VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

Müller Cell—Localized G-Protein—Coupled Receptor 81 (Hydroxycarboxylic Acid Receptor 1) Regulates Inner Retinal Vasculature via Norrin/Wnt Pathways

Ankush Madaan,^{*†} Prabhas Chaudhari,^{*‡} Mathieu Nadeau-Vallée,^{*§} David Hamel,^{*} Tang Zhu,^{*} Grant Mitchell,^{*} Mark Samuels,^{*} Sheetal Pundir,^{*} Rabah Dabouz,^{*} Colin Wayne Howe Cheng,[†] Mohammad A. Mohammad Nezhady,^{*} Jean-Sébastien Joyal,^{*†§} José Carlos Rivera,^{*¶} and Sylvain Chemtob^{*†§¶}

From the Departments of Pediatrics, Ophthalmology and Pharmacology,^{*} Centre Hospitalier Universitaire Sainte-Justine Research Center, Montréal, Quebec; the Departments of Pharmacology and Therapeutics[†] and Experimental Medicine,[‡] McGill University, Montréal, Quebec; the Department of Pharmacology,[§] Université de Montréal, Montréal, Quebec; and the Research Center,[¶] Maisonneuve-Rosemont Hospital, Montréal, Quebec, Canada

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Address correspondence to
Sylvain Chemtob, M.D., Ph.D.,
Departments of Pediatrics,
Ophthalmology and Pharma-
cology, CHU Sainte-Justine
Research Center, 3175 Chemin
Côte Ste-Catherine, Montréal,
QC, Canada H3T 1C5; or José
Carlos Rivera, Ph.D., Depart-
ment of Ophthalmology, Mai-
sonneuve-Rosemont Hospital,
Research Center, 5690 Boule-
vard Rosemont, Montréal, QC,
Canada H1T 2H2 E-mail:
sylvain.chemtob@umontreal.ca
or jc.rivera@umontreal.ca.

Ischemic retinopathies are characterized by a progressive microvascular degeneration followed by a postischemic aberrant neovascularization. To reinstate vascular supply and metabolic equilibrium to the ischemic tissue during ischemic retinopathies, a dysregulated production of growth factors and metabolic intermediates occurs, promoting retinal angiogenesis. Glycolysis-derived lactate, highly produced during ischemic conditions, has been associated with tumor angiogenesis and wound healing. Lactate exerts its biological effects via G-protein—coupled receptor 81 (GPR81) in several tissues; however, its physiological functions and mechanisms of action in the retina remain poorly understood. Herein, we show that GPR81, localized predominantly in Müller cells, governs deep vascular complex formation during development and in ischemic retinopathy. Lactate-stimulated GPR81 Müller cells produce numerous angiogenic factors, including Wnt ligands and particularly Norrin, which contributes significantly in triggering inner retinal blood vessel formation. Conversely, GPR81-null mice retina shows reduced inner vascular network formation associated with low levels of Norrin (and Wnt ligands). Lactate accumulation during ischemic retinopathy selectively activates GPR81—extracellular signal-regulated kinase 1/2—Norrin signaling to accelerate inner retinal vascularization in wild-type animals, but not in the retina of GPR81-null mice. Altogether, we reveal that lactate via GPR81-Norrin participates in inner vascular network development and in restoration of the vasculature in response to injury. These findings suggest a new potential therapeutic target to alleviate ischemic diseases. (*Am J Pathol* 2019, 189: 1878–1896; <https://doi.org/10.1016/j.ajpath.2019.05.016>)

Ischemic retinopathies, such as retinopathy of prematurity and diabetic retinopathy, are characterized by an initial phase of microvascular degeneration, which is usually most pronounced in the deep vascular network¹ and is preceded by a secondary phase of exaggerated aberrant preretinal neovascularization.^{2–6} The initial inner retinal vessel depletion leads to retinal hypoxia,^{7,8} which results in damage to retinal neurons, as has been described experimentally

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and clinically^{9,10}; this observation is consistent with evidence that prompts postischemia revascularization lessens adverse outcome.^{11–19} Hence, to diminish the duration of intraretinal ischemia, it is requisite to determine the factors that govern the development of the deep intraretinal vascular network. Notwithstanding the paramount and widespread role of hypoxia-triggered vascular endothelial growth factor in neovascularization, not surprisingly other factors also partake in this process.^{2,4} Of interest, intermediates of carbohydrate metabolism significantly affected by hypoxic ischemia have generally been overlooked. For instance, the Krebs cycle metabolite succinate has been shown by us to exert an important role in regulating retinal angiogenesis.²⁰ Specifically, G-protein–coupled receptor 91 (GPR91), present on retinal ganglion cells (RGCs), governs the release of numerous angiogenic factors implicated mostly in the development of the superficial retinal vasculature.²⁰

It has been proposed that the deep retinal vascular network is formed in response to a hypoxic state that triggers the release of factors from the pan-retinal Müller cells.^{21,22} It is now established that Norrin, specifically secreted by Müller cells, guides deep vascular complex development.²³ Norrin exerts its vasoproliferative effect by binding with high affinity to a receptor, Frizzled-4 (Fzd-4), which interacts with its coreceptor low density lipoprotein receptor-related protein 5 (Lrp5) and the transmembrane polypeptide, tetraspanin 12 (Tspan12), present on the surface membrane of vascular cells.^{24,25} Mutation in any of these genes encoding these four proteins leads to insufficient inner retinal vascular development.²⁵ Norrin has been shown to be involved in promoting vascular regrowth after oxygen-induced vessel loss and suppresses ischemic retinopathy.²⁶ However, to date, the factors that control Norrin expression in the retina are still not well known.

Lactate is a glycolysis-derived metabolite, abundantly generated by the highly glycolytic Müller cells in the retina.²⁷ Lactate, produced by Müller cells, can act in an autocrine manner, be exported to ganglion cells and photoreceptor as fuel, or be exchanged in the retinal blood vessels.^{28,29} In the circumstance of reduced tissue oxygenation or an increased metabolic rate, lactate rapidly increases before any changes in downstream Krebs cycle activity.^{30,31} Interestingly, lactate is not simply a glycolytic end product, but its increases have been associated with angiogenesis in tumors and wound healing,^{32–34} and vasodilatation in the inner retina,³⁵ suggesting a link between capillary function and tissue metabolic needs. Furthermore, a neuroprotective role for exogenous lactate in pathologic situations, such as diabetes and cerebral ischemia, has been documented.^{36–38} Lactate was recently found to exert its biological effects specifically via GPR81 [alias hydroxycarboxylic acid receptor (HCA1 or HCAR1)], mostly expressed in fat, as well as in the central nervous system.^{39–41} GPR81 has been demonstrated to be implicated in several functions, including antilipolytic effects in the adipose tissue,^{39,42}

protection against ischemic brain injury,⁴³ cancer cell survival,⁴⁴ attenuation of inflammation,^{45,46} and, recently, angiogenesis in breast tumors.⁴⁷ However, its physiological functions in governing neuroretinal vascularization during development and ischemic retinopathy are not known. Herein, we propose that lactate, acting through its GPR81 receptor present in retina, is implicated in the regulation of retinal angiogenesis. Our findings reveal, for the first time, that lactate-induced Norrin production by activating GPR81 receptor present in Müller cells regulates deep vascular complex development and promotes revascularization in ischemic retinopathy.

