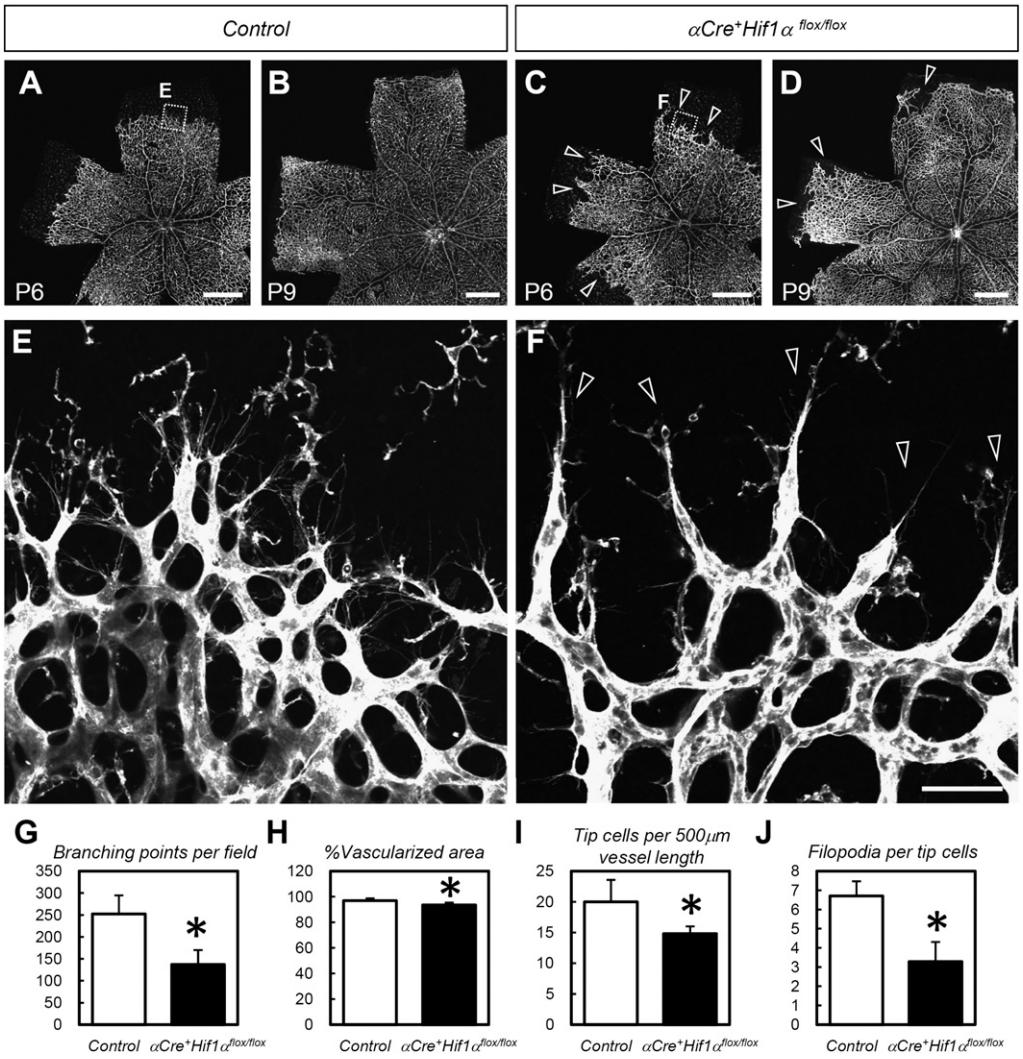

Fig. 3. *αCre$^+$Hif1α$^{flox/flox}$* mice show decreased vessel branching and tip cells. (A–F) iB4 staining for P6 and P9 retinas. Decreased vessel branching and irregular growing edges were detected in *αCre$^+$Hif1α$^{flox/flox}$* mice (arrowheads). High magnification views of sprouting edges (E, F), indicated by the boxes in A, C, revealed a decrease in the number of endothelial tip cells and their filopodia (arrowheads) in *αCre$^+$Hif1α$^{flox/flox}$* mice. (G–J) Quantification of isolectin-positive vascular structures at P6 in G, I, J and P9 in H (n = 8). Quantification in G was performed in the images taken for the peripheral half of the retina. Scale bars: 500 μm in A–D; 50 μm in E, F; *p<0.05.

