The Role of the Hypoxia Response in Shaping Retinal Vascular Development in the Absence of Norrin/Frizzled4 Signaling

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PURPOSE. To define the role of hypoxia and vascular endothelial growth factor (VEGF) in modifying the pattern, density, and permeability of the retinal vasculature in mouse models in which Norrin/Frizzled4 signaling is impaired.

METHODS. Retinal vascular structure was analyzed in mice with mutation of *Ndp* (the gene coding for Norrin) or *Frizzled4* (*Fz4*) with or without three additional perturbations: (1) retinal hyperoxia and reduction of VEGF, (2) reduced induction of VEGF in response to hypoxia, or (3) reduced responsiveness of vascular endothelial cells (ECs) to VEGF. These perturbations were produced, respectively, by (1) genetic ablation of rod photoreceptors in the *retinal degeneration 1* (*rd1*) mutant background, (2) conditional deletion of the gene coding for hypoxia-inducible factor (HIF)-2alpha either in all neural retina cells or specifically in Müller glia, and (3) conditional deletion of the VEGF coreceptor neuropilin1 (NRP1) in ECs.

RESULTS. All three conditions reduced vascular proliferation. Eliminating HIF2-alpha in Müller glia blocked VEGF induction in the inner nuclear layer, identifying HIF2-alpha as the transcription factor responsible for the hypoxia response in these cells. When Norrin/Frizzled4 signaling was eliminated, a secondary elevation in VEGF levels was required to compromise the barrier to transendothelial movement of high molecular weight compounds.

CONCLUSIONS. In the absence of Norrin or Frizzled4, the vascular phenotype is determined by the primary defect in Norrin/Frizzled4 signaling (i.e., canonical Wnt signaling) and compensatory responses resulting from hypoxia. This work may be useful in guiding therapeutic strategies for the treatment of familial exudative vitreoretinopathy (FEVR).

Keywords: endothelial cell, canonical Wnt signaling, Norrie disease, familial exudative vitreoretinopathy

Vascular development is controlled by a variety of signaling systems that regulate endothelial cell (EC) proliferation, migration, and differentiation. Among these are the VEGF, Notch, Semaphorin, Integrin, Angiopoietin, and canonical Wnt pathways.¹ The retina presents an especially important system for studying angiogenesis both because of the clinical significance of retinal vascular disease and because the precise architecture and stereotyped development of the retinal vasculature facilitate experimental analysis.²

Investigations over the past two decades have significantly clarified the molecular basis of inherited defects in retinal vascular development. Norrie disease (ND), a severe retinal hypovascularization disorder, is an X-linked trait caused by loss-of-function mutations in the gene coding for Norrin, a distantly related member of the TGF-beta superfamily of secreted ligands.³ Osteoporosis-pseudoglioma syndrome arises from loss-of-function mutations in both alleles of the gene coding for low-density lipoprotein receptor-related protein (Lrp)5, one of two closely related coreceptors for canonical Wnt signaling.^{4,5} Both disorders are characterized by persistence of the hyaloid vasculature and severe gliosis of the retina, resulting in

congenital blindness. The milder retinal hypovascularization disorders, referred to as familial exudative vitreoretinopathy (FEVR), are caused by mutations in the genes coding for the Wnt receptor Frizzled4 (*Fz4*), the integral membrane protein Tspan12, or Lrp5.⁶⁻¹⁰ Familial exudative vitreoretinopathy is associated with retinal folds, tears, and detachments and variable extents of vision loss.¹¹

A mechanistic unification of the retinal hypovascularization disorders has emerged with the experimental demonstration that (1) Norrin functions as a high-affinity *Fz4* ligand, (2) Norrin-*Fz4* binding activates the coreceptor Lrp5 and mediates canonical Wnt signaling, and (3) Tspan12 promotes the formation of the Norrin/*Fz4*/Lrp5 signaling complex.^{12,13} The role of these proteins in retinal vascular development appears to be conserved between mice and humans since targeted mutations in the corresponding mouse genes lead to hypovascularization of the mouse retina.¹²⁻¹⁶ Reporter knock-in experiments have shown that Norrin is produced by Müller glia,¹⁷ and cell type-specific gene deletion experiments have shown that *Fz4* signaling is required in ECs.^{16,18} In addition to its role in promoting angiogenesis, Norrin/*Fz4*/Lrp5 signaling is

required for maintenance of the blood-retina barrier (BRB), as seen by the acute loss of BRB integrity that occurs when the *Fz4* gene is inactivated in adult ECs.¹⁸ The *Norrin/Fz4/Lrp5/Tspan12* system appears to be poised at a signal strength that is just above the threshold required for normal vascular development, since halving the dose of *Fz4*, *Lrp5*, *Tspan12*, or *Ndp* (*Norrie disease protein*, the gene coding for *Norrin*) leads to mild defects in vascular architecture and/or BRB integrity in mice and humans.^{7,8,10,19}

The hallmarks of *Ndp* or *Fz4* inactivation are failure of the vasculature to invade the retina, vascular proliferation at the vitreal face of the retina, and loss of the BRB.^{12,14–16,18} The general assumption has been that the first of these represents a primary consequence of defective *Norrin/Fz4* signaling; the second represents a response to VEGF produced by the hypoxic retina; and the third may reflect a combination of defective *Norrin/Fz4* signaling and increased VEGF signaling. These assumptions are in accord with the known actions of VEGF in promoting EC proliferation and increasing vascular permeability.^{20–22} To experimentally define the role of the VEGF response in shaping the vascular phenotype of retinas deficient in *Norrin/Fz4/Lrp5* signaling, we have studied retinal vascular structure and BRB integrity in mice in which the genetic background leads to (1) decreased retinal VEGF due to an increase in retinal oxygenation, (2) decreased retinal VEGF, despite retinal hypoxia, due to the loss of hypoxia-inducible factor (HIF2)- α either in all retinal layers or exclusively in Müller glia, or (3) decreased responsiveness to VEGF due to loss of the VEGF coreceptor neuropilin1 (NRP1) in ECs.