Materials and Methods

Animals

The use of mice for this study was first approved by the Animal Care Committee of Sainte-Justine's Hospital (Montréal, Canada) following the principles of the *Guide for the Care and Use of Experimental Animals* developed by the Canadian Council on Animal Care. C57BL/6J mice were obtained from Charles River Inc. (Montreal, Canada) with pups at postnatal day (P) 2 and were allowed to acclimatize for 2 days before experiments. The animals were maintained on standard laboratory chow under a 12:12 light/dark cycle and allowed free access to chow and water. GPR81^{−/−} mice were obtained from Lexicon Pharmaceuticals (The Woodlands, TX) and were used following the same aforementioned guidelines. The GPR81^{−/−} mice were backcrossed with adult C57BL/6J mice to obtain a pure C57BL/6J suitable for oxygen-induced retinopathy (OIR) experiments. The transmembrane domain 2 of mice GPR81 coding region (100 bp) is replaced by a 4-kb IRES-lacZ-neo cassette. All experimental protocols were approved by the Animal Care Committee of Sainte-Justine's Hospital.

Chemical Data

Lactate was purchased from Sigma-Aldrich (L1750; Oakville, Canada).

OIR Mice Model

The vaso-obliteration and neovascularization models used in this study were designed on the basis of a well-established mice model of retinopathy of prematurity—the OIR model.⁴⁸ C57BL/6J mice were placed with an adoptive breastfeeding CD1 mother at P5 to avoid cannibalism in hyperoxia compared with the C57BL/6J mothers. These litters were placed in a hyperoxic chamber with 75% ± 2% oxygen from P7 to P12. As mothers are more vulnerable to the toxic effects of oxygen, they were exchanged between normoxic and hyperoxic cages every day. The litters of mice were returned to normoxia or 21% O₂ 5 days later, on P12.

The mice were anesthetized with isoflurane (2%) and sacrificed by decapitation. The eyes from P12 pups were enucleated, and retinas were dissected to evaluate vaso-obliteration in retinal flat mounts. At P17, the mice were sacrificed, and eyes were enucleated to analyze preretinal neovascularization. The weight of mice was considered to exclude underdeveloped mice, thereby eliminating the difference in weight gain as a variable factor.⁴⁹ Control animals were maintained in room air (21% O₂). All other conditions (eg, light exposure, temperature, and feeding) were similar for both treatment groups.

Retinal Flat Mounts

Mice eyes were collected and processed for retinal flat mounts, as in previously published studies.^{50,51} Briefly, eyes were fixed in 4% paraformaldehyde and then retinas were isolated and incubated overnight at 4°C in a solution containing 1% Triton X-100 and 1 mmol/L CaCl₂/phosphate-buffered saline (PBS) and tetramethylrhodamine isothiocyanate—conjugated lectin endothelial cell marker *Bandeiraea simplicifolia* (1:100; Sigma-Aldrich, St. Louis, MO). Retinas were mounted and imaged under a confocal microscope (Olympus, Richmond Hill, Canada) using a 30× objective or a Zeiss AxiObserver Z1 motorized inverted microscope (Zeiss, San Diego, CA) using a 10× objective. Retinal vascular densities were calculated using the AngioTool software version 0.5 (National Cancer Institute, Bethesda, MD).⁵² Vascular density in study groups was normalized with that of untreated groups raised in 21% O₂. Vaso-obliterated areas were assessed as the retinal area devoid of vasculature/the total retinal area. Neo-vascularization was analyzed using the SWIFT-NV method,⁵³ which consists of a set of macros that was developed to quantify all of the pixels represented by neo-vascular tufts and clusters, but not normal vessels in lectin-stained retinal whole mounts.

Electroretinography

Electroretinograms (ERGs) were recorded from wild-type (WT) and age-matched knockout mice (6 months old) on an Espion ERG Diagnosys machine and a ColorDome Ganzfeld stimulator (Diagnosys LLC, Lowell, MA). Mice were dark adapted overnight and anesthetized intraperitoneally with a mixture of 100 mg/kg ketamine and 20 mg/kg xylazine solution. Pupils were dilated using atropine and phenylephrine. A drop of methylcellulose was placed onto the corneal surface to prevent corneal dehydration. Mouse body temperature was maintained at 37°C using a heated water pad. The protocol for the analysis of retinal function was similar to the version of the human ERG standard by the International Standard for Clinical Electrophysiology of Vision.

Flash ERGs were measured using a gold wire corneal electrode, and the electrodes were placed onto the surface of the cornea. A needle electrode on the forehead served as a

reference electrode, and a needle electrode near the base of the tail served as ground. Scotopic responses were simultaneously recorded from both eyes of the dark-adapted animals at the following increasing light intensities: 0.01, 3.0, and 10.0 cd-seconds/m². Photopic responses were recorded at the light intensity of 3.0 cd-seconds/m² in the presence of a rod-saturating background. Ten waveform responses were averaged in each step. All procedures were performed in a dark room under dim red-light illumination. The amplitude and latency of major ERG components were measured with Espion software version 4.0 (Diagnosys LLC). The ERG a-wave amplitudes were measured from the baseline to the negative peak, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the positive wave.

Lv Production and Intravitreal Injection

Lentivirus (Lv) was generated by transiently transfecting lentivector and packaging vectors into 293FT cells (Invitrogen, Carlsbad, CA), as previously described.⁵⁴ Five different shRNA sequences were used against *Gpr81* (RMM4534-EG243270; Dharmacon, Lafayette, CO), and the most effective were selected [442: 5'-TTGACCGAGCAGAACAAGATG-3' (forward) and 5'-CATCTTGTCTCTGCTCGGTCAA-3' (reverse); and 448: 5'-AAGATGACCAAAGTCCAGAGG-3' (forward) and 5'-CCTCTGGACTTTGGTCATCTT-3' (reverse)]. Separately, Lv Norrin or Lv green fluorescence protein (GFP; under cytomegalovirus promoter) was also injected in GPR81-null animals. *In vivo* infections were performed in mice at P4 with a single intravitreal injection. Briefly, mice were steadily anesthetized with an isoflurane mask. After anesthesia, 1 to 2 µL of vehicle, Lv scrambled or Lv short hairpin (sh)GPR81, was injected intravitreally in the vitreous humor. Lv was allowed to infect the retina for 72 hours. The vascular density of retinas was evaluated by retinal flat mounts and cryosections, as described above.

