VEGF Secreted by Hypoxic Müller Cells Induces MMP-2 Expression and Activity in Endothelial Cells to Promote Retinal Neovascularization in Proliferative Diabetic Retinopathy

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In proliferative diabetic retinopathy (PDR), retinal ischemia promotes neovascularization (NV), which can lead to profound vision loss in diabetic patients. Treatment for PDR, panretinal photocoagulation, is inherently destructive and has significant visual consequences. Therapies targeting vascular endothelial growth factor (VEGF) have transformed the treatment of diabetic eye disease but have proven inadequate for treating NV, prompting exploration for additional therapeutic options for PDR patients. In this regard, extracellular proteolysis is an early and sustained activity strictly required for NV. Extracellular proteolysis in NV is facilitated by the dysregulated activity of matrix metalloproteinases (MMPs). Here, we set out to better understand the regulation of MMPs by ischemia in PDR. We demonstrate that accumulation of hypoxia-inducible factor-1α in Müller cells induces the expression of VEGF, which, in turn, promotes increased MMP-2 expression and activity in neighboring endothelial cells (ECs). MMP-2 expression was detected in ECs in retinal NV tissue from PDR patients, whereas MMP-2 protein levels were elevated in the aqueous of PDR patients compared with controls. Our findings demonstrate a complex interplay among hypoxic Müller cells, secreted angiogenic factors, and neighboring ECs in the regulation of MMP-2 in retinal NV and identify MMP-2 as a target for the treatment of PDR. ***Diabetes*** **62:3863–3873, 2013**

Diabetic retinopathy (DR) is the most common microvascular complication in the diabetic population. Development of DR is directly related to the duration of diabetes; 20 years after diagnosis, nearly all diabetic patients have DR (1). Current approaches to prevent and/or treat DR include optimizing control of blood glucose levels and screening of high-risk patients for early identification of retinopathy (2). Despite these efforts, DR is the leading cause of blindness among working-age adults in the developed world (3).

DR is classified as nonproliferative or proliferative (PDR). Sustained hyperglycemia is the major initiator for the development of nonproliferative PDR (4). However, PDR is an ischemic retinopathy; it develops when the oxygen demand of the inner retina exceeds oxygen supply, and results in the upregulation of angiogenic factors that promote neovascularization (NV) (5). PDR manifests clinically with retinal NV (Supplementary Fig. 1*A*), which, if left untreated, can result in profound vision loss.

Retinal NV in PDR occurs at the junction between perfused and nonperfused (ischemic) retina (Supplementary Fig. 1*B*), and results in the growth of fibrovascular tissue on the surface of the retina (Supplementary Fig. 1*C*). The standard of care for patients with PDR is panretinal laser photocoagulation (PRP), a destructive procedure in which peripheral (ischemic) retina is killed to preserve central vision (6). PDR can progress even after PRP treatment (Supplementary Fig. 1*D*) and remains the most difficult consequence of diabetic eye disease to treat, highlighting the need for new therapeutic options for patients with PDR (6).

Work from several laboratories has resulted in our current appreciation for the central role of the transcriptional activator hypoxia-inducible factor (HIF) in pathological angiogenesis (7). HIF regulates the expression of hypoxia-inducible genes that promote NV. The remarkable success of therapies targeting the HIF-regulated gene product vascular endothelial growth factor (VEGF) for the treatment of diabetic macular edema highlights the importance of HIF in diabetic eye disease (8). Although anti-VEGF therapy can delay and even prevent the progression to PDR (9), alone it has not yet been shown to be sufficient to treat PDR (10). Recent evidence further suggests that chronic inhibition of VEGF activity may have unwanted long-term consequences for the vulnerable neurosensory retina (11), prompting exploration for additional HIF-dependent therapeutic targets for PDR patients.

In this regard, the extensive interplay among endothelial cells (ECs), secreted factors, and the extracellular matrix (ECM) is an emerging target for antiangiogenic therapies. In particular, ECM proteolysis has been implicated as one of the first and most sustained activities involved in pathological angiogenesis (12). Proteolysis by matrix metalloproteinases (MMPs), zinc-dependent endopeptidases

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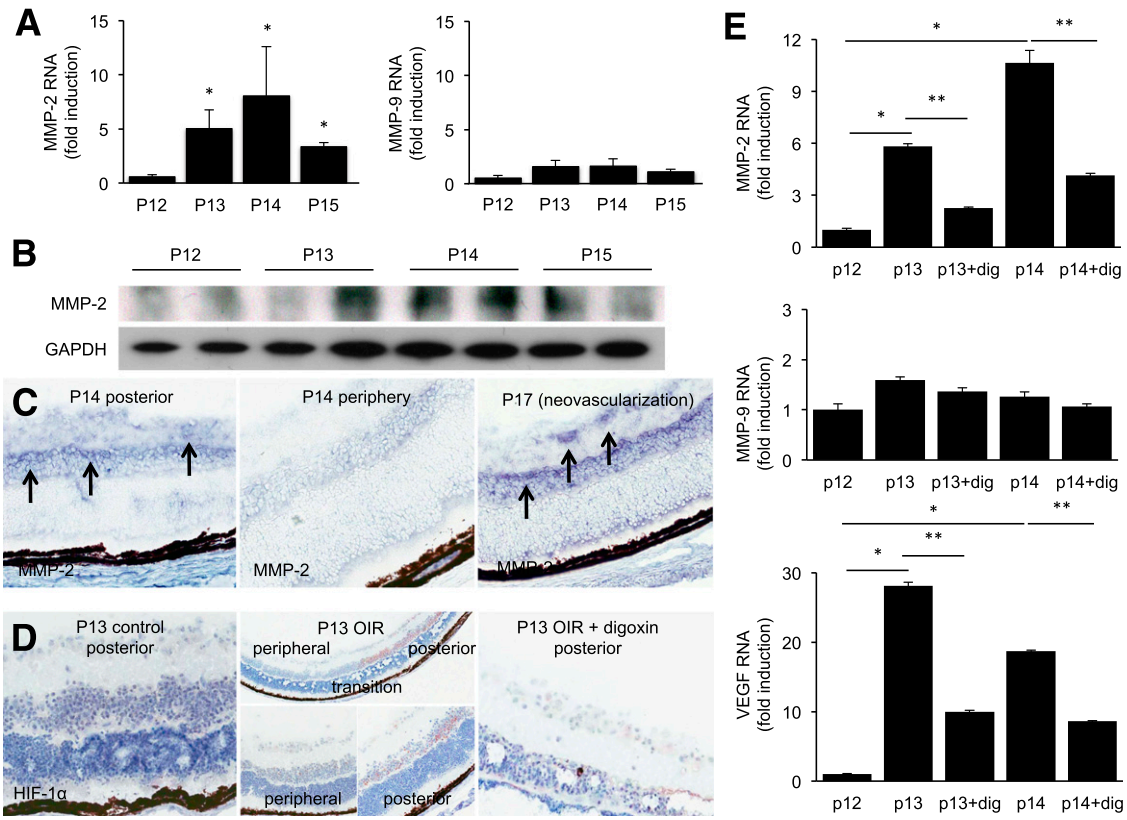

FIG. 1. HIF-dependent MMP-2 expression during the ischemic phase in the OIR model. *A*: RT-PCR of *Mmp-2* and *Mmp-9* mRNA from the neurosensory retina of OIR animals at P12–P15 normalized to cyclophilin *B* mRNA and reported as fold induction compared with P12. *B*: Representative Western blot of MMP-2 protein accumulation from the neurosensory retina of OIR animals at P12–P15. Paired lanes represents lysates from two eyes from two separate animals. GAPDH was used as a loading control. *C*: Representative immunohistochemical analysis of MMP-2 (black arrows; blue chromogenic substrate) in the retina of OIR eyes during the ischemic phase at P14 (posterior retina, *left*; peripheral retina, *middle*) and during the neovascular phase at P17 (*right*). *D*: Representative immunohistochemical analysis of HIF-1α (brown chromogenic substrate) in the retina of P13 OIR eyes (*middle*) compared with P13 control eyes (*left*). HIF-1α expression was inhibited with daily intraperitoneal injections of digoxin (*right*). *E*: RT-PCR of *Mmp-2*, *Mmp-9*, and *Vegf* mRNA from the neurosensory retina of OIR animals at P12–P14 with daily intraperitoneal injection of digoxin (+dig) or without injection, normalized to *cyclophilin B* mRNA, and reported as fold induction compared with P12. All experiments were performed in duplicate and are representative of at least three independent experiments. $n = 3$ animals in each group. Student *t* test: *$P < 0.05$; **$P < 0.01$.

that degrade various components of the ECM, has been reported to play an important role in PDR (13). However, despite well-designed studies aimed at improving our understanding of the role of these proteases in eye disease, contradictory results from several investigations have made it difficult to determine the relative contribution of MMPs to ocular NV. Consequently, reasonable disagreement remains as to whether—and which—MMPs are valid therapeutic targets for the treatment of PDR. Here, we investigated the relationship among hypoxia, HIF, and MMP regulation in the ischemia-driven retinal NV observed in patients with PDR, with the goal of identifying targets for the treatment of this vision-threatening disease.