MATERIALS AND METHODS

Mice

The following mouse alleles were used: *platelet-derived growth factor B* (*Pdgfb*)-*CreER*²³; *Fz4*^{−24}; *Fz4*^{CKO16}; *Ndp*^{KO16}; *Pax6-alpha*-*Cre*²⁵; *rd1*²⁶; *GLAST*-*CreER*²⁷; *Z/Edn2*²⁸; *mTmG*²⁹; and *R26-LSL-SUN1-GFP* (Mo A, Nathans J, unpublished observations, 2014). For *GLAST*-*CreER* experiments, 200 μ g 4-hydroxytamoxifen (4HT; Sigma-Aldrich Corp., St. Louis, MO, USA) was delivered by intraperitoneal (IP) injection at postnatal days (P)6, P8, and P10. For *Pdgfb*-*CreER* experiments, the dose and timing of 4HT (delivered by IP injection) are indicated. All procedures were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antibodies and Other Reagents

Antibodies used in this study were as follows: mouse anti-claudin-5, clone 4C3C2, Alexa 488 conjugate (352588; Invitrogen, Carlsbad, CA, USA); rat anti-PLVAP/MECA-32 (553849; BD Pharmingen, San Jose, CA, USA); mouse anti-alpha smooth muscle actin, clone 1A4, Cy3 conjugate (C6198; Sigma-Aldrich Corp.); goat anti-NRP1 (AF566; R&D Systems, Minneapolis, MN, USA); and rabbit anti-pimonidazole antibodies (PAb2627; NPI, Burlington, MA, USA). Alexa Fluor-labeled secondary antibodies and GS-lectin (isolectin GS-IB4) were from Invitrogen. Hypoxyprobe (pimonidazole) was from NPI.

Immunohistochemistry, In Situ Hybridization, Microscopy, and Image Analysis

In situ hybridization to VEGF transcripts and Hypoxyprobe labeling and detection were performed as described previously.²⁸ Within each figure, retinal sections that were subjected to VEGF in situ hybridization were processed in parallel and

imaged with identical settings. Immunohistochemistry on retina sections and flat mounts was performed as described previously.^{18,28} Images were collected with a Zeiss Apotome microscope (Oberkochen, Germany) or a Zeiss LSM700 confocal microscope using Zeiss ZEN software, and they were further processed using Adobe Photoshop (San Jose, CA, USA).

Quantification of Vascular Structure

To quantify retinal vascular density, four 640- $\times$ 640- μ m-square grid images were overlaid on a Z-stacked image of a flat-mounted retina as described previously.³⁰ One grid was placed over each quadrant in the midperiphery at a distance of $\sim$ 1 mm from the optic disc. Each grid consisted of six evenly spaced horizontal lines and six evenly spaced vertical lines (7.68 mm total line length per grid). The number of times that blood vessels and lines crossed was recorded for all grid lines and summed for each grid. A vessel with a trajectory coincident with a line for an extended distance was counted as crossing once for each 107- μ m length of coincident trajectory. Counting of glomeruloids was performed with four 640- $\times$ 640- μ m-square territories overlaid in the midperiphery as described for the grids. Statistical analysis was performed in MatLab (Mathworks, Natick, MA, USA) and Microsoft Excel (Redmond, WA, USA).

RESULTS

The Effect of Hyperoxia on Vascular Growth in the *Ndp*^{KO} Retina

As one approach to suppressing the retinal hypoxia that accompanies loss of *Norrin/Fz4* signaling, we generated mice that harbored loss-of-function mutations in both *Ndp* and *retinal degeneration 1* (*rd1*). *rd1*^{−/−} mice lack the beta subunit of the rod outer segment cGMP phosphodiesterase, which leads to a rapid and selective loss of rod photoreceptors ($\sim$ 80% loss by $\sim$ P15], coincident with the period of intraretinal vascular development.^{2,26} Photoreceptors are far more metabolically active than inner retinal neurons; they account for $\sim$ 80% of all retinal oxygen consumption,³¹ and their metabolic needs are normally met by the choroidal vasculature, which has a blood flow many times greater than the intraretinal vasculature. In consequence, the loss of photoreceptors in the *rd1*^{−/−} retina bathes the surviving inner retina in high levels of choroid-derived oxygen, which leads to regression of the intraretinal vascular plexus.^{32–34} We note that a tacit assumption in using the *rd1*^{−/−} retina as a model for inner retinal hyperoxia is that the degeneration of photoreceptors does not perturb the inner retina in ways that complicate the analysis of the hyperoxia response.

As determined by in situ hybridization, the large increase in the abundance of VEGF transcripts in the inner nuclear layer in adult *Ndp*^{KO} retinas was eliminated in *Ndp*^{KO};*rd1*^{−/−} retinas (Fig. 1A; compare Figs. 1Ad, 1Aj). (Genotypes and retinal phenotypes are shown in Table 1, and the number of retinas and mice examined is shown in Table 2.) Indeed, VEGF transcript levels in *Ndp*^{+/−};*rd1*^{−/−} and *Ndp*^{KO};*rd1*^{−/−} retinas appear to be below the level observed in the *Ndp*^{+/−};*rd1*^{+/−} control retina (Fig. 1A; compare Figs. 1Aa, 1Ag, 1Aj). (*Ndp* is an X-linked gene. To simplify the text that follows, we refer to both *Ndp*^{−/−} females and *Ndp*^{−/Y} males as *Ndp*^{KO}. The breeding scheme used for these experiments produced littermates that served as controls.) Hypoxyprobe uptake showed that the tissue hypoxia that characterizes *Ndp*^{KO};*rd1*^{+/−} retinas was missing in *Ndp*^{KO};*rd1*^{−/−} retinas, despite a lack of intraretinal capillaries in both cases (Figs. 1Ab, 1Ae, 1Ah, 1Ak). These results validate the working assumption that photoreceptor

