Role of the Norrie Disease Pseudoglioma Gene in Sprouting Angiogenesis during Development of the Retinal Vasculature

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PURPOSE. To characterize developmental defects and the time course of Norrie disease in retinal and hyaloid vasculature during retinal development and to identify underlying molecular angiogenic pathways that may be affected in Norrie disease, exudative vitreoretinopathy, retinopathy of prematurity, and Coats’ disease.

METHODS. Norrie disease pseudoglioma homologue (Ndph)-knockout mice were studied during retinal development at early postnatal (p) stages (p5, p10, p15, and p21). Histologic techniques, quantitative RT-PCR, ELISA, and Western blot analyses provided molecular data, and scanning laser ophthalmoscopy (SLO) angiography and electroretinography (ERG) were used to obtain in vivo data.

RESULTS. The data showed that regression of the hyaloid vasculature of Ndph-knockout mice occurred but was drastically delayed. The development of the superficial retinal vasculature was strongly delayed, whereas the deep retinal vasculature did not form because of the blockage of vessel outgrowth into the deep retinal layers. Subsequently, microaneurysm-like lesions formed. Several angiogenic factors were differentially transcribed during retinal development. Increased levels of hypoxia inducible factor-1α (HIF1α) and VEGFA, as well as a characteristic ERG pattern, confirmed hypoxic conditions in the inner retina of the Ndph-knockout mouse.

CONCLUSIONS. These data provide evidence for a crucial role of Norrin in hyaloid vessel regression and in sprouting angiogenesis during retinal vascular development, especially in the development of the deep retinal capillary networks. They also suggest an early and a late phase of Norrie disease and may provide an explanation for similar phenotypic features of allelic retinal diseases in mice and patients as secondary consequences of pathologic hypoxia. (Invest Ophtalmol Vis Sci. 2005;46:3372–3382) DOI:10.1167/iovs.05-0174

Norrie disease is a rare X-linked recessive disease characterized by retinopathy, deafness, and mental retardation. It is caused by mutations in the Norrie disease gene (NDP), which is involved in the development of the retina. The knockout model of the mouse orthologue Ndph (NDP homologue) resembles the human phenotype in eye and ear. In mutant mice, the most prominent defects are found within retinal vasculature, but the persistence of the hyaloid vessels has also been described. Because of these human and mouse phenotypes a causal relationship between Norrin function and sprouting angiogenesis has been suggested.

We characterized blood vessel development in the eye of Ndph-knockout mice (Ndph−/−) histologically and, for the first time, in vivo. We correlated morphologic and functional de-
effects in retinal vascularization with the expression of genes encoding important angiogenic factors. In addition, we gathered data elucidating the process of hyaloid vessel regression in vivo. Our results reveal primary lesions in sprouting angiogenesis in the retina due to the absence of Norrin, a secondary development of hyoxia in the Ndpb+/− retina, and a delayed regression of the hyaloid vasculature.

METHODS

Animals

The Ndpb knockout mouse line has been described elsewhere.13 Dark-adapted (overnight) mice with dilated pupils were examined with scanning laser ophthalmoscopy (SLO) and electroretinography (ERG). Anesthesia was performed according to a published method.16 The research was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Histologic Examination

Dissected eyes were fixed overnight at 4°C in Serras fixative (60% ethanol, 11.1% formaldehyde, and 10% acetic acid), dehydrated in 70%, 95%, and 100% isopropanol, embedded in paraffin, and sectioned (7 μm). Histologic examination was performed (MGB–3, Man Probes and Primers Used for Expression Assays). The Relative Quantification of Gene Expression Assay Mm00493179-m1 was used.

Wholemount Retinal Staining

Collagen type IV and lectin staining of retinal wholemount preparations has been described.17 Briefly, eyes were fixed in 4% PBS-buffered paraformaldehyde. Dissected retinas were labeled either with collagen type IV (polycyclonal Col IV antibody, rabbit anti mouse, 1:300, no. 2150-1470 Anowa, Wangen, Switzerland), isolectin (Bandeira simplicifolia; Sigma-Aldrich, Deisenhofen, Germany), or glial fibrillary acidic protein (GFAP; polyclonal GFAP antibody; rabbit anti-mouse 1 μg/mL; Dako, Hamburg, Germany). Retinal pericytes were labeled with a polyclonal NG-2 (2 μg/mL; Chemicon, Hoehheim, Germany) and vascular matrix with a polyclonal fibronectin antibody (3.5 μg/mL; Sigma-Aldrich), as published.18 Secondary antibodies were FITC-labeled anti-rabbit (1:20, Dako) or Cy3-labeled anti rabbit (1:300, no. 111-165-003; Dianova, Hamburg, Germany).

