von Hippel-Lindau protein regulates transition from the fetal to the adult circulatory system in retina

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SUMMARY

In early neonates, the fetal circulatory system undergoes dramatic transition to the adult circulatory system. Normally, embryonic connecting vessels, such as the ductus arteriosus and the foramen ovale, close and regress. In the neonatal retina, hyaloid vessels maintaining blood flow in the embryonic retina regress, and retinal vessels take over to form the adult-type circulatory system. This process is regulated by a programmed cell death switch mediated by macrophages via Wnt and angiopoietin 2 pathways. In this study, we seek other mechanisms that regulate this process, and focus on the dramatic change in oxygen environment at the point of birth. The von Hippel-Lindau tumor suppressor protein (pVHL) is a substrate recognition component of an E3-ubiquitin ligase that rapidly destabilizes hypoxia-inducible factor αs (HIF-αs) under normoxic, but not hypoxic, conditions. To examine the role of oxygen-sensing mechanisms in retinal circulatory system transition, we generated retina-specific conditional-knockout mice for VHL (Vhl\(-\text{CreKO}\) mice). These mice exhibit arrested transition from the fetal to the adult circulatory system, persistence of hyaloid vessels and poorly formed retinal vessels. These defects are suppressed by intraocular injection of FLT1-Fc protein [a vascular endothelial growth factor (VEGF) receptor-1 (FLT1)/Fc chimeric protein that can bind VEGF and inhibit its activity], or by inactivating the HIF-1α gene. Our results suggest that not only macrophages but also tissue oxygen-sensing mechanisms regulate the transition from the fetal to the adult circulatory system in the retina.

KEY WORDS: Angiogenesis, Circulatory system, Hypoxia-inducible factor 1, Mouse

INTRODUCTION

In early neonates, the fetal circulatory system undergoes rapid and dramatic transition to the adult circulatory system to adapt to detachment from the maternal blood supply. Normally, embryonic connecting vessels such as ductus arteriosus, ductus venosus and the foramen ovale close and regress (Merkle and Gilkeson, 2005). In the neonatal retina, it is generally known that the hyaloid vessels that maintain blood flow in the embryonic retina regress, and that retinal vessels assume the role of supplying blood to the retina (Lang, 1997; Lobov et al., 2005). This process is regulated by the programmed cell death switch mediated by macrophages via Wnt and angiopoietin 2 pathways (Lang and Bishop, 1993; Lobov et al., 2005; Rao et al., 2007).

Oxygen concentration is vital to nearly all forms of life on earth via its role in energy homeostasis (Yun et al., 2002). Mammalian embryos develop in a low (~3%) oxygen environment inside the maternal body (Rodesch et al., 1992). However, pups are abruptly exposed to atmospheric oxygen levels (~21%) after birth. This dramatic change in oxygen environment at the point of birth indicates some crucial roles of oxygen concentration in triggering the transition from the fetal to the adult circulatory system.

The von Hippel-Lindau tumor suppressor protein (pVHL) is a substrate recognition component of an E3-ubiquitin ligase that rapidly destabilizes hypoxia-inducible factor αs (HIF-αs) under normoxic, but not hypoxic, conditions (Nicholson et al., 1976). The von Hippel-Lindau disease is an autosomal dominant syndrome that causes the development of various benign and malignant disorders (Nicholson et al., 1976). The...
major disorders include retinal, brain and spinal cord angioma, pheochromocytoma, renal cell carcinoma and pancreatic cystadenoma. In the majority of VHL patients, retinal angioma is the first sign of the disease to appear. However, the molecular mechanism of retinal angioma development in VHL disease still remains unknown.

In this study, by using retina-specific conditional-knockout technology, we explore the precise in vivo function of VHL in retinal vascular development, and show that not only macrophages but also tissue oxygen-sensing mechanisms regulate the transition from the fetal to the adult circulatory system in the retina.

