HIF1A Is Essential for the Development of the Intermediate Plexus of the Retinal Vasculature

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PURPOSE. HIF1A is one of the major transcription factors that regulate tissue response to low oxygen tension. It controls expression of a large number of genes involved in cell survival, proliferation, angiogenesis, and other cellular processes. HIF1A is present at increased levels in the early postnatal retina. In this study its potential function during postnatal development of the mouse retina and retinal vasculature was analyzed.

METHODS. A mouse line was generated with a Cre-mediated Hif1a knockdown in the peripheral retina. Retinal morphology and vasculature were analyzed in sections and flat mount preparations. Gene and protein expression were determined by real-time PCR and Western blot analysis.

RESULTS. The Cre-mediated knockdown caused a significant reduction in Hif1a gene expression and HIF1A protein levels in the early postnatal retina. Retinal morphology was normal but the Hif1a knockdown prevented the formation of the intermediate vascular plexus in the peripheral retina. The primary plexus and the outer plexus were less affected. The Hif1a knockdown did not affect expression of such angiogenesis-related genes as vascular endothelial growth factor (Vegf) but strongly induced expression of erythropoietin (Epo). At the protein level, EPAS1 (HIF2A) was stabilized in the Hif1a knockdown mice.

CONCLUSIONS. The results suggest that HIF1A may be directly or indirectly required for normal development of the retinal vasculature, especially of the intermediate plexus. EPO but not VEGF may play a significant role in the development of this phenotype. HIF1A may not be the main factor that regulates Vegf expression during retinal development in the mouse. (Invest Ophthalmol Vis Sci. 2011;52:2109–2117) DOI:10.1167/iovs.10-6222

The retina is one of the most metabolically active tissues in the body and is therefore highly sensitive to alterations in oxygen tension. Hypoxia is defined as a state in which oxygen tension is below the normal limits in tissue beds. Systemic hypoxemia caused by lung or cardiac disease or occlusive vascular diseases in the eye may result in retinal hypoxia, with negative effects on retinal cell viability and function.

Hypoxia-inducible factors (HIFs) are the primary signaling proteins responsible for driving the cellular response to hypoxia. They regulate the transcription of more than 70 genes that encode proteins with a wide array of actions.2 HIF1, the most studied example of the HIF protein family, is a heterodimeric transcription factor consisting of a constitutively expressed subunit ARNT (aryl hydrocarbon receptor nuclear translocator) and an oxygen-regulated α-subunit (HIF1A). In the presence of normal oxygen levels, the HIF1A subunit is hydroxylated by proline-hydroxylase-2 (PHD-2/EGLN1), polyubiquitinated by the von-Hippel-Lindau (VHL) ubiquitin ligase complex, and subsequently degraded by proteasomes. Therefore, HIF-1 does not normally activate transcription of target genes in the presence of sufficient oxygen.3 However, in hypoxic conditions, HIF1A is stable and active, as hydroxylases and VHL proteins are inhibited by the lack of oxygen. In these conditions, HIF1A is able to interact with its co-activators and to dimerize with the constitutively expressed β-subunit. Once stabilized and assembled, the HIF1 complex can regulate the transcription of its target genes involved in cell survival, adaptation, anaerobic metabolism, immune reaction, cytokine production, vascularization, and general tissue homeostasis.4

The vasculature is of primary importance for tissue oxygenation. The vascular network that supplies the inner portion of the retina with oxygen and nutrients undergoes dramatic changes and reorganization during development. Initially, the hyaloid vasculature metabolically supports the inner part of the eye. In the later stages of development, the hyaloid vessels are replaced by the retinal vasculature. This switch occurs in humans at midgestation and in mice at birth. During the regression of the hyaloid vasculature, a vascular plexus emerges from the optic nerve head and spreads in the nerve fiber layer across the inner surface of the retina. In mice, the primary plexus reaches the periphery of the retina within 8 to 10 days after birth.5 This primary plexus eventually remodels into three parallel but interconnected networks located in the nerve fiber layer and the two plexiform layers. The deeper plexi of the retinal vasculature develop by sprouting from the primary plexus. Angiogenic sprouts start to penetrate the retina along the Müller cells, perpendicular to the primary plexus. They turn at the outer (forming the outer plexus) and inner (forming the intermediate plexus) boundaries of the inner nuclear layer (INL), to establish two additional capillary networks parallel to the primary plexus. This process is preceded by a transient expression of VEGF in cells of the INL. In contrast to the formation of the primary plexus, the deeper plexi develop independently of retinal astrocytes.6 Vascular differentiation occurs in a central-to-peripheral gradient, with vessels at the growing edge of the vascular network being less mature than the more central vessels.7 However, the mechanisms that in-
duce and guide deeperplexusangiogenesisare still largely unknown.

Because the retina of the newborn mouse is virtually avascular, lacking especially the two inner vascularplexi that do not form until the first 2 weeks after birth, reduced tissue oxygenation is expected to trigger an appropriate molecular response. Indeed, HIF1A levels are high in the young mouse retina and decrease as the retinal vasculature develops. In the present study, we used a conditional knockdown mouse with a selective downregulation of Hif1a in the retinal periphery to study the role of Hif1a for retinal development and specifically for the formation of the vascular network in the retina.

METHODS

Animals

All animal experiments were conducted in accordance with the regulations of the Cantonal Veterinary Authority of Zurich and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Conditional Hif1a knockdowns were generated by breeding Hif1a<sup>fl<sup>x</sup>/<sup>flox</sup></sup> mice<sup>*</sup> to mice expressing Cre recombinase under control of the α-element of the Pax6 promoter (α-Cre),<sup>9</sup> resulting in Hif1a<sup>fl<sup>x</sup>/<sup>flox</sup></sup> gene expression in retinal nerve head. (A) Detection of the Cre expression pattern as indicated by the expression of RFP in ROSA-flox-RFP;α-Cre mice (Table 1). cDNA levels were normalized to β-actin and are expressed relative to that at PND1 in the Hif1a<sup>fl<sup>x</sup>/<sup>flox</sup></sup> mice, which was set to 1. Differences in gene expression levels between the knockdown and control mice at individual time points were tested for significance with Student’s t-test; *P < 0.05; **P < 0.01. (C) Western blot showing retinal HIF1A protein levels at PND10 in Hif1a<sup>fl<sup>x</sup>/<sup>flox</sup></sup> mice without (Cre -) or with (Cre +) α-Cre. β-Actin served as loading control. Scale bar, 200 μm.

For study of the spatial expression of the Cre recombinase in α-Cre mice, a ROSA-flox-RFP reporter line<sup>10</sup> was crossed to Hif1a<sup>fl<sup>x</sup>/<sup>flox</sup></sup>;α-Cre animals. Retinas were cryosectioned and analyzed for red fluorescence protein (RFP) expression by fluorescence microscopy.

RNA Isolation, cDNA Synthesis, and Real-Time PCR

Retinas were isolated from the cornea, immediately frozen in liquid nitrogen, and stored at −80°C. Total retinal RNA was extracted with a kit (RNeasy isolation kit; Qiagen, Hilden, Germany), including a DNase treatment to digest residual genomic DNA. cDNA was prepared from equal amounts of total retinal RNA with oligo(dT) primers and M-MLV reverse transcriptase (Promega, Madison, WI). Ten nanograms of cDNA were amplified in a thermocycler (LightCycler 480, Roche Diagnostics AG, Rotkreuz, Switzerland) with a master mix (SYBR Green I; Roche Diagnostics AG) and the appropriate primer pairs (Table 1). cDNA levels were normalized to β-actin, and relative values were calculated with a respective calibrator sample.
**Western Blot Analysis**