FIGURE 1. Effects of hyperoxia, secondary to early photoreceptor degeneration, on vascular architecture in Ndp^{KO} retinas. (A) Vascular endothelial growth factor transcripts were detected by in situ hybridization (top row); retinal hypoxia was visualized by Hypoxyprobe localization (middle row); the Müller glial stress response was visualized with anti-GFAP (bottom row); and vascular anatomy was visualized with GS-lectin (middle and bottom rows). Genotypes analyzed: $Ndp^{+/+};rd1^{+/+}$ (phenotypically WT control), $Ndp^{KO};rd1^{+/+}$, $Ndp^{+/+};rd1^{-/-}$, and $Ndp^{KO};rd1^{-/-}$. Since $rd1$ is recessive, $rd1^{+/+}$ retinas closely resemble $rd1^{+/+}$ retinas. The vascular architecture in $Ndp^{+/+};rd1^{-/-}$ retinas closely approximates that of WT retinas. The ONL is missing from $rd1^{-/-}$ retinas. In these and all other retina cross sections, the outer retina is up and the inner retina is down. In all figures,

retinas are from young adult mice unless indicated otherwise. Ch, choroid; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. *Scale bars*: 100 μ m. (B) PLVAP and claudin-5 expression in ECs in the *rd1*^{+/-}, *Ndp*^{KO}/*rd1*^{+/-}, *rd1*^{-/-}, and *Ndp*^{KO}/*rd1*^{-/-} retinal vasculature. Retinas are shown in cross section as in (A). *Scale bar*: 200 μ m. (C) Vascular density and artery/vein differentiation at the vitreal surface of *rd1*^{+/-}, *Ndp*^{KO}/*rd1*^{+/-}, *rd1*^{-/-}, and *Ndp*^{KO}/*rd1*^{-/-} retinas shown as flat mounts. Smooth muscle actin (SMA) is a marker of arterial pericytes; GS-lectin labels all ECs. A, artery; V, vein. *Scale bar*: 200 μ m. (D) Retina flat mounts showing that claudin-5 is suppressed in all blood vessels in *Ndp*^{KO}/*rd1*^{+/-} retinas and is suppressed in veins and capillaries but not in arteries in *Ndp*^{KO}/*rd1*^{-/-} retinas. A, artery; V, vein. *Scale bar*: 200 μ m.

TABLE 1. Summary of Retinal and Vascular Phenotypes

Genotype	Retinal Structure	Intraretinal Capillaries	Retinal Hypoxia	VEGF RNA Production by Müller Glia	Hyperproliferation of Vasculature on the Vitreal Surface
WT	Normal	Normal	Minimal	Minimal	No
<i>Ndp</i> ^{KO}	Normal	Absent	Yes	Elevated	Yes
<i>rd1</i> ^{-/-}	ONL missing	Reduced	No	Reduced	No
<i>Ndp</i> ^{KO} / <i>rd1</i> ^{-/-}	ONL missing	Absent	No	Reduced	No
<i>Ndp</i> ^{KO} / <i>Pax6-alpha-Cre</i> ; <i>Hif2-alpha</i> ^{CKO/-}	Normal	Absent	(Yes)*	Eliminated, in the periphery	Reduced
<i>Ndp</i> ^{KO} / <i>GLAST-CreER</i> ; <i>Hif2-alpha</i> ^{CKO/-}	Normal	Absent	(Yes)*	Eliminated	Reduced
<i>Nrp1</i> ^{CKO/-} ; <i>Pdgfb-CreER</i>	Normal	Reduced	(Yes)*	(Elevated)†	No
<i>Fz4</i> ^{CKO/-} ; <i>Nrp1</i> ^{CKO/-} ; <i>Pdgfb-CreER</i>	Normal	Absent	(Yes)*	(Elevated)†	No

* Retinal hypoxia phenotypes shown in parentheses are inferred based on the paucity of intraretinal capillaries, but hypoxia was not measured with Hypoxyprobe.

† Vascular endothelial growth factor RNA levels shown in parentheses are inferred based on the paucity of intraretinal capillaries, but VEGF transcripts were not measured by in situ hybridization.

loss in *rd1*^{-/-} mice leads to abundant oxygenation of the inner retina. Müller glial stress, measured by GFAP induction, was prominent with loss of *Ndp*, *rd1*, or both (Figs. 1Ac, 1Af, 1Ai, 1Al). In the text that follows, we will refer for convenience to VEGF as if it is the only mediator of the vascular insufficiency/hypoxia response, but we note that other intercellular signaling molecules could also play a role in this response.

In the absence of elevated VEGF, that is, in an *rd1*^{-/-} background, loss of *Ndp* did not result in hyperproliferation of vessels on the vitreal face of the retina (Fig. 1B; compare Figs. 1Bd–Bf, 1Bj–Bl; Fig. 1C; compare Figs. 1Cb, *Ndp*^{KO} phenotype of aberrant vein-associated mis-expression of smooth muscle actin (SMA), which is normally enriched in artery- and arteriole-associated mural cells relative to venous mural cells (Fig. 1C). The conversion of blood vessels from a BRB-competent state (characterized by the presence of the tight junction protein claudin-5 and the absence of the endothelial fenestration structural protein plasmalemma vesicle-associated protein [PLVAP]³⁵; hereafter “PLVAP+/claudin-5+”) to a BRB-incompetent state (PLVAP+/claudin-5–) was observed in veins and capillaries to a greater extent than in arteries in *Ndp*^{KO}/*rd1*^{-/-} retinas, implying that in veins and capillaries this conversion is not dependent on elevated VEGF signaling (Figs. 1B, 1D).

To functionally assess BRB integrity, we visualized the accumulation of IgG (molecular weight ~150 kDa) in retinal tissue. Figures 2a through 2l show that endogenous IgG, which is normally confined largely to the intravascular space, accumulated in retinal tissue in *Ndp*^{KO} retinas but not in *wild-type* (WT), *rd1*^{-/-}, or *Ndp*^{KO}/*rd1*^{-/-} retinas. Overexpression of *endothelin-2* (*Edn2*) in *Z/Edn2*; *Pax6-alpha-Cre* retinas produces a retinal phenotype similar to *Ndp*^{KO}, with suppression of intraretinal vascular invasion, retinal hypoxia, elevated VEGF expression, and proliferation of blood vessels on the vitreal face of the retina.²⁸ Interestingly, *Edn2* overexpression was not associated with accumulation of IgG in retinal tissue (Figs. 2m–2o).

Earlier studies of genetically mosaic retinal vasculature demonstrated that loss of Norrin/Fz4 signaling in only one or a few ECs leads to a conversion of ECs from a PLVAP+/claudin-5+ state to a PLVAP+/claudin-5– state with an associated leakage of low molecular weight tracers (~300 Da) from the intravascular space to the surrounding tissue.¹⁸ The data in

Figure 2 imply that transendothelial passage of IgG is facilitated by the combination of absent Norrin/Fz4 signaling and high VEGF levels (*Ndp*^{KO}), whereas intact Norrin/Fz4 signaling with elevated VEGF levels (*Z/Edn2*; *Pax6-alpha-Cre* peripheral retina) or absent Norrin/Fz4 signaling without elevated VEGF levels (*Ndp*^{KO}/*rd1*^{-/-}) is insufficient to induce IgG permeability. We note that the *Z/Edn2*; *Pax6-alpha-Cre* phenotype should be interpreted cautiously since *Edn2* overexpression could perturb vascular permeability, either directly or indirectly.