Capillary Morphometry

Fluorescent microphotographs were taken with the microscope and camera used for histology or with a second microscope (DMRE) and digital camera (equipped with IM50 software; Leica, Wetzlar, Germany). Retinal vascular network outgrowth and vascular diameters were measured as reported (Qwin software; Leica).20 The relative coverage of retinal area by the vasculature was analyzed (AnalysisPro; Olympus Optical, Hamburg, Germany). Confocal laser scanning micrographs were taken with a microscope (DM IRE2; Leica) and processed with confocal software (Leica).

SLO Angiography

To follow vascular changes in the eyes of the Ndpb+/− mice in vivo at p14 and p21, we used both fluorescein (FLA; argon blue laser, 488 nm; barrier, 500 nm) and indocyanine green (ICGA; infrared laser, 795 nm; barrier, 800 nm) SLO angiography (Heidelberg Retina Angiograph; Heidelberg Engineering GmbH, Dossenheim, Germany). FLA followed subcutaneous injection of 75 mg/kg body weight fluorescein-Na (University Pharmacy; University of Tubingen, Germany), and ICGA followed subcutaneous injection of 50 mg/kg body weight ICG (ICG-Pulsion; Pulsion Medical Systems AG, Munich, Germany).

RNA Isolation, DNasel Treatment, and Quantitative Real-Time PCR

Retina-RNA (n = 4–6 per stage and genotype) was prepared (Absolutely RNA Microprep Kit; Stratagene, La Jolla, CA). DNaseI-treated (Invitrogen, Basel, Switzerland) RNA was reverse transcribed (SuperScript II RNase H− Reverse Transcriptase; Invitrogen) and random primers (Hexamer Primers pd(N)6; Amersham Bioscience, Freiburg, Germany). Quantitative real-time PCR (Prism 7000 Sequence Detection System; Applied Biosystems, Inc. [ABI] Rotkreuz, Switzerland) was performed (MGB-TaqMan probes from Assay by Design; ABI, Table 1). Three replicates per sample were included. For relative quantification with the ΔCt-method, 18S rRNA (TaqMan Ribosomal RNA Control Reagent; ABI) was used as the internal standard, and the data were analyzed (Prism 7000 SDS Software; ABI; and Excel; Microsoft, Redmond, WA). The Mann-Whitney test was used for statistical analysis (SPSS 13 for Windows; SPSS Inc., Chicago, IL).
Delayed and reduced formation of the superficial capillary network. Morphometric quantitation of vessel outgrowth toward the periphery in lectin-stained retinal wholemount preparations of Ndph<sup>y/y</sup> (Ko) compared with wild-type (WT) mice (n = 5). The delay in formation of the superficial capillary network was present from p5 onward in the Ndph<sup>y/y</sup> mice and remained delayed and reduced until p21. Data are the mean ± SD of radius (%), which is radius (vasculature)/radius (retina), of the retinal arteries in the wild-type and Ndph<sup>y/y</sup> mice.

protein content measured by Bradford reagent. VEGF levels were determined in duplicate, according to the manufacturer’s instructions for an ELISA kit (no. MMV00; R&D Systems, Minneapolis, MN).

**Western Blot Analysis for Hif1α**

Retinal protein (40 μg) was used for Western blot analysis according to standard protocols. For immunodetection, chicken anti-HIF-1α<sup>y/y</sup> and rabbit anti-actin (no. sc1616; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were applied, followed by horseradish peroxidase-conjugated secondary anti-chicken (G135A; Promega, Madison, WI) and anti-rabbit (no. sc2004; Santa Cruz Biotechnology) antibodies, respectively. Immunoreactivity was visualized with a Western blot detection kit (Renaissance; Perkin Elmer Life Sciences, Emeryville, CA).