MATERIALS AND METHODS

Mice
All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. In the expression analysis shown in Fig. 1, we used C57BL/6 mice (Clea Japan). Transgenic mice expressing Cre recombinase under control of the Pax6 retina-specific regulatory element α-promoter (α-Cre) (Marquardt et al., 2001) were mated with Vhl floxed/floxed mice (Ryan et al., 2000), or HIF-2α floxed/floxed mice (Gruber et al., 2007). As control littermates for Vhl floxed/floxed mice, HIF-1α floxed/floxed mice, HIF-1α Cre– mice, and HIF-2α Cre–KOMP, Vhl floxed/floxed mice and HIF-2α floxed/floxed mice, HIF-1α CRE–/Vhl floxed/floxed mice, HIF-2α CRE–/Vhl floxed/floxed mice and HIF-1α CRE–/Vhl floxed/floxed mice without the α-Cre transgene, respectively. In a preliminary study, we found no detectable difference in retinal vascular structures between α-Cre+ and α-Cre– mice, confirming the validity of these control litters. To monitor Cre expression in α-Cre mice or Vhl floxed/floxed mice, we mated these mice with CAG-CAT-EGFP transgenic mice (Kawamoto et al., 2000). All mice used in this study were maintained on a C57BL/6J background.

Preparation of whole-mount samples and cryosections of retinas
Enucleated eyes were fixed for 20 minutes in 4% paraformaldehyde (PFA) in PBS and then dissected as previously described (Fruttiger et al., 1996; Gerhardt et al., 2003; Kubota et al., 2008). The obtained tissues were post-fixed overnight in 4% PFA in PBS and stored in methanol at –20°C. Cryosections of retinas (10 μm) were prepared as previously described (Kurthara et al., 2006), after eyeballs were immersed overnight in 4% PFA.

Immunostaining and in situ hybridization
Immunohistochemistry (IHC) for whole-mount retinas and other tissues was performed as described previously (Fruttiger et al., 1996; Gerhardt et al., 2003; Kubota et al., 2008; Kurthara et al., 2006). The primary antibodies used were: monoclonal anti-PECAM1 (2H8, Chemicon), actin (1A4; FITC-conjugated; Sigma-Aldrich), desmin (DAKO), F4/80 (A3-1; Serotec, Oxford, UK), GFAP (G-A-5; Cy3-conjugated; Sigma-Aldrich) or Dako), polyclonal type IV collagen (Cosmo Bio), HIF-1α (originally established by immunizing purified fusion proteins encompassing amino acids 416 to 785 of mouse HIF-1α into guinea pigs), HIF-2α (Santa Cruz Biotechnology), glutamine synthetase (Molecular Probes), opsin (Cosmo Bio) and pVHL (Santa Cruz Biotechnology). The secondary antibodies used were Alexa Fluor 488-conjugated IgGs (Molecular Probes) or Cy3/Cy5-conjugated IgGs (Jackson ImmunoResearch). Nuclei were stained with 10 μg/ml Hoechst bisbenzimide 33258 (Sigma-Aldrich) or DAPI (Molecular Probes). For whole-mount in situ hybridization (ISH), retinas were briefly digested with proteinase K and hybridized with digoxigenin (DIG)-labeled antisense RNA probes. When ISH was combined with IHC, IHC was performed after all the ISH procedures were completed. For the BrdU incorporation assay, 100 μg per body weight (g) of BrdU (BD Pharmingen) dissolved in sterile PBS was injected intraperitoneally 2 hours before sacrifice. Isolated retinas were stained using a BrdU immunohistochemistry system (Calbiochem) according to the manufacturer’s instructions. When BrdU assays were combined with IHC, the application of the first antibody for each procedure was done simultaneously. FITC-conjugated Dextran (Sigma-Aldrich) was injected into the left cardiac ventricle and allowed to circulate for 2 minutes. After staining, samples were mounted using a Prolong Antifade Kit (Molecular Probes).

Assessment of tissue hypoxia
Detection of hypoxic cells in cryosections was performed using a Hypoxyprobe-1 Plus Kit (Chemicon). In brief, 60 mg/kg of pimonidazole was injected intraperitoneally 30 minutes before sacrifice, and samples were stained with Hypoxyprobe MbI-FITC.