(Figs. 4A–D, G). These mice showed no significant changes in the amount of empty basement membrane sleeves highlighting vessel regression (Phng et al., 2009) (Figs. 4E, F, H). These features indicate the vascular phenotypes in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice are different from those seen in the condition of the VEGF inhibition or Notch gain-of-function (Phng et al., 2009). The numbers of macrophages (Fantin et al., 2010; Kubota et al., 2009), pericytes (Lindblom et al., 2003), arterial smooth muscle cells, and ganglion cells which potentially affect tip cell activity, were not affected in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice (Figs. 4I–P, R). *Colony stimulating factor 1 receptor (csf1r)* expression, which largely correlates with the number of macrophages (Kubota et al., 2009) showed no significant reduction in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice (Fig. 4Q).

$\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice show a hypoplastic astrocyte network

Next we examined the astrocyte network which provides guidance cues for endothelial tip cells. $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice showed a sparse network of astrocytes and decreased proliferation of astrocytes compared with control mice (Figs. 5A–F, I). Transcription factor, Pax2 is specifically expressed in astrocytes in the retina (Chu et al., 2001). $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ showed a significant decrease in the number of $Pax2^{+}$ astrocytes (Figs. 5G, H, J). Immunostaining for cleaved caspase3 only marked some ganglion cells, and the number of these cells did not differ in control and $\alpha Cre^{+};Hif1\alpha^{flox/flox}$ mice (data not shown), suggesting that apoptosis in endothelial cells and astrocytes is not the primary cause of their defects in $\alpha Cre^{+};Hif1\alpha^{flox/flox}$ mice. Consistent with the spatial diversity of α-Cre expression, hypoplasticity of astrocyte network was not detected in the proximal region of $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice (Figs. 5M–P). Accordingly, the vascular defects in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice were not apparent at P3 when the vascular plexus has not extended into the area where Cre recombination had occurred (Figs. 5K, L).

Decreased vegfa-expressing cells and a sparse meshwork of extracellular matrix in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice

In accordance with a decrease in the number of astrocytes (Figs. 5G, H, J), *vegfa*-expressing cells are reduced in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice, although *vegfa* expression in each astrocyte was normal (Figs. 6A, B). In quantitative RT-PCR analysis, $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice showed a slight but significant decrease in *Vegfa* expression (Fig. 6E), presumably