Intravitreal Injections of Lactate

On P5 or P7, mice pups were anesthetized with isoflurane (2%) and intravitreally injected with 10 mmol/L lactate buffered to pH ≈ 7.3 (to avoid effects induced simply by low pH of lactic acid) or sterile PBS as control (final intravitreal concentration based on estimated eye volume) using a Hamilton syringe (Hamilton Company, Franklin, MA) with glass capillaries of approximately 60 gauge (total volume injected, approximately 1 µL). The concentration of lactate is consistent with that observed in pathophysiologic settings (5 to 20 mmol/L).⁵⁵ Forty-eight hours after the administration of lactate, the animals were euthanized, the eyes were removed, retinal flat mounts were stained by the lectin staining technique described above, and vascular density was evaluated using ImageJ software version 1.51d (NIH, Bethesda, MD; <https://imagej.nih.gov/ij>).

Figure 1 Lactate receptor GPR81 localization in mouse retina. **A:** Representative confocal images of retinal sagittal sections from P17 wild-type (WT) mice showing colocalization of GPR81 immunoreactivity (red) with the specific Müller cell (MC) marker glutamine synthetase (green) in yellow (merged image; nuclei were counterstained with DAPI in blue). **B:** GPR81 immunoreactivity is also strongly corroborated in primary Müller cells extracted from P8 WT mice. **C:** Western blot analysis in homogenates from immortalized retinal precursor cells (IRPCs), MCs, endothelial cells [rat brain endothelial cells (RBECs)], and adult retinal pigment epithelium cell line—19 (ARPE-19) showing GPR81 protein (39-kDa) expression. Immunoreactivity to GPR81 was also present in retinal homogenates from WT mice, but absent in retinas of GPR81-null mice. β -Actin (42 kDa) was used as an internal control. **D:** Real-time quantitative PCR analysis shows abundant *GPR81* mRNA expression in Müller glia and IRPCs, but lower and minimal expression in retinal pigment epithelium cells (ARPE-19) and endothelial cells (RBECs), respectively. Data are expressed as means $\pm$ SEM. $n = 3$ (**C** and **D**). *** $P < 0.001$ (by one-way analysis of variance with a Dunnett postanalysis compared with RBECs set at 1). Scale bars: 50 μ m (**A**); 100 μ m (**B**). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; KO, knockout; ONL, outer nuclear layer; OPL, outer plexiform layer.

Cell Culture

Primary Müller cells from WT and GPR81^{-/-} mice (extracted and characterized, as described before⁵⁶) and immortalized retinal precursor cells (originally called RGC-5⁵⁷) were cultured in Dulbecco's modified Eagle's medium growth medium supplemented with 10% serum, 50 U/mL penicillin, and 50 mg/mL streptomycin. Cells were propagated in regular conditions (37°C, 5% CO₂). For *in vitro* experiments, cells (serum starved overnight) from WT or GPR81^{-/-} mice were treated with 10 mmol/L lactate (pH balanced), consistent with effective concentrations used

in young subjects,⁴⁶ and were pretreated with 2 μ mol/L extracellular signal-regulated kinase (Erk)1/2-specific inhibitor U0126 for 1 hour, followed by treatment with lactate or PBS for 8 hours. Cells and tissues were then collected in RiboZol (AMRESCO, LLC, Solon, OH) and stored at -80°C for mRNA extraction and real-time quantitative PCR.

RNA Extraction and Real-Time Quantitative PCR

RNA from retinal tissues or cultured cells was extracted according to manufacturer's protocol. Cells were pretreated with Lv scrambled or Lv shGPR81 for 72 hours. Total RNA was

isolated with an RNase TM mini kit (Qiagen, Germantown, MD). The RNA concentration and integrity were measured with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA (500 ng) was used to synthesize cDNA using iScript Reverse Transcription SuperMix (Bio-Rad, Hercules, CA). Primers were designed using the National Center for Biotechnology Information's Primer Blast: GPR81, 5'-CCGGTTCATCATGGTGGTGGCT-3' (forward) and 5'-CTCTTCTGACCTCCGCGTCTTC-3' (reverse); *Lrp5*, 5'-AAGGGTGCTGTGTACTGGAC-3' (forward) and 5'-AGAAGAGAACCTTACGGGACG-3' (reverse); *Fzd4*, 5'-TTCCTTTGTTTCGGTTTATGTGCC-3' (forward) and 5'-CTCTCAGGACTGGTTCACAGC-3' (reverse); TSPAN12, 5'-CCCCGTCATGATTGCTGTCT-3' (forward) and 5'-CCTCCTGCTCGTATGTCCAC-3' (reverse); *Norrin*, 5'-CGCTGCATGAGACACCATTAT-3' (forward) and 5'-CTCAGAGCGTGATGCCTGG-3' (reverse); *Wnt3*, 5'-GGTTCGTGCGGATGGGTGGG-3' (forward) and 5'-GGGGCCAGATGGGAGCTGGA-3' (reverse); *Wnt7*, 5'-CACTTGTGGTCTCAGGGGT-3' (forward) and 5'-GCATCTGAGTTTCACCAGCA-3' (reverse); *Wnt10*, 5'-GCTCAACGCCAACACAGTG-3' (forward) and 5'-CGAAAACCTCGGCTGAAGATG-3' (reverse); plasma-membrane vesicle-associated protein (*PLVAP*), 5'-CGTCAAGGCCAAGTCGCT-3' (forward) and 5'-CATC-CACAGGTGGGCGATT-3' (reverse); and *Claudin-5*, 5'-GCAAGGTGTATGAATCTGTGCT-3' (forward) and 5'-GTCAAGGTAACAAAGAGTGCCA-3' (reverse). Quantitative gene expression analysis was performed on the Stratagene MXPro3000 (Stratagene, San Diego, CA) with SYBR Green Master Mix (Bio-Rad). Gene expression levels were normalized to 18S universal primer (Ambion Life Technology, Burlington, Canada). Dissociation curves were also acquired to test primer specificity.