RT-PCR analysis
Total RNA was prepared from retinal tissues and reverse-transcribed using Superscript II (Invitrogen). A quantitative PCR assay for Vegfa was performed on an ABI 7500 Fast Real-Time PCR System using TaqMan Fast Universal PCR master mixture (Applied Biosystems) and TaqMan Gene Expression Assay Mix of Vegfa (Mm00437304_m1), Csf1r (Mm00432689_m1), and angiopoietin 2 (Mm00545822_m1). Mouse β-actin (Mm00607939_s1) assay mix served as an endogenous control. Data were analyzed with 7500 Fast System SDS Software 1.3.1. All experiments were done with four replicates.

Intravitreous injections
Injections into the vitreous body were performed using 33-gauge needles, as described previously (Gerhardt et al., 2003; Kubota et al., 2008). Sterile PBS (0.5 μl) with or without 1 mg/ml Flt1-Fc chimera proteins (R&D Systems) was injected at postnatal day (P) 4.

Confocal microscopy and quantification
Fluorescence images were obtained using a confocal laser scanning microscope (FV1000; Olympus) at room temperature. Quantification of the cells or substances of interest was usually done in eight random 200 μm × 200 μm fields just behind the sprouting edges of each retina. To construct three-dimensional projections, multiple slices horizontally imaged from the same field of view were integrated by FV10-ASW Viewer (Olympus).

Retinal explant culture
Retinal explant culture was performed using P6 mouse neural retinas based on the protocol we previously described (Ozawa et al., 2007; Ozawa et al., 2004). Briefly, eyes were enucleated and neural retinas were isolated and placed on a Millicell chamber filter (Millipore; pore size, 0.4 Am) with the culture plate, containing 50% MEM (GIBCO), 25% HBSS (GIBCO), 25% glucose. Explants were incubated at 34°C in 5% CO2. Twenty-four hours after placement on the protocol we previously described (Ozawa et al., 2007; Ozawa et al., 2004), Retinal explant culture was performed using P6 mouse neural retinas based on the protocol we previously described (Ozawa et al., 2007; Ozawa et al., 2004). Briefly, eyes were enucleated and neural retinas were isolated and placed on a Millicell chamber filter (Millipore; pore size, 0.4 Am) with the

Statistical analysis
Comparison between the average variables of two groups was performed by Student’s t-test.

RESULTS

HIF-1α expression is rapidly downregulated after birth
As a first step in the investigation of the cellular responses in the retina to the dramatic change in the oxygen environment at birth, we examined the expression patterns of pVHL, HIF-1α and HIF-2α in the developing retina. Expression of pVHL was detected ubiquitously from the inner to the outer side in the retina, and did not change between before and after birth (Fig. 1A-E). By contrast, nuclear staining of HIF-1α was downregulated in the deep retinal layer [the neuroblastic layer (NBL) at this stage] after birth (Fig. 1H-J), despite its ubiquitous expression in embryonic stages (Fig. 1F,G). HIF-2α immunoreactivity was predominantly detected in endothelial cells of hyaloid vessels in the vitreal space (see Fig.
S1A-C in the supplementary material) and in retinal vessels in the ganglion cell layer (GCL) (see Fig. S1D,E in the supplementary material). In embryonic retina, hypoxic areas were visualized by a hypoxic probe, pimonidazole, through all layers (Fig. 1K,L), although a relatively hypoxic area after birth was limited to the GCL (Fig. 1M-O), which still expressed HIF-1α (Fig. 1H-J).