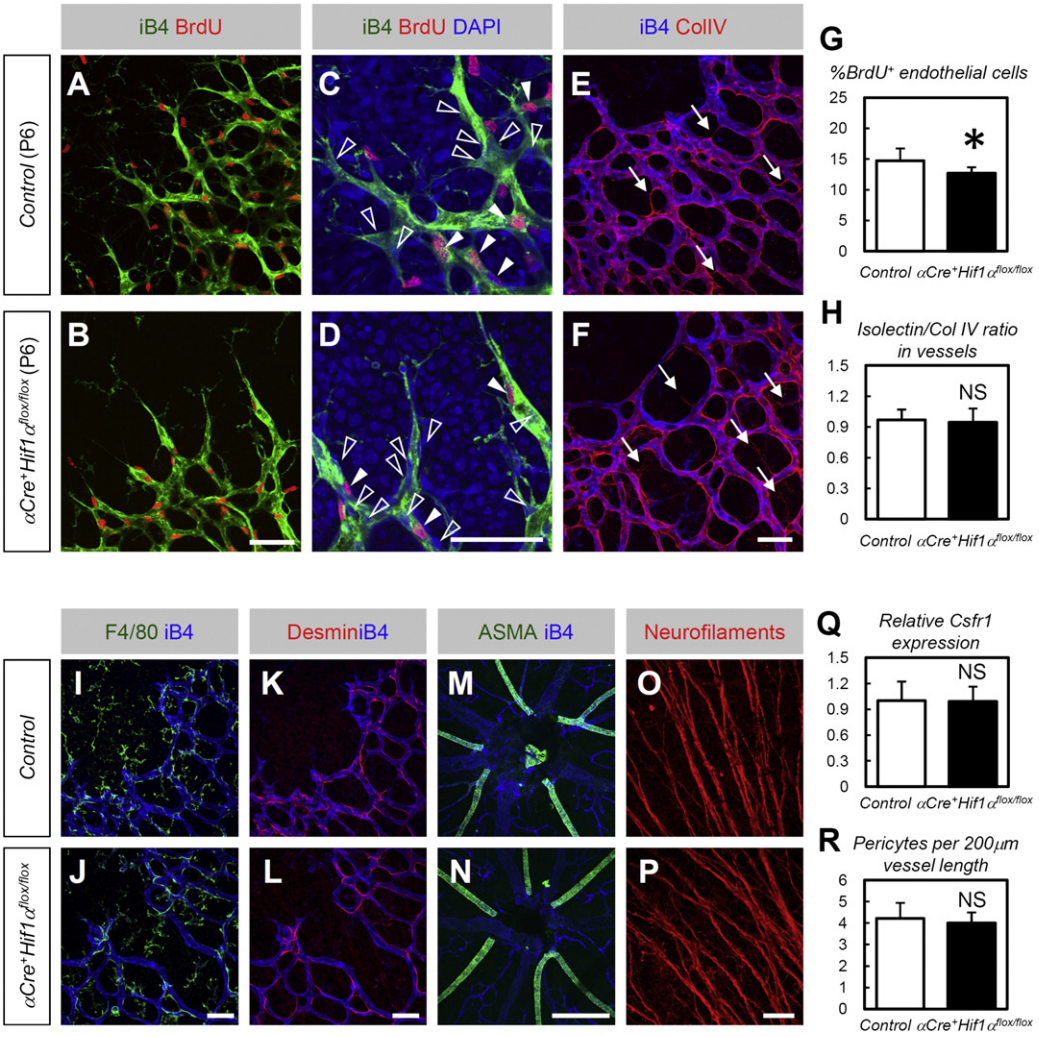

Fig. 4. $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice show moderately decreased endothelial proliferation and no alteration in perivascular cells. (A–D) Short-term (2 h) BrdU incorporation assay for P6 retinas. The ratio of $BrdU^{+}$ endothelial cells (solid arrowheads in C, D) to $BrdU^{-}$ endothelia cells (clear arrowheads in C, D) was moderately decreased in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. (E, F) Immunofluorescence of P6 retinas. $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice showed no alteration in the amount of empty basement membrane sleeves (arrows). (G, H) Quantification in the images taken for the vascular front of P6 retinas (n = 8). (I–P) Immunofluorescence of P6 (I–L, O, P) or P9 (M, N) retinas. $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice showed no alteration in macrophages (I, J), pericytes (K, L), arterial smooth muscle cells (M, N), and ganglion cell axons (O, P). (Q) Quantitative RT-PCR analysis in the P6 retina (n = 8). (R) Quantification in the images taken for the vascular front of P6 retinas (n = 6). Scale bars: 50 μm; *$p<0.05$; NS, not significant.