For protein extraction, isolated retinas were sonified in 0.1 M Tris-HCl (pH 8.0) at 4°C, and protein content was determined with a Bradford assay. Protein extracts were mixed with sodium dodecylsulfate (SDS) sample buffer and incubated for 10 minutes at 95°C. Proteins were separated by SDS polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. After the membranes were blocked with 5% nonfat dry milk (Bio-Rad, Munich, Germany) in 10 mM Tris-HCl (pH 8), 150 mM NaCl, and 0.05% Tween-20, they were incubated with primary antibodies at 4°C overnight. The primary antibodies used were as follows: rabbit anti-HIF1α (1:1000; cat no. 100-479; Novus Biologicals, Littleton, CO); rabbit anti-EPAS1 (HIF2A, 1:500; cat. no. 100-480; Novus Biologicals); and mouse anti-β-actin (sc1616, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). After the membranes were incubated with horseradish peroxidase–labeled secondary antibodies for 1 hour at room temperature, the protein bands were visualized by the application of a chemiluminescent substrate (PerkinElmer, Boston, MA) and exposure to autoradiograph film (Super RX; Fujifilm, Dielsdorf, Switzerland).

**Morphology and Retinal Thickness**

Eyes were enucleated, fixed in glutaraldehyde, embedded in Epon, and processed for light microscopy, as previously described. A digitalized microscope (Axiovision; Carl Zeiss Meditec, Jena, Germany) was used to examine the slides. Retinal thickness was measured on 0.5-μm-thick nasotemporal sections at fixed distances (0, 150, 500, 1000, 1500, 1750, 2000, and 2250 μm) from the optic nerve head using ImageJ (ver. 1.43; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) for both Hif1α<sup>flk/flk</sup>;α-Cre mice and control littermates (n = 3).

**Immunofluorescence**

Eyes were isolated and incubated for 10 minutes in phosphate-buffered saline (PBS) containing 4% paraformaldehyde. Cornea and lens were removed, and the remaining tissue, including the retina, was incubated for another 15 minutes in 4% paraformaldehyde. The eye cups were then immersed in 30% sucrose, kept at 4°C overnight, and embedded in cryoprotective medium (Jung, Nussloch, Germany). Twelve-micrometer sections were cut (nasotemporal orientation), and the cryoslices

**Figure 2.** Normal retinal morphology in Hif1α knockdown mice. (A) Retinal morphology of Hif1α<sup>flk/flk</sup>;α-Cre mice, and Hif1α<sup>flk/flk</sup> littermates at PND10, -35, and -60 were analyzed. Shown are representative sections of the retinal periphery of at least three animals per time point. RPE, retinal pigment epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 100 μm. (B) The thickness of the retina and of the photoreceptor layer (ONL plus photoreceptor segments) in Hif1α<sup>flk/flk</sup>;α-Cre mice and control littermates. Thickness was measured at 0, 150, 500, 1000, 1500, 1750, 2000, and 2250 μm from the optic nerve head (ONH) in both nasal and temporal hemispheres, as indicated. Mean ± SD; n = 3. Ticks on the x-axis correspond to 500 μm.
were blocked in a humid chamber with PBS containing 3% normal goat serum and 0.3% Triton X-100. The slides were incubated with rabbit anti-VSX2 (CHX10; 1:500) antibody (the generous gift of Connie Cepko, Harvard University, Boston, MA), rabbit anti-GNAT1 (sc-389, 1:500; Santa Cruz Biotechnology), rabbit anti-GNAT2 (sc-390, 1:500; Santa Cruz Biotechnology), mouse anti-BRN3A (MAB1585, 1:100; Chemicon, Billerica, MA), mouse anti-GS (B302, 1:500; Millipore, Billerica, MA), mouse anti-GFAP (G-3893, 1:500; Sigma-Aldrich, St. Louis, MO), mouse anti-SYP (synaptophysin, NCL-L-SYNAP-299, 1:100; Novocastra, Newcastle, UK), Griffonia simplicifolia isolecitin IB4-Alexa594 (I21413, 1:50; Invitrogen, Basel, Switzerland), and rabbit anti-CALB1 (calbindin, AB1778, 1:500; Chemicon). After being washed with PBS, the slides were incubated with Cy3-labeled secondary antibodies (Jackson ImmunoResearch Europe, Newmarket, UK), for 1 hour at room temperature, washed with PBS, counterstained with 4,6-diamidino-2-phenylindole dilactate (Molecular Probes, Invitrogen, Carlsbad, CA) and mounted with polyvinyl alcohol (Mowiol 4-88 Reagent; VWR International AG, Lucerna, Switzerland). Immunofluorescent stainings were analyzed with the digitalized microscope (Axiovision; Carl Zeiss Meditec).