**Vhl-lox/lox-CreKO** mice show persistence of the hyaloid vessels independently of macrophages

To elucidate the function of pVHL in postnatal retinal vascularization, we employed a retina-specific mouse Cre line, α-Cre, which shows Cre expression in the deep retinal layer, including in retinal progenitor cell-derived neural cells, under the control of a neural retina-specific regulatory element of murine Pax6 gene, but not in astrocytes and endothelial cells at P10 (Marquardt et al., 2001). We examined the past events of Cre expression in α-Cre mice until early postnatal age by crossing α-Cre mice with a reporter transgenic line, CAG-CAT-EGFP (Kawamoto et al., 2000). At P6, GFP expression was detected in neural cells in the deep retinal layer, but not in astrocytes or endothelial cells of both retinal and hyaloid vasculature (see Fig. S2A-T in the supplementary material). Then, we crossed Vhl-lox/lox mice (Haase et al., 2001) with α-Cre mice to generate α-Cre-specific conditional-knockout mice for Vhl (Vhl-lox/lox-CreKO mice). As control littersmates for Vhl-lox/lox-CreKO mice, we used Vhl-lox/lox mice without the α-Cre transgene. At P6, blood flow visualized by intracardiac injection of fluorescein isothiocyanate (FITC)-dextran was detected in both hyaloid and retinal vessels of control mice (Fig. 2A,B,E,G,I). In Vhl-lox/lox-CreKO mice, however, there was abundant blood flow in hyaloid vessels, poor flow in retinal vessels, and abundant collateral flow from hyaloid vessels to retinal vessels (Fig. 2C,D,F,H,J). Nonetheless, despite the impaired blood flow, the retinal vascular structure could be detected by immunohistochemistry for platelet/endothelial cell adhesion molecule 1 (PECAM1) in Vhl-lox/lox-CreKO mice (Fig. 2F,H). The persistent blood flow in hyaloid vessels in Vhl-lox/lox-CreKO mice prompted us to examine the well-known mechanisms of hyaloid vessel regression: reduced perivascular macrophages or angiopoietin 2 deficiency lead to persistence of the hyaloid vessels (Lobov et al., 2005; Rao et al., 2007). Macrophages around the hyaloid vessels were not significantly changed (Fig. 2K-M), and colony stimulating factor 1 receptor (Csf1r) expression, which correlates with the number of macrophages (Kubota et al., 2009), showed no significant reduction in Vhl-lox/lox-CreKO mice (Fig. 2N). Angiopoietin 2 expression was rather increased in Vhl-lox/lox-CreKO mice (Fig. 2O). Collectively, Vhl-lox/lox-CreKO mice show persistence of the hyaloid vessels independently of macrophages and angiopoietin 2.

**Vhl-lox/lox-CreKO** mice show poorly formed retinal vessels characterized by excessive vessel regression

As abnormal hyaloid vessel persistence could affect retinal vascularization, we examined the retinal vessels in Vhl-lox/lox-CreKO mice. In these mice, the entire growth of the retinal vasculature spreading from the optic disc and the branching points were significantly reduced compared with control mice (P=0.013 for spreading distance; P=0.00012 for branching points; Fig. 3A,B,D,E,M,N; see also Fig. S3A,B in the supplementary material). Endothelial tip cells and their filopodia, which are controlled by VEGF and the Delta-like 4/Notch pathway (Gerhardt et al., 2003; Hellstrom et al., 2007; Phng et al., 2009), were also significantly reduced in Vhl-lox/lox-CreKO mice (P=0.0012 for tip cells; P=0.011 for filopodia; Fig. 3C,F,O,P). A significant decrease in endothelial proliferation (Fig. 3G,J,Q) and excessive vessel regression (Fig. 3H,K,R), characterized by empty vascular sleeves (Phng et al., 2009), were also detected in Vhl-lox/lox-CreKO mice (P=7.9×10⁻⁴ for BrdU⁺ endothelial cells; P=0.0044 for empty sleeves). Empty sleeves were abundantly detected not only in their stalk cell area but also at their leading edge, suggesting that vessel regression occurs in both tip cells and stalk cells in Vhl-lox/lox-CreKO mice. Although it is well known that pVHL regulates the expression of various extracellular matrices (Ohh et al., 1998), Col IV expression was not impaired in Vhl-lox/lox-CreKO mice. Although the number of
pericytes associated with endothelial tubes was not changed in Vhl\textsuperscript{-CreKO} mice (P=0.395; Fig. 3I,L,S), detached pericytes were frequently detected just beyond their sprouting edges. Previously, injection of angiopoietin 2 into the eyes of normal rats was shown to induce a dose-dependent pericyte loss (Hammes et al., 2004), suggesting that upregulated angiopoietin 2 (Fig. 2O) might contribute to pericyte defects in Vhl\textsuperscript{-CreKO} mice. Collectively, Vhl\textsuperscript{-CreKO} mice showed poorly formed retinal vessels, presumably owing to persistence of the hyaloid vessels.