**Hypoxic Exposure**

Wild-type mice were exposed to reduced oxygen levels by altering the O<sub>2</sub>-N<sub>2</sub> ratio. O<sub>2</sub> was reduced to 10% in steps of 2% over a 1-hour period. Retinas were isolated from mice exposed for 6 hours to 10% oxygen. Normoxic controls were kept in normal room air.

**Electroretinography**

ERG consisted of a Ganzfeld bowl, a DC amplifier, and a computer-based control and recording unit (Toennies Multiliner Vision; Viasys Healthcare, Höchberg, Germany). The ERGs were obtained according to published procedures. 16

**RESULTS**

**Defects in the Superficial Retinal Vasculature**

Morphometric quantitation of lectin-stained retinal vasculature of the Ndph<sup>y/y</sup> mice and age-matched control animals at postnatal day (p)5, p10, p15, and p21 revealed a delayed and reduced outgrowth of the superficial capillary network toward the periphery in the Ndph<sup>y/y</sup> mice (Fig. 1). This delay was observed as early as p5, when superficial vessels covered 55.1% ± 3.5% of the Ndph<sup>y/y</sup> retinas compared with 75% ± 4.6% in the wild-type mice. In mutant mice, the percentage coverage remained reduced through p21 (Fig. 1). Using an antibody against collagen type IV (Col IV), a basal matrix component of endothelial cells, we confirmed the delayed and reduced outgrowth of the superficial retinal vasculature (Fig. 2A). Large periorbita capillary-free zones (Fig. 2A, arrowheads), narrowed capillaries and a wider meshed network of the superficial vasculature were observed as early as p5 in the Ndph<sup>y/y</sup> mice (Figs. 2A, 2B), suggesting a defect in sprouting angiogenesis in the retinal vasculature, even at that early developmental stage. Between p10 and p15, however, the mutant capillaries increased in diameter (Figs. 2B, 3B) representing ongoing irregular remodeling within the superficial capillary network of the Ndph<sup>y/y</sup> mice between p10 and p21, similar to small aneurysm-like lesions, which were observed at p15 (Figs. 2A, 3B, arrows). Their size and number were increased at p21, and they were filled with ICG in vivo during ICG angiography. Confocal microscopy revealed drum-stick–like, lectin-positive, dead-ended vascular sprouts, with accumulation of cells as the likely cause for the clublike capillary malformation (Fig. 2C; left). Retinal digest preparations (Fig. 2C, right) and HE staining (Fig. 3Ce, arrow) confirmed the phenotype.

In addition, FLA revealed substantial leakiness of the mutant retinal vasculature, because the fluorescein left the vessels, which led to a quick loss of image contrast, and the entire retina turned diffusely white (Fig. 3A; p21, FLA). This occurred primarily around microaneurysm-like lesions (not shown) but the contribution of other vessels cannot be excluded.

The pattern of the large vessels remained rather unaffected in its radial arrangement (Fig. 3B). However, in vivo imaging with the ICGA in the Ndph<sup>y/y</sup> mice revealed that these vessels were not correctly attached to the underlying tissues, particularly over the mesh holes (Fig. 3B; open arrows). At these sites, they were commonly lifted and moved relatively freely, some of them with the frequency of the heartbeat. Although these vessels were part of the hyaloid circulatory system, some of them protruded substantially toward the vitreal space. They correspond well to ectopic vessels found with HE staining (Fig. 3Cd) that were part of membranelike structures between the vitreous and the NFL.

**No Development of Deep Retinal Vascular Systems Due to Impaired Angiogenic Sprouting**

Formation of the deep retinal vasculature, which normally occurs at ~p7, was not observed at any stage in the Ndph<sup>y/y</sup> mice. Whereas in the wild-type animals, the intermediate (not shown) and the deep retinal capillary networks developed normally (Fig. 2B; second row), in the Ndph<sup>y/y</sup>-knockout mice, neither the intermediate nor the deep capillaries were visible, as indicated by the absence of capillaries in the background of the superficial vasculature (Fig. 2B). Initial branching of vessels, which normally form deep retinal networks, seems to occur, but their subsequent outgrowth into the deep retinal layers is blocked (Fig. 2C). This and the absence of deep capillary networks are also visualized in a 3-D reconstruction of the retinal vasculature of the Ndph knockout mouse at p21 (Movie 1, http://www.iovs.org/cgi/content/full/46/9/3572/DC1). In summary, these data suggest that sprouting angiogenesis into the deep retinal layers is initiated (branching) but not completed (blockage of outgrowth and tube formation).

