Hypoxia Inducible Factor-2α Regulates the Development of Retinal Astrocytic Network by Maintaining Adequate Supply of Astrocyte Progenitors

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Abstract

Here we investigate the role of hypoxia inducible factor (HIF)-2α in coordinating the development of retinal astrocytic and vascular networks. Three Cre mouse lines were used to disrupt floxed Hif-2α, including Rosa26(CreERT2), Tie2(Cre), and GFAP(Cre). Global Hif-2α disruption by Rosa26(CreERT2) led to reduced astrocytic and vascular development in neonatal retinas, whereas endothelial disruption by Tie2(Cre) had no apparent effects. Hif-2α deletion in astrocyte progenitors by GFAP(Cre) significantly interfered with the development of astrocytic networks, which failed to reach the retinal periphery and were incapable of supporting vascular development. Perplexingly, the abundance of strongly GFAPα mature astrocytes transiently increased at P0 before they began to lag behind the normal controls by P3. Pax2α and PDGFRα astrocyte progenitors and immature astrocytes were dramatically diminished at all stages examined. Despite decreased number of astrocyte progenitors, their proliferation index or apoptosis was not altered. The above data can be reconciled by proposing that HIF-2α is required for maintaining the supply of astrocyte progenitors by slowing down their differentiation into non-proliferative mature astrocytes. HIF-2α deficiency in astrocyte progenitors may accelerate their differentiation into astrocytes, a change which greatly interferes with the replenishment of astrocyte progenitors due to insufficient time for proliferation. Rapidly declining progenitor supply may lead to premature cessation of astrocyte development. Given that HIF-2α protein undergoes oxygen dependent degradation, an interesting possibility is that retinal blood vessels may regulate astrocyte differentiation through their oxygen delivery function. While our findings support the consensus that retinal astrocytic template guides vascular development, they also raise the possibility that astrocytic and vascular networks may mutually regulate each other’s development, mediated at least in part by HIF-2α.

Introduction

Retinal vascular development is closely associated with the development of an astrocytic template. Earlier studies found that retinal astrocytes were present in animal species with vascularized retinas but absent in animals with avascular retinas [1]. More recent studies employed gene targeting approach in mice to address the relationship between astrocytic and vascular development. Tlx null mutation led to poor astrocytic and vascular development in the retina [2]. Besides reduced astrocyte numbers, Tlx null mice also displayed poor assembly of extracellular fibronectin matrices [3], and astrocyte specific Tlx disruption further demonstrated that the expression of both fibronectin and heparan-sulfate was compromised [4]. These extracellular components were thought to mediate retinal vascularization by regulating VEGF-A binding and distribution [4].

In rodents, the development of retinal astrocytic network begins at birth with immigration of Pax2-positive cells from the optic nerve, spreading in a centrifugal direction in the retinal inner surface [5,6]. Pax2+ cell population gives rise to both optic nerve astrocytes and retinal astrocytes, with the progenitors to the latter also expressing PDGFRα in addition to Pax2 [7]. PDGFRα expression is critical to the proliferation of immature retinal astrocytes in response to stimulation by PDGFA from retinal ganglion cells [8]. As astrocyte progenitors migrate towards the retinal periphery, vascular structures emerge from the optic nerve, forming a vascular network which expands towards retinal periphery behind the PDGFRα astrocytic network. In vascularized areas, astrocyte maturation occurs, presumably mediated by endothelial cell derived leukemia inhibitory factor (LIF) [9–11]. Mature astrocytes exhibit high level expression of glial fibrillary acidic protein (GFAP), whereas Pax2 expression is lost [12–14].

How the astrocytic network facilitates retinal vascular development remains incompletely understood. In spite of the important role of VEGF-A in vascular development [15–17], astrocyte specific disruption of VEGF-A expression did not interfere with retinal vascular growth, although vascular stability was compromised [18]. Thus, VEGF-A for retinal vascular development is presumably derived from non-astrocytic cells. However, VEGF-A extracellular distribution in developing retinas may be controlled by astrocyte-derived fibronectin and heparan sulfate [3,4]. In addition, R-cadherin in retinal astrocytes is also important for...
constitutively active. The Rosa26CreERT2 mouse line was a gift from A. Joyner (New York University School of Medicine, New York, New York, USA) and was similar to a related mouse line described by Sheher et. al. [27,28]. Rosa26CreERT2-encoded CreER T2 was activated by tamoxifen. Unless specifically indicated, neonatal mice were treated with tamoxifen by daily oral gavage from postnatal day 1 (P1) through P3 (40 mg/kg of body mass, with tamoxifen dissolved in corn oil). In some experiments, pregnant mothers were treated with tamoxifen by oral gavage at 17.5 days post coitum, followed by two more doses delivered to neonates at P1 and P2. In addition, some mice were treated with tamoxifen at P0 through P2. In all cases, pups with or without Rosa26CreERT2 were treated with tamoxifen in parallel.