**Vascular defects in Vhl\textsuperscript{-CreKO} mice are attributable to ectopic VEGF expression**

The intact macrophages and preserved angiopoietin 2 expression in Vhl\textsuperscript{-CreKO} mice caused us to suppose that some other mechanism led to the persistence of the hyaloid vessels. As retinal vascular growth depends on the appropriate spatial distribution of heparin-binding VEGF within the retina (Gerhardt et al., 2003; Kubota et al., 2008; Ruhrberg et al., 2002; Stalmans et al., 2002), we suspected that the vascular defects in Vhl\textsuperscript{-CreKO} mice were attributable to the abnormal expression pattern of VEGF. In control mice, VEGF expression was predominantly detected in astrocytes beyond the sprouting edge (Fig. 4A,B,E-H). In Vhl\textsuperscript{-CreKO} mice, marked expression of VEGF was detected in the deep retinal layer (Fig. 4C,D,I-L), and was coincidental with the area of persistent hyaloid vessels. However, typical VEGF expression in astrocytes beyond the sprouting edge was diminished in Vhl\textsuperscript{-CreKO} mice, despite the fact that their astrocyte plexus was normal (Fig. 4M,N). This VEGF expression pattern in Vhl\textsuperscript{-CreKO} mice was similar to normal VEGF expression in the embryonic retina (Saint-Geniez et al., 2006). By quantitative RT-PCR analysis, a more than 4-fold significant increase (P=0.00071) in Vegfa expression was detected in the entire retina of Vhl\textsuperscript{-CreKO} mice (Fig. 4O). Because these expression patterns of VEGF suggested that the vascular defects in Vhl\textsuperscript{-CreKO} mice could be attributable to ectopic and abundant VEGF expression, we injected a potent VEGF inhibitor, FLT1-Fc chimeric protein (Gerhardt et al., 2003; Kubota et al., 2008), into the eyes of Vhl\textsuperscript{-CreKO} mice. These FLT1-Fc injections reduced the amount of both collateral blood flow and vascular structures from hyaloid vessels to retinal vessels (Fig. 4P-U). As genetic inactivation of the Vhl gene results in HIF-1\textalpha~stabilization (Kaptisouinou and Haase, 2008) and the upregulation of Vegfa expression, which is widely known as a HIF-1\textalpha~target gene (Forsythe et al., 1996), we examined the expression of pVHL, HIF-1\textalpha~and HIF-2\textalpha~in Vhl\textsuperscript{-CreKO} mice. We found that pVHL immunoreactivity was greatly reduced in the deep layer of the peripheral retina (Fig. 4V,W), where the strong \(\alpha\)-Cre-mediated recombination is detected (see Fig. S2 in the supplementary material), except for in astrocytes and endothelial cells (see Fig. S4A-J in the supplementary material). HIF-1\textalpha~immunoreactivity was increased in this area of Vhl\textsuperscript{-CreKO} mice (Fig. 4X,Y; see also Fig. S4K-O in the supplementary material). In addition, strong HIF-2\textalpha~immunoreactivity was detected in invaginating hyaloid vessels, but not in the area of reduced pVHL expression (Fig. 4Z,AA; see also Fig. S4P-T in the supplementary material).

**Increased VEGF expression in Vhl\textsuperscript{-CreKO} mice is due to their impaired oxygen-sensing mechanism via HIF-1\textalpha**

As the VEGF expression pattern in postnatal Vhl\textsuperscript{-CreKO} retina is similar to normal VEGF expression in the embryonic retina (Saint-Geniez et al., 2006), we expected abnormal VEGF expression in the Vhl\textsuperscript{-CreKO} retina to be attributable to an impaired oxygen-sensing
we generated HIF-1α (Ryan et al., 2000) (Vhl-α-CreKO) mice. Persistent hyaloid vessels in the lower left lobe of Vhl-α-CreKO mice were removed mechanically before immunostaining (D). (G-L) Immunostaining with the indicated antibodies. Note the decreased BrdU+ endothelial cells (arrows, G,J), type IV collagen (Col IV) isolectin-empty sleeves (arrowheads, H,K), and detached pericytes beyond sprouting edges (open arrowheads, L) in Vhl-α-CreKO mice, although most pericytes in control mice are closely contacted with vasculature (closed arrowheads, L)).