**Delayed Regression of Hyaloid Vessels**

To study regression versus persistence of hyaloid vasculature during early postnatal development of Ndph<sup>y/y</sup> mice in vivo, we used ICGA (Fig. 3A, left and right). The difference between the Ndph<sup>y/y</sup> mice and the wild type was the preservation of many hyaloid vessels at p14 (Fig. 3A, left) and p21 (Fig. 3A, right), whereas in the wild type they normally had regressed completely at these stages, except in very rare cases, in which
one or two vessels were left. Mutant vessels, which lost their function between p14 and p21 (obliterated vessels), still
looked very much like their functional counterparts but had a rather dark appearance, due to the lack of blood flow (Fig. 3A; p21, FLA and ICG; filled arrows). In FLA we could see the nonperfused vessels and other vitreous structures (Fig. 3A; center) because the extravascular fluorescein in the superficial retinal layer produced a background light. In the Ndpb\textsuperscript{−/−} mice, comparing p14 and p21 ICG, a reduction in the number and diameter of the hyaloid vessels was found (Fig. 3A, left versus right). This represents an ongoing obliteration, continuing until the age of 6 to 8 weeks, when most vessels in the vitreous were nonfunctional, and remnants of vessels and cell bodies remained visible (data not shown). Occasionally, at p15, hyaloid vessels grew into the peripheral retina in the Ndpb\textsuperscript{−/−} mice only (Figs. 3Cb, c).

**Figure 2.** Defects in the superficial retinal vasculature and lack of deep capillary networks. (A, B) Fluorescence microscopy of anti-collagen IV vascular wholemounts of the postnatal retina in Ndpb\textsuperscript{−/−} mice at p5, p10, p15, and p21. (A) A reduced and delayed outgrowth of the superficial vasculature, larger periarteriolar capillary-free zones (arrowheads), and a wider meshed superficial capillary network were observed. (B) The images in the first (Wt) and third rows (Ko) were focused on the optical plane of the superficial layer, whereas images in the second row (set) were focused on the deep capillary network. At p5, narrow superficial capillaries were visible in the Ndpb\textsuperscript{−/−} mice, which at p15 and p21 became larger than in the wild type (Ko, third row, versus Wt, first row). At p10, the deep network had not formed, even in proximity to the optic disc (shown for Ndpb\textsuperscript{−/−} mice) and does not develop later in mutant mice. At p15, drum-stick-like capillary lesions formed in the Ndpb\textsuperscript{−/−} mice, and they increased in number and size at p21 (arrows). (C) Enlargements of dead-ended microaneurysm-like capillary lesions at p21 in lectin-stained retinal wholemount and digest preparations. The vessels in the Ndpb\textsuperscript{−/−} mice appeared flattened and pathologic compared with the wild type. Scale bar: (A) 200 μm; (B) 100 μm; (C, lectin) 16 μm; (C, digestion) 20 μm.
Periphery; (Ndph and p15 (Fig. 4D). Around vessels in the superficial layers of the retina were also found for the ECM molecule FN, which accumulated suggesting a secondary activation of glial cells. Similar results was not altered until p15, and then it increased at p21 (Fig. 4B).

Transcriptional Analysis of Angiogenic Factors

To study the molecular processes underlying the vascular defects in the Ndph-knockout retinas, we analyzed the transcription of genes involved in retinal angiogenesis (Fig. 5). Overall, this revealed moderate changes in transcript levels at p5 and p10, whereas for several genes at p15 and many genes at p21 transcriptional alterations at p5 and p10 were found for Pdgfb, Pdgfrb, Tie1, Tie2, and Vegfa (Table 2). All but Vegfa were reduced in expression compared with the wild-type (Figs. 5A, 5B). Vegfa in contrast was significantly increased at p10 and later on (Figs. 5B–D). Notably, the expression of Pdgfb, Pdgfrb, Tie1, and Tie2 also became upregulated in the Ndph<sup>y−/−</sup> retinas until p21 (Figs. 5C, 5D; Table 2). Besides the dramatic alteration in Vegfa expression during retinal development, the most prominent alteration in mRNA level was found for integrin β3 (Itgb3). Its expression was approximately five times higher in the Ndph<sup>y−/−</sup> retinas than in the wild type at p15 and p21 (Figs. 5C, 5D, Itgb5). All but Vegfa were reduced in expression compared with the wild-type (Figs. 5A, 5B). Vegfa in contrast was significantly increased at p10 and later on (Figs. 5B–D). Notably, the expression of Pdgfb, Pdgfrb, Tie1, and Tie2 also became upregulated in the Ndph<sup>y−/−</sup> retinas until p21 (Figs. 5C, 5D; Table 2).