Genotyping

Mouse ear punch (for weaned mice) and toe clips (for neonatal mice younger than P8) were used for DNA extraction. To determine deletion efficiency in the retina, P3 retinal tissues were used for DNA isolation and PCR analysis. Various alleles were identified using the following primer pairs: Hif-1α primers were as described previously [29]; Hif-2α primers: EPASlsoF (AGTTCTGCTCCGTCAAGAA) and EPASlsoR (TTGGCA-GAGGGGAGATCTAATTG), wt, 638 bp; floxed, 877 bp; deleted, 260 bp; Rosa26CreERT2 knock in allele was determined as described previously [27]; Tie2Creα transgenic: Tie2CreF (CCTGCTTTCCACGTGCTC) and Tie2CreR (GGCTGCGGATCATGCCATGG), 260 bp; GFAPCreα transgenic: GFAPCreF (ACTCTCTCCAAAGGCCCTTG) and GFAPCreR (ATCACTCGTTGGCATGACCG), 290 bp. All PCR reactions were carried out under the following conditions: 94°C for 9 minutes to activate Taq DNA polymerase (Life Technologies), followed by 35 cycles at 55°C for 1 minute, 72°C for 4 minutes, and 94°C for 30 seconds.

Whole mount staining of neonatal retinas with isolectin B4 conjugated to Alexa fluor®-594 (B4-Alexa 594)

IB3 staining of neonatal retinas was carried out as described before [30]. Briefly, neonatal mice were euthanized by decapitation at indicated stages between P0 and P8, and eyes were isolated by enucleation. Isolated eyes were fixed with 4% paraformaldehyde (PFA) for 45 minutes at room temperature, following which retinas were dissected out. Retinas were cut by four incomplete radial incisions, leaving 4 pedals attached to one another at the center. Retinas were incubated at 4°C overnight with B4-Alexa 594 (Life Technologies) at 1:100 dilution in Retina Staining Buffer (RSB), which consisted of phosphate buffered saline (PBS), 1 mM CaCl2, 1 mM MgCl2, 1% Triton X100, and 1% BSA. Stained retinas were washed three times in RSB (1 hour each with rocking), and flat-mounted in 50% glycerol in PBS. Imaging was carried out by confocal microscopy.

Immunofluorescence (IF) staining of flat mount retinas

Retinas were prepared as described for B4-Alexa 594 staining, and incubated with primary antibodies in RSB at 4°C overnight. Primary antibodies included rabbit anti-GFAP (1:200, Life Technologies), rat anti-GFAP (2 μg/ml, Life Technologies), rabbit anti-Pax2 (1 μg/ml, Life Technologies), and goat anti-PDGFRα (1 μg/ml, R&D Systems). For Pax2 and GFAP double IF staining, rabbit anti-Pax2 and rat anti-GFAP were used. Following incubation with primary antibodies, retinas were washed, and incubated overnight with appropriate secondary antibodies including goat anti-rabbit IgG-Alexa fluor®-488 (1:200, Life Technologies), donkey anti-rat IgG-Cy3 (2 μg/ml, Jackson ImmunoResearch, West Grove, PA), and donkey anti-goat IgG-Alexa fluor®-594. Stained retinas were washed thoroughly, and mounted in 50% glycerol in PBS. Images were taken with a Zeiss LSM 510 confocal microscope.

Detection of proliferative and apoptotic astrocyte progenitors

For proliferation assay, neonatal mice were injected with 5-bromo-2-deoxyuridine (BrdU, 120 mg/kg of body mass; Roche Applied Science, Indianapolis, IN), and euthanized after 60 minutes. Retinas were fixed in 4% paraformaldehyde with the rest of...
the eyes still attached, and stored in 70% ethanol overnight. Thereafter, fixed retinas were dissected away from other eye structures, treated with 1% Triton X-100 in PBS for 30 minutes at room temperature, and then incubated in 2 mol/L HCl for 1 hour at 37°C. Processed retinas were double stained with mouse anti-BrdU antibody (BD Biosciences, San Jose, CA) and rabbit anti-Pax2, followed by goat anti-mouse IgG-Alexa fluor®-594 and goat anti-rabbit IgG-Alexa fluor®-488.

Apoptosis was monitored by IF staining with anti-active Caspase 3 (Abcam, Cambridge, MA). For positive control, neonatal mice were exposed to 75% oxygen at P7-P8 for 16 hours, returned to room air, and euthanized. Retinas from oxygen exposed mice were dissected, fixed, and stained with anti-active Caspase 3.

**Immunofluorescence staining of retinal cryosections**

Retinas prepared as above were embedded in OCT®, and cut at 6 μm. Sections were stained with rabbit anti-GFAP, rabbit anti-Pax2, or mouse anti-neurofilament (anti-NF, University of Iowa Developmental Studies Hybridoma Bank) in RSB for 2 hours. Afterwards, sections were washed, and stained with appropriate secondary antibodies, including goat anti-rabbit IgG-Alexa fluor®-488, or goat anti-mouse IgG-DyLight 488 (1:200, Jackson ImmunoResearch). After washing and mounting, images were analyzed by confocal microscopy.

**Preparation of nuclear protein extracts and Western blotting**

Nuclear protein extract were prepared as described previously [31]. Briefly, neonatal mice were euthanized by decapitation, and retinas were isolated immediately in pre-chilled PBS, which was frequently replaced with additional aliquots of PBS precooled on ice. Nuclear protein extracts were prepared by homogenizing retinas in ice cold nuclear extraction buffer (10 mM HEPES-KOH [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM deferoxamine (Sigma), 0.1% NP-40, and 1× complete protease inhibitor cocktail (Roche)). Nuclei were collected by centrifugation, and resuspended in ice cold re-suspension buffer containing 20 mM HEPES-KOH [pH 7.9], 420 mM NaCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.2 mM deoxyferroxamine, 1× protease inhibitor cocktail, 0.2 mM phenylmethylsulfonyl fluoride, and 25% glycerol. The following antibodies were used for Western blotting: anti-HIF-1α (NB100-449, Novus Biologicals), anti-HIF-2α (NB100-132, Novus Biologicals), and anti-β-actin (sc-1616; Santa Cruz Biotechnology).