Western Blot Analysis

Proteins from homogenized retinal fragments, lysed in radio-immunoprecipitation assay buffer, were quantified using Bradford's method (Bio-Rad). Protein sample (50 µg) was loaded onto SDS-PAGE gel and electrotransferred onto polyvinylidene difluoride membranes. Müller cells from WT and GPR81-null mice were plated up to 80% confluency and incubated overnight in serum-free Dulbecco's modified Eagle's medium. Cells were treated with PBS or lactate (10 mmol/L) for 10 minutes, and the ligand binding reaction was inhibited

by addition of 2 mL ice-cold PBS. Ice-cold radio-immunoprecipitation assay buffer (300 µL) was added to lyse the cells. The cells were scraped off and agitated on a rocking platform for 30 minutes at 4°C. The lysate was further centrifuged for 30 minutes at 13,000 × *g* at 4°C. The supernatant was electrophoresed on 15% polyacrylamide gel for 2 hours, after which it was transferred to a polyvinylidene difluoride membrane. The membranes were immunoblotted using anti-GPR81 (SAB1300790; Sigma-Aldrich, Oakville, Canada) antibody (dilution 1:250), monoclonal anti-phosphorylated Erk1/2 (Thr202/Tyr204) antibody (dilution 1:1000; Cell Signaling, Danvers, MA), or Claudin-5 (dilution 1:300; sc-374221; Santa Cruz Biotechnology Inc., Dallas, TX). The chemiluminescence was detected with their respective secondary antibodies conjugated to horseradish peroxidase substrate (PerkinElmer, Waltham, MA). The blots were stripped and reprobed using an anti-total Erk1/2 antibody (dilution 1:1000) and an anti-β-actin monoclonal antibody (dilution 1:10,000) as a control for protein loading. The levels of Erk1/2 phosphorylation were normalized to total Erk1/2 and quantified to the ratio of phosphorylated Erk1/2/total Erk1/2 by ImageJ software. Enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK) was used for detection using the ImageQuant LAS-500 (GE Healthcare).

Lactate Quantification Assay

Age-matched mice from the OIR model and control (room air) were sacrificed, and their retina was snap frozen in liquid nitrogen and stored at -80°C. A fragment of 50 mg of each retina was homogenized and used to quantify tissue lactate concentration using a colorimetric assay, following manufacturer's protocol (K627; BioVision, Milpitas, CA). Readings were made on a microplate reader (EnVision Multilabel reader; PerkinElmer) adjusted for 450 nm. Results were then converted into a concentration unit (mmol/L) using a standard curve.

Immunohistochemistry

Müller cells were plated onto coverslips precoated with poly-D-lysine and fixed in 4% paraformaldehyde. After blocking, cells were coincubated overnight with a rabbit anti-glutamine synthetase antibody (ab228590; Abcam, Eugene, OR; dilution 1:500) for Müller cells and an anti-rabbit antibody for GPR81 (SAB1300790; Sigma-Aldrich,

Figure 2 Intraretinal vascular development is delayed in GPR81 knockout (KO) mice. **A:** Western blot analysis showing GPR81 (39-kDa) expression in mice retinas during the second and third postnatal weeks (P8 to P21). β-Actin (42 kDa) was used as an internal control. Densitometry quantification is illustrated in the histogram. **B:** Representative confocal images of retinal flat mounts stained with lectin, showing superficial (red) and inner (green) vasculature in age-matched GPR81 KO and wild-type (WT) animals collected at different postnatal days (P8, P9, and P14). Merged images show both superficial and inner vasculature networks. **Dashed lines** show the localization of the optic nerve. Histograms represent the quantification of the inner and superficial vasculature networks in WT versus GPR81 KO retinas at P9 and P14. **C:** Delayed intraretinal vasculature in GPR81 KO mice was corroborated on representative images of lectin-stained (green) retinal cross-sections (P9); in WT mice, abundant vasculature was detected, as highlighted by **white arrows** pointing to microvessels. **D:** Illustrations of superficial (red) and intraretinal (green) vascularization in age-matched WT and GPR81 KO mice retina during P8, P9, and P14. Data are expressed as means ± SEM. *n* = 12 to 16 retinas per time point (**B**); *n* = 14 to 20 retinas (**C**). **P* < 0.05 compared with P8 (using two-tailed *t*-test); ***P* < 0.01 compared with corresponding WT (using two-tailed test). Scale bars = 300 µm (**B** and **C**).

Oakville, Canada; dilution 1:500). After primary antibody incubation, the cells underwent 2 hours of incubation at room temperature with a secondary antibody conjugated with Alexa Fluor (Molecular Probes, Eugene, OR). For tissue immunohistochemistry, retinas were dissected to remove cornea and lens, fixed in 4% paraformaldehyde for 1 hour, and transferred in 30% sucrose overnight. Localization of GPR81 was determined on retina cryosections (12 μ m thick). Sections were blocked with 1% bovine serum albumin, 1% goat serum, and 0.1% Triton X-100 (T-8787; Sigma-Aldrich, Oakville, Canada) in PBS and were subsequently incubated overnight with the primary antibodies. Secondary antibodies conjugated with Alexa Fluor (Molecular Probes) directed against rabbit were incubated for 2 hours at ambient temperature. Nuclei were stained with DAPI (Invitrogen; dilution 1:5000). Images were captured using 10 $\times$ (for whole retina imaging) or 30 $\times$ (for cells and magnified retina images) objective with an Eclipse E800 (Nikon, Melville, NY) fluorescence microscope. Whole retina images were captured using a Zeiss AxioObserver.Z1 (Zeiss, San Diego, CA). Images were merged into a single file using the MosiaX option in the AxioVision software version 4.6.5 (Zeiss, San Diego, CA).

Microvascular Sprouting from Aortic Explants

Microvascular sprouting from aortic explants was prepared from adult (1-month-old) C57BL/6J mice. Aortic rings (1 mL thick) were placed in 24-well tissue culture plates, covered with 40 μ L growth-factor-reduced Matrigel (BD Biosciences, San Jose, CA), and cultured for 4 days in endothelial cell growth medium-2 medium (Lonza Inc., Walkersville, MD). To establish the role of Müller cells expressing GPR81 in guiding angiogenesis, they were treated with lactate (pH controlled to 7.2) or vehicle and the respective conditioned medium from these cells was extracted after 24 hours to treat aortic explants. Photomicrographs of individual explants were taken over 72 hours, and microvascular sprouting was quantified by measuring the area covered by outgrowth of the aortic ring by using ImageJ software.

Differentiation Assay/Tube Formation Assay

Differentiation assays essentially measure the formation of capillary-like tubules by endothelial cells cultured on matrices consisting of fibrin, collagen, or a basement membrane substance like Matrigel that enhances the attachment, migration, and differentiation of endothelial cells into tubules in a manner analogous to the *in vitro* situation. Rat brain endothelial cells were plated in 24-well plates on growth-factor-reduced Matrigel and treated with lactate alone (pH controlled to 7.2) or incubated with conditioned medium from Müller cells and RGC-5. Images were captured using phase contrast microscopy (Eclipse TE300; Nikon), and tube formation was quantified in nonoverlapping fields with ImageJ software.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay

Retinal cryosections (10 μ m thick) from WT and GPR81-null mice at different time points (P8, P11, and P30) were subject to the *in situ* cell-death detection kit (ApopTag Plus; Millipore-Sigma, Oakville, Canada) following the manufacturer's instructions. The retinal cryosections were counterstained with 0.1 μ g/mL DAPI (Molecular Probes). Images were captured using confocal microscopy (Eclipse E800; Nikon), and the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling—positive cells were quantified using ImageJ software.