Progressive retinal degeneration in adult Vhl-α-CreKO mice

Next, we examined retinal function and circulation in adult Vhl-α-CreKO mice. Increased apoptosis of photoreceptors and other neuronal cells was seen in Vhl-α-CreKO mice after P14 (Fig. 7A-H) induced decrease of the outer nuclear layer (ONL; Fig. 7I,J), despite the normal development of photoreceptors at P7 (Fig. 7E,J). Gliosis, accompanied by vessels invading into the deep retinal layer, destroyed the construction of the ONL (Fig. 7K,L) at P14. As a result, a significant (P<0.00028) decrease of the outer nuclear layer (ONL; Fig. 7I,J) was observed in Vhl-α-CreKO mice compared with control (Fig. 7S). Consistent with the in vivo results (Fig. 1A-E), the expression pattern of pVHL was stable in the deep retinal layer during ex vivo culture, and was not affected by the oxygen concentration (Fig. 5E,G,L,N,P,R). These data in the ex vivo culture system suggest that the increased VEGF expression in Vhl-α-CreKO mice is due to their impaired oxygen-sensing mechanism via HIF-1α.

Deletion of HIF-1α, but not HIF-2α, rescues vascular defects in Vhl-α-CreKO mice

The expression patterns of pVHL, HIF-1α, HIF-2α and VEGF in Vhl-α-CreKO mice (Fig. 4) and the data from the ex vivo culture system (Fig. 5) suggested that the increased activity of HIF-1α was responsible for the vascular defects in Vhl-α-CreKO mice. Therefore, we generated α-Cre-specific double-knockout mice for VHL and HIF-1α (Ryan et al., 2000) (HIF-1α;Vhl-α-CreKO mice). Vascular defects seen in Vhl-α-CreKO mice were suppressed in HIF-1α; HIF-2α;Vhl-α-CreKO mice (Fig. 6A,B,D-G,M-P). The abnormal expression of VEGF in Vhl-α-CreKO was fairly normalized in HIF-1α; Vhl-α-CreKO mice (Fig. 6J,K; see also Fig. S5A in the supplementary material; 1.15±0.08-fold compared with control). Conversely, α-Cre-mediated VHL and HIF-2α (Gruber et al., 2007) double-knockout mice (HIF-2α;Vhl-α-CreKO) showed similar vascular defects and a similar VEGF expression pattern to Vhl-α-CreKO mice (Fig. 6C,H,L; see also Fig. S5B in the supplementary material). Taken together, HIF-1α, but not HIF-2α, plays essential roles in the appearance of vascular defects in the Vhl-α-CreKO retina.

DISCUSSION

In the present study, we showed that retina-specific conditional-knockout mice for the Vhl gene exhibit persistent hyaloid vessels independently of macrophage function, which are
sustained until adulthood. These vascular defects in Vhlα-CreKO mice are rescued by either local VEGF inhibition or genetic deletion of HIF-1α, but not of HIF-2α. These results suggest not only macrophages but also tissue oxygen-sensing mechanisms regulate the transition from the fetal to the adult circulatory system in retina.