**Statistical analysis**

Data were evaluated by two tailed Student’s t-tests using Excel, and are presented as means ± standard error of means (SEM). All n values refer to number of mice. p < 0.05 was considered significant.

**Results**

**Differential but partially overlapping roles of HIF-1α and HIF-2α in retinal vascular development**

To compare the roles of HIF-1α and HIF-2α, we generated HIF-1α<sup>f/f</sup>/Rosa26<sup>CreERT2</sup> (f/f floxed) and HIF-2α<sup>f/f</sup>/Rosa26<sup>CreERT2</sup> mice. To activate CreERT2, neonatal mice were treated with tamoxifen by daily oral gavage at P1–P3. This procedure was previously shown to result in highly efficient deletion of floxed exons in a variety of tissues including the neonatal retina [27,31]. In this study, we were also able to demonstrate significant deletion in retinal tissues (Figure S1).

At P8, the development of primary vascular plexus was examined by IB, staining of retinas from mice treated with tamoxifen at P1–P3. Vascular development was indistinguishable between HIF-1α<sup>f/f</sup> and HIF-1α<sup>f/f</sup>/Rosa26<sup>CreERT2</sup> mice (Figure 1A, D, G, and J), but was significantly reduced in HIF-2α<sup>f/f</sup>/Rosa26<sup>CreERT2</sup> mice (Figure 1B, E, H, and K). The number of vascular branches was quantified in 0.2 x 0.5 mm areas midway between the optic nerve head and the periphery (Figure 1M). HIF-1α<sup>f/f</sup> and HIF-1α<sup>f/f</sup>/Rosa26<sup>CreERT2</sup> mice both had similar number of vascular branches (104.3±7.4 and 95.2±5.7 per 0.1 mm², respectively; n = 6, p=0.14). By contrast, HIF-2α<sup>f/f</sup>/Rosa26<sup>CreERT2</sup> mice had substantially reduced branching activities (102.1±5.2 in HIF-2α<sup>f/f</sup> mice, 49.3±11.1 in HIF-2α<sup>f/f</sup>/Rosa26<sup>CreERT2</sup> mice, n = 7, p<0.01). When tamoxifen treatment was performed at earlier time points (starting at P0 or E17.5), vascular morphogenesis was further reduced in HIF-2α<sup>f/f</sup>/Rosa26<sup>CreERT2</sup> mice (Figure S2).

To determine if HIF-1α and HIF-2α might have any overlapping functions, we generated HIF-1α<sup>f/f</sup>/HIF-2α<sup>f/f</sup>/Rosa26<sup>CreERT2</sup> mice. Vascular development in double disrupted mice was not only defective compared to floxed controls (Figure 1C, F, I, and L), but was also more severely compromised than in HIF-2α<sup>−/−</sup>/Rosa26<sup>CreERT2</sup> mice (Figure 1H, I, K and L). While the number of vascular branches in HIF-2α<sup>−/−</sup>/HIF-2α<sup>f/f</sup>/Rosa26<sup>CreERT2</sup> mice was about half of floxed controls, the corresponding value in HIF-1α<sup>−/−</sup>/HIF-2α<sup>f/f</sup>/Rosa26<sup>CreERT2</sup> mice was further reduced to about 26.0% (Figure 1M).

**Apparent normally retinal vascular development following Tie2<sup>Cre</sup>-mediated disruption of floxed HIF-1α or HIF-2α in endothelial cells**

Next, we investigated if HIF-1α or HIF-2α disruption in endothelial cells might cause vascular deficiency. For this test, we used Tie2<sup>Cre</sup> mice, which was well documented for its high efficiency in deleting floxed DNA sequences in endothelial and hematopoietic cells [26,32]. For example, we showed in a previous study that Tie2<sup>Cre</sup> mediated disruption of floxed Vegf-1 led to vascular defects identical to those in Vegf-1 germline null embryos [30].

To directly test Cre activity in the context of retinal vascular development, we crossed Tie2<sup>Cre</sup> mice with tdTomato reporter mice, the latter of which carried a Cre-inducible tdTomato expression cassette integrated into the Rosa26 locus [25]. Tie2<sup>Cre</sup> specifically activated the expression of tdTomato in retinal vascular structures (Figure 2A and B), demonstrating that within the retina, Cre activity was most predominantly located in vascular endothelial cells. In HIF-1α<sup>f/f</sup>/Tie2<sup>Cre</sup> or HIF-2α<sup>f/f</sup>/Tie2<sup>Cre</sup> mice, retinal vascular patterns were indistinguishable from those in floxed mice (Figure 2C to F), suggesting that endothelial HIF-1α or HIF-2α was not essential for normal vascular development in neonatal retinas.