**Flat Mount Immunofluorescence and Quantification of Vasculature**

Eyes were isolated and incubated for 3 to 5 minutes in phosphate-buffered saline (PBS) containing 2% paraformaldehyde. The cornea and lens were removed, and the retina was dissected from the sclera and flat mounted in PBS. The retina was then stored in methanol at −20°C. Before immunofluorescence analysis, retinal flat mounts were incubated another 10 minutes in 4% paraformaldehyde in PBS and blocked with PBS containing 3% normal goat serum and 0.3% Triton X-100. Flat mounts were incubated with the vascular-specific G. simplicifolia isolecitin IB4-Alexa594 (I21413, 1:50; Invitrogen) overnight. After they were washed with PBS, the flat mounts were mounted with polyvinyl alcohol (Mowiol 4-88 Reagent; VWR International AG). Immunofluorescent staining was analyzed with the digitalized microscope (Axiovision; Carl Zeiss Meditec) or with a laser scanning confocal microscope (model SP5; Leica, Wetzlar, Germany). Image-analysis software (Imaris; Bitplane AG, Zurich, Switzerland) was used to analyze confocal microscope z-stacks and to generate x–z projections.

![Image of retinal cell types](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932973/)
For quantification of vascular coverage, three regions (770 μm²) at a distance of 2000 μm from the optic nerve head were analyzed for each flat-mounted retina (PND60) by laser scanning confocal microscopy (n = 3 for both the Hif1a^floxflox;α-Cre mice and the control littermates). The image-analysis software (Imaris; Bitplane AG) was used to analyze confocal microscope z-stacks and to generate x–y-sections for each vascular layer. The sections layer were then postprocessed (thresholded and binarized), with ImageJ used to obtain binary data from which vascular coverage was determined (ratio of black pixels to total pixels).

RESULTS

Peripheral Hif1a Knockdown Does Not Lead to Abnormal Retinal Architecture

Cre recombinase in the α-Cre mouse is expressed starting around E10.5 and leads to deletion of floxed sequences in most cells of the peripheral but not of the central retina.9 Visualization of expressed RFP protein after Cre-mediated deletion of a floxed STOP sequence in 21-day-old ROSA-flox-RFP;α-Cre mice confirmed the spatial distribution of Cre activity and revealed that virtually every cell type in the peripheral retina expressed Cre recombinase (Fig. 1A). In contrast, the central retina in proximity to the optic nerve head did not show RFP protein, indicating that Cre recombinase was not expressed in this region (Fig. 1A). The Hif1a^floxflox;α-Cre knockdown mice showed a reduced retinal expression of Hif1a mRNA as early as PND5 (Fig. 1B). Similarly, HIF1A protein levels were severely downregulated in the knockdown retina (Fig. 1C). The strong downregulation of HIF1A protein at PND10 suggests that in the early postnatal retina, HIF-1A is normally stabilized mainly in the periphery, the region of Cre recombinase activity in knockdown mice. This possibility is conceivable because the central retina is already fully vascularized at PND10, whereas the outer and intermediate plexi are not yet formed in the peripheral retina,10,11 which may lead to hypoxic stabilization of HIF1A in wild-type retinas.