Statistical Analysis

All data were analyzed using GraphPad Prism software version 7.0 (GraphPad Software, San Diego, CA). Normal distribution of data was tested using the Shapiro-Wilk test.⁵⁸ Accordingly, parametric analysis was performed in line with the fact that power analysis is also generally greater for continuous parametric variables and given the spread of data between groups.⁵⁹ Two variants were compared by *t*-test as data were normally distributed. Comparisons between several groups were performed using one-way variance analysis; Dunnett's multiple comparison method was used as a post-hoc test when many treatments were compared with one control. Descriptive statistics, including means $\pm$ SEM, have been reported. *P* < 0.05 was considered statistically significant.

Results

GPR81 Receptor Is Mainly Localized in Müller Cells

Immunohistochemistry using specific cell markers in retinal cryosections and primary isolated cell cultures revealed predominance of GPR81 in Müller cells (Figure 1, A and B). GPR81 mRNA (real-time quantitative PCR) and protein (Western blot analysis) expression on primary cell cultures or immortal cell lines corroborated the presence of GPR81 primarily on Müller cells, albeit also on RGCs, as reported,⁶⁰ and (possibly) to a lesser extent on retinal pigment epithelium. However, the presence of GPR81 on endothelium was negligible (Figure 1, C and D). GPR81 antibody specificity was appreciated on Western blot analysis (Figure 1C) and by immunohistochemistry (Supplemental Figure S1) as no immunoreactivity was detected in retinas from GPR81-null mice.

GPR81 Affects Intraretinal Vascular Development and Retinal Function

GPR81 protein expression increased gradually from P8 to P12 in wild-type mice, inferring a prominent effect of GPR81 on formation of the deeper vascular network rather

Figure 3 Delayed intraretinal vascular development results in retinal dysfunction in GPR81-null mice. **A:** Flash electroretinograms of age-matched wild-type (WT) and GPR81-null mice show a significant reduction in scotopic (0.01 cd-seconds/m²) and photopic (3.0 cd-seconds/m²) b-wave amplitude responses in GPR81 knockout (KO) compared with WT mice; a-wave amplitudes hardly differed. **B:** Real-time quantitative PCR analysis showing the mRNA expression of *Wnt* ligands and *Norrin* in retinas from WT and GPR81 KO mice at P9. Data are expressed as means $\pm$ SEM. $n = 4$ to 7 eyes (**A**); $n = 10$ retinas per group (**B**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with control values in WT mice (using two-tailed *t*-test).

than intermediate network.⁶¹ By P17, GPR81 expression decreased and continued to do so by P21, at a time when the deep vascular network has been completed (Figure 2, A and B). Correspondingly, intraretinal vascularization was significantly delayed in GPR81-null mice, as shown at P8, P9, and P14 using confocal microscopy of retinal flat mounts (Figure 2B). By P14, retinal vascularization caught up and was similar in wild-type and GPR81-null mice (Figure 2B). On the other hand, GPR81 contributed modestly to the development of the superficial vasculature, as attested by comparable superficial retinal vascular development in WT and GPR81-null animals (Figure 2, B–D). Curtailed intraretinal vascular development was also observed at P9 in mice knocked down of GPR81 using a lentiviral-encoded shRNA targeting *Gpr81* (Lv shGPR81) (Supplemental Figure S2, A and B), injected intravitreally in WT mice at P4 (Supplemental Figure S2C); GFP-encoding Lv exerted no effects.

A delay in vascular development is reported to likely interfere with neuroretinal function.^{62,63} Concordant to vascular changes, a significant number of apoptotic cells was also detected at P11 in the inner nuclear layer of GPR81-null mice during the preceding delayed vascular development (Supplemental Figure S3). Consistently, as a possible consequence of this inner nuclear cell death, ERG in these animals revealed a marked reduction (abnormality) in scotopic (0.01 cd-seconds/m²) and photopic (3.0 cd-seconds/m²; inner retinal function, Müller cell–contributed) b-wave amplitudes at P30 (Figure 3A); photoreceptor-generated a waves were hardly affected.

The expression of selective genes implicated in the development of the inner retinal vascular network regulated by the Wnt signaling pathway,^{23,25,64} such as *Wnt3A*, *Wnt7A*, and *Wnt10A* and robustly *Norrin* and its coreceptors *Lrp5*, as well as the protein *Tspan12* but not *Fzd-4*, were coincidentally significantly diminished in the retina of GPR81-null mice in comparison with WT mice at P9 (Figure 3B). Genes associated with the Wnt signaling pathway that play an important role in the blood-retinal barrier integrity, such as *PLVAP*⁶⁵ and *Claudin-5*,⁶⁶ were also altered in the GPR81-null mice at P9 (Supplemental Figure S4), consistent with their respective roles and profiles related to *Norrin* signaling.^{65,67} Overall, these findings suggest that GPR81 could be implicated in governing the expression of *Norrin* (and various Wnt factors) involved in inner retinal angiogenesis and blood-retinal barrier integrity during development.

GPR81 Activation Accelerates Intraretinal Vascular Development

To further establish the role of GPR81 on inner retinal angiogenesis, lactate [10 mmol/L (final ocular concentration)] was injected intravitreally^{68,69} to P5 wild-type mice; this age corresponds to that of an incomplete superficial and absent deep retinal vasculature. Note that lactate binds GPR81 in the low millimolar range ($EC_{50} = 1.3$ to 4.8 mmol/L),^{39,42} consistent with physiological and pathophysiological concentrations of lactate (5 to 20 mmol/L).⁵⁵ As expected, superficial vascularization was marginally affected by lactate at P8 (when superficial vasculature is

Figure 4 Lactate acts via GPR81 to augment vascular density of the intraretinal vascular plexus. **A:** Representative confocal images of flat mount petals (**top row**) and retinal cryosections (**bottom row**) showing retinal microvascular density and intraretinal vascular plexus on wild-type (WT) and GPR81 knockout (KO) mice intravitreally injected with lactate (10 mmol/L at P5) and stained with tetramethylrhodamine isothiocyanate-labeled lectin *Bandeiraea simplicifolia* at P8. Lactate is ineffective on intraretinal microvasculature from GPR81 KO mice (P8 KO Lactate). Reduced vascularization is evident in GPR81 KO mice (P8 KO Vehicle) compared with age-matched WT (P8 WT vehicle). **Dashed lines** in **top row** show the localization of the optic nerve. **White arrows** in **bottom row** point to deep vascular network. The histograms show the percentage of vascular area in the inner and superficial vasculature networks from retinas treated with lactate versus vehicle in WT and GPR81 KO mice. **B:** mRNA expression of Wnts and *Norrin* on retina of WT animals with and without lactate treatment. Values are presented as fold increases over control. Data are expressed as means $\pm$ SEM. $n = 14$ to 18 retinas (**A**); $n = 6$ to 10 retinas per group (**B**). ** $P < 0.01$ compared with corresponding WT control; †† $P < 0.01$, ††† $P < 0.001$ compared with control values (using two-tailed t -test). Scale bar = 300 μ m (**A**). ONH, optic nerve head.

completed), whereas inner retinal vascularization (which normally begins at P8⁷⁰) was significantly accelerated (Figure 4A); lactate was ineffective in the retina of GPR81 gene knockout mice.