**Reduced development of the astrocytic network in association with HIF-2α deficiency**

The above findings suggested that the requirement for HIF-2α proteins in retinal vascular development might reside in non-endothelial cells. Thus, we examined the development of the retinal astrocytic network by anti-GFAP IF staining in whole mount retinas. At P8, the morphology of astrocytic networks was essentially the same between HIF-1α<sup>f/f</sup> and HIF-1α<sup>f/f</sup>/Rosa26<sup>CreERT2</sup> mice (Figure 3A, D, G, and J). However, HIF-2α<sup>f/f</sup>/Rosa26<sup>CreERT2</sup> mice displayed substantially reduced astrocyte development compared to HIF-2α<sup>f/f</sup> mice (Figure 3B, E, H, and K). In HIF-2α<sup>f/f</sup>
Rosa26CreERT2 mice, retinal tissues were less densely populated with GFAP+ astrocytes, and individual astrocyte branches in the network appeared thinner than their counterparts in Hif-2αf/f mice. Interestingly, astrocyte development was further reduced in Hif-1αf/f and Hif-1αf/f/Rosa26CreERT2 mice (Figure 3C, F, I, and L).

The development of astrocytic networks was quantified by calculating the percentage of GFAP+ tissues in areas midway between the optic nerve and the periphery (Figure 3M). In Hif-1αf/f and Hif-1αf/f/Rosa26CreERT2 mice, GFAP+ areas occupied 47.4±2.8% and 44.9±3.8% of total areas, respectively (n=6 per group, p=0.61). In Hif-2αf/f and Hif-2αf/f/Rosa26CreERT2 mice, the corresponding values were 46.7±1.2% and 32.7±3.9% (n=7 per group, p<0.05). In Hif-1αf/f/Hif-2αf/f/Rosa26CreERT2 mice, GFAP+ area was down to 21.6±2.1%, which was not only significantly lower than 45.4±3.1% in Hif-1αf/f/Hif-2αf/f mice (n=7 per group, p<0.001), but also lower than in Hif-2αf/f/Rosa26CreERT2 mice (p<0.05).

Figure 1. Differential but partially overlapping roles of HIF-1α and HIF-2α in retinal vascular development. A to L. Confocal images of flat-mounted retinas stained with IB4-Alexa 594. A to C and G to I are representative images from areas midway between the optic nerve head and periphery; D to F and J to L each displays one of the 4 pedals of flat-mounted retinas. Images were tiled from multiple panels due to the size of retinas. Genotypes at the Hif-α and Rosa26 loci are indicated on top and to the left of the images, respectively. For example, panels G and J are Hif-1αf/f/Rosa26CreERT2. All mice were treated with tamoxifen by daily oral gavage at P1 through P3 (40 mg/kg in corn oil). At P8, retinas were dissected and stained with IB4-Alexa 594. Note that vascular development was reduced in H and K relative to G and J, respectively, and further reduced in I and L. Hyaloid vessels were removed during dissection. Scale bars: A and G, 100 µm; D and J, 500 µm. M. Quantification of vascular branches. LoxP modifications at Hif-1α and Hif-2α loci are indicated below the bar graph, and presence or absence of Rosa26CreERT2 (R26CreERT2) is indicated by solid or open bars, respectively. Quantification was carried out with the assistance of NIH ImageJ. To average out local variations, vascular branches were counted in 3 rectangular areas (0.2×0.5 mm²) areas midway between the optic nerve head and the periphery, with an example shown in D. Where a single pedal was not cut wide enough to encompass all three rectangular areas, the third area was counted in an adjacent pedal. Average values from three areas were used as one data point for statistical analysis. n=6. * p<0.05; ** p<0.01; *** p<0.001.

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Figure 2. Apparently normal development of the primary layer of retinal vasculature in Hif-1αf/f/Tie2Cre and Hif-2αf/f/Tie2Cre mice. A and B. Confocal images of flat-mounted P7 retinas from tdTomato reporter mice carrying Tie2Cre transgene. B is expanded from the red rectangle in A. Clearly visible vascular patterns indicate that Tie2Cre is fully functional in retinal blood vessels. C to F. P8 retinas stained with IB4-Alexa 594. Areas in red rectangles are shown at higher magnifications below each source image. Images are representative of 4 to 6 mice in each group. Scale bars are 400 µm in A, 125 µm in B, and 500 µm in C-F.

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Rosa26CreERT2 mice, retinal tissues were less densely populated with GFAP+ astrocytes, and individual astrocyte branches in the network appeared thinner than their counterparts in Hif-2αf/f mice. Interestingly, astrocyte development was further reduced in Hif-1αf/f/Hif-2αf/f/Rosa26CreERT2 mice (Figure 3C, F, I, and L).

The development of astrocytic networks was quantified by calculating the percentage of GFAP+ tissues in areas midway between the optic nerve and the periphery (Figure 3M). In Hif-1αf/f and Hif-1αf/f/Rosa26CreERT2 mice, GFAP+ areas occupied 47.4±2.8% and 44.9±3.8% of total areas, respectively (n=6 per group, p=0.61). In Hif-2αf/f and Hif-2αf/f/Rosa26CreERT2 mice, the corresponding values were 46.7±1.2% and 32.7±3.9% (n=7 per group, p<0.05). In Hif-1αf/f/Hif-2αf/f/Rosa26CreERT2 mice, GFAP+ area was down to 21.6±2.1%, which was not only significantly lower than 45.4±3.1% in Hif-1αf/f/Hif-2αf/f mice (n=7 per group, p<0.001), but also lower than in Hif-2αf/f/Rosa26CreERT2 mice (p<0.05).
Requirement of astrocyte-derived HIF-2α for vascular development