Analysis of the retinal morphology by light microscopy revealed no abnormalities or degeneration in the Hif1a^floxflox;α-Cre mice (Fig. 2A). The architecture of the Hif1a knockdown retina at PND10 appeared normal in the center (not shown) as well as in the periphery, where Cre-mediated deletion of HIF1A genomic DNA led to a knockdown of HIF1A protein. Maturation of the retina also proceeded normally, resulting in a retinal morphology indistinguishable from that of the control mice, at least up to PND60, the latest time point analyzed (Fig. 2A). Normal retinal morphology was further supported by a similar thickness of the retina and of the photoreceptor layer in the knockdown and wild-type mice at PND60 (Fig. 2B).

In line with normal retinal morphology, no appreciable differences in localization and spatial arrangement of the main neuronal and glial cells were observed between the knockdown and control retinas (Fig. 3). It is important to note that expression of GFAP was not upregulated in Müller cells of the Hif1a^floxflox;α-Cre mice. This finding suggests that the Hif1a knockdown retinas were not gliotic and indicates that the absence of Hif1a did not lead to retinal stress or injury.

Hif1a Knockdown Results in a Reduced Peripheral Vascular Density

Since HIF1A levels are high in the early postnatal retina of wild-type mice and are reduced concomitantly with the development of the retinal vasculature,7 we analyzed the vessel network in retinal flat mounts of the Hif1a knockdown and wild-type mice. At PND5, vessels of the primary plexus covered about two thirds of the retinal surface in both the control and knockdown mice (Fig. 4). The superficial capillary network of the primary plexus extended toward the periphery with approximately the same kinetics and reached the periphery at ~PND10 in both the Hif1a knockdown and control mice. Thus, the lack of HIF1A in the peripheral retina did not affect the correct timing of the development of the postnatal retinal vasculature.

When we analyzed the vasculature in more detail, the Hif1a^floxflox;α-Cre mice showed an incomplete development of the intermediate plexus in the peripheral retina at PND21 (Fig. 5). In contrast, the primary plexus and the outer plexus were well established. In the central retina of the Hif1a^floxflox;α-Cre mice (where Cre recombinase is not expressed and floxed gene sequences are not deleted6), both deeper plexi were present and showed a pattern comparable to that in the control mice (Fig. 5A). The incomplete vasculature in the periphery persisted at least until PND60 (the last time point observed). Confocal imaging of flat-mounted retinas of the wild-type controls at PND60 showed the dense capillary network of the intermediate plexus (Fig. 5B). In the Hif1a knockdown mice, however, horizontally extending vessels were largely absent in the intermediate plexus and only individual spots were visible in the peripheral retina. The x–z-stacks of the area shown in the flat mounts revealed that these spots were most likely vessels connecting the primary plexus with the outer plexus without establishing the intermediate plexus (Fig. 5B). The area shown for the Hif1a knockdown retina in Figure 5B shows the transition zone from the central to the peripheral retina. The asterisk demarcates the more central area where the vascular network of the intermediate plexus was still visible. In contrast, horizontally extending capillaries were missing in the adjacent peripheral retina, resulting in an x–z stack with no horizontal intermediate plexus visible in the more peripheral region of the retina (left side on x–z stack) but with an intermediate plexus formed in the region of the more central retina (right side on the x–z stack). The two yellow lines in each x–z stack show the focal plane of the flat mounts above the stacks. In contrast to the intermediate plexus, vessel density in the primary and outer plexi was less affected in the knockdown retinas. When the extent of vascular coverage in 21-day-old retinas was quantified for each individual plexus, the knockdown mice showed a reduction of 75% ± 0.06% SD (P < 0.01) in the extent of vascularization of the intermediate plexus compared with the controls.