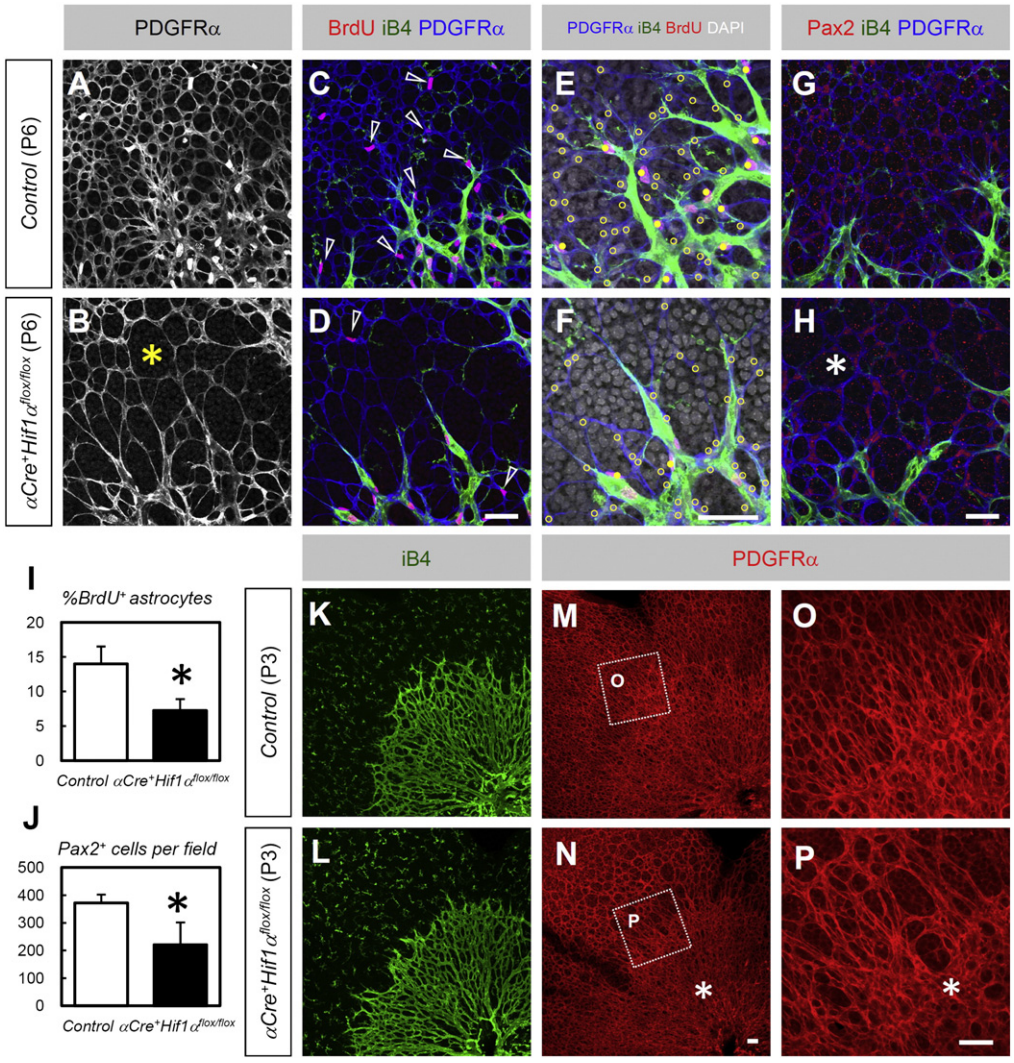

Fig. 5. $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice show a hypoplastic astrocyte network. (A–H) Immunofluorescence of P6 retinas. Panels A, C and B, D show the same field, respectively. $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice show a sparse network of PDGFRα^{+} astrocytes (asterisk in B), decreased proliferation of astrocytes (arrowheads in C, D) and a decrease in the number of Pax2^{+} astrocytes (asterisk in H). The ratio of BrdU^{+} astrocytes (dots in E, F) to BrdU^{-} astrocytes (circles in E, F) was decreased in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. (I, J) Quantification in the vascular front of P6 retinas (n = 6). (K–P) Immunofluorescence of P3 retinas. Hypoplasticity of astrocyte network was not detected in the proximal region of $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice (asterisk in N, P). The vascular defects in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice were not apparent at P3. Scale bars: 50 μm; *$p<0.05$.

reflecting a decrease in the number of astrocytes rather than altered *vegfa* expression in the neuroretina. $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ showed unchanged levels of *Flk-1* and *Flt-1*, receptors for the VEGF protein (Figs. 6F, G). RT-PCR analysis showed unchanged VEGF isoform (Ruhrberg et al., 2002) ratios in control and $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice (data not shown). As another guidance cue provided by astrocytes, extracellular matrices (ECM) such as fibronectin and heparan-sulfate are known to be important for retinal vascular growth (Stenzel et al., 2011). $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice showed a sparse proangiogenic fibronectin scaffold (Figs. 6C, D), which is sufficient to cause their vascular defects independently of VEGF. Expression of angiogenic factors such as *Ang-1*, *Ang-2*, *and Lif*, which are secreted from endothelial cells or pericytes (Gale et al., 2002; Kubota et al., 2008; Uemura et al., 2002), was not affected in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice (Figs. 6H–J). Interestingly, the expressions of erythropoietin (*Epo*) and endothelin2 (*Edn2*), recently reported to be upregulated in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice at later stages (after P15) (Caprara et al., 2011), were not significantly altered at P6 (Figs. 6K, L). As increased expressions of *Epo* and *Edn2* may be a potential mechanism for defects in the intermediate plexus formation of $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice, the impact of neuroretinal HIF-1α-deficiency on the primary plexus and intermediate plexus may differ mechanistically.