Delayed regression of the hyaloid vessel system in Ndph<sup>y−/−</sup> mice. Hyaloid vessels were present and functional at p14 in Ndph<sup>y−/−</sup> mice, whereas at p21 some vessels appeared rather dark (filled arrows) because they had been obliterated. More degraded hyaloid vessels appear merely as a linear formation of dark cell bodies (open arrow, inset). Images of the wild-type control are not shown, because representative ICGA and FLA images were made blank by the complete regression of the hyaloid vessels. Only in very rare cases were one or two vessels of the hyaloid vasculature left at p14 and p21. In FLA of wild-type retinas, no background light was visible, because the fluorescein remained in the retinal vasculature. (B) A full view and a detailed view of ICG angiography at p14 and p21 at the optical section of the nerve fiber layer (NFL). Defects in the retinal vessels are obvious, and small dots represent microaneurysm-like lesions. At p14 larger retinal vessels were lifted over the network holes (open arrows). The network structure observed at p14 is still visible (open arrows) but fine vessels seem to have formed, representing irregular and ongoing remodeling (filled arrow). (C) HE staining of wild-type and Ndph<sup>y−/−</sup> retinas at p15 (shown in an extreme example). (Ca) Wild-type control. (Cb, Cc) Ndph<sup>y−/−</sup> hyaloid vessels entering the retinal periphery; (Cd) lifted retinal vessels between the NFL and vitreous; (Ce) defective vascular sprouts in the NFL (arrow). Scale bar, 50 μm.

Contribution of Other Retinal Cell Types and of the Extracellular Matrix to the Vascular Phenotype

Defects in retinal vascular development may indicate a functional deficit of glial cells, which are important for guiding outgrowing vessels. Similarly, defects in the pericyte recruitment of the extracellular matrix (ECM) may lead to impaired angiogenesis. Therefore, double staining of endothelial cells (lectin) in combination with antibodies for glial cells (anti-GFAP), pericytes (anti-NG-2), or fibronectin (anti-FN), an abundant ECM component, were studied in retinal wholemount preparations.

For GFAP, similar patterns were observed in the wild-type and Ndph<sup>y−/−</sup> mice from p5 to p15. At p21, however, the astrocytic network was disturbed (Fig. 4A). Western blot results confirmed that GFAP expression in the Ndph<sup>y−/−</sup> retinas was not altered until p15, and then it increased at p21 (Fig. 4B), suggesting a secondary activation of glial cells. Similar results were also found for the ECM molecule FN, which accumulated around vessels in the superficial layers of the Ndph<sup>y−/−</sup> retina at p15 (Fig. 4D).

At earlier stages (from p5 on), pericytes followed closely the tips of the endothelial sprout in the superficial vessel layer of both the wild-type and Ndph<sup>y−/−</sup> mice (data not shown). At p15 the capillaries were properly invested by pericytes (Fig. 4C). Even within the microaneurysm-like lesion, the pericytes colocalized with endothelial cells (Fig. 4C) indicating an intact communication between these two cell types.
not show significantly altered mRNA levels in knockout retinas (Figs. 5C, 5D).

Overall, these molecular data suggest two stages of disease progression in the Ndph<sup>y/</sup>H11002 mice. One phase with minor transcriptional changes until p10 was followed by another phase, in which an activation of a variety of molecular signaling pathways involved in sprouting angiogenesis was found. Despite those prominent molecular changes and hence a highly activated angiogenic capacity in the Ndph<sup>y/</sup> retinas at p15 and p21, we neither observed active neovascularization nor the compensatory later formation of deep retinal capillary networks.