TABLE 2. Numbers of Retinas and/or Mice Analyzed per Genotype

Genotype	Number of Retinas† and/or Number of Mice*
WT	4†
<i>Ndp</i> ^{+/-} / <i>rd1</i> ^{+/-}	4†, 2*
<i>Ndp</i> ^{+/-} / <i>rd1</i> ^{-/-}	2†
<i>Ndp</i> ^{KO} / <i>rd1</i> ^{+/-}	2†, 2*
<i>Ndp</i> ^{KO} / <i>rd1</i> ^{-/-}	5†, 3*
<i>rd1</i> ^{-/-}	5†
<i>Ndp</i> ^{KO}	7†
<i>Ndp</i> ^{+/-}	4†
<i>Z/Edn2</i> ; <i>Pax6-alpha-Cre</i>	6*
<i>Ndp</i> ^{KO} ; <i>Pax6-alpha-Cre</i> ; <i>Hif2-alpha</i> ^{CKO/-}	Whole mount: 10†, 6* Sections: 4†, 4*
<i>Ndp</i> ^{KO} ; <i>Pax6-alpha-Cre</i> ; <i>Hif2-alpha</i> ^{CKO/+}	Whole mount: 11†, 6* Sections: 3†, 4*
<i>Ndp</i> ^{KO} ; <i>GLAST-Cre</i> ; <i>Hif2-alpha</i> ^{CKO/-}	Whole mount: 4†, 4* Sections: 2†, 2*
<i>Ndp</i> ^{KO} ; <i>GLAST-alpha-Cre</i> ; <i>Hif2-alpha</i> ^{CKO/+}	Whole mount: 4†, 3* Sections: 2†, 2*
<i>Nrp1</i> ^{CKO/-} ; <i>Pdgfb-CreER</i>	>10†
<i>Fz4</i> ^{CKO/+} ; <i>Nrp1</i> ^{CKO/+} ; <i>Pdgfb-CreER</i>	>10†
<i>Fz4</i> ^{CKO/-} ; <i>Nrp1</i> ^{CKO/+} ; <i>Pdgfb-CreER</i>	>10†
<i>Fz4</i> ^{CKO/+} ; <i>Nrp1</i> ^{CKO/-} ; <i>Pdgfb-CreER</i>	>10†
<i>Fz4</i> ^{CKO/-} ; <i>Nrp1</i> ^{CKO/-} ; <i>Pdgfb-CreER</i>	>10†

* Number of mice analyzed.

† Number of retinas analyzed.

FIGURE 2. Effects of hyperoxia, secondary to early photoreceptor degeneration, on BRB integrity in *Ndp*^{KO} retinas. Cross sections of young adult retinas immunostained for mouse IgG and incubated with GS-lectin and DAPI. High levels of endogenous mouse IgG have accumulated in extravascular tissue in *Ndp*^{KO} retinas, but not in WT, *rd1*^{-/-}, *Ndp*^{KO};*rd1*^{-/-}, or *Z/Edn2*;*Pax6-αCre* retinas. Abbreviations as in Figure 1. Scale bar: 50 μm.

HIF2-Alpha Controls VEGF Induction, Which Increases Vascular Density in *Ndp*^{KO} Retinas

As a second approach to assessing the role of the hypoxia response in general and VEGF induction in particular in sculpting the *Ndp*^{KO} vasculature, we sought to blunt this response by conditional knockout of the responsible transcription factor(s). The hypoxia response is controlled by HIF1-α and HIF2-α, each of which is regulated by oxygen-dependent proline hydroxylation and ubiquitin-mediated proteolysis of the nonhydroxylated polypeptide.³⁶ Previous work with neural retina-specific deletion of the *Hif1-α* gene demonstrated that this transcription factor regulates erythropoietin expression, but it is not the major regulator of VEGF expression.³⁷ With this result in mind, we asked whether retina-specific deletion of *Hif2-α* could eliminate VEGF induction in response to hypoxia.

In these experiments we have used *Pax6-αCre* to recombine a *Hif2-α*^{KO} allele in the neural retina. As reported by Marquardt et al.,²⁵ this transgene drives Cre expression in a spatial gradient beginning at ~embryonic day

(E)9.5, with nearly all neurons and Müller glia in the peripheral retina exhibiting Cre-mediated recombination and a low frequency of Cre-mediated recombination in the central retina (Fig. 3A; note that the reporter used here is silenced, not activated, by Cre-mediated recombination). There is also a lower frequency of Cre-mediated recombination in the dorsal/ventral retina compared to the medial/lateral retina, although our observations suggest substantial retina-to-retina variability in the extent of this asymmetry. In cross sections of control *Ndp*^{KO};*Pax6-αCre*;*Hif2-α*^{KO/+} retinas from 4- to 5-week old mice, high levels of VEGF transcripts were found in the inner nuclear layer (INL) uniformly across the retina (Fig. 3B, left). In contrast, cross sections of age-matched *Ndp*^{KO};*Pax6-αCre*;*Hif2-α*^{KO/-} retinas showed a small reduction in the number of VEGF-expressing cells in the central retina and a severe reduction in the number of VEGF-expressing cells in the peripheral retina (Fig. 3B, right). Control *Ndp*^{KO};*Hif2-α*^{KO/-} mice have a retinal phenotype indistinguishable from *Ndp*^{KO} mice, and control *Hif2-α*^{KO/-} mice have a retinal phenotype indistinguishable