Considering that HIF-1α in the outer side of the retina is dramatically downregulated after birth but that pVHL expression in the retina does not differ between embryos and neonates (Fig. 1), the change of environmental oxygen concentration must be important for the role of pVHL in this transition (Rodesch et al., 1992). VEGF expression in the deep retinal layer during embryonic but not

Fig. 4. Vascular defects in Vhlα-CreKO mice are attributable to ectopic VEGF expression. (A-L) Whole-mount (A-D) or section (E-L) in situ hybridization for VEGF combined with immunostaining with the indicated antibodies. Although VEGF expression is detected in astrocytes located in the avascular area (arrows) of control mice, abundant VEGF expression is detected in the deep retinal layer (open arrowheads) where persistent hyaloid vessels invaginate in Vhlα-CreKO mice. (M,N) Immunostaining with PECAM1 (green) and PDGFRα (red) on P6 retinas. Despite a normal astrocyte plexus (asterisks), vessel regression (open arrowheads) occurs in Vhlα-CreKO mice. (O) Quantitative PCR of Vegfa RNA isolated from P6 retinas (n=6). (P-U) Fluorescence microscopic images in P6 retinas perfused with FITC-dextran (P-R), and confocal images labeled with PECAM1 (S-U). FLT1-Fc injection into the eyes of Vhlα-CreKO mice reduces collateral flow (arrows, R) and vascular structures (arrows, U) that exist abundantly in Vhlα-CreKO mice injected with vehicle (open arrowheads in Q,T). (V-AA) Immunostaining of P6 retinal sections with the indicated antibodies. Although pVHL expression is greatly reduced (open arrowheads, W), HIF-1α immunoreactivity is increased (closed arrowheads, Y) in the deep retinal layer of Vhlα-CreKO mice. HIF-2α staining is detected in invaginating hyaloid vessels in Vhlα-CreKO mice (arrows, AA).

**P<0.01. Error bars indicate mean±s.d. Scale bars: 500 μm in A-L; 200 μm in P-AA; 100 μm in M,N.

Fig. 5. Increased VEGF expression in Vhlα-CreKO mice is due to their impaired oxygen-sensing mechanism via HIF-1α. (A) Schematics show the experimental procedure: isolated retinas from control or conditional-knockout mice were unfolded, placed on a chamber filter, and exposed to each concentration of oxygen. Retinal explants of control mice (B-E,K-N), Vhlα-CreKO mice (F-I), or HIF-1αa-CreKO mice (O-R) were exposed to 21% oxygen (B,C,F,G,K,L,O,P) or 1% oxygen (D,E,H,I,M,N,Q,R). (J,S) Quantitative PCR of Vegfa RNA isolated from retinal explants (n=6, respectively). Note, strong HIF-1α staining (red) is induced by 1% oxygen (D,H,M) except in HIF-1αa-CreKO retina (Q), and by genetic inactivation of VHL (F,H). pVHL expression was not detected in Vhlα-CreKO retina (G,I). (J) Correlated with the expression of HIF-1α, Vegfa expression was upregulated even under normoxia conditions in Vhlα-CreKO retina. (S) In HIF-1αa-CreKO retina, hypoxia-induced Vegfa expression was significantly suppressed. **P<0.01. Error bars indicate mean±s.d. Scale bars: 50 μm in B-I,K-R.
postnatal retinal stages (Saint-Geniez et al., 2006) supports the idea that the abnormal VEGF distribution in postnatal Vhl\(^{-}\)CreKO retina represents a defective VEGF expression switch from the embryonic pattern to the postnatal one. Mice selectively expressing single isoforms of VEGF (VEGF\(_{120}\) or VEGF\(_{188}\)) or overexpressing VEGF pattern to the postnatal one. Mice selectively expressing single isoforms of VEGF (VEGF\(_{120}\) or VEGF\(_{188}\)) or overexpressing VEGF (bidirectional arrows) decreased branching points, tip cells and filopodia] seen in Vhl\(^{-}\)CreKO mice are abolished by deletion of HIF-1\(\alpha\), but not HIF-2\(\alpha\), in Vhl\(^{-}\)CreKO mice. (J-L) Whole-mount in situ hybridization for VEGF (blue) combined with immunostaining with Col IV (green). Ectopic VEGF expression seen in Vhl\(^{-}\)CreKO mice is abolished by deletion of HIF-1\(\alpha\), but not HIF-2\(\alpha\), in Vhl\(^{-}\)CreKO mice. (M-T) Quantification of percentage spreading distance from optic discs (M,O), the number of branching points in the area behind sprouting edges (N,R), tip cells per field (O,S), filopodia per tip cell (P,T; n=6 for each genotype). *P<0.05, **P<0.01. Error bars indicate mean ± s.d. Scale bars: 500 μm in A-C,D,F,H; 100 μm in E,G,I-J,L.