**Severe Hypoxia in Retinas of the Ndph<sup>y/</sup>H11002 Mice**

The lack of vascularity in the inner retina observed in the Ndph<sup>y/</sup> mice suggests the manifestation of severe hypoxia. We measured VEGFA protein levels in the Ndph<sup>y/</sup> and wild-
type retinas at different ages (Fig. 6A). Observed mRNA levels were consistently accompanied by similar changes in amounts of VEGF protein (Table 3), suggesting that regulation of VEGF occurs predominantly at the transcriptional level. Hypoxia stabilized the HIF-1α leading to the activation of the heterodimeric transcription factor HIF-1 and to the expression of a variety of hypoxia-regulated genes, including Vegf. Therefore, we tested HIF-1α by Western blot analysis (Fig. 6B). At p5 and p10, HIF-1α levels in the $\text{Ndphp}^+/−$ retinas were not different from those in the wild-type retinas, but they increased at p15 and p21 (Fig. 6B). They correlated with HIF-1α protein levels, which were artificially induced by hypoxia in wild-type retinas (Fig. 6C).

Further support of hypoxia comes from changes in ERG waveforms in 6-week-old $\text{Ndphp}^+/−$ mice (Fig. 6D). They matched closely those obtained from retinas in known states of retinal, but not choroidal, hypoxia. The almost complete loss of oscillatory potentials together with the subtotal reduction of the b-wave (negative ERG) are characteristic of hypoxia.

**DISCUSSION**

This study provides molecular and functional evidence that divides the clinical course of Norrie disease into two phases. In an early phase, the absence of functional Norrin causes a defect in sprouting angiogenesis affecting capillary network formation in the retina, whereas in a later phase, the developmental lack of the deep vasculature leads to inner retinal hypoxia.

Sprouting angiogenesis is a common process in the formation of retinal capillaries, which is guided by glial cells. Endothelial cells in the superficial network are guided by a preceding astrocytic template, whereas the guidance of the deep retinal vascular networks is performed by Müller cells and ECM components such as R-cadherin.
As shown in the current study, the absence of Norrin affects sprouting angiogenesis in both guidance processes in distinct ways. From p5 on, vessels in the superficial layer showed a delayed outgrowth and therefore exhibited a clear defect in sprouting angiogenesis. Nevertheless, they branched, fused, and formed a vascular network in close association with the astrocytes until p15. As this association and the activity of glial cells were only affected in the hypoxic phase, the lack of Norrin influenced the astrocytic network rather secondarily, and astrocytes may have compensated partially for the defect in angiogenic sprouting.

In contrast, the vessels that normally form the deep retinal capillaries are more drastically affected by the absence of Norrin. Although these vessels branch out and initiate sprouting angiogenesis, they cannot finish this process, suggesting a crucial role of Norrin in the astrocyte-independent vessel guidance into the deep retinal layers. These findings show that the lack of deep retinal networks in adult Ndph<sup>y/H11002</sup> mice<sup>14</sup> is due to a developmental defect in sprouting angiogenesis and is not due to a secondary loss of retinal capillaries.

Although similar to astrocytes the ECM, as shown by double labeling for FN and endothelial cells, was affected secondarily.

### Table 2. Transcriptional Alterations in Ndph<sup>y/H11002</sup> Mice of Selected Genes Involved in Sprouting Angiogenesis during Retinal Development

<table>
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<th>Gene Symbol</th>
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<td>—</td>
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<td>Pdgfb</td>
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<td>PdgfrB</td>
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Only significantly altered mean values ± SD of genes marked in Figure 5 are given. Data were obtained by real-time PCR (n = 4–6 per stage) and are the relative expression (RE) ± SD in Ndph<sup>y/H11002</sup> mice vs. wild-type mice (Wt = 1).