Consistent with stimulation of inner retinal angiogenesis, *in vivo* lactate administration induced expression of

corresponding relevant angiogenic factors, *Wnt3A*, *Wnt7A*, *Wnt10A*, and *Norrin* (Figure 4B). Lactate-stimulated isolated Müller cells exhibited a similar profile of gene expression seen *in vivo* in retina (Figure 5A). It was, therefore, determined if lactate-generated Müller cell products elicited angiogenesis (including migration,

Figure 5 Proangiogenic effects of lactate acting via GPR81. **A:** mRNA expression, measured by real-time quantitative PCR, of lactate (10 mmol/L)–stimulated primary [wild-type (WT)] Müller cells (MCs) induced an increment in expression of Wnt ligands *Wnt3A*, *Wnt7A*, and *Wnt10*, and especially of *Norrin* but not of *Ang2*; this increase is not observed in primary Müller cells from GPR81 knockout (KO) mice; values are presented as fold increases over control (vehicle stimulated) and normalized for 18S. **B:** Representative micrographs showing endothelial cell (EC) tube formation (white arrows; measured at 24 hours after plating) induced by conditioned media from WT and GPR81 KO Müller cells previously stimulated with lactate or vehicle. Histograms show the experiments in triplicates. **C:** Conditioned media from WT Müller cells (MCs) stimulated with lactate (10 mmol/L) induced EC migration but not in Müller cells of GPR81 KO (KO MC) mice, and in those knocked down for *Norrin* (WT MC shRNA *Norrin*). Fetal bovine serum (FBS) was used as a positive control. Histograms represent the experiments in triplicates. **D:** Representative microvascular sprouting of Matrigel-embedded adult mice aortic explants treated with conditioned media from WT- and GPR81 KO mice–derived Müller cells previously stimulated with lactate or vehicle. Histograms are the experiments in triplicates. FBS (10%) was used as a positive control. Data were analyzed by one-way analysis of variance with Dunnett's postanalysis. Data are expressed as means $\pm$ SEM. $n = 3$ separate experiments (**A–D**). $**P < 0.01$, $***P < 0.001$ compared with Müller cells from WT mice; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ compared with corresponding control. Scale bars: 1 mm (**B**, top row, and **D**); 10 mm (**B**, bottom row). Original magnifications: $\times 10$ (**B**, top row); $\times 4$ (**B**, bottom row). MC-L, Müller cells–lactate stimulated.

Figure 6 Increased lactate concentration in oxygen-induced retinopathy (OIR). **A:** Lactate concentrations (mmol/L) in retinas of wild-type mice subjected to OIR compared with normoxia-raised animals at different postnatal days. **B:** Lactate levels in peripheral (less ischemia) and central (more ischemia) retina of mice subjected to normoxia and OIR. **C:** Real-time quantitative PCR analysis showing the mRNA expression of *GPR81* and *Norrin* at P12, P14 (midprogression of revascularization), and P17 (peaking revascularization) of mice exposed to OIR; values are presented as fold increases over control (P8) normalized to 18S. **D:** Western blot analysis of GPR81 (39-kDa) expression in mice retina subjected to OIR (P12 to P21) compared with normoxia-raised animals; β -actin (42 kDa) was used as an internal control. Densitometry quantification is illustrated in the histogram. Data were analyzed by one-way analysis of variance with Dunnett's postanalysis. Data are expressed as means $\pm$ SEM. $n = 14$ to 18 retinas (**B**); $n = 5$ to 7 retinas (**C**); $n = 3$ separate experiments (**D**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with normoxia.

Figure 7 Lactate attenuates vaso-oblation and consequently preretinal neovascularization in oxygen-induced retinopathy (OIR). **A:** Representative images from lectin-stained retinal flat mounts at P9, showing attenuation in retinal vaso-oblation (outlined by the **fine yellow line**) in wild-type (WT) mice subjected to OIR retina after intravitreal injection of lactate at P7; lactate was ineffective in GPR81 knockout (KO) mice; vaso-oblation areas are outlined with **solid yellow lines**. Lactate-unresponsive GPR81 KO mice display increased vaso-oblation. Changes in vaso-oblation were calculated as percentage unvascularized regions relative to total retina at P9. Histogram quantification of vaso-oblation areas is presented. **B:** Retinal cross-sections confirm lactate-induced stimulation of intraretinal angiogenesis (**white arrows**) at P9; GPR81 KO animals were unresponsive to lactate. **C:** Retinal flat mounts stained with lectin showing attenuation in preretinal neovascular tufts (white areas) in lactate-treated animals subjected to OIR with respect to vehicle at P17. Histogram quantification of neovascularization is presented. **D:** Real-time quantitative PCR mRNA analysis of *Norrin* expression in retinas of WT and GPR81 KO mice injected with vehicle or lactate during vaso-oblative phase of OIR (P7); note increased *Norrin* levels at P9 in retinas injected with lactate during OIR. Data are expressed as means $\pm$ SEM. $n = 12$ to 16 retinas (**A** and **C**); $n = 6$ to 10 retinas (**D**). $*P < 0.05$, $**P < 0.01$ compared with WT vehicle; $^{\dagger}P < 0.01$ compared with vehicle OIR. Scale bar = 300 μ m (**B**). PBS, phosphate-buffered saline.

Figure 8 Norrin (up-regulation) restores intraretinal vascularization in GPR81 knockout (KO) mice. **A:** To investigate the mechanism of action of GPR81 via Norrin, Western blot analysis of extracellular signal-regulated kinase (Erk) 1/2 and phosphorylated Erk1/2 (p-Erk1/2) was studied in wild-type (WT) and KO primary Müller cells. Representative images show an increase in Erk1/2 phosphorylation after lactate stimulation in Müller cells from WT mice and not in KO Müller cells. **B:** mRNA expression of *Norrin* in WT and KO primary Müller cells treated with or without lactate in the presence of Erk1/2-specific inhibitor (U0126). Values are presented as fold increases over control (vehicle stimulated). Data were analyzed by one-way analysis of variance with Dunnett's postanalysis. **C:** RT-PCR (top panel) and real-time quantitative PCR (bottom panel) confirming *in vivo* Norrin mRNA expression of a lentivirus (Lv) encoded with the *Norrin* gene (Lv Norrin) or green fluorescent protein (GFP; Lv GFP; negative control). S18 was used as internal control. **D:** Representative confocal images showing the superficial (red) and inner (green) retinal vasculature in WT and GPR81 KO mice. Retinas from GPR81 KO mice intravitreally injected with Lv Norrin increase intraretinal vascularization with respect to the mice injected with Lv GFP (negative control) at P9. Histogram quantification is presented as percentage increase in intraretinal vascularization relative to age-matched noninjected mice at starting point (set at 100%). Data are expressed as means $\pm$ SEM. $n = 3$ separate experiments (**B**); $n = 8$ to 12 retinas (**D**). * $P < 0.05$ quantified to the ratio of p-Erk1/2/total Erk1/2; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ compared with control values. Scale bar = 250 μ m (**D**).