To determine if HIF-2α was required in astrocytes, we decided to disrupt floxed Hif-2α with GFAP promoter-driven Cre. Several different GFAPCre lines had been reported previously [23,24,33], two of which were tested in this study, including line 77.6 from the Sofroniew lab and the line from the Messing lab. To examine Cre activity in neonatal retinas, these Cre lines were crossed with tdTomato reporter mice. Unexpectedly, despite reported expression in astrocytes in the brain, line 77.6 failed to activate any tdTomato expression in the retina (data not shown). In contrast,

Figure 3. Reduced retinal astrocyte development in Hif-2αf/f/Rosa26CreERT2 mice. A to L. Confocal images showing retinal astrocytic networks of P8 neonatal mice. All mice were treated with tamoxifen by daily oral gavage at P1 through P3. At P8, retinas were dissected, fixed, and stained with rabbit anti-GFAP followed by goat anti-rabbit IgG-Alexa 488. Boxed areas in A–C and G–I are shown at higher magnifications below each source image. Scale bars are 500 μm in A and G and 100 μm in D and J. M. Percentage of retinal area occupied by GFAP+ cells. Quantification was carried out midway between the optic nerve and periphery (white boxes). Equivalent areas in three different pedals were counted for each retina, and the average value was used as one data point. Hif-1α and Hif-2α alleles are indicated below each bar; presence of R26CreERT2 is indicated by solid bars. n = 6; * p < 0.05; *** p < 0.001.
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the line from the Messing lab activated tdTomato expression predominantly in Pax2+ astrocyte progenitors and GFAP+ astrocytes at P0, P3 and P5 (Figure S3 and Figure S4). Since we have not investigated GFAPCre activities at developmental stages beyond the focus of this study, our data do not rule out likely GFAPCre activity in other cell types in older mice.

We generated Hif-1αCre/GEAPflox and Hif-2αCre/GEAPflox mice using GFAPflox Cre mice originated from the Messing lab, and visualized retinal vascular structures at P8 by whole mount staining with IB4-Alexa 594. As shown in Figure 4A to C, vascular patterns were similar among Hif-1αCre, Hif-1αCre/GEAPflox and GFAPflox mice. In sharp contrast, Hif-2αCre/GEAPflox mice displayed drastically reduced vascular development relative to floxed controls (Figure 4D to F). Among 22 Hif-2αCre/GEAPflox mice examined at P8, only 2 had remnant amounts of blood vessels as shown in Figure 4D, but the remaining 20 were completely devoid of blood vessels (Figure 5F). While the primary retinal vascular network was absent in most of Hif-2αCre/GEAPflox mice, hyaloid vascular structures were abundantly present (Figure S5).

Precocious but tapered retinal astrocyte differentiation in Hif-2αCre/GEAPflox mice

Data presented above strongly pointed to the possibility that retinal astrocyte development might be defective in Hif-2αCre/GEAPflox mice. Unexpectedly, retinas from Hif-2αCre/GEAPflox mice exhibited significantly more active astrocyte differentiation at P0 (Figure 5A, B, I, and J). Strong GFAP staining was mostly limited to the optic nerve head in floxed mice but extended much further in Hif-2αCre/GEAPflox mice. By P3, the development of astrocytic networks started to lag behind in Hif-2αCre/GEAPflox mice (Figure 5C, D, and J). While strongly GFAP structures extended similar distances from optic nerves in Hif-2αCre/+ and Hif-2αCre/GEAPflox mice, astrocytes in the latter failed to form complex network organization and occupied less percentage of retinal areas. Further away from the optic nerve head, weakly GFAP structures were abundantly present in Hif-2αCre/+ but not Hif-2αCre/GEAPflox mice (Figure 5C and D, upper rectangles and insets).

By P8, Hif-2αCre/+GEAPflox mice were completely overtaken by floxed controls (Figure 5E to H). Differences in Hif-2αCre/+ and Hif-2αCre/+GEAPflox mice were manifested in three ways. First, whereas GFAP cells had spread out over the entire retina area in Hif-2αCre/+ pups, they only reached approximately midway in Hif-2αCre/+GEAPflox mice (Figure 5E and F). Second, within GFAP territories, Hif-2αCre/+GEAPflox mice were less densely populated with astrocytes (Figure 5G, H, I and J). Third, astrocytes in Hif-2αCre/+GEAPflox mice were much thinner and had fewer branches (Figure 5G and H). In contrast to the dramatic effects of GFAPCre-mediated Hif-2α disruption on astrocyte development, little difference was found between Hif-1αCre/+ and Hif-1αCre/+GEAPflox mice. As shown in Figure S6A and B, anti-GFAP staining patterns at P8 were indistinguishable between floxed and Hif-1α disrupted mice.

We also evaluated retinal astrocyte development by anti-PDGFRA staining. At P0, PDGFRA+ positive astrocyte networks expanded towards the retinal periphery by similar distances in Hif-2αCre/+ and Hif-2αCre/+GEAPflox mice (Figure 6A, B and E). Within PDGFRA territories however, the percentage of area occupied by PDGFRA structures is much lower in Hif-2αCre/+GEAPflox mice (Figure 6A, B and F). At P3, PDGFRA astrocytes covered essentially the entire retinal inner surface in Hif-2αCre/+ mice, but were limited to the more central half in Hif-2αCre/+GEAPflox mice (Figure 6C, D and E). Within PDGFRA retinal areas, percentage area occupied by PDGFRA astrocytes was also significantly lower in Hif-2αCre/+GEAPflox mice (Figure 6C, D and F).