**FIGURE 6.** Severe hypoxia in the retina of Ndph<sup>y/H11002</sup> mice. (A) Relative amounts of VEGFA protein in 40 μg retinal protein extract of Ndph<sup>y/H11002</sup> mice (n = 5) normalized to wild-type mice (n = 4) were determined during retinal development from p5 to p21 by VEGFA ELISA. (B) Anti-HIF1α Western blot analyses of retinal protein homogenates of wild-type (n = 4) and Ndph<sup>y/H11002</sup> mice (n = 5) at p5, p10, p15, and p21. The same blot was probed with β-actin antibody to confirm equal amounts of total protein. (C) HIF1α protein levels in wild-type retinas under normoxic (N) and hypoxic (H) conditions. (D) ERG studies in the Ndph<sup>y/H11002</sup> mice versus the control mice at the age of 6 weeks confirmed physiologic hypoxia in the inner retina of the Ndph<sup>y/H11002</sup> mice. y-axis, amplitude in 200 μV per division; x-axis: time in 40 ms per division. (Open arrow: diminished oscillatory potentials; filled arrow: b-wave reduction.)
in the hypoxic phase, pericyte recruitment, visualized by NG-2 staining, was not disturbed in the Ndph<sup>y/y</sup> mice. Thus, the lack of Norrin affects astrocytes and pericytes, if even only secondarily, and defects in their development are not the primary cause of the malformation of the retinal vasculature.

Instead, our data suggest that lack of Norrin primarily affects endothelial cells. Endothelial transcripts, such as the Tie-2 and Tie-1 receptors, as well as Pdgfb<sup>y/y</sup>, showed significant lower levels at p5 and p10. These findings may either reflect the decreased number of endothelial cells in the retina or a decreased activation of the angiopoietin-2/Tie pathway, which may contribute to the delayed formation of the superficial network. Overall, these data suggest that Norrin plays an important role in sprouting angiogenesis in retinal vascular development and that this defect affects capillary development in the superficial and the deep retinal layers in different ways. Although Norrin plays a less important role in astrocyte-dependent blood vessel guidance during postnatal retinal development, it is crucial for the astrocyte-independent guidance of retinal blood vessels for the formation of the deep retinal capillary networks.

Upregulation of HIFα and VEGF clearly indicates that lack of the deep retinal capillary networks leads to hypoxic conditions. Furthermore, the ERG recordings in the current study strongly resemble those recorded in hypoxic conditions. However, previously obtained negative ERGs in adult Ndph<sup>y/y</sup> mice (age, >7 months) were similar but were attributable to a retinoschisis-like alteration of the retina. Together with our findings, these results suggest that retinal hypoxia remains during adulthood and is the main factor that causes negative ERGs in Ndph<sup>y/y</sup> mice.

Hypoxia and VEGF are strong regulators of many of the studied angiogenic factors, including Pdgfb<sup>y/y</sup>. Therefore, many transcriptional changes—in particular Pdgfb—observed in the Ndph<sup>y/y</sup> mice after p15 may be secondary.

Elevated VEGF provides an explanation for the leakiness of the superficial retinal vasculature of the Ndph<sup>y/y</sup> mice after p10. Subsequent extravasation of fluid from leaky superficial retinal vasculature may lead to displacement of large vessels from the superficial network and to disturbance of the organization of inner retinal layers as described in Ndph<sup>y/y</sup> mice previously. Therefore, major phenotypic features in the Ndph<sup>y/y</sup> retinas may be explained as secondary consequences of increased VEGF, as may also be true of the increased deposition of FN in the extracellular matrix of vessels at p15.

Despite the upregulation of VEGF and other proangiogenic factors, no induction of pathologic neovascularization was observed in the Ndph<sup>y/y</sup> retinas before p21. This finding is in contrast to those showing that increased VEGF levels correlate with the occurrence of neovascularization in the mouse model of oxygen-induced retinopathy, the induction of neovascularization during normal retinal development, and the observation that pathologic neovascularization can be induced, even in adult mice after transgene overexpression of VEGF. Thus, our findings suggest that high VEGF levels alone are not sufficient to induce neovascularization in the absence of Norrin until p21. Additional factors may be essential for the VEGFA-induced neovascularization. Alternatively, the complete absence of the deep retinal capillaries in Ndph knockout mice, where retinal neovascularization normally originates, may explain the lack of this pathologic process in the absence of Norrin.