FIGURE 3. A reduction in the retinal hypoxia response blunts vascular proliferation in response to loss of Norrin/Fz4 signaling. (A) X-gal staining of a retina flat mount in which a constitutively expressed lacZ reporter with flanking *loxP* sites was knocked into the *Ubiquitin B (Ubb)* locus ("*Z/X*") and subsequently excised by Cre-mediated recombination after crossing to the Pax6- α -Cre locus. Note that the reporter is expressed in regions where Cre is absent, and it is deleted in regions where Cre is present. D, dorsal; V, ventral. (B) Vascular endothelial growth factor transcripts visualized by in situ hybridization (red) and vascular anatomy visualized with GS-lectin (green) in cross sections from adult $Ndp^{KO};Pax6\alpha$ -Cre;Hif2 $\alpha^{CKO/+}$ (control) versus $Ndp^{KO};Pax6\alpha$ -Cre;Hif2 $\alpha^{CKO/-}$ retinas. In the peripheral $Ndp^{KO};Pax6\alpha$ -Cre;Hif2 $\alpha^{CKO/-}$ retina, the abundance of VEGF transcripts in the INL is reduced relative to the control, and vascular proliferation is also reduced. In these images, the nitro blue tetrazolium (NBT)-based in situ hybridization signal has been false colored red. The choroidal melanin also appears red when the image is processed in this manner. Abbreviations as in Figure 1. Scale bar: 100 μm . (C) Retina flat mounts showing reduced numbers of vascular glomeruloids in the medial and temporal sides of young adult control $Ndp^{KO};Pax6\alpha$ -Cre;Hif2 $\alpha^{CKO/+}$ (left) versus $Ndp^{KO};Pax6\alpha$ -Cre;Hif2 $\alpha^{CKO/-}$ (right) retinas. The number of glomeruloids was counted in four 640 $\times$ 640- μm territories from each retina (one per quadrant; red squares); these regions are enlarged in a through d and e through h, with each glomeruloid circled in red. D, dorsal; V, ventral. Scale bars: 1 mm (upper); 100 μm (lower). (D) Quantification of glomeruloid density from $Ndp^{KO};Pax6\alpha$ -Cre;Hif2 $\alpha^{CKO/+}$ and $Ndp^{KO};Pax6\alpha$ -Cre;Hif2 $\alpha^{CKO/-}$ retinas, scored as shown in (C). Vascular density was scored from the same cohort of retinas, as described in the Methods section. The number of territories analyzed (N) is shown in the figure. Bars: Mean $\pm$ standard deviation. P values were calculated with a two-tailed Student's *t*-test.

from WT mice. These data imply that HIF2- α is the major regulator of VEGF expression in the INL.

Flat-mounted $Ndp^{KO};Pax6\alpha$ -Cre;Hif2 $\alpha^{CKO/-}$ retinas showed low vascular density, whereas control $Ndp^{KO};Pax6\alpha$ -Cre;Hif2 $\alpha^{CKO/+}$ retinas showed high vascular den-

sity (Figs. 3C, 3D). In addition, quantification of vascular tufts/glomeruloids, which appear to represent abortive angiogenic invasion of the inner retina from the surface vasculature, revealed a reduction in $Ndp^{KO};Pax6\alpha$ -Cre;Hif2 $\alpha^{CKO/-}$ retinas compared to $Ndp^{KO};Pax6\alpha$ -Cre;Hif2 $\alpha^{CKO/+}$

controls (Figs. 3C, 3D). Within *Ndp^{KO};Pax6-alpha-Cre;Hif2-alpha^{CKO/-}* retinas, vascular density did not show significant regional variation when assessed across the cohort of retinas. Taken together, these data imply that in the hypoxic *Ndp^{KO}* retina, HIF2-alpha induces the production of signaling molecules, including VEGF, that stimulate vascular proliferation on the vitreal surface of the retina but are unable to correct the defect in vascular invasion.

Müller Glial Cells Are the Source of HIF2-Alpha-Regulated VEGF

The localization of VEGF transcripts to the INL is consistent with expression of VEGF in Müller glia, bipolar cells, horizontal cells, and/or amacrine cells. However, significant VEGF expression in horizontal or amacrine cells seems unlikely since these cells reside in the outermost and innermost layers of the INL, respectively, and the VEGF in situ hybridization signal is centered in the INL (Figs. 1A, 3B). The loss of VEGF induction with neural retina deletion of the *Hif2-alpha* gene (Fig. 3B), together with an earlier report that HIF2-alpha localizes to Müller glia and astrocytes, whereas HIF1-alpha localizes to neurons,³⁸ implies that Müller glia are the principal source of VEGF. To directly test this hypothesis, we used a glutamate/aspartate transporter (*EAAT1;SLC1A3;GLAST*)-*CreER* bacterial artificial chromosome (BAC) transgene to eliminate *Hif2-alpha* expression selectively in Müller glia in the *Ndp^{KO}* retina.

Figure 4A shows the cell type specificity of the *GLAST-CreER* transgene. In *SUN1-GFP;GLAST-CreER* retinas, Cre-mediated recombination activated expression of the *SUN1-GFP* reporter in a row of nuclei in the center of the INL, the expected subcellular localization, since SUN1 is a nuclear membrane protein (Fig. 4A, left). In these retinas, there were rare cells in other retinal layers that also expressed the reporter (data not shown). In *mTmG;GLAST-CreER* retinas, Cre-mediated recombination following low-dose 4-hydroxytamoxifen (4HT) activated expression of the *mTmG* reporter (a membrane-anchored GFP) in cells with the distinctive morphology of Müller glia, which span the full thickness of the retina (Fig. 4A, right).

In retina cross sections, there was a nearly complete absence of VEGF transcript induction in the INL in *Ndp^{KO}* mice in which the *Hif2-alpha* gene had been selectively deleted in Müller glia (*Ndp^{KO};GLAST-CreER;Hif2-alpha^{CKO/-}* mice that were treated with high-dose 4HT in early postnatal life; Fig. 4B). Rare INL cells that showed VEGF transcript induction are likely to represent rare failures of Cre-mediated recombination. In comparing retinas from *Ndp^{KO};GLAST-CreER;Hif2-alpha^{CKO/-}* and control *Ndp^{KO};GLAST-CreER;Hif2-alpha^{CKO/+}* mice, we observe in *Ndp^{KO};GLAST-CreER;Hif2-alpha^{CKO/-}* retinas the same pattern of reduced vascular density and reduced numbers of vascular glomeruloids as observed in *Ndp^{KO};Pax6-alpha-Cre;Hif2-alpha^{CKO/-}* retinas (compare Figs. 3C, 3D to Figs. 4C, 4D).