RESEARCH DESIGN AND METHODS

Constructs and reagents. Recombinant mouse VEGF (rmVEGF), recombinant human VEGF (rhVEGF), MMP-2, and VEGF ELISA kits were obtained from R&D Systems. Predesigned control (scrambled) and VEGF small interfering RNA sequences were obtained from Qiagen. Digoxin and desferrioxamine (DFO) were obtained from Sigma-Aldrich. 1,4-dihydrophenonthrolin-4-one-3-carboxylic acid (1,4-DPCA) and dimethyloxalylglycine (DMOG) were obtained from Cayman Pharmaceuticals.

Cell culture. Immortalized human Müller cells (MIO-M1 cells) were a gift from Astrid Limb (Institute of Ophthalmology, University College London, London, U.K.). Isolation of primary Müller cells was performed as previously described (14). Immortalized human umbilical vein ECs (iHUVECs) were obtained from Lonza and cultured according to the manufacturer's protocols.

Mice. Eight-week-old, pathogen-free female C57BL/6 mice (The Jackson Laboratory) and timed pregnant C57BL/6 mice (embryonic day 14) (Charles River Laboratories) were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Johns Hopkins University Animal Care and Use Committee. Oxygen-induced retinopathy (OIR) experiments were performed as previously described (15). A subset of mice was given daily intraperitoneal injections of vehicle or 2 mg/kg digoxin, a dose previously demonstrated to decrease HIF expression in the eye (16). Intraocular injections with 1 μL rmVEGF (200 ng/μL) were performed on 8-week-old female C57BL/6 mice with a NanoFil syringe (World Precision Instruments) using a 36-gauge beveled needle under sterile conditions.

Western blot and zymography. Cell and neurosensory retina lysates were subjected to 4–15% gradient SDS-PAGE (Invitrogen). Immunoblot assays were performed with primary antibodies specifically recognizing HIF-1α and MT1-MMP (Abcam), MMP-2 (Millipore), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fitzgerald). MMP-2 enzymatic (gelatinase) activity in conditioned media was measured by zymographic assay as previously described (17), using Novex Zymogram Gel (Invitrogen)

Quantitative real-time RT-PCR. Messenger RNA (mRNA) was isolated from cultured cells or isolated retinas with RNeasy Mini Kit (Qiagen), and cDNA was prepared with MuLV Reverse Transcriptase (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) and MyiQ Real-Time PCR Detection System (Bio-Rad).

Immunohistochemistry and immunofluorescence. Immunohistochemical detection of HIF-1α (Abcam), MMP-2 (Santa Cruz Biotechnology), CD31 (BD Pharmingen; BD Biosciences), and CD34 (Covance) was performed in paraffin-embedded human tissue and cryopreserved mouse tissue sections using the ABC system (Dako) as previously described (18). Immunofluorescence

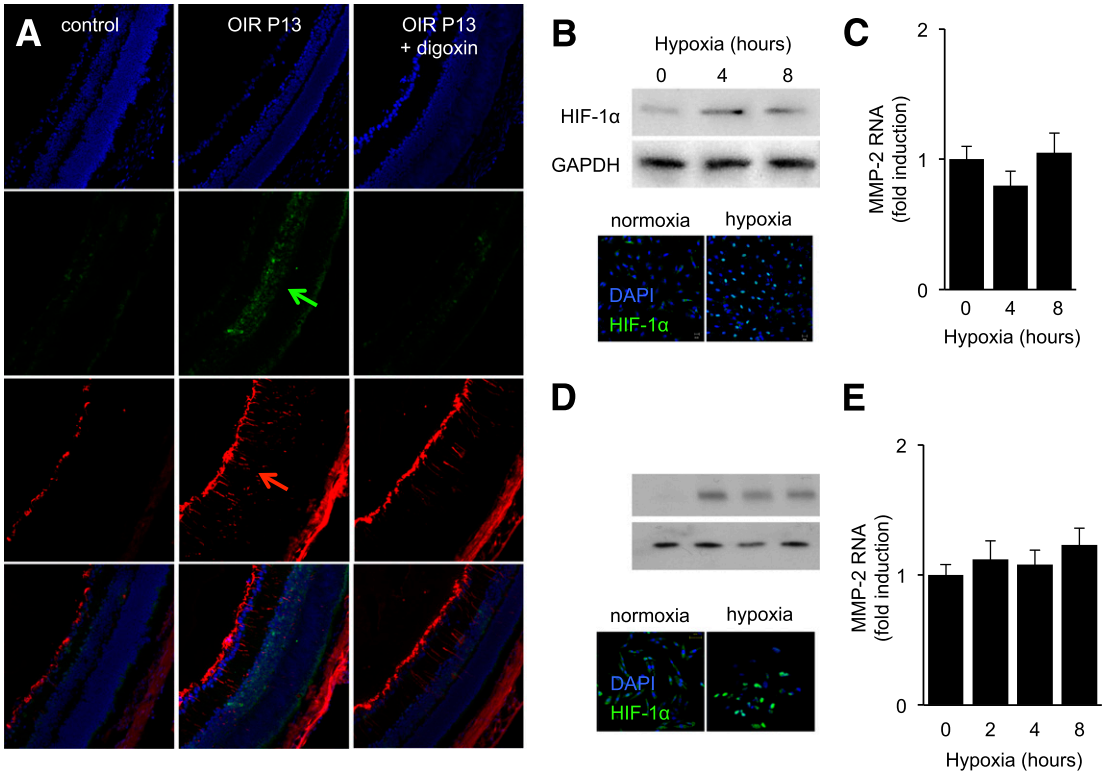

FIG. 2. Stabilization of HIF-1α in hypoxic Müller cells does not lead to MMP-2 expression. *A*: Representative immunofluorescence analysis of HIF-1α in the retina of P13 OIR eyes compared with control P13 eyes. *B*: Western blot and immunofluorescence for HIF-1α in primary murine Müller cells exposed to hypoxia. GAPDH was used as a loading control. *C*: *Mmp-2* mRNA levels from primary murine Müller cells exposed to hypoxia, normalized to cyclophilin *B* mRNA, and reported as fold induction compared with cells exposed to 20% O_2. *D* and *E*: Similar studies were performed using MIO-M1 cells. All experiments were performed in duplicate are representative of at least three independent experiments. *n* = 3 animals in each group. The differences in the fold induction in *C* and *E* were not statistically significant (*P* >0.05).

detection of CD31, glial fibrillary acidic protein (GFAP) (Sigma), HIF-1α, VEGF (Santa Cruz Biotechnology), and MMP-2 was performed on retina flat mounts or cryopreserved mouse tissue sections as previously described (19,20). Immunofluorescence was performed using goat anti-mouse Alexa Fluor 555, goat anti-rabbit Alexa Fluor 488, and goat anti-rat Alexa Fluor 647 (Invitrogen) in combination with DAPI (Invitrogen). Images were captured using the LSM 710 Meta confocal microscope (Carl Zeiss).

Patient samples. Institutional Review Board approval from the Johns Hopkins University School of Medicine was obtained for all patient samples and images used in this study. Aqueous and serum samples were collected from consenting patients undergoing cataract or vitrectomy surgery. Serum samples were allowed to clot for 30 min prior to centrifugation (1,200*g* for 10 min).

ELISAs. ELISAs for MMP-2 and VEGF were performed according to the manufacturer's protocols. Aqueous humor and serum were diluted 1:10 for the MMP-2 ELISA. Aqueous humor was diluted 1:10 for the VEGF ELISA. Undiluted serum was used for the VEGF ELISA.

Statistical analysis. In all cases, results are shown as mean ± SD from at least three independent experiments. Western blot scans are representative of at least three independent experiments. Statistical analysis was performed with Prism 4.2 software (GraphPad).

RESULTS

Hypoxia and HIF induce MMP-2 expression in the OIR mouse model. The OIR mouse model has previously been shown to reproduce the retinal ischemia that characterizes PDR (15). Briefly, vascularization of the posterior retina is impaired during the hyperoxia phase (postnatal day 7 [P7] to P12), leading to the ischemic phase (P13–P15), in which the oxygen demand of the inner retina exceeds oxygen supply (Supplementary Fig. 2*A*). The subsequent angiogenic response results in retinal NV at the junction of the perfused and nonperfused retina that is remarkably similar to that observed in patients with PDR (Supplementary Fig. 2*B*). In a recent screen for inhibitors of the transcriptional enhancer HIF-1, the cardiac glycoside digoxin was observed to potently inhibit HIF-1α protein synthesis and, in turn, the expression of HIF-regulated genes (21). Daily treatment of mice with intraperitoneal injections of digoxin at doses previously demonstrated to decrease HIF expression in the eye (16) results in a decrease in retinal NV (Supplementary Fig. 2*C*) (16). The OIR model therefore provides a model to examine the role of hypoxia, HIF, and HIF-regulated gene products in the pathogenesis of retinal NV in PDR.

ECM proteolysis has been implicated as one of the first and most sustained activities required for pathological angiogenesis (12). MMP-2 (gelatinase A) and MMP-9 (gelatinase B) play an important role in ECM degradation, an essential step for new vessels to escape from the retina and invade the vitreous cavity, which is a hallmark of PDR. *Mmp-2*, but not *Mmp-9*, mRNA levels were markedly increased in the neurosensory retina during the ischemic phase (P13–P15) compared with levels immediately after hyperoxia exposure (P12) (Fig. 1*A*). MMP-2 protein levels also peaked during the ischemic phase (Fig. 1*B*). Immunohistochemical analysis of P14 OIR eyes demonstrated an increase in MMP-2 in the posterior (ischemic) but not the peripheral (perfused) inner retina (Fig. 1*C*). Localized MMP-2 expression persisted adjacent to areas of NV after the ischemic phase (P17) (Fig. 1*C* and Supplementary Fig. 3*A*).

To assess whether ischemic upregulation of MMP-2 in the inner retina is a result of stabilization of the oxygen-sensitive

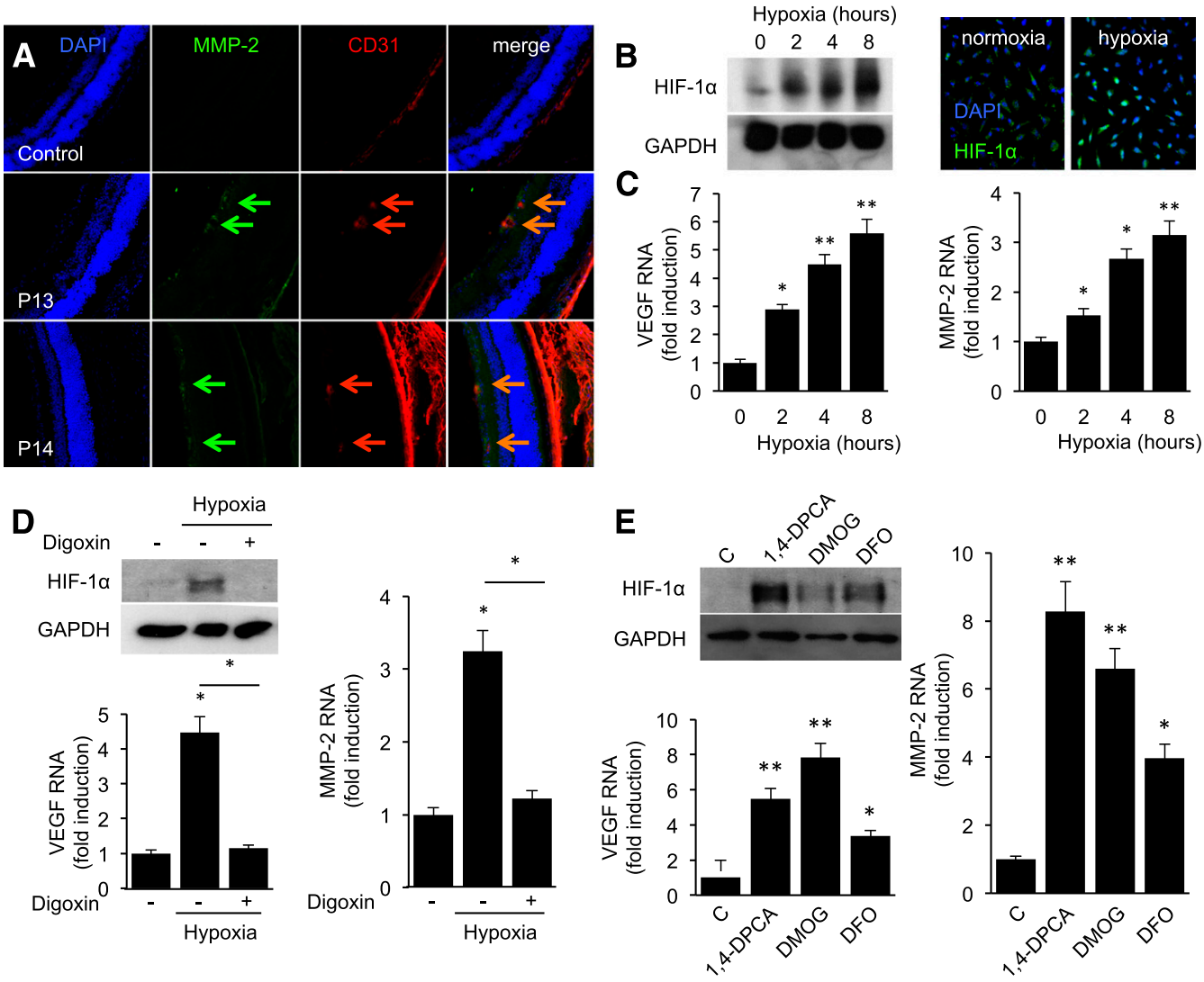

FIG. 3. Stabilization of HIF-1α in hypoxic vascular ECs leads to increased *MMP-2* mRNA levels. *A*: Representative immunofluorescence analysis of MMP-2 in the retina of P13 and P14 OIR eyes compared with P13 control eyes. Retinal vasculature was labeled with anti-CD31, an EC marker. *B*: Western blot and immunofluorescence for HIF-1α in iHUVECs exposed to hypoxia. GAPDH was used as a loading control. *C*: *VEGF* and *MMP-2* mRNA normalized to *β-actin* mRNA from iHUVECs exposed to hypoxia, reported as fold induction compared with control cells exposed to 20% O_2. *D*, *left*: Western blot for HIF-1α (*top*) and *VEGF* mRNA normalized to *β-actin* mRNA and reported as fold induction compared with control cells exposed to 20% O_2 (*bottom*) from iHUVECs exposed to hypoxia that had been pretreated with digoxin. GAPDH was used as a loading control for the Western blot. *D*, *right*: *MMP-2* mRNA normalized to *β-actin* mRNA from iHUVECs exposed to hypoxia, pretreated with digoxin, and reported as fold induction compared with control cells. *E*, *left*: Western blot for HIF-1α (*top*) and *VEGF* mRNA normalized to *β-actin* mRNA and reported as fold induction compared with control cells (*bottom*) in iHUVECs treated with 1,4-DPCA and DMOG or DFO for 8 h. GAPDH was used as a loading control for the Western blot. *E*, *right*: *MMP-2* mRNA normalized to *β-actin* mRNA from iHUVECs treated with 1,4-DPCA, DMOG, or DFO for 8 h, and reported as fold induction compared with control cells. All experiments were performed in duplicate are representative of at least three independent experiments. $n = 3$ animals in each group. Student t test: $^*P < 0.05$; $^{**}P < 0.01$.