Reduced PDGF-A expression underlies the hypoplastic astrocyte meshwork in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice

Retinal astrocytes are immigrants from the optic nerve but are not derived from RPCs (Marquardt et al., 2001; Watanabe and Raff, 1988). As *α-Cre* is not expressed in astrocytes, and HIF-1α expression in astrocytes is normal in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice (Figs. 2O, P), we suspected that a paracrine factor secreted by α-Cre^{+} cells, responsible for the proliferation of astrocytes, is altered in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. The promoter of *Pdgfa*, the gene for the PDGF-A chain, is known to be activated in hypoxic liver cells (Moon et al., 2009). PDGF-A is well known as a potent mitogen for astrocytes (Fruttiger et al., 1996; Selmaj et al., 1990). Using flow cytometry, we isolated GFP^{+} cells from *α-Cre*; *flox-CAT-EGFP*; $Hif1\alpha^{+/flox}$ mice ($Hif1\alpha^{+/-}$ cells) or *α-Cre*; *flox-CAT-EGFP*; $Hif1\alpha^{flox/flox}$ mice ($Hif1\alpha^{-/-}$ cells) and examined *Pdgfa* expression. Quantitative RT-PCR analysis showed a significant decrease in *Pdgfa* expression in $Hif1\alpha^{-/-}$ cells (Fig. 7A). Consistent with the spatial diversity of astrocyte hypoplasticity, decrease in *Pdgfa* expression was limited to the distal retina of $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice (Fig. 7B). Immunoreactivity for PDGF-A was decreased in the distal retina of $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice (Figs. 7C, D). Next, we tested whether supplementation of PDGF-A by intra-ocular injection restores the astrocytic and vascular defects