Depletion of Pax2+ astrocyte progenitors by GFAPCre-mediated Hif-2α disruption

To further investigate retinal astrocyte differentiation in Hif-2αCre/+GEAPflox mice, we analyzed the behavior of Pax2+ cells, which represent retinal astrocyte progenitors. At P0, the presence of Pax2+ cells was significantly reduced in Hif-2αCre/+GEAPflox retinas (Figure 7A, B, and K). This difference became more dramatic by P4 (Figure 7C, D and K). We reasoned that increased astrocyte differentiation at early stages might have occurred at the expense of Pax2+ progenitor population growth, happening too early and too fast, which resulted in the depletion of astrocyte progenitors and immature astrocytes. To test this hypothesis, we directly compared the development of Pax2+ and GFAP+ cells by anti-Pax2 and anti-GFAP double IF staining at P0. In Hif-2αCre/+ mice, GFAP signals were mostly limited to the optic nerve head area, but Pax2+ cells were abundantly present both near and further...
beyond the optic nerve head (Figure 7E and F). Pax2\(^{+}\) and GFAP\(^{+}\) domains did not colocalized, but instead formed a complementary pattern (Figure 7G), demonstrating that Pax2\(^{+}\) population was expanding beyond the optic nerve head without significant astrocyte differentiation at this stage of development. In Hif-2\(\alpha\)\(^{f/f}/GFAP\text{Cre}\) littermates, retinas had already developed numerous GFAP\(^{+}\) sprouts but were less densely populated with Pax2\(^{+}\) cells than in Hif-2\(\alpha\)\(^{f/f}\) controls (Figure 7H and I). Merged images also revealed that both Pax2\(^{+}\) and GFAP\(^{+}\) cells were present off the optic nerve head in Hif-2\(\alpha\)\(^{f/f}/GFAP\text{Cre}\) mice (Figure 7J), suggesting that Pax2\(^{+}\) cells were quickly differentiating into GFAP\(^{+}\) mature astrocytes.

We also assessed proliferation of Pax2\(^{+}\) cells at P3. While Hif-2\(\alpha\)\(^{f/f}/GFAP\text{Cre}\) mice contained less abundant Pax2\(^{+}\) cells, the percentages of BrdU-labeled Pax2\(^{+}\) cells did not differ between Hif-2\(\alpha\)\(^{f/f}\) and Hif-2\(\alpha\)\(^{f/f}/GFAP\text{Cre}\) mice (Figure 8A–I). Apoptotic cells were not detectable by anti-active Caspase 3 staining in both Hif-2\(\alpha\)\(^{f/f}\) and Hif-2\(\alpha\)\(^{f/f}/GFAP\text{Cre}\) mice (Figure 8J and K). As a positive control, we performed the same analysis in retinas from hyperoxia treated mice. Apoptotic cells were readily detectable by anti-active Caspase 3 staining in the control (Figure 8L). Based on these findings, we conclude that loss of Pax2\(^{+}\) cells in Hif-2\(\alpha\)\(^{f/f}/GFAP\text{Cre}\) retinas was unlikely a result of decreased proliferation or increased apoptosis.

Discussion

Roles of HIF-\(\alpha\) proteins in the development of retinal primary vascular plexus

HIF-1\(\alpha\) and HIF-2\(\alpha\) have well known roles in angiogenesis in a variety of tissues including embryos, ischemic tissues, and tumors [21,34–41]. In the retina, both HIF-1\(\alpha\) and HIF-2\(\alpha\) are linked to pathological neoangiogenesis [42–44], and HIF-1\(\alpha\) in peripheral retinal tissues mediates the development of the intermediate plexus [45]. In this study, global but not endothelial/hematopoietic HIF-2\(\alpha\) deficiency led to diminished retinal vascular development. The lack of an essential role for HIF-2\(\alpha\) in endothelial cells was not completely unexpected, because mice used in this study were in mixed CD1/B6 background whereas endothelial role of HIF-2\(\alpha\) is mostly limited to the 129 strain [35,36,46].
HIF-2α deficiency in the astrocytic lineage led to severe vascular defects. This finding contrasts sharply to normal vascular development a previous study which used a different GFAPCre line to disrupt Hif-2α [44]. In the study described here, a GFAPCre line originally generated in the Messing lab was used [23], which robustly activated the expression of Cre-inducible tdTomato reporter in astrocyte progenitors as early as P0. We presume that early expression of GFAPCre in astrocyte progenitors may be responsible for the phenotypes presented in this manuscript.

Figure 6. Reduced number of PDGFRα astrocytes in Hif-2αff/GFAPCre mice. A to D. Retinas dissected at P0 and P3 were subject to anti-PDGFRα IF staining. Wavefronts of PDGFRα astrocytes are marked by white lines. Red lines represent the approximate average positions of the zigzag white lines. In C, no red line is provided because the front of PDGFRα astrocyte network was relatively even. Areas indicated by white boxes were quantified for percentage of PDGFRα tissues. E. Distance from optic nerve head to the front of PDGFRα areas (white line in C, or red lines in A, B, and D). F. Percentage of PDGFRα retinal areas. Quantifications were carried for areas indicated by white boxes, taking the average of three such areas from the same mouse as one data point. Scale bars are 500 μm. n = 4. ** p < 0.01. doi:10.1371/journal.pone.0084736.g006