HIF-1α subunit, beginning at P12 we treated OIR mice with daily intraperitoneal injections of digoxin, which resulted in decreased HIF-1α protein accumulation in the posterior inner retina compared with untreated animals within 24 h of initiating treatment (Fig. 1*D* and Supplementary Fig. 3*B*). *Mmp-2* mRNA expression was potently inhibited by digoxin treatment compared with untreated OIR mice (Fig. 1*E*), suggesting that induction of *Mmp-2* mRNA expression requires HIF.

Hypoxia stabilizes HIF-1α but does not affect MMP-2 expression in Müller cells. We next set out to identify the cells that express MMP-2 in the ischemic inner retina. Although the inner retina is composed of several cell types, increased HIF-1α protein levels were observed in the posterior ischemic inner retina in areas in which there was also an increase in the expression of the intermediate filament protein GFAP (Fig. 2*A*). GFAP is expressed in astrocytes but also in injured or "activated" Müller glial cells. We therefore hypothesized that hypoxic Müller cells in the mouse inner retina were responsible for HIF-1α protein stabilization and *Mmp-2* mRNA and protein expression in the OIR model. To directly assess whether HIF-1α regulates the expression of MMP-2 in injured (hypoxic) retinal Müller cells, we isolated primary murine Müller cell cultures from the neurosensory retinas of P0–P5 C57BL/6 mice and exposed these cells to relative hypoxia (3% O_2), which led to increased HIF-1α protein levels and nuclear localization (Fig. 2*B*). Nonetheless, *Mmp-2* mRNA levels did not increase after exposure of the primary murine Müller cells to hypoxia (Fig. 2*C*). These results were

confirmed using MIO-M1 cells, a previously characterized immortalized human Müller cell line (Fig. 2*D* and *E*). These results suggested that hypoxic Müller cells may not be responsible for *Mmp-2* mRNA and protein expression in ischemic retinal disease.

MMP-2 expression is induced by hypoxia in ECs. Inspection of the inner retina in OIR eyes demonstrated that MMP-2 expression colocalized with the EC marker CD31 (Fig. 3*A*). We therefore examined whether hypoxia induces MMP-2 expression in iHUVECs. Exposure of iHUVECs to hypoxia resulted in increased HIF-1α protein accumulation and nuclear localization (Fig. 3*B*). *MMP-2* mRNA levels were also increased after exposure of iHUVECs to hypoxia (Fig. 3*C*). This induction was markedly decreased in iHUVECs exposed to hypoxia that were pretreated with digoxin (Fig. 3*D*), suggesting that hypoxic induction of *MMP-2* mRNA expression in ECs requires HIF.

In addition to HIF-1α, a number of transcription factors (e.g., nuclear factor-κB, CREB, AP-1, p53, SP-1, and SP-3) are also activated either directly or indirectly by hypoxia. To confirm that hypoxic stabilization of HIF-1α was responsible for the induction of MMP-2 expression in ECs, we induced endogenous HIF stabilization in iHUVECs by inhibiting prolyl hydroxylases (negative regulators of HIF-1α) with 1,4-DPCA, DMOG, or DFO (Fig. 3*E*). Pharmacological stabilization of endogenous HIF-1α resulted in the induction of *MMP-2* mRNA expression in iHUVECs cultured under nonhypoxic (20% O_2) conditions (Fig. 3*E*). Collectively, these observations demonstrate that HIF stabilization is necessary and sufficient for the promotion of MMP-2 expression in hypoxic ECs.

MMP-2 expression by ECs is induced by secreted factors from Müller cells. Although hypoxia stabilizes HIF-1α in both hypoxic Müller glial cells and ECs, these results suggest that upregulation of MMP-2 expression by HIF-1 occurs primarily in ECs. However, retina vasculogenesis is a complex process that involves interplay among glial cells and ECs (22,23). Indeed, careful examination of the retinal vasculature of adult mice reveals an intimate relationship between glial cells and retinal blood vessels (Fig. 4*A*). A similar relationship is also seen between retinal glial cells and neovascular buds in OIR mice (Fig. 4*B*). We therefore explored whether MMP-2 expression may be further enhanced through cooperation between Müller glia and ECs. To address this question, we cocultured MIO-M1 cells with iHUVECs under nonhypoxic or hypoxic conditions, and measured the levels of *MMP-2* mRNA expression in these cocultures. Coculture of MIO-M1 cells with iHUVECs was sufficient to induce a modest increase in *MMP-2* mRNA expression levels under normoxic (20% O_2) conditions (Fig. 4*C*). Exposing these cells to hypoxia (1% O_2) further increased *MMP-2* mRNA expression levels.

To test the hypothesis that increased *MMP-2* mRNA expression in ECs was increased in response to factors released by hypoxic Müller cells, we exposed iHUVECs to conditioned media from MIO-M1 cells that were cultured