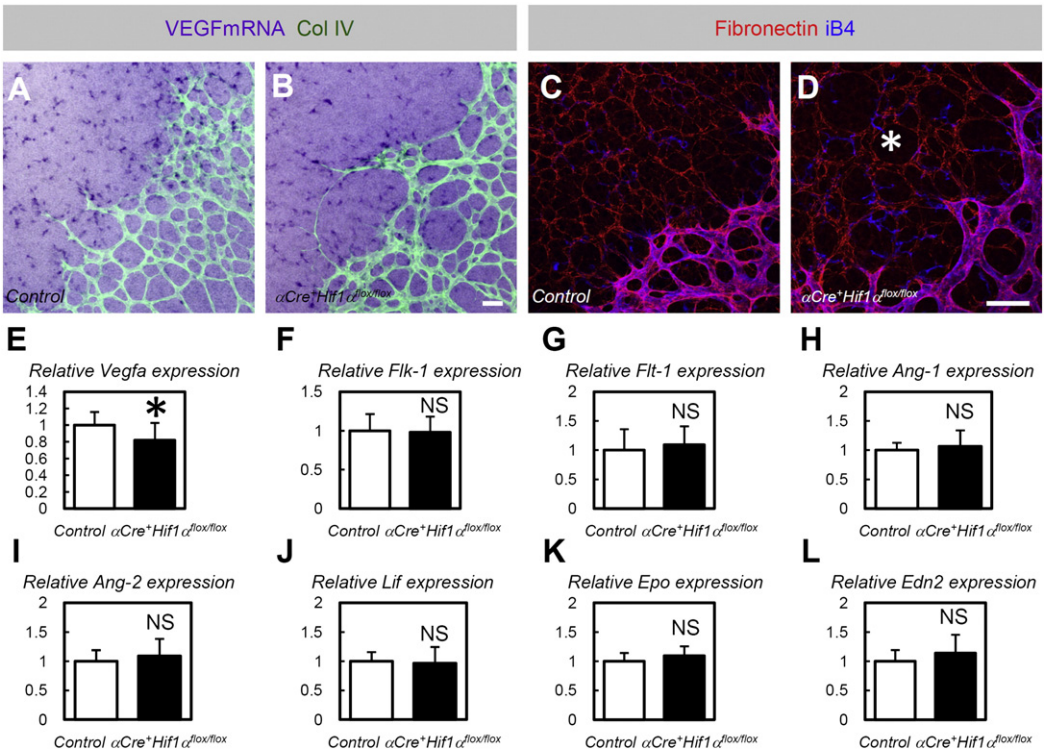

Fig. 6. Decreased *vegfa*-expressing cells and a sparse meshwork of extracellular matrix in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. (A, B) Images for in situ hybridization of *Vegfa*. $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ showed a decrease in the number of *Vegfa*-expressing astrocytes (purple cells in A, B), although *Vegfa* expression in each astrocyte was normal. (C, D) Immunofluorescence of P6 retinas. $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ showed a sparse meshwork of fibronectin (asterisk in D). (E–L) Quantitative RT-PCR analysis in the P6 retina ($n=8$). Scale bars: 50 μm; *$p<0.05$; NS, not significant.

in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice (Figs. 7E–T). Consistent with the reported phenotypes in GFAP-PDGFA transgenic mice (Fruttiger et al., 1996; Gerhardt et al., 2003), PDGF-A injection into control retinas leads to a dense astrocytic meshwork and multilayered blood vessels 3 days after injection (Figs. 7G, K, O). $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice showed a similar astrocytic and vascular response to PDGF-A injection (Figs. 7H, L, P), suggesting that reduced PDGF-A expression is associated with the formation of the hypoplastic astrocytic and vascular network in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. As an acute response (24 h) to PDGF-A injection, reduced proliferation in astrocytes but not endothelial cells in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice was restored (Figs. 7Q–T), suggesting that mitogenic activity of PDGF-A in astrocytes primarily contributes to the rescue effects and thus secondarily affects the endothelial hyperplasia.

Discussion

In this study, we have shown that HIF-1α, a key transcription factor involved in cellular responses to hypoxia, is abundantly expressed in the neuroretina, especially RPCs, rather than astrocytes. Conditional knockout of *Hif1α* in the neuroretina showed impaired vessel growth and a hypoplastic astrocyte network. In addition, we observed that PDGF-A, a potent mitogen for astrocytes, showed decreased expression in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice, which could be an explanation for this defect. Supplementation of PDGF-A, a potent mitogen for astrocytes, restored reduced astrocytic and vascular density in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. Our data suggest that the neuroretina, which acts as a key player in the oxygen-sensing mechanisms, regulates the formation of the angiogenic astrocyte template by secreting PDGF-A.

It has been believed that retinal astrocytes sense hypoxia and drive the HIFs/VEGF cascade to promote angiogenesis into the avascular retina (Gariano and Gardner, 2005; Gerhardt et al., 2003; West et al., 2005). However, two recent papers (Scott et al., 2010; Weidemann et al., 2010) presented quite unexpected results. The papers show that not only GFAP-Cre mediated *hif1α* but also *vegfa* knockouts have a trivial impact on retinal vascular development. Although it is unclear whether all of VEGF-A is depleted in neonatal retinas of GFAP-Cre-specific *Vegf-a* knockout mice, a small amount of residual VEGF-A proteins, if at all, is sufficient to complete proper vascular development. Apart from the issue regarding the requirement of astrocyte-derived VEGF-A, recent genetic evidences demonstrated that deposition of ECM is fundamentally important as an angiogenic function of retinal astrocytes (Stenzel et al., 2011). Our data support this notion; astrocytes, independently of VEGF secretion, are essential for retinal vascular development. In retinas of $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice, the vascular plexus is sparse regardless of normal *vegfa* expression in each astrocyte.