The difference in vascular density between the experimental and control retinas shown in Figures 3 and 4 likely represents an underestimate of the true magnitude of the *Hif2-alpha* effect because Cre-mediated recombination at the *Hif2-alpha^{CKO}* allele is less than complete in the periphery of *Ndp^{KO};Pax6-alpha-Cre;Hif2-alpha^{CKO/-}* retinas (Fig. 3A) and in Müller glia in *Ndp^{KO};GLAST-CreER;Hif2-alpha^{CKO/-}* retinas (Fig. 4B). Taken together, the data in Figures 1, 3, and 4 imply that in the absence of Norrin/Fz4 signaling, developmental hypovascularization leads to retinal hypoxia, which triggers HIF2-alpha-mediated VEGF production by Müller glia, which then induces a compensatory increase in vascular density on and near the vitreal surface of the retina.

A Role for Neuropilin1 in Regulating Vascular Growth in Retinas Deficient in Norrin/Fz4 Signaling

Neuropilin1 plays a dual role as a cell surface receptor: It acts as a receptor for semaphorin3A (SEMA3A) and as a coreceptor for VEGF in cooperation with the tyrosine kinase receptor VEGFR2.³⁹ *Neuropilin1* is required in ECs for normal angiogenesis, and in its absence embryonic angiogenesis is disrupted, although the disruption is not as severe as that seen with loss of VEGF.^{40–43} Recent experiments in which mice were engineered to express a hypomorphic *Nrp1* allele that lacks VEGF binding but retains SEMA3A binding in place of the *WT Nrp1* allele indicate that VEGF binding to NRP1 is largely dispensable in the context of embryonic angiogenesis but is required for rapid radial growth of ECs on the surface of the early postnatal retina and for the full neovascularization response to oxygen-induced retinopathy.⁴⁴

To define the vascular phenotype associated with a complete loss of *Nrp1* in retinal ECs and to explore the role that NRP1 plays in the response to a deficiency in Norrin/Fz4 signaling, we characterized retinas with early postnatal EC-specific deletion of a conditional *Nrp1* allele (*Nrp1^{CKO/-};Pdgfb-CreER* mice treated with 4HT before P6). A conditional mutant strategy was required because conventional *Nrp1* null mutations lead to embryonic lethality. In the presence of *Pdgfb-CreER* and following a single 4HT injection, the *Nrp1^{CKO}* allele used here was observed to recombine with nearly 100% efficiency (Fig. 5E). Early postnatal loss of *Nrp1* (4HT at P3 or P4) leads to a severe disruption of retinal vascular invasion with multiple glomeruloid structures near the vitreal surface and endothelial extensions from INL capillaries that protrude into but fail to ramify in the outer nuclear layer (ONL; Figs. 5A, 5B). As a result, overall vascular density is reduced compared to that in *WT* retinas, as quantified in Figure 5F, left. The arteries and veins of the *Nrp1^{CKO/-};Pdgfb-CreER* retinal vasculature exhibit a high degree of tortuosity (a snake-like appearance of the vessels; Fig. 5C), a finding characteristic of some hypoxic retinal conditions.⁴⁵ In the absence of *Nrp1* there is also an increase in the diameter of veins (Fig. 5C).

We next characterized retinal vascular architecture in mice in which both *Nrp1* and *Fz4* were eliminated during the early postnatal period using *Pdgfb-CreER*. In the NRP1 experiments described here, we used mutations in *Fz4* rather than *Ndp* to eliminate Norrin/Fz4 signaling. As shown previously,¹⁶ *Fz4^{-/-}* and *Ndp^{KO}* retinas exhibit indistinguishable retinal vascular phenotypes. We have used *Fz4^{+/-}* and *Nrp1^{+/-}* genotypes as controls, since we observe little or no effects on retinal vascular architecture of heterozygosity for *Fz4* or *Nrp1* (or both) compared to *WT* (Fig. 5D, left). As shown earlier, loss of *Fz4* is marked by a cell-autonomous conversion of ECs from a PLVAP-/claudin-5+ state to a PLVAP+/claudin-5- state¹⁸ (Figs. 5D, 5E). In contrast to the high-efficiency deletion of the *Nrp1^{CKO}* allele, the *Fz4^{CKO}* allele recombines at lower efficiency, generating mosaic retinal vasculatures with patches of *Fz4^{+/-}* (unrecombined; PLVAP-) and *Fz4^{-/-}* (recombined; PLVAP+) vasculature (Fig. 5E).

Figure 5D shows a set of four littermate retinas from a cross that generated all of the genotypes of interest: *Fz4^{CKO/+};Nrp1^{CKO/+};Pdgfb-CreER* (*WT* phenotype), *Fz4^{CKO/-};Nrp1^{CKO/+};Pdgfb-CreER* (mosaic for loss of *Fz4*), *Fz4^{CKO/+};Nrp1^{CKO/-};Pdgfb-CreER* (nearly complete loss of *Nrp1*), and *Fz4^{CKO/-};Nrp1^{CKO/-};Pdgfb-CreER* (mosaic for loss of *Fz4* with nearly complete loss of *Nrp1*). In *Fz4^{CKO/+};Nrp1^{CKO/-};Pdgfb-CreER* retinas, occasional zones of hyperdense vascular growth occur on the retinal surface (white arrow in Fig. 5D, third image), which could represent a clonal expansion of rare ECs that did not undergo Cre-mediated deletion of the *Nrp1^{CKO}* allele; that is,