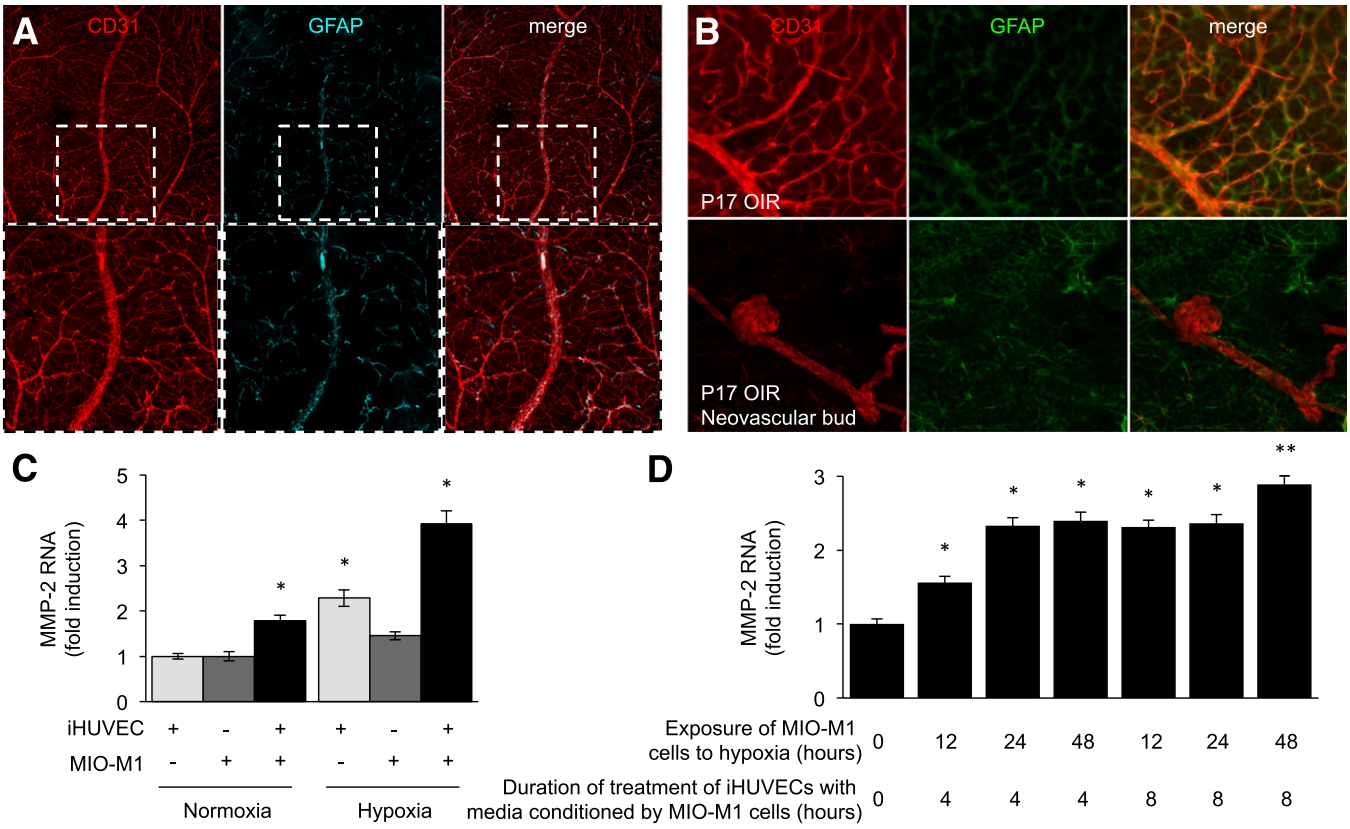

FIG. 4. Cooperation between retinal Müller glial cells and ECs increases MMP-2 mRNA levels in cocultured cells. Representative immunofluorescent staining for the EC marker CD31 and the glial cell marker GFAP in the retina of adult (*A*) and OIR (*B*) mice. *C*: *MMP-2* mRNA levels by RT-PCR in cocultures of MIO-M1 cells with iHUVECs under normoxic or hypoxic conditions. *D*: iHUVECs were cultured at 20% O_2 in the presence of conditioned media (for 4 or 8 h) from MIO-M1 cells exposed to 20% O_2 or 1% O_2 (for 12–48 h, as indicated). Levels of *MMP-2* mRNA (normalized to *β-actin* mRNA) are reported as fold induction compared with untreated controls. All experiments were performed in duplicate and are representative of at least three independent experiments. $n = 3$ animals in each group. Student t test: *$P < 0.05$; **$P < 0.01$.

at 20 or 1% O_2. iHUVECs treated with conditioned media from hypoxic MIO-M1 cells demonstrated significantly increased *MMP-2* mRNA levels compared with iHUVECs treated with conditioned media from normoxic MIO-M1 cells (Fig. 4*D*). This result suggested that a secreted factor produced by hypoxic Müller cells promotes increased MMP-2 expression in ECs.

VEGF is necessary and sufficient for Müller cells to promote MMP-2 expression by ECs. We next set out to identify the factors secreted by hypoxic Müller cells that induce MMP-2 expression in neighboring ECs. Previously, it was suggested that VEGF may play an important role in regulating the expression of MMPs during tumor angiogenesis (24). VEGF expression was detected in the inner retina adjacent to retinal vessels in the OIR model (Fig. 5*A*). Primary murine and immortalized human Müller cells exposed to hypoxia had increased *Vegf* mRNA and protein levels compared with control cells (Fig. 5*B* and *C*).

We therefore examined whether the inhibition of VEGF secretion by hypoxic Müller cells with RNA interference (RNAi) would affect the ability of media conditioned by these cells to promote MMP-2 expression in ECs. We observed that the inhibition of *VEGF* mRNA expression in hypoxia-treated MIO-M1 cells with RNAi potently blocked the ability of conditioned media from these cells to induce *MMP-2* mRNA expression in treated iHUVECs (Fig. 5*D*). Moreover, treatment of iHUVECs with rhVEGF resulted in an increase in *MMP-2* mRNA expression and MMP-2 protein secretion (Fig. 5*E* and *F*), but did not affect *MMP-9* mRNA expression (Fig. 5*G*).

VEGF induces MMP-2 enzymatic activity in ECs. Induction of *MMP-2* mRNA results in the expression of the inactive pro-MMP-2. Affecting MMP-2 enzymatic activity further requires the participation of membrane type 1 (MT1)-MMP (also known as MMP-14), which is encoded by a known HIF target gene (25). MT1-MMP initiates the activation pathway by converting pro-MMP-2 into an activation intermediate that undergoes autocatalytic conversion to generate the mature (active) MMP-2 (25). Conversely, endogenous tissue inhibitors of metalloproteinases (TIMPs) inhibit MMP-2 activity. Specifically, active TIMP-2

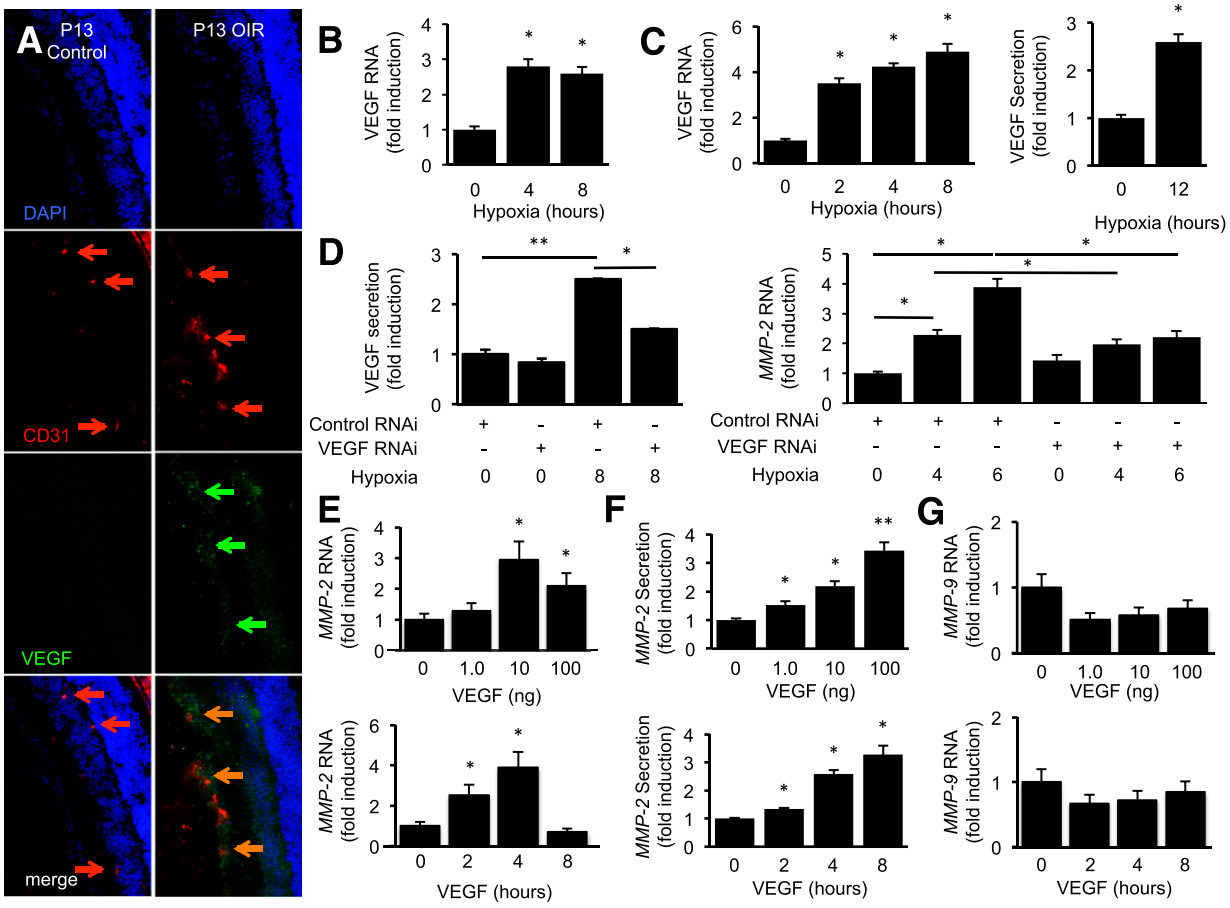

FIG. 5. VEGF is necessary and sufficient for Müller cells to induce *MMP-2* mRNA expression in ECs. *A*: Representative immunofluorescence analysis of VEGF in the retina of P13 OIR eyes compared with P13 control eyes. The EC marker CD31 highlights retinal vasculature. *B*: *Vegf* mRNA levels by RT-PCR in primary murine Müller cells exposed to hypoxia (normalized to *cyclophilin B* mRNA) are reported as fold induction compared with untreated controls. *C*: *VEGF* mRNA and protein secretion in MIO-M1 cells exposed to hypoxia reported as fold induction compared with untreated controls. *D*: *VEGF* and *MMP-2* mRNA levels in iHUVECs treated with conditioned media from MIO-M1 cells exposed to hypoxia (0–6 h) with or without RNAi against *VEGF* mRNA reported as fold induction compared with untreated controls. *E* and *F*: *MMP-2* mRNA (*E*) and secreted protein (*F*) levels after treatment of iHUVECs with increasing doses of rhVEGF (for 6 h) or increasing duration of treatment (with 10 ng rhVEGF) reported as fold induction compared with untreated controls. *G*: *MMP-9* mRNA levels after treatment of iHUVECs with increasing doses of rhVEGF (for 6 h) or increasing duration of treatment (with 10 ng rhVEGF) reported as fold induction compared with untreated controls. All experiments were performed in duplicate and are representative of at least three independent experiments. $n = 3$ animals in each group. Student t test: $^*P < 0.05$; $^{**}P < 0.01$.