proliferation, and tube formation of endothelial cells). Conditioned medium from primary Müller cells stimulated with lactate significantly induced endothelial tube formation and migration and vascular sprouting of aortic explants

(Figure 5, B–D); lactate-stimulated Müller cells from GPR81-null mice exerted no effects. More important, Müller cells infected with a Lv containing shRNAs targeting Norrin interfered with lactate-triggered endothelial cell

migration necessary in angiogenesis (Figure 5C). Collectively, these observations are consistent with *in vivo* angiogenic properties of lactate in retina and suggest that angiogenic factors, particularly Norrin, released from lactate-stimulated Müller cells, have an important contribution in retinal angiogenesis.

Role of Lactate/GPR81 in Ischemic Retinopathy

The role of lactate/GPR81 was also studied in a model of ischemic retinopathy, specifically in OIR, which mimics the two phases of ischemic retinopathy (notably, the vaso-obliteration, followed by an aberrant intravitreal neovascularization).⁴⁸ Lactate levels in retina were measured at different time points during the (ischemic) vasoproliferative phase (P12 to P17) and the subsequent phase of neovascular regression (P18 to P21). Although levels of lactate in OIR-subjected animals were slightly increased at P12 (end of ischemic vaso-obliterative phase) (Figure 6A), both *GPR81* and *Norrin* expression were low (compared with values in normoxic animals) (Figure 6, C and D). The highest values of retinal lactate were detected at P16 in the peaking revascularization phase⁷¹ (as well as in extraretinal neovascularization, which is contributed by superficial vascular metabolism²⁰) when GPR81 as well as Norrin expression concomitantly increased (at P14 to P17) to restore OIR-depleted retinal vascularization, as reported^{48,49}; thereafter, lactate levels approach basal values (by P21) along with GPR81 expression (Figure 6D), as normal vascularization is reestablished. Changes in lactate were equivalently affected at the periphery and center of the retina (Figure 6B), suggesting a marked diminution in metabolic rate of the central retina, predominantly vaso-obliterated in OIR.

Lactate/GPR81 Reestablishes Norrin Expression in Ischemic Retinopathy

The ischemic retinopathy model is initially associated with a prominent retinal vaso-obliteration detectable within 24 to 48 hours after exposure of animals to hyperoxia¹³ when levels of Norrin are low.⁷² It was, therefore, determined if exogenous lactate can attenuate retinal vaso-obliteration while reestablishing Norrin expression during ischemic retinopathy. Exposure of mice to 75% O₂ at P7 (to elicit OIR) led to a more marked retinal vaso-obliteration, a compromised intraretinal vascular development, and consequently predisposed to augmented preretinal neovascularization in GPR81-null mice compared with WT mice, at P17 (Supplemental Figure S5, A and B). These vascular changes in OIR coincide with timing for low expression of Norrin, critical for intraretinal vascular development. Intravitreal injection of exogenous lactate [10 mmol/L (intraocular concentration)] in mice subjected to OIR at the beginning of the vaso-obliteration phase (at P7) increased Norrin levels in the retina at P9, reduced vaso-obliteration in WT littermates (but not in GPR81-null mice), and consequently mitigated extraretinal

neovascularization (at P17) (Figure 7). These findings corroborate the angiogenic properties of lactate acting via GPR81 to stimulate not only normal retinal vascularization during development (Figure 4), but also that in OIR (Figure 7), and highlight the importance of lactate/GPR81 in regulating Norrin.

GPR81 Activates Norrin via Erk1/2 Phosphorylation, and Norrin Promotes Vascular Regrowth in GPR81-Null Mice

In view of the evidence presented herein that GPR81 governs Norrin, which, in turn, regulates intraretinal vascularization, the downstream signaling of GPR81 causing induction of Norrin was investigated. On the basis of reported information, Erk1/2 activity^{73,74} in primary Müller cells from WT and GPR81-null mice was studied. Phosphorylation of Erk1/2 was activated on lactate stimulation in WT but not in cells from GPR81-null animals (Figure 8A). Moreover, lactate-induced Norrin expression was significantly inhibited by the specific Erk1/2 inhibitor, U0126 (Figure 8B).

Furthermore, it was determined if Norrin can restore vascular depletion in Norrin-depleted GPR81-null mice (Figure 8, C and D). Norrin [or GFP (negative control)] gene-expressing Lv (Lv Norrin or Lv GFP) was injected intravitreally at P5, as previously reported.⁷² Up-regulation of Norrin was ascertained by measuring mRNA of mouse retina injected with Lv Norrin (Figure 8C) and was associated with an increase in inner retinal vascular sprouting at P9 (Figure 8D).

Discussion

Retinal microvascular degeneration and ensued tissue hypoxia in ischemic retinopathies predispose to excessive aberrant intravitreal neovascularization and defective retinal function. Prompt restoration of normal retinal vascularization in vascular depleted regions is paramount to preserve retinal integrity.⁷⁵ Herein, we have discovered an unprecedented mechanism in the development of the inner retinal microvasculature. Relevant hypoxia-generated lactate and its newly described receptor GPR81 present primarily on Müller cells, govern the expression of Norrin to fulfill metabolic demands of the inner retina by stimulating vascular development, and ensure adequate oxygen supply during physiological development as well as in ischemic retinopathy. A schematic representation of our findings is depicted in Figure 9.

Traditionally, lactate was considered as a waste product of anaerobic metabolism. Lactate is now known to be a valuable intermediate metabolite implicated in several functions, including angiogenesis^{69,76,77}; abrogation of GPR81 also reduces tumor progression and metastasis *in vivo*.⁷⁸ Herein, we show that lactate, a biologically active molecule acting through its receptor GPR81, bridges the gap between hypoxic retina and the ensuing vascularization