FIGURE 4. Müller glia-specific deletion of *Hif-2alpha* blunts vascular proliferation in response to loss of Norrin/Frizzled4 signaling. **(A)** Cross sections of retinas with a *R26-loxP-stop-LoxP(LSL)-SUN1-GFP* reporter or a *R26-loxP-membrane-tdTomato-loxP-membrane-GFP (mTmG²⁹)* reporter show Müller glia-specific Cre-mediated recombination in the presence of the *GLAST-CreER* BAC transgene. Scale bar: 50 μm . **(B)** Loss of *Hif2-alpha* in Müller glia leads to loss of induction of VEGF transcripts (dark signals in the center of the INL) in an *Ndp^{KO}* background. Top: In situ hybridization signal (nitro blue tetrazolium [NBT]-based alkaline phosphatase reaction product) is shown in a darkfield image. Bottom: The NBT signal is false colored red and is shown with GS-lectin (blood vessels) and DAPI. Rare Müller glia that show VEGF transcript induction in the *Ndp^{KO}* background (arrows at right) presumably represent cells in which Cre failed to delete the *Hif2-alpha^{CKO/-}* allele. Scale bar: 100 μm . **(C)** Retina flat mounts showing reduced vascular density and reduced numbers of vascular glomeruloids in adult *Ndp^{KO}; GLAST-CreER; Hif2-alpha^{CKO/-}* (right) versus control *Ndp^{KO}; GLAST-CreER; Hif2-alpha^{CKO/+}* (left) retinas. The vascular glomeruloids and the number of glomeruloids were quantified in four 640 $\times$ 640- μm territories from each retina (one per quadrant; red squares); these regions are enlarged in a through d and e through h, with each glomeruloid circled in red. Scale bars: 1 mm (upper); 100 μm (lower). **(D)** Quantification of glomeruloid density in adult *Ndp^{KO}; GLAST-CreER; Hif2-alpha^{CKO/-}* versus control *Ndp^{KO}; GLAST-CreER; Hif2-alpha^{CKO/+}* retinas, scored as shown in (C). Vascular density was scored from the same cohort of retinas, as described in the Methods section. Sixteen territories were analyzed for each sample. Bars: Mean $\pm$ standard deviation. *P* values were calculated with a two-tailed Student's *t*-test.

they are *Nrp1^{+/-}* in a vasculature that is predominantly *Nrp1^{-/-}*. Eliminating *Fz4* together with *Nrp1* (*Fz4^{CKO/-}; Nrp1^{CKO/-}; Pdgfrb-CreER*) is permissive for the production of vascular glomeruloids but results in a lower vascular density at the vitreal face of the

retina compared to the density observed with loss of *Fz4* alone (compare Fig. 5D, second and fourth images) or loss of *Nrp1* alone (compare Fig. 5D, third and fourth images). These data are quantified in Figure 5E. These observations are most easily

FIGURE 5. Vascular defects in adult retinas in response to early postnatal loss of *Nrp1* and/or *Fz4*. **(A)** GS-lectin-stained flat mount of a P27 retina from a *Nrp1^{CKO/-};Pdgfb-CreER* mouse that was treated with 400 µg 4HT at P4. Scale bar: 1 mm. **(B)** GS-lectin-stained flat mounts of P30 retinas from control *Nrp1^{CKO/-}* and *Nrp1^{CKO/-};Pdgfb-CreER* mice treated with 400 µg 4HT at P4. Depth is color coded. Arrowheads point to dead end capillary protrusions in the OPL. Scale bar: 200 µm. **(C)** GS-lectin- and anti-smooth muscle actin (SMA)-stained flat mounts of P35 retinas from WT and *Nrp1^{CKO/-};Pdgfb-CreER* mice treated with 200 µg 4HT at P5 and P7 showing increased tortuosity of arteries and veins in the *Nrp1^{CKO/-};Pdgfb-CreER* retina. The white boxed region in the center is enlarged at the right. The traced images in the lower three images show the trajectories of arteries and veins. Scale bar: 1 mm. **(D)** P22 retina flat mounts immunostained for PLVAP and claudin-5 following mosaic EC-specific postnatal deletion of *Fz4* alone (second image), *Nrp1* alone (third image), or both *Fz4* and *Nrp1* (right image). Heterozygous genotypes serve as controls. The mice were littermates, and each mouse received IP injections of 200 µg 4HT at P6, P9, and P10. Loss of *Fz4* expression is marked by the cell-autonomous conversion of ECs from PLVAP-/claudin-5+ to PLVAP+/claudin-5-. White arrow in the third image (*Fz4^{CKO/+};Nrp1^{CKO/-};Pdgfb-CreER*) marks a localized cluster of high vascular density. Scale bar: 500 µm. **(E)** The *Nrp1^{CKO}* allele recombines with high efficiency, and the *Fz4^{CKO}* allele

recombines with lower efficiency. A single IP injection of 400 μ g 4HT at P8 leads to nearly complete loss of *Nrp1* in *Fz4^{CKO/-};Nrp1^{CKO/-};Pdgfb-CreER* as seen in a retina flat mount stained with anti-NRP1, whereas the control *Fz4^{CKO/-};Nrp1^{CKO/-};Pdgfb-CreER* littermate shows widespread vascular expression of *Nrp1* from the remaining *Nrp1* allele. Loss of *Fz4* expression in ECs (marked by the cell-autonomous conversion from PLVAP+/claudin-5+ to PLVAP+/claudin-5-) is seen in ~50% of ECs in both retinas. Scale bar: 500 μ m. (F) Quantification of vascular density in 640- $\times$ 640- μ m squares (four per retina) (left) for WT versus *Nrp1^{CKO/-};Pdgfb-CreER* retinas at P25 and P35, and (right) for the four retinas (and additional littermates) shown in (D). All littermates received the same regimen of 4HT. Vascular density was scored as described in the Methods section. The number of territories analyzed (*N*) is shown in the figure. Bars: Mean $\pm$ standard deviation. *P* values were calculated with a two-tailed Student's *t*-test.

explained by positing that NRP1 promotes VEGF signaling to stimulate vascular proliferation in hypoxic retinas that lack Norrin/Fz4 signaling. This explanation is consistent with the lower vascular density observed in retinas with reductions in Müller glial-derived VEGF, as shown in Figures 3 and 4.

DISCUSSION

This study clarifies the role of the hypoxia/VEGF response to a loss of Norrin/Fz4 signaling during retinal vascular development, as summarized in Figure 6. The principal findings are that in the absence of Norrin/Fz4 signaling, (1) Müller glia exhibit a hypoxia response, as measured by the accumulation of VEGF transcripts, secondary to the failure of inner retinal vascularization; (2) the hypoxia response in Müller glia can be suppressed by deletion of *Hif2-alpha*, which decreases vascular proliferation at the vitreal face of the retina and the density of vascular glomeruloids; (3) hyperoxygenation of the inner retina, secondary to an early loss of photoreceptors in the *rd1* mutant retina, suppresses the hypoxia response, VEGF induction, and compensatory vascular proliferation; and (4) loss of the VEGF coreceptor NRP1 reduces compensatory vascular proliferation without significantly altering the formation of vascular glomeruloids. We also describe the distinctive retinal vascular phenotype that results from EC-specific loss of *Nrp1*. This phenotype includes a partial deficiency in the development of the deep vascular plexus, an increase in the diameter of veins, and an increase in the tortuosity of arteries and veins. While the chain of events leading to each of these *Nrp1* phenotypes remains to be determined, the severity of the defects implies a major role for NRP1 in retinal vascular development.