Figure 7. Depletion of Pax2+ astrocyte progenitors in Hif-2αff/GFAPCre mice. A to D. Anti-Pax2 IF staining of retinas at P0 (A and B) and P4 (C and D). The abundance of Pax2+ cells was dramatically reduced in Hif-2αff/GFAPCre mice at both P0 and P4. Scale bars are 500 μm. E to J. Double IF staining of P0 retinas with rabbit anti-Pax2 and rat anti-GFAP, followed by goat anti-rabbit IgG-Alexa 488 and donkey anti-rat IgG-Cy3. In Hif-2αff retinas, Pax2+ (green) are abundantly present in the vicinity of strongly GFAP+ (red) optic nerve head, but mature GFAP+ astrocytes are virtually absent. In Hif-2αff/GFAPCre mice, Pax2+ cells less abundantly present off the optic nerve, accompanied by many GFAP+ cells. Scale bars in E to J are 200 μm. K. Percentage (%) of retinal areas occupied by Pax2+ cells. n = 5. * p < 0.05, *** p < 0.001. doi:10.1371/journal.pone.0084736.g007
Regulation of retinal astrocyte development by HIF-2α

In Hif-2α<sup>f/f</sup>/GFAP<sup>Cre</sup> mice, astrocytic templates were only partially developed, and the morphology of HIF-2α deficient astrocytes was abnormally thin and elongated. These defects were associated with almost complete absence of retinal vascular development, suggesting that HIF-2α deficiency compromised not only the abundance but also the function of astrocytes. While the co-existence of vascular and astrocytic defects echoes previous findings that the development of retinal vasculature depends on the astrocytic template [2–4], a role of HIF-2α in retinal astrocyte differentiation may have further implications on the relationship between retinal astrocytic and vascular development. Since the stability of HIF-2α is sensitively regulated by prolyl hydroxylase domain proteins in an oxygen dependent manner [47–50], our findings raise the possibility that regulation of astrocytic and vascular development may be a two way process. In addition to the role of astrocytic templates in regulating retinal vascular growth, their own development may be regulated by oxygen diffusing out of retinal blood vessels. The defects in Hif-2α<sup>f/f</sup>/GFAP<sup>Cre</sup> mice may reflect the exaggeration of an inherently important mechanism.

Interpretation of retinal astrocytic phenotypes in Hif-2α<sup>f/f</sup>/GFAP<sup>Cre</sup> mice

Despite reduced astrocyte abundance in Hif-2α<sup>f/f</sup>/GFAP<sup>Cre</sup> retinas after P3, astrocyte differentiation from their progenitors was initially increased instead of being decreased, proliferation index of astrocyte progenitors was not reduced, and apoptosis was not increased. These apparently paradoxical phenomena may be understood by proposing precocious and accelerated differentiation of astrocyte progenitors. Because mature astrocytes are non-proliferative, normal development of the astrocytic network

![Figure 8. Proliferation and apoptosis assays. A to H. Proliferation assays. Mice were injected with BrdU at P3, and retinas were dissected after an hour. BrdU incorporation in astrocyte progenitors was detected by anti-BrdU and anti-Pax2 double IF staining. A to F show representative images from areas indicated in G and H. Both Pax2<sup>+</sup> and BrdU<sup>+</sup> cells were counted with the assistance of NIH ImageJ program. I. Proliferation index was calculated as % of Pax2<sup>+</sup> cells that were also BrdU<sup>+</sup>. Cre refers to GFAP<sup>Cre</sup>. n = 4. No significant difference was found between Hif-2α<sup>f/f</sup> and Hif-2α<sup>f/f</sup>/GFAP<sup>Cre</sup> mice. J to L. Apoptosis assay by anti-active Caspase 3 IF staining. J and K are P3 retinas. No apoptotic cells were detected in either Hif-2α<sup>f/f</sup> or Hif-2α<sup>f/f</sup>/GFAP<sup>Cre</sup> mice. L is from mice treated with 75% oxygen for 16 hours between P7 and P8. The same anti-active Caspase 3 staining procedure detected large numbers of apoptotic cells in oxygen-treated mice. Images are representative of data from 4 mice in each group. Scale bars are 50 μm for A to F, 300 μm for G and H, and 50 μm for J to L.

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depends on the proliferation of astrocyte progenitors and immature astrocytes. If progenitor cells differentiate into mature astrocytes too fast, the supply of the progenitor stock may be depleted because progenitor cells are deprived of the time needed for proliferation. Eventually, dwindling supply of progenitor cells may interrupt astrocyte development. In support of this viewpoint, Hif-2α deficiency disturbs the balance between progenitor proliferation and astrocyte differentiation. Accelerated astrocyte differentiation may cause rapid loss of astrocyte progenitors due to inadequate time for proliferation, leading to unsustainable astrocyte development.

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Figure 9. Model for HIF-2α regulated retinal astrocyte differentiation. In wild-type mice, physiological level of HIF-2α may partially suppress differentiation of mature astrocytes (green stars) from their progenitors (orange ovals). Thus, while a proportion of astrocyte progenitors differentiate into non-proliferative mature astrocytes, the rest may undergo active proliferation to replenish progenitor populations. HIF-2α deficiency disturbs the balance between progenitor proliferation and astrocyte differentiation. Accelerated astrocyte differentiation may cause rapid loss of astrocyte progenitors due to inadequate time for proliferation, leading to unsustainable astrocyte development.