binds to MMP-2 in a 1:1 stoichiometric ratio (26). Normally, there is a tight balance between MMP-2 and TIMP-2, but in pathological conditions this balance can be altered, resulting in an excess of activated MMP-2. Exposure of iHUVECs to hypoxia induced both *MT1-MMP* and *TIMP-2* mRNA expression (Fig. 6*A*). Regulation of *Mt1-mmp* and *Timp-2* mRNA in the OIR model during the ischemic phase was less robust, but was also suggestive of an increase in *Timp-2* mRNA (Supplementary Fig. 4). This suggested that hypoxic induction of MMP-2 (and MT1-MMP) expression may be compensated for by a balanced induction of TIMP-2 expression.

Of note, postnatal development of the normal mouse retinal vasculature is also driven by hypoxia, yet NV is not observed; rather, there is an orderly and predictable progression of retinal angiogenesis. Interestingly, *Vegf* (and *Mmp-2*) mRNA levels increased only modestly (less than fivefold and threefold, respectively) during the ischemic phase (P1–P5) of postnatal retinal development (Supplementary Fig. 5). This increase was balanced by a similar increase in *Timp-2* mRNA levels. In contrast, during the ischemic phase of the OIR model, there is a more marked increase in *Vegf* (almost 30-fold) and *Mmp-2* (5- to 10-fold; see Fig. 1) mRNA expression. This suggests that a marked (and rapid) increase of VEGF may promote MMP-2 expression and activity, which then leads to the development of retinal NV.

To further examine the regulation of MMP-2 activity by VEGF in vitro, we treated iHUVECs with rhVEGF, and observed an increase in *MT1-MMP* mRNA levels (Fig. 6*B*) and protein expression (Fig. 6*C*). However, unlike exposure to hypoxia, the treatment of iHUVECs with rhVEGF did not affect *TIMP-2* mRNA levels, suggesting that VEGF induction of MMP-2 (and MT1-MMP) expression is not compensated for by an increase in TIMP-2 expression. Similarly, the treatment of iHUVECs with conditioned media from hypoxic Müller cells also resulted in an increase in *MT1-MMP* mRNA levels but did not affect

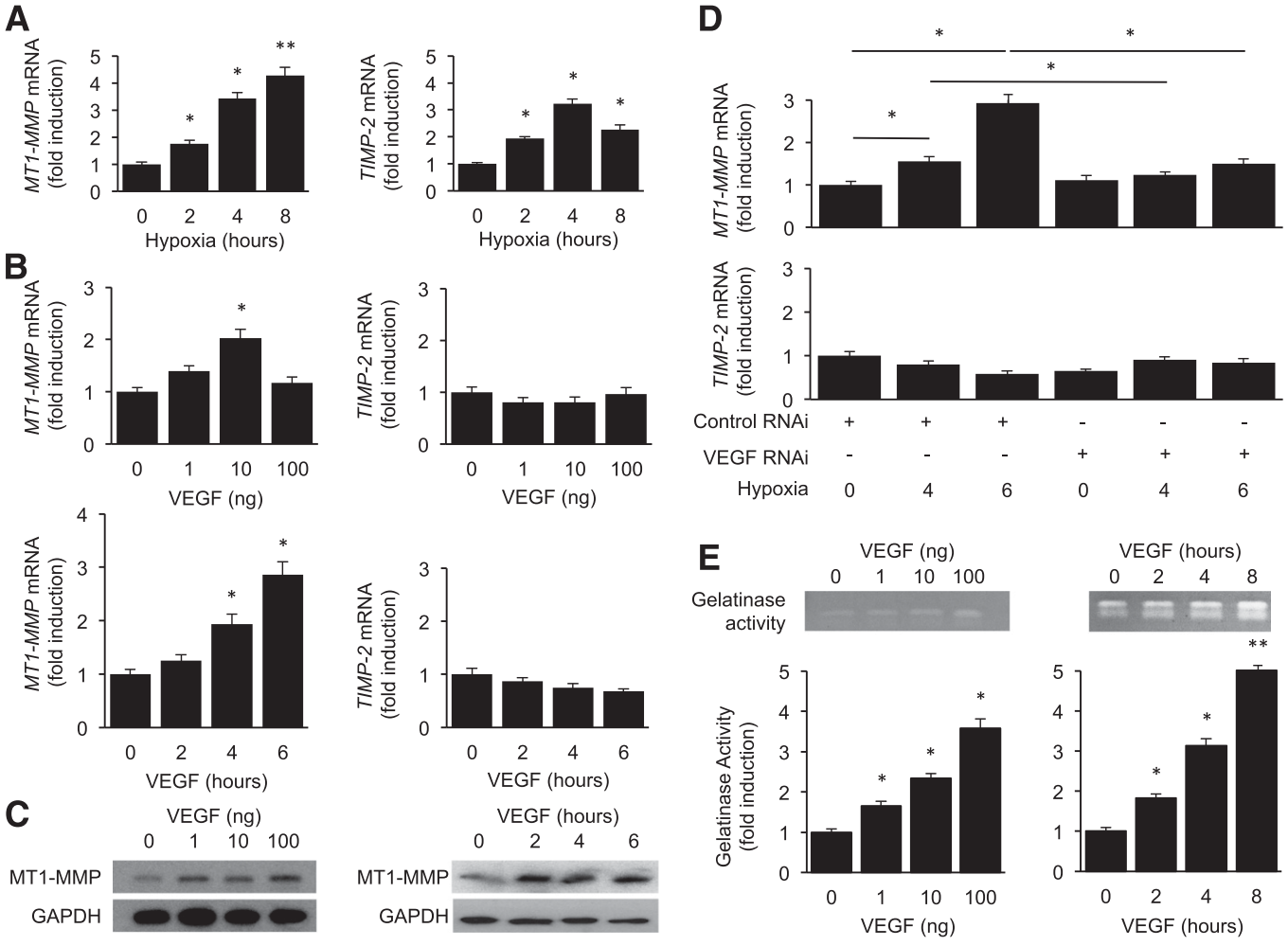

FIG. 6. VEGF promotes increased MMP-2 activity by increasing MT1-MMP expression in ECs. *A*: *MT1-MMP* and *TIMP-2* mRNA levels, normalized to *β-actin* mRNA after exposure of iHUVECs to hypoxia for 0–8 h, reported as fold induction compared with untreated controls. *B*: *MT1-MMP* and *TIMP-2* mRNA levels, normalized to *β-actin* mRNA after treatment of iHUVECs with increasing doses of rhVEGF for 6 h or increasing duration of treatment with 10 ng rhVEGF, reported as fold induction compared with untreated controls. *C*: Western blot for MT1-MMP in iHUVECs treated with increasing doses of rhVEGF (for 8 h) or increasing duration of treatment (with 10 ng rhVEGF). GAPDH was used as a loading control. *D*: *MT1-MMP* and *TIMP-2* mRNA levels (RT-PCR) in iHUVECs treated with conditioned media from MIO-M1 cells exposed to hypoxia (0–6 h) with or without RNAi against *VEGF* mRNA reported as fold induction compared with untreated controls. *E*: MMP-2 enzymatic (gelatinase) activity after treatment of iHUVECs with increasing doses of rhVEGF (for 6 h) or increasing duration of treatment (with 10 ng rhVEGF), reported as fold induction compared with untreated controls. All experiments were performed in duplicate and are representative of at least three independent experiments. $n = 3$ animals in each group. Student t test: $^*P < 0.05$; $^{**}P < 0.01$.