Although astrocytes display the strongest VEGF expression, with particularly high levels distally to the growing vascular plexus (Ruhrberg et al., 2002; Gerhardt et al., 2003), VEGF is also expressed in retinal ganglion cells and cells in INL, suggesting that the minor effects of astrocyte VEGF deletion on retinal angiogenesis (Scott et al., 2010) are compensation of VEGF production by these cells. $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice exhibited vascular defects characterized by a decrease in the number of endothelial tip cells and branching points. Theoretically, it is possible that reduced HIF-1α-dependent VEGF expression in the neuroretina caused these vascular defects in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. However, BrdU incorporation assay showed only moderate decrease in endothelial proliferation and no significant changes in the vessel regression, both of which are highly dependent on the VEGF-Notch signaling (Phng et al., 2009). These features indicate that the vascular phenotypes in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice are different from those seen in the condition of the VEGF inhibition or Notch gain-of-function. Instead, they fit into the abnormalities caused by a sparse ECM scaffold. Although our data suggest that decreased PDGF-A expression is significantly involved, alteration in the other paracrine factors may contribute to the astrocyte defects

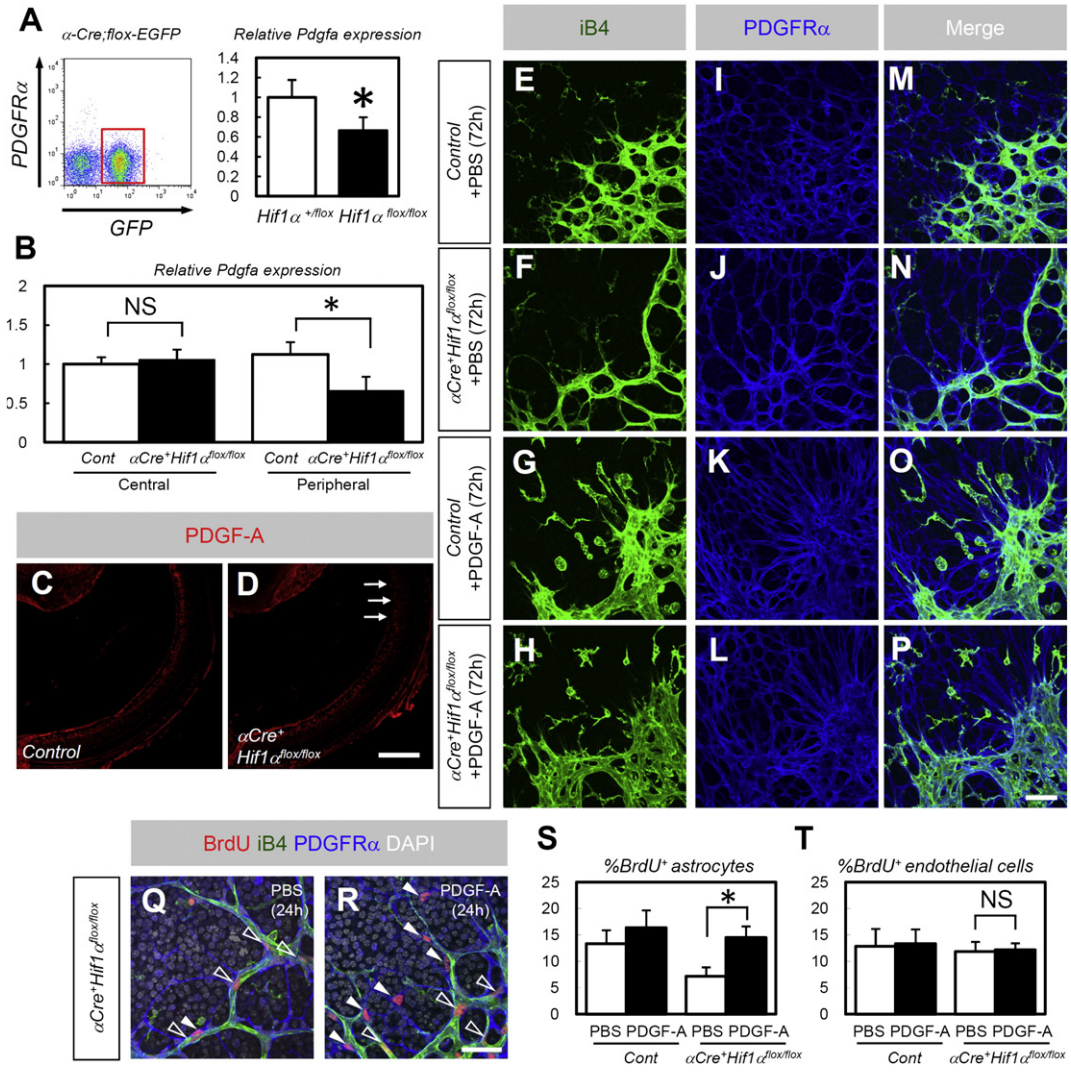

Fig. 7. Reduced PDGF-A expression underlies the hypoplastic astrocyte meshwork in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. (A) Quantitative RT-PCR analysis in GFP^{+} cells in *α-Cre*; *flox-CAT-EGFP*; $Hif1\alpha^{+/flox}$ or *α-Cre*; *flox-CAT-EGFP*; $Hif1\alpha^{flox/flox}$ mice (n = 6). (B) Quantitative RT-PCR analysis in retinal tissues harvested from central or peripheral area (n = 4). (C, D) Immunofluorescence of P6 retinas. Immunoreactivity for PDGF-A was decreased in the distal neuroretina of $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice (arrows in D). (E–P) Immunofluorescence on P6 retinas injected with PDGF-A or vehicle only at P3. $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice showed astrocytic and vascular response (H, L, P) to injected PDGF-A similar to control mice (G, K, O). (Q–T) Immunofluorescence on P6 retinas injected with PDGF-A or vehicle at P5, and quantification (n = 5). As an acute response (24 h) to PDGF-A injection, reduced proliferation in astrocytes (solid arrowheads) but not endothelial cells (clear arrowheads) in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice was restored. Scale bars: 200 μm in C, D; 50 μm in E–R; *p<0.05; **p<0.01; NS, not significant.