![Hif1a Knockdown Results](image-url)

**FIGURE 4.** The development of the primary plexus in Hif1a knockdown mice. Retinal flat mounts of the Hif1a^floxflox;α-Cre mice (right) and the Hif1a^floxflox littermates (left) at PND5 (top) and PND10 (bottom). Vessels were stained with isoelectric IB4 coupled to Alexa594. Shown are representative images. Scale bar, 500 μm.

**FIGURE 5.** The development of the intermediate plexus in Hif1a knockdown mice. (A) PND5, vessels formed in the more central retina (right side on the x–z stack). The two yellow lines in each x–z stack show the focal plane of the flat mounts above the stacks. In contrast to the intermediate plexus, vessel density in the primary and outer plexi was less affected in the knockdown retinas. When the extent of vascular coverage in 21-day-old retinas was quantified for each individual plexus, the knockdown mice showed a reduction of 75% ± 0.06% SD (P < 0.01) in the extent of vascularization of the intermediate plexus compared with the controls.

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A mildly increased vascular density of 130% ± 0.11% SD (P < 0.05) of that of the control littermates was observed for the primary plexus in the Hif1a\textsuperscript{flox/flox} knockdown mice. At the level of the outer plexus, no significant differences were detected. This result suggests that HIF1A has a direct or indirect role in the development of a correct retinal vasculature and especially of the intermediate plexus.

**Erythropoietin (Epo) Is Upregulated in Hif1a Knockdown Mice**

Even though HIF1A protein levels (Fig. 1B) and vessel density (Fig. 5) were reduced in the Hif1a\textsuperscript{flox/flox} knockdown mice, no significant differences were observed between the knockdown and control littermates at PND21. Images of the vascular layers in flat mounts were taken by adjusting the focal plane of the confocal microscope accordingly. Vessels were highlighted by artificial coloring (primary plexus: blue; intermediate plexus: green; outer plexus: red). Note that the density of the vessels in the intermediate plexus was strongly reduced in the peripheral retina of the Hif1a knockdown mice. (B) Representative images of the intermediate plexus in flat mounts of Hif1a\textsuperscript{flox/flox} littermates (left), and Hif1a\textsuperscript{flox/flox};\alpha-Cre mice (right) at PND60. The focal plane was adjusted to visualize exclusively the intermediate plexus (top). The \(x\)-z-stacks (bottom) are artificial sections and show the sum of all signals (including signals in the primary, the intermediate, and the outer plexi) of the area shown in the flat mount panels above the stacks. For better recognition of the different plexi, the \(z\)-value of the \(z\)-stacks was increased five times (\(x = 1; y = 1; z = 5\)). Yellow lines depict the focal planes used for the flat mount pictures. Note the absence of a continuous capillary network in the intermediate plexus of Hif1a\textsuperscript{flox/flox};\alpha-Cre mice. In these mice, such a network is only formed in the lower right corner of the flat-mounted retina, which is the more central area of the retina and presumably the region where HIF1A was not knocked down. (*) The more central retina. Scale bar: (A) 200 \(\mu\)m (flat mounts); 100 \(\mu\)m (sections); (B) 100 \(\mu\)m.