TIMP-2 mRNA levels, whereas the inhibition of VEGF secretion by hypoxic Müller cells with RNAi blocked the ability of conditioned media to induce *MT1-MMP* mRNA expression (Fig. 6*D*). Zymography assays on the conditioned media from iHUVECs treated with rhVEGF demonstrated an increase in MMP-2 gelatinase activity (Fig. 6*E*). Similar results were observed in primary human retinal ECs (Supplementary Fig. 6). Collectively, these results suggest that in pathological NV, VEGF induces MMP-2 enzymatic activity by inducing its expression, while also upregulating the expression of the MMP-2 activating protein MT1-MMP, but not affecting the expression of the MMP-2 inhibitor TIMP-2.

VEGF is sufficient to induce MMP-2 expression in retinal NV in vivo. In NV, MMPs enable the advancing front of the retinal endothelial tip cells to invade through the basement membrane and ECM (12). To examine whether, in the absence of hypoxia, VEGF alone was sufficient to promote MMP-2 expression and function by retinal endothelial tip cells in vivo, we injected rmVEGF into the vitreous of adult mice (Fig. 7*A*). *Mmp-2* mRNA levels were increased in the neurosensory retina of mice after intravitreal injection with rmVEGF compared with control (PBS-treated) mice (Fig. 7*A*). The expression of MMP-2 was detected in the inner retina of VEGF-treated eyes (Fig. 7*B*) and localized to the tip cells of new vessels (Fig. 7*C*).

MMP-2 expression is elevated in patients with PDR. MMP-2 expression was detected in four of four autopsy eyes from patients with a known diagnosis of PDR (Fig. 8*A*), but it was not detected in adjacent normal retinal vessels in these patients (Fig. 8*A*, inset). Motivated by these results, we next examined the levels of VEGF and MMP-2 in the eyes of patients with known PDR compared with diabetic patients without DR or compared with healthy control subjects (Supplementary Table 1). The levels of cytokines measured in the aqueous humor of patients with diabetic eye disease had previously been reported to reflect the levels measured in the vitreous of these patients (27). We observed increased VEGF and MMP-2 levels in the aqueous of diabetic patients with PDR compared with control patients ($P < 0.05$) (Fig. 8*B* and *C*). Moreover, MMP-2 levels were significantly elevated in PDR patients whether or not they had received prior treatment with PRP ($P < 0.01$) (Fig. 8*D*). Neither VEGF nor MMP-2 levels were increased in the serum of diabetic patients (with or without PDR) compared with control patients (Fig. 8*E*).

DISCUSSION

Proteolytic activity of the ECM by both MMPs and non-MMPs facilitates degradation of the basement membrane, matrix remodeling, and cell migration and invasion, all of which are essential for pathological angiogenesis (28). Work on MMPs from several laboratories has helped broaden our appreciation for the contribution of this group of proteases to pathological angiogenesis in the eye. MMPs appear to play a dual role in the development of DR. In the earlier stages (prior to the development of NV), MMPs may have an intracellular role in which they facilitate apoptosis of retinal capillary cells, resulting in pericyte and EC loss, which is characteristic of early DR (29–33). However, in later stages, MMPs likely function as extracellular proteases facilitating the development of NV in PDR (13).

Much of our appreciation for the role of the MMPs in retinal NV has come from work examining knock-out mice; however, results from these studies have proven difficult to interpret. Retinal NV in the OIR model was reported to be reduced in *Mmp-2*-null mice by two studies (34,35), but was unaffected in a third study (36). Studies examining the role of MMP-9 in retinal NV have also been conflicting: NV

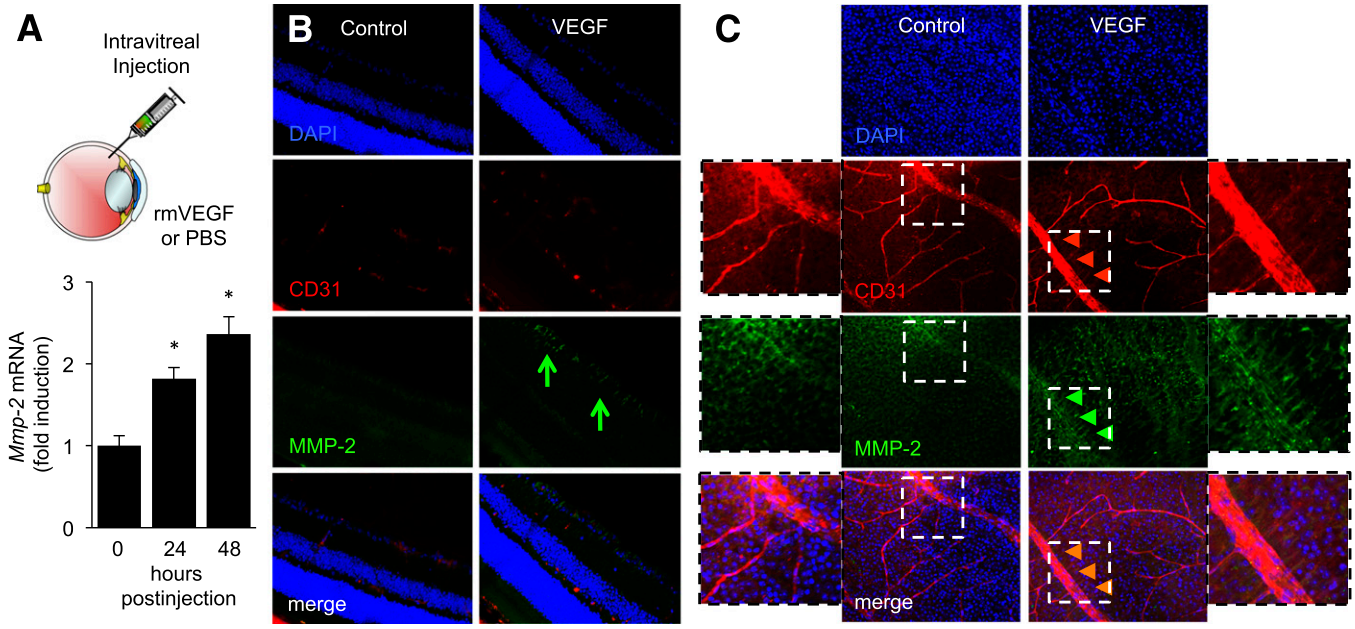

FIG. 7. VEGF promotes increased MMP-2 expression in retinal NV in vivo and in human PDR tissue. *A*, *top*: Schematic demonstrating intravitreal injections of rmVEGF or PBS control. *A*, *bottom*: *Mmp-2* mRNA from the neurosensory retina of animals injected with rmVEGF over time normalized to *cyclophilin B* mRNA and reported as fold induction compared with control (PBS-injected) eyes. Representative immunofluorescence analysis of MMP-2 (green arrows) in cross-section (*B*) and flat mount (*C*) from eyes injected with rmVEGF or PBS (control). The EC marker CD31 highlights tip cells of retinal microvasculature (arrowheads). All experiments were performed in duplicate and are representative of at least three independent experiments. $n = 3$ animals in each group. Student t test: $^*P < 0.05$.