We hypothesize that in patients with Norrie disease, retinal hypoxia with elevated VEGF levels occurs as a secondary consequence of the lack of Norrin and thus may lead to defects in vascular development. This would explain clinical features such as few retinal blood vessels and disarranged retinal ganglion cell and inner nuclear layers and may provide an explanation for the high phenotypic variability due to variations in VEGF levels. Thus, inner retinal hypoxia may be a main pathogenic mechanism in Norrie disease and may lead to phenotypic similarities to familial exudative vitreoretinopathy, Coats’ disease, and retinopathy of prematurity.

Although elevated VEGF levels in the retina did not induce neovascularization in the absence of Norrin, they may provide a sufficient antiapoptotic signal for endothelial cells of the hyaloid vasculature, which could explain the dramatic delay and the incomplete regression of the hyaloid vasculature in the Ndph<sup>y/y</sup> mice. This observation of hyaloid vessel obliteration and regression in vivo is in contrast to the conclusion of Ohlmann et al., who suggested a functional persistence of the hyaloid vessels until adulthood. Persistence of the hyaloid system to compensate for defects in outgrowth of the primary retinal capillary system has been found in other genetically modified mice, such as the angiopoietin-2 lacZ mouse or a VEGF isof orm–deficient mouse. Similar to the observations in these mouse models, in Ndph<sup>y</sup>-knockout mice, too, hyaloid vessels occasionally grew into the retinal periphery trying to compensate for the results of retinal hypoxia; but, as shown by the elevated HIFα and VEGFA levels as well as in later stages by the ERG findings, this was not sufficient until p15, when most of the hyaloid vasculature is still functional. Therefore, we hypothesize that early defects in the retinal vasculature, observed as early as p5, cause delayed hyaloid regression. This view is supported by findings in animals that show normal development of the retinal vasculature, although the hyaloid system persists, indicating that hyaloid persistence does not necessarily lead to retinal vascular defects.

Because of the incomplete removal of vessels and cells from the vitreous, it may be that the lack of Norrin also affects the process of hyaloid vessel regression directly. The function of macrophages, which are responsible for clearing the vitreous from cellular debris, may be impaired. This could be mediated by Norrin-Lrp5 signaling as suggested by findings in Lrp5–knockout mice, which show persistent hyaloid vessels due to the failure of Lrp5-expressing macrophages to induce apoptosis of endothelial cells.

Indeed, it has become apparent that the Wnt-receptor Frizzled-4 (Fzd4) and its coreceptor LRP5, which are also mutated in cases of familial exudative vitreoretinopathy (FEVR), are receptors for Norrin and that the classic Wnt-β-catenin pathway becomes activated on binding of Norrin. These findings put both clinical phenotypes, Norrie disease and FEVR, into the context of one signaling pathway.
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We found an increase of Fzd4 expression between p15 and p21, whereas the absence of Norrin did not significantly affect Lrp5 transcription. This suggests a compensatory upregulation of Fzd4 expression in response to the lack of Norrin or may be due to hypoxic regulation of Fzd4 transcription.

Ndph<sup>−/−</sup> and Fzd4<sup>−/−</sup> mice share some remarkable similarities, including the delayed regression of the hyaloiodia and an overall absence of deep capillary networks in the retina. However, some phenotypic differences, such as more severe intraretinal hemorrhages in eyes of Fzd4<sup>−/−</sup> mice are also apparent. In addition, the formation of perpendicular vessels from the superficial network into deep layers was blocked in the Ndph<sup>−/−</sup> mice, whereas this vessel ingrowth occurs in Fzd4<sup>−/−</sup>-knockout mice. In the Ndph<sup>−/−</sup> mice, first signs of a disturbed association between astrocytes and endothelial cells were found at approximately p21, whereas in Fzd4<sup>−/−</sup> mice this is apparent at p5. Therefore, we conclude that defects in the Fzd4<sup>−/−</sup> mice are more severe than those observed in the Ndph<sup>−/−</sup> mice, suggesting additional molecules to be involved in normal signaling.

Our study provides the basis for defining two phases of Norrie disease and thus enables a better understanding of the function of Norrin in guidance of endothelial cells in the superficial versus the deep retinal layers during normal retinal development as well as in pathologic neovascularization. Further experiments are needed to clarify whether Norrin exclusively acts as an LRP5-dependent signaling molecule via the Frizzled-4 receptor or whether it interacts with other molecules to modulate the ECM by opening the gate for vessels to grow into the deep retinal layers.

References