Role of the Hypoxia Response in Compensatory Vascularization

Previous work from multiple laboratories has explored the role of the HIF system in mediating normal retinal vascular development and the response to hypoxia. Mowat et al.³⁸ showed that HIF1-alpha immunoreactivity is predominantly localized to the nuclei of inner retinal neurons and HIF2-alpha immunoreactivity is predominantly localized to nuclei that very likely correspond to Müller glia. Mowat et al.³⁸ further showed that both HIF1-alpha and HIF2-alpha proteins accumulate during the hypoxic phase of oxygen-induced retinopathy, whereas the abundances of their transcripts were unaffected. *Hif2-alpha* knockout mice—which survive beyond birth only on particular genetic backgrounds⁴⁶—show severely reduced retinal vascularization,⁴⁷ but the *Hif2-alpha*-expressing cells relevant to this phenotype have not been defined. In mice, eliminating retinal *Hif1-alpha* blocks only the development of the intermediate vascular plexus, although the interpretation of this observation is complicated by the presence of a compensatory induction of *Hif2-alpha*.³⁷ Taken together, these observations point to essential but nonequivalent roles of HIF1-alpha and HIF2-alpha in normal retinal vascular development.

In the context of retinal stress associated with hyperoxia or hypoxia, reducing Hif1-alpha expression in Müller glia led to a

reduced neovascular response to oxygen-induced retinopathy (OIR),⁴⁸ and eliminating the prolyl hydroxylase-1 (*Pdh-1*) gene (which codes for the enzyme that hydroxylates and destabilizes HIF1-alpha and HIF2-alpha) led to reduced hyperoxia-induced vascular obliteration.⁴⁹ Similarly, a partial reduction in Müller glial-derived VEGF leads to a reduction in ischemia-induced neovascularization.⁵⁰ Our observations with Müller glial-specific *Hif2-alpha* deletion clarify a number of these findings by identifying HIF2-alpha as the central regulator of the hypoxia response in Müller cells.

Comparison of Norrin/Fz4 Deficiency and the Oxygen-Induced Retinopathy Model of Neovascularization

Oxygen-induced retinopathy represents a major source of morbidity in preterm infants, and as a result it has been studied intensively for more than half a century.⁵¹ Over the past 20 years, the mouse has become the most widely used experimental animal for OIR studies, and this model has been used to assess a wide variety of genetic and pharmacologic perturbations for their effects on neovascularization.⁵² The present study explores a highly reproducible form of hypoxia that is developmentally and anatomically distinct from the hypoxia produced by the OIR model. In the standard mouse OIR model, exposure to 75% oxygen between P7 and P12 leads to vessel loss near the center of the retina (the vaso-obliterative phase). Following a return to normoxia at P12, there is rapid growth of disorganized surface vessels (the neovascular phase). Over the ensuing 2 weeks in normoxic conditions, the neovascular tufts

FIGURE 6. Schematic showing the Müller glial HIF2-alpha/VEGF feedback loop to the growing retinal vasculature. *Left:* A WT retina in which low-level hypoxia elicits a low level of HIF2-alpha-dependent VEGF production from Müller glia, which acts on VEGFR2/NRP1 on vascular ECs. *Right:* A retina in which Norrin/Fz4 signaling is defective or absent, and in which severe hypoxia elicits a high level of HIF2-alpha-dependent VEGF production from Müller glia, which binds to VEGFR2/NRP1 on vascular ECs.

regress and the pair of intraretinal vascular beds develop. By contrast, the loss of Norrin/Fz4 signaling leads to complete and relatively uniform vascular coverage over the vitreal face of the retina with a complete absence of intraretinal vascular beds. The resulting retinal hypoxia increases during the second postnatal week as retinal neurons mature, and it remains high for the life of the mouse. In light of the differences in these two hypoxia models, it would be interesting to compare their responses to various pharmacologic perturbations.

Arterial and venous tortuosity and venous dilation are seen in both the *Nrp1* mutant retina and during the neovascular phase of OIR in the mouse, and quantification of arterial tortuosity has recently been used as an early outcome measure in the mouse OIR model.⁴⁵ The generation of tortuous vessels in the mouse OIR model is associated with interstitial EC proliferation.⁵³ In humans, dilated and tortuous vessels in the posterior pole are associated with retinopathy of prematurity,^{54–56} and tortuous veins are associated with branch vein occlusion.⁵⁷ A recent study suggests that quantitative assessment of retinal microvascular tortuosity may have predictive value for cardiovascular health in the general population, as there is a statistically significant increase in tortuosity in subjects who have had an ischemic stroke compared to an age-matched control cohort.⁵⁸ It would be interesting to explore the mechanisms responsible for the development of vessel tortuosity in the *Nrp1* mutant retina to determine whether it arises simply as a consequence of hypoxia due to underdevelopment of the deep vascular plexus or whether it also reflects aberrant regulation of EC proliferation due to loss of *Nrp1* function.

Implications for Norrie Disease, Osteoporosis-Pseudoglioma Syndrome, and FEVR

The present study has implications for understanding the pathophysiology of human disorders of Norrin/Fz4 signaling. In particular, the restoration of BRB impermeability for high molecular weight molecules (IgG) with suppression of the hypoxic response in *Ndp^{KO};rd1^{-/-}* retinas (Fig. 2) is intriguing. Our earlier studies of genetically mosaic retinal and brain vasculature demonstrated a cell-autonomous loss of vascular barrier integrity for a low molecular weight tracer (sulfo-NHS-biotin) when Norrin/Fz4 signaling was eliminated in a subset of ECs.^{18,19} In the most informative mosaic animals studied by Wang et al.,¹⁸ only a small minority of ECs were rendered *Fz4^{-/-}* by Cre-mediated recombination. In these retinas, vascular architecture—and, presumably, tissue oxygenation—was unaffected, while leakage of the low molecular weight intravascular tracer was observed at the site of individual *Fz4^{-/-}* capillary ECs. Extrapolating from the mouse experiments, the data suggest that in FEVR patients, anti-VEGF therapy could reduce vascular permeability to high molecular weight compounds. In contrast, permeability to low molecular weight compounds may reflect cell-autonomous and VEGF-independent mechanisms.

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