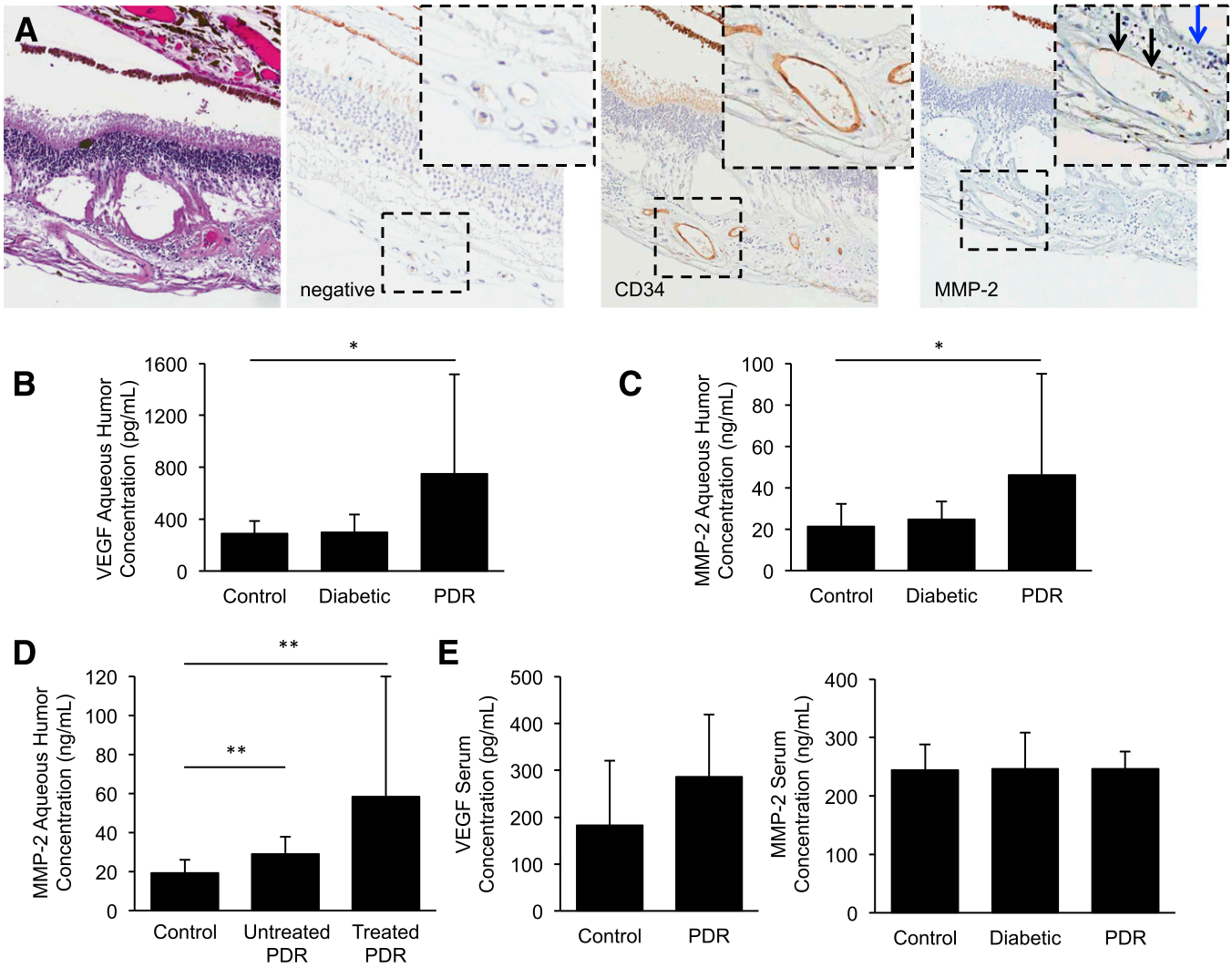

FIG. 8. Increased MMP-2 expression in patients with PDR. *A*: Representative images from immunohistochemical analysis of MMP-2 expression in NV (black arrows) but not in normal retinal vessels (blue arrow) in the retina of four of four eyes with NV and a known diagnosis of PDR (see inset). No primary antibody was used for the negative control. *B*: VEGF levels in the aqueous humor of nondiabetic patients, diabetic patients without DR, and diabetic patients with PDR. *C*: MMP-2 levels in the aqueous humor of these patients. *D*: MMP-2 levels in the aqueous humor of nondiabetic patients compared with diabetic patients with PDR who were not previously treated with PRP or those who had previous PRP treatment. *E*, *left*: VEGF levels in the serum of nondiabetic patients and diabetic patients with PDR. *E*, *right*: MMP-2 levels in the serum of nondiabetic patients, diabetic patients without DR, and diabetic patients with PDR. All experiments were performed in duplicate. Control, nondiabetic patients; Diabetic, diabetic patients without DR; PDR, diabetic patients with PDR; Untreated, diabetic patients with PDR who were not previously treated with PRP; Treated, diabetic patients who had received previous PRP treatment. Student *t* test: *$P < 0.05$. Multiple *t* tests: *$P < 0.05$; **$P < 0.01$.

in the OIR model was reported to be reduced in *Mmp-9* knock-out mice in one study (34) but unaffected in a second study (35). These contradictory results may be due to differences in the strains of mice and to compensatory upregulation of other genes. Recent work also suggests that MMP-9 is required for the release from the bone marrow of endothelial progenitor cells, which are essential for the development of NV (37). Thus, MMP-9 may not play a direct role in retinal NV but, rather, may be required for the release of the endothelial progenitor cells that populate NV lesions. Ultimately, studies of retinal NV using knock-out mice have not provided a clear picture as to the role of MMPs in retinal NV.

The findings of studies demonstrating the expression of specific MMPs in PDR tissue are similarly not sufficient to conclude that these MMPs are active in retinal NV. The promotion of ECM proteolysis by MMPs is very tightly regulated; it is, therefore, equally important to determine whether their inhibitors or activators—and their enzymatic activity—are also modulated. It is also essential that key events that trigger the expression of these genes also be delineated. Understanding the role of MMPs in retinal NV is further complicated by the participation of multiple cell types that may contribute different components of the proteolysis machinery. The consequence of these obstacles is that reasonable disagreement remains as to whether —and which—MMPs are valid therapeutics targets for the treatment of PDR.

Here, we provide evidence demonstrating that the transcriptional activator HIF-1 can induce MMP-2 expression in ischemic retinopathies in the following two ways: *1*) stabilization of HIF-1α in hypoxic ECs leads to increased expression of MMP-2 in these cells and *2*) accumulation of HIF-1α in hypoxic Müller cells leads to increased VEGF

secretion, which, in turn, upregulates MMP-2 expression in neighboring ECs (Supplementary Fig. 7). This may help to explain further why *Mmp-2* mRNA levels peak later than *Vegf* mRNA levels in the OIR model. We further demonstrate that whereas hypoxic induction of MMP-2 expression in ECs is balanced by the simultaneous upregulation of TIMP-2, a negative regulator of MMP-2, VEGF induction of MMP-2 is not balanced by an increase in TIMP-2. Accordingly, MMP-2 enzymatic activity is markedly increased by the treatment of ECs with VEGF, which was sufficient to promote MMP-2 expression in endothelial tip cells in vivo. Of note, TIMP-2 has also been shown to play a role in promoting the activation of MMP-2 by MT1-MMP (38,39). Also, MT1-MMP itself is a collagenase and could play a direct (MMP-2–independent) role in the development of fibrovascular proliferation in PDR (40). Additional studies will be necessary to further characterize the complex regulation of the function of these proteases in diabetic eye disease.

Elevated expression of MMPs was previously reported within retinal neovascular tissue in patients with PDR, as well as in the aqueous of patients with PDR (41–48). We demonstrate here that MMP-2 levels are increased in PDR patients compared with control patients, regardless of whether or not PDR patients had prior treatment with PRP, suggesting that MMP-2 is a therapeutic target even in patients who have received prior treatment for PDR. Serum levels of MMP-2 in diabetic patients (with or without PDR) were almost identical to those in control patients, indicating that the increased levels of MMP-2 in the eyes of PDR patients were an ocular (not systemic) consequence of diabetes.

Collectively, our findings provide molecular and cellular mechanisms underlying the regulation of MMP-2 in retinal NV and suggest that MMP-2 is a valid target for the treatment of PDR. In support of the latter, prior work has demonstrated that broad-spectrum pharmacological inhibition of MMPs prevents retinal NV in animal models (34,49,50). In light of our results, we propose that therapies specifically targeting MMP-2 could become an important and effective part of the complex approach to prevent or treat NV in patients with PDR.

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M.R., X.X., K.J., and T.M. performed experiments and interpreted data. S.B.-F., F.K., I.B., and S.J.H. performed experiments. Y.D., D.B., and S.S. provided clinical samples. G.L. and G.L.S. reviewed and edited the manuscript. S.M. interpreted data, reviewed and edited the manuscript, and designed experiments. A.S. provided clinical samples, interpreted data, designed experiments, and wrote the manuscript. A.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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