OXYGEN-INDUCED RETINOPATHY

Figure 9 Illustration of mechanism of action of GPR81 in the retina. Schematic overview of lactate acting through its GPR81 receptor localized in Müller cells (MCs) governing deep intraretinal vessel formation during development and in ischemic retinopathy. Lactate/GPR81 activation stimulates expression of Wnt ligands, in particular Norrin, that act through its receptor Frizzled-4 localized on the endothelium to regulate the formation of the intraretinal vasculature during development. During the first phase in the oxygen-induced retinopathy, a progressive microvascular degeneration occurs associated with low levels of GPR81 and corresponding low levels of Norrin (regulated by GPR81). In the second phase, as an attempt to reinstate vascular supply to the hypoxic/ischemic retina, an augmentation in the production of lactate occurs. Lactate acts through increased GPR81 expression to trigger the generation of Wnt ligands (Wnt3A, Wnt7A, Wnt10A) and especially Norrin, which elicits revascularization. Conversely, down-regulation of GPR81 interferes with lactate actions in reinstating an appropriate intraretinal vascular supply, thus aggravating hypoxia and consequently preretinal neovascularization. On the other hand, administration of lactate during the vaso-obliteration phase stimulates revascularization, which accelerates the restoration of the inner retinal vasculature and subsequently decreases pathologic preretinal neovascularization. RPE, retinal pigment epithelium.

process. Lactate has been shown to exert vasoproliferative effects in tumors and skin.^{32–34} Of relevance, oxygen concentrations are lowest in the deep retina (inner and outer plexiform layers and inner nuclear layer), particularly before vascular development and after retinovascular injury, as in ischemic retinopathies.^{79–81} It was, thus, tempting to speculate that lactate (generated during hypoxia/ischemia) may act as a mediator to trigger neovascularization of the deep vascular complex in the retina. In this model of ischemic retinopathy, lactate levels started to increase at the beginning of ischemic retinopathy and reached maximum levels [comparable to those in tumors (6 to 15 mmol/L)]⁸² before the peak pathologic neovascularization. These results suggest that lactate is implicated in rapid revascularization of affected tissue, as previously reported,^{33,34} but in this case

specifically of the inner retinal vascular plexus, so far never described. Accordingly, exogenous lactate administered during the vaso-obliterative phase of OIR stimulates angiogenesis to promote vascular repair and, thus, reinstates metabolic supply to prevent ischemic retinopathy and ensue preretinal neovascularization. Conversely, deficiency in GPR81 accrues vaso-obliteration and, consequently, preretinal neovascularization, which seems more dependent on RGC metabolic consequences.²⁰

An important feature of this study is that Müller cells, as classic sensors of hypoxic stress in the retina⁸³ and major lactate secretory retinal cells,^{28,84} express GPR81 abundantly⁶⁰ and respond to its ligand lactate by releasing several Wnt ligands, such as Wnt3, Wnt7, Wnt10, and more important, Norrin, which is implicated in establishing

vascular supply during physiological developmental ischemia and early in pathologic ischemic retinopathy. Notably, the Wnt ligands (Wnt3, Wnt7, and Wnt10) are angiogenic factors that play a critical role in the formation of the vascular networks during development.⁶⁴ Wnt ligands have also been implicated in the development of pathologic angiogenesis during OIR.⁸⁵ Hence, Wnt ligands are also likely to participate in physiological and pathologic intraretinal vascular development. Nonetheless, increased Norrin expression in GPR81-null mice was sufficient to significantly rescue intraretinal vascularization.

Müller cells, which exhibit primarily a glycolysis-dependent metabolism, are the major producers of lactate in retina²⁷; accordingly, Müller cells have the highest expression of the lactate transporter monocarboxylate transporter 1 in the retina,⁸⁶ allowing its export to exert its actions on cell surface GPR81. In addition, Müller cells harbor by far the greatest expression of Norrin in retina,⁶¹ and this elongated cell, which spans the whole retina, is suitably localized to control intraretinal vascularization. This study showed a direct role for Müller cells in influencing vascular development via GPR81, as lactate stimulated Müller cells to elicit Norrin (and Wnt) expression and angiogenesis, and Norrin silencing interfered with the latter, emphasizing its importance.

Retinal angiogenesis is a complex process that involves several retinal cell types^{21,87,88} and, in particular, a large number of proangiogenic factors generated by neurons and glia, which have been found to govern superficial retinal vascularization.^{87,89} However, the factors that specifically govern the intraretinal vascular network are only now beginning to be determined. In this study, lactate was observed acting via GPR81 in governing the expression of Wnt ligands and particularly Norrin to accelerate revascularization, thereby ensuring an adequate oxygenation to the ischemic tissue. Norrin was further studied because it is a chemoattractant guiding protein almost exclusively produced by Müller cells⁶¹ that plays a critical role in the development of the intraretinal vasculature. Norrin is known to exert its vasoproliferative effects²⁴ through the Wnt/ β -catenin pathway by binding to Frizzled-4, which interacts with coreceptors Lrp5 (or Lrp6)⁹⁰ and Tspan12.²⁴ Relevantly in the present context, it has also been shown that a deficiency in Norrin, Fzd-4, Lrp5/6, or Tspan12 leads to a marked deficiency in intraretinal vascular development.^{24,25,61,91} Consistent with these findings, it was found that although GPR81 receptor depletion hardly affected Fzd-4 expression in the retina, Lrp5 and Tspan12 expression levels were decreased to a similar extent as Norrin, in line with delayed formation of the inner retinal vascular network in GPR81-null mice. More important, the activation of the GPR81 receptor by intraocular lactate stimulated Norrin expression and accelerated corresponding inner microvascular formation during retinal development in WT animals, but not in GPR81-null mice, which indicates that lactate acts via GPR81 to govern retinal angiogenesis by regulating the

formation of several major angiogenic factors, including notably Norrin. Moreover, knockdown of Norrin in Müller cells interferes with endothelial cell migration, whereas rescue with Norrin of Norrin-depleted GPR81-null mice restores intraretinal vascularization. Altogether, these observations highlight the link between GPR81, Norrin, and intraretinal vascularization.

Finally, these data reveal that a deficiency in GPR81 is manifested by a depression of the b-wave amplitude, contributed by Müller cells.⁹² This ERG functional deficit could be attributed to the GPR81-mediated retinal cell death that occurs early during development. These deleterious effects of GPR81 deficiency, culminating in delay of inner vascular development leading to perturbed hemodynamics and retinal cell death, cannot be ignored. Conversely, lactate stimulates inner retinal vascularization as it induces Norrin expression.

All in all, we show, for the first time, that lactate exerts proangiogenic effects via GPR81 in the retina and participates in the formation of the inner retinal vascular network during development, as well as in the restoration of the vasculature in response to ischemic injury. These findings suggest lactate/GPR81 as a new potential therapeutic target to alleviate ischemic diseases.

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A.M., J.C.R., and S.C. conceptualized and designed the study and prepared the manuscript; A.M. performed most of the experiments and analyzed the data; P.C., M.N.-V., R.D., C.W.H.C., and M.A.M.N. performed single blinded experiments; T.Z. designed and prepared lentivirus for G-protein-coupled receptor 81 and Norrin; J.C.R. drew the graphical illustration for Figure 8; D.H. helped troubleshoot in preliminary stages; S.P. and J.-S.J. performed electrophysiological procedures; G.M. and M.S. provided scientific advice; all authors read and approved the final manuscript.

Supplemental Data

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