**FIGURE 5.** Lack of the intermediate plexus in the peripheral retina of Hif1a knockdown mice. (A) Immunostaining of blood vessels (isolectin IB4, coupled to Alexa594) in Hif1a\textsuperscript{flox/flox} littermates (left) and Hif1a\textsuperscript{flox/flox};\alpha-Cre mice (right). Shown are flat mounts (top) and retinal cryosections (bottom) at PND21. Images of the vascular layers in flat mounts were taken by adjusting the focal plane of the confocal microscope accordingly. Vessels were highlighted by artificial coloring (primary plexus: blue; intermediate plexus: green; outer plexus: red). Note that the density of the vessels in the intermediate plexus was strongly reduced in the peripheral retina of the Hif1a knockdown mice. (B) Representative images of the intermediate plexus in flat mounts of Hif1a\textsuperscript{flox/flox} littermates (left), and Hif1a\textsuperscript{flox/flox};\alpha-Cre mice (right) at PND60. The focal plane was adjusted to visualize exclusively the intermediate plexus (top). The \(x\)-z-stacks (bottom) are artificial sections and show the sum of all signals (including signals in the primary, the intermediate, and the outer plexi) of the area shown in the flat mount panels above the stacks. For better recognition of the different plexi, the \(z\)-value of the \(z\)-stacks was increased five times (\(x = 1; y = 1; z = 5\)). Yellow lines depict the focal planes used for the flat mount pictures. Note the absence of a continuous capillary network in the intermediate plexus of Hif1a\textsuperscript{flox/flox};\alpha-Cre mice. In these mice, such a network is only formed in the lower right corner of the flat-mounted retina, which is the more central area of the retina and presumably the region where HIF1A was not knocked down. (*) The more central retina. Scale bar: (A) 200 \(\mu\)m (flat mounts); 100 \(\mu\)m (sections); (B) 100 \(\mu\)m.
**DISCUSSION**

The development of the retinal vasculature in mice starts shortly after birth at the optic nerve head in the center of the retina and expands toward the periphery. First, the primary plexus develops and reaches the retinal periphery after 8 to 10 days. Vessel growth is guided by a preestablished network formed by astrocytes. From the primary plexus, angiogenic sprouts emerge, and vessels start to penetrate the retina, perpendicular to the primary plexus. Transient expression of VEGF precedes formation of the intermediate and outer plexi, which develop from the center toward the periphery, independent of the retinal astrocytes. This process was obviously disturbed in mice lacking the Hif1a knockdown and control mice at PND10. At this time point, also Epo was expressed at similar low levels in the two mice (Fig. 6).
HIF1A in the peripheral retina. Whereas vessels of the primary plexus and of the outer plexus developed similarly in the knockdown and control mice, the capillaries of the intermediate plexus were significantly reduced in the retinal periphery of the Hif1a knockdowns. It is interesting to note that the intermediate plexus, which is incompletely formed in the knockdown retina, is the last vascular plexus to develop in the normal postnatal mouse retina. The significance of this observation must be established, but it may suggest regulatory mechanisms specific for each vascular plexus.

The most prominent candidate for involvement in vessel formation is VEGF, a potent angiogenic and vasopermeability factor, whose contribution to general and ocular angiogenesis has been extensively studied.13,14 The gene for VEGF is strongly regulated by HIFs, which leads to increased production and secretion of VEGF under hypoxic conditions.15 Several retinal cell types, including endothelial cells, astrocytes, pericytes, Müller cells, and retinal pigment epithelial cells, upregulate VEGF expression under conditions of low oxygen tension.16,17 Although the Hif1a<sup>flox/flox</sup>-Cre<sup>-</sup> mice had severely reduced levels of HIF1A in the developing retina (Fig. 1) and a pronounced vessel phenotype, no reduction in VEGF expression was detected. This surprising observation is in line with recent results of Weidemann et al.,18 who showed that astrocyte- and Müller cell-specific Hif1a knockdown also did not influence VEGF expression in the retina. Given the strong knockdown of HIF1A and the unaltered VEGF expression levels in the Hif1a<sup>flox/flox</sup>-Cre<sup>-</sup> mice, our results support the conclusion that transcription factors other than HIF1A are the main regulators of VEGF expression during retinal development. Although the identity of these factors has yet to be established, recent results suggest a prominent role of EPAS1 in the regulation of VEGF expression. Knockdown of the von Hippel-Lindau (VHL) protein during retinal development led to the activation of HIF1 and -2 and to increased VEGF expression.19,20 Whereas the simultaneous knockdown of Vbl and Hif1a also led to increased VEGF levels, simultaneous ablation of Vbl and Epas1 (Hif2a) did not.18 Furthermore, functional studies in a mutant cell line expressing neither HIF1A nor EPAS1 showed stronger transactivation of the VEGF promoter by EPAS1.21 In addition, the introduction of Epas1 cDNA into 293 fetal kidney cells22 or an adenovirus-mediated delivery of the Epas1 gene in mouse wound-healing models significantly induced the expression of VEGF.23 These data suggest that EPAS1 may be able to regulate VEGF expression, either alone or in conjunction with HIF1A. Therefore, the increased expression of EPAS1 in our model may compensate for the downregulation of HIF1A, resulting in unaltered expression levels of VEGF. However, despite normal VEGF expression, the intermediate plexus did not develop, suggesting an essential role for other, HIF1A-controlled factors in this process. Importantly, knocking down Hif1a in astrocytes and Müller cells did not lead to alterations in the vascular organization.18 This result is in contrast to the reduced vessel density and the lack of the peripheral intermediate plexus in our pan-Hif1a knockdown mouse line, suggesting that the correct formation of the retinal vasculature, or at least of the intermediate plexus, may be regulated by a correct Hif1a-expressing profile in cells other than Müller cells or astrocytes.