Previously, Lang and colleagues clearly demonstrated that macrophages mediate hyaloid vessel regression through the paracrine action of WNT7B (Lobov et al., 2005). They showed that a lack of macrophages in P.U.\(^{Lc}\) mice caused a significant delay in hyaloid vessel regression, and that intraocularly injected wild-type, but not Wnt7b-mutant, macrophages ameliorated this delay. Moreover, angiopoietin 2 has also been shown to be involved in hyaloid vessel regression via the dual effect of suppressing survival signaling in the endothelial cells of hyaloid vessels and stimulating Wnt ligand production by macrophages (Rao et al., 2007). Interestingly, the macrophage/microglia number in Vhl\(^{-}\)CreKO mice was not affected (Fig. 2K-M), but angiopoietin 2 expression was increased (Fig. 2O), which suggests that an oxygen-sensing mechanism mediated by the VHL/HIF-1\(\alpha\)/VEGF system operates hyaloid vessel regression independently of macrophages, Wnt and angiopoietin 2.

A possible concern with our data might be the slightly, but significantly, increased branching points observed in HIF-1\(\alpha\); Vhl\(^{-}\)CreKO mice (Fig. 6G,N). Independently of HIF-\(\alpha\) proteolysis, pVHL is known to be involved in extracellular matrix assembly and turnover (Ohh et al., 1998). Moreover, erythropoietin has been reported to be regulated by VHL and HIFs (Chen et al., 2008; Rankin et al., 2007). These previous findings suggest the involvement of multiple candidates in addition to HIF-1\(\alpha\) and VEGF, and might explain the minor vascular changes observed in HIF-1\(\alpha\); Vhl\(^{-}\)CreKO mice. However, the dramatic rescue effects obtained by FLT1-Fc injection (Fig. 4P-U), or by the genetic
inactivation of HIF-1α, but not HIF-2α (Fig. 6), show that the VHL/HIF-1α/VEGF cascade plays crucial roles in the transition from the fetal to the adult circulatory system in the retina.

Our current study has possible clinical implications. Adult Vhl flox/−CreKO mice show similar characteristics (Fig. 8) to human ischemic retinopathies, such as diabetic retinopathy, retinal vessel occlusion, and retinopathy of prematurity (Hayreh, 2007), and could provide clues for exploring the mechanisms of these human diseases. It has been recently shown that systemic administration of a pro-hydroxylase inhibitor protects from retinal vaso-oblitration in response to hyperoxia (Sears et al., 2008), suggesting that pVHL functions in retinopathy of prematurity.

Retinal hemangioma in VHL patients and ischemic retinopathies are usually treated by laser photocoagulation that disrupts photoreceptors and increases the oxygen supply for the other neural cells from the choroidal vasculature bed, although it may cause night blindness and visual field defects (Yu and Cringle, 2001). Recently, various kinds of VEGF inhibitors (Brown and Regillo, 2007) have become possible candidates for use against ophthalmic diseases, although destruction of the physiological vasculature (Fischer et al., 1997) and neuronal dysfunction (Saint-Geniez et al., 2008) caused by long-term VEGF inhibition caution against the continuous administration of these reagents. Suppression of HIF-1α might be an alternative strategy against these diseases in the future.

Finally, all of the results presented suggest that oxygen-sensing mechanisms mediated by VHL/HIF-1α/VEGF regulate the transition from the fetal to the adult circulatory system in the retina, and could represent a theoretical basis for the retinal vascular abnormalities in human VHL disease and the development of ischemic retinopathies. It will be interesting to determine whether this oxygen-sensing system, the VHL/HIF-1α pathway, is involved in the transition of the circulatory system in other parts, such as ductus arteriosus, which would confirm its generality for future studies.

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VHL regulates circulatory system transit

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