in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. For example, TNFα and IL-6 are known to promote astrocyte proliferation in vitro or in vivo (Burrows et al., 1997; Selmaj et al., 1990); however, to our knowledge, the genes for these cytokines are not targets of HIF-1α, and are unlikely, therefore, to be affected directly by HIF-1α deletion.

Retinal ganglion cells (RGCs) is one of the major population of neuroretinal cells in close proximity to retinal vasculature. RGC-deficient ($Brn3^{bZ\text{-}dta/+}$; Six3-Cre) mice show complete absence of retinal vasculature, suggesting a key role of RGCs in retinal vascularization (Sapieha et al., 2008). Sema3A and GPR91 (G protein-coupled receptor binding succinate) expressed in RGCs govern both developmental and pathological angiogenesis in retina (Joyal et al., 2011; Sapieha et al., 2008). As *α-Cre* is also expressed in RGCs, HIF-1α deletion in RGCs as well as RPCs may also contribute to our observed phenotypes.

The importance of neuroendothelial interactions is not limited to development. Malfunctioning of this cross-talk can cause, or influence, several vascular and neuronal disorders such as Alzheimer's disease (Zlokovic, 2005) and amyotrophic lateral sclerosis (Lambrechts et al., 2003). In the regenerating brain tissue after stroke, neural progenitor cells migrate along blood vessels towards the post-stroke striatum (Kojima et al., 2010; Yamashita et al., 2006). Interestingly, VEGF has direct effects on neural stem cells and motor neurons, as well as acting indirectly through its effects on endothelial cells (Segura et al., 2009; Storkebaum et al., 2005).

Finally, our data provide a novel mechanism for retinal hypoxic response required for proper vascular development. Our data suggest that the neuroretina, especially RPCs, which act as key players in the oxygen-sensing mechanisms, regulates the formation of the astrocyte template by secreting PDGF-A in a HIF-1 dependent manner.

Conclusion

We show that HIF-1α is most abundantly expressed in the neuroretina, especially RPCs. A neuroretina-specific knockout of HIF-1α ($\alpha Cre^{+}Hif1\alpha^{flox/flox}$) showed impaired vascular development characterized by decreased tip cell filopodia and reduced vessel branching. The astrocyte network was hypoplastic in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. Mechanistically, PDGF-A, a mitogen for astrocytes, was downregulated in the neuroretina of $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice. Supplementing PDGF-A

restored reduced astrocytic and vascular density in $\alpha Cre^{+}Hif1\alpha^{flox/flox}$ mice.

These data demonstrates that the neuroretina, especially RPCs, but not astrocytes acts as a primary oxygen sensor which ultimately controls the retinal vascular development by regulating an angiogenic astrocyte template.

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