Our results are also in line with those in recent studies that elucidated the role of Hif1a in the postnatal vascular development and/or oxygen-induced neovascularization by pharmacologic approaches. Yoshida et al.24 showed that digoxin reduces HIF1A levels in ischemic tissues in vivo and suppresses retinal and choroidal neovascularization. Similarly, DeNiro et al.25 reported that another inhibitor of HIF1A, YC-1, decreases basal expression levels of HIF1A protein in normoxia and inhibits HIF1A protein synthesis, stability, and nuclear translocation in hypoxia. This effect was accompanied by a significant influence on HIF1 target gene expression that finally resulted in a reduction of vascular density in the retina.25 Both of these studies confirm that inhibition of HIF1 results in a reduction of retinal vascularization. These results, along with our data and the availability of HIF1A inhibitors, validate the potential of modulating the Hif1a signaling pathway as a therapeutic strategy to inhibit vessel growth.

Although the lack of the capillaries in the intermediate plexus most likely affects delivery of nutrients and oxygen to cells especially of the INL, retinal architecture, and the number and distribution of cells were not affected by the Hif1a knockdown. Compensatory mechanisms may thus be in place, allowing the cells to cope with this special situation. A possible candidate for involvement in such an adaptive response is EPO, which was shown to be neuroprotective of retinal cells.26,27 Epo expression was upregulated in the knockdown retinas (Fig. 6) around the time when the retinal vasculature should be mature. EPO may thus be part of the retinal response to improper vascularization. Interestingly, EPO was not only shown to be neuroprotective, but it was also suggested to be involved in angiogenesis and thus in the regulation of the growth of blood vessels.28,29 It was also suggested that EPO is a main factor in the development of retinopathy of prematurity (ROP)30–32 and is involved in vessel stability.33 The upregulation of Epo expression in the Hif1a knockdown mice may thus protect cells from damage but may correct the vascular abnormalities. Increased expression of Epo was probably caused by the increased presence of EPAS1 in the knockdown retinas. EPAS1 has been shown to be the main regulator of Epo expression in other organs,34,35 and EPAS1, like HIF1A, is regulated through stabilization in hypoxic conditions. Lack of the development of the intermediate plexus may have led to reduced oxygen tension in the retina and thus to the stabilization of EPAS1.

In general, our results establish and support the importance of a correct spatial and temporal expression profile of Hif1a for the development of a normal retinal vasculature and especially for the formation of the intermediate plexus.

**Acknowledgments**

The authors thank Coni Imsand for excellent technical assistance with retinal morphology and genotyping, the Center for Microscopy and Image Analysis of the University of Zurich for assistance with confocal microscopy analysis, and Hans Joerg Fehling (University Clinics Ulm, Germany) for providing us the ROSA-Iox-RFP reporter mice.
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