The model

Our data led to a model schematically illustrated in Figure 9, which states that HIF-2α regulates retinal astrocyte development by guarding the rate of astrocyte differentiation from immature progenitors. This function is important because astrocyte progenitors are proliferative whereas mature astrocytes are not. Without HIF-2α, accelerated astrocyte differentiation depletes astrocyte progenitors by disallowing them the time needed for proliferation. As a result, retinal astrocyte development ceases prematurely due to the lack of astrocyte progenitors. It should be pointed out that the physiological HIF-2α level in wild-type retinas is apparently insufficient to completely inhibit astrocyte differentiation, but maintains a balance between progenitor population growth and astrocyte maturation.

Supporting Information

Figure S1 Disruption of Hif-1α and Hif-2α. Pups were obtained by crossing Hif-1αf/f/Rosa26creERT2 males with Hif-1αf/f females, or Hif-2αf/f/Rosa26creERT2 males with Hif-2αf/f females. At P1–P3, pups were treated with tamoxifen by daily oral gavage. At P5, pups were euthanized, and retinas were dissected. Deletion of floxed Hif-1α (A) and Hif-2α (B) in retinal tissues was assessed by PCR of retinal DNA extracts. Floxed Hif-1α, 270 bp, deleted allele, 877 bp, deleted allele, 260 bp. HIF-1α and HIF-2α protein levels were determined by anti-HIF-1α (C) or anti-HIF-2α (D) Western blotting of retinal nuclear protein extracts.

Figure S2 Induction of more severe vascular defects in Hif-2αf/f/Rosa26creERT2 mice by tamoxifen treatment at earlier time points. A and B. Hif-2αf/f and Hif-2αf/f/Rosa26creERT2 neonatal mice were treated with tamoxifen at P0 through P2. C and D. Pregnant Hif-2αf/f females mated with Hif-2αf/f/Rosa26creERT2 males were treated with a single dose of tamoxifen at 17.5 d.p.c.. Following birth, neonatal mice were treated with two more doses at P1 and P2. All retinas were stained with IB4 -Alexa 594 at P8. n = 3. Scales bar represents 50 μm. (TIF)

Figure S3 GFAFcre activity in retinal astrocytes. GFAFcre mice were crossed with transgenic mice carrying a CAG promoter-locP-Stop-loxP-tdTomato transgene targeted into the ubiquitously expressed Rosa26 locus. A to D. Co-localization of tdTomato expression with GFAP+ astrocytes. Retinas were dissected from neonatal mice at P5, and cryosections were cut at 6 μm. Sections were stained with rabbit anti-GFAP and anti-Rabbit IgG-Alexa 488, and analyzed by confocal imaging for tdTomato expression (A) or GFAP+ cells (B). Merged images are shown in C and D at different magnifications. tdTomato expression and GFAP+ cells colocalized at the inner surface of retinal tissue. E to H. Confocal images of tdTomato expression and anti-NF (neurofilament) immunofluorescence staining (green). Cryosections were prepared as in A to D, but stained with mouse anti-NF followed by goat anti-mouse IgG-DyLight 488. It is evident that tdTomato expression does not colocalize with NF+ signals. Scales bars, A–C and E to G, 100 μm; D and H, 50 μm. (TIF)

Figure S4 GFAFcre activity in astrocyte progenitors. GFAFcre and Cre-inducible tdTomato transgenic mice were crossed, and pups carrying both transgenes were subject to anti-Pax2 or anti-GFAP IF staining at P0 and P3, and visualized by confocal imaging. The vast majority of tdTomato positive cells were also Pax2+ and GFAP+, demonstrating early onset of Cre activity in retinal astrocyte progenitors. n = 3. All images are at the same magnification. Scale bar represents 50 μm. (TIF)

Figure S5 Hyaloid vessels in Hif-2αf/f/GFAFcre mice. Retinas were dissected at P0, care being taken to preserve hyaloid vessels. A and B. IB4-Alexa 594-stained retinas. C and D. Hyaloid vessels are more prominent in Hif-2αf/f/GFAFcre mice, presumably to compensate for the loss of retinal blood vessels. n = 3. (TIF)

Figure S6 Apparently normal astrocyte development in Hif-1f/f/GFAFcre mice. At P8, retinas were dissected from Hif-1f/f (A) and Hif-1f/f/GFAFcre (B) mice, fixed, and stained by rabbit anti-GFAP followed by goat anti-rabbit IgG-Alexa 488.
Data shown are representative confocal images from 4 mice in each genotype. Scales bars are 100 μm. Images are representative of data from 3 mice per group.

Figure S7 Astrocyte progenitors and immature astrocytes in P3 retinas. A to C are confocal images of wild-type (Hif-2a/+) retinas stained with indicated antibodies at P3. Boxed areas are expanded and shown in D to F. Images in D to F were brightened to show more details at the periphery. These data confirm that by P3, astrocyte progenitors and immature astrocytes are already present throughout the retina. Images are representative of at least 3 mice each. Scales bars are 500 μm for A–C, and 100 μm for D to F.

Table S1 Breeding strategy and strain background of relevant mouse lines.

Author Contributions
Conceived and designed the experiments: GHF. Performed the experiments: LJD KT. Analyzed the data: LJD KT GHF. Contributed reagents/materials/analysis tools: LJD KT GHF. Wrote the paper: LJD GHF.

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