Hypoxia-Regulated Retinal Glial Cell-Specific Promoter for Potential Gene Therapy in Disease

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PURPOSE. Retinal Müller cells span the retina and secrete several trophic factors and represent the functional link between blood vessels and neurons, making them attractive targets for gene therapy. Therefore, a hypoxia-regulated, retinal glial cell-specific vector was constructed and tested for its response to hypoxia.

METHODS. A hybrid promoter containing domains of human glial fibrillary acidic protein (GFAP) and several hypoxia-responsive and aerobically silenced elements (HRSE) was incorporated separately into plasmid vectors for generation of self-complementary adeno-associated virus. Müller cells transfected with plasmids or virus were compared with other cell lines using standard methods. The mouse model of oxygen-induced retinopathy (OIR) was used to analyze retinas from mice exposed to high oxygen or room air to evaluate the induction of the regulated promoter.

RESULTS. The regulated promoter was silenced under aerobic conditions in comparison with unregulated promoter in Müller cells. Hypoxia induced a 12-fold and 16-fold increase in promoter activity in primary Müller cells and human Müller cell lines, respectively. In the OIR model, intravitreal injection of the regulated promoter at postnatal day 7 (P7) resulted in high levels of green fluorescent protein expression only in retinal Müller cells at P17. GFP expression was absent in retinas of mice only exposed to room air. In vivo studies confirm normoxia silencing, hypoxic induction, and cell specificity of the regulated promoter in the mouse retina.

CONCLUSIONS. This hypoxia-regulated, retinal glial cell-specific AAV vector provides a platform for gene therapy within regions of retinal hypoxia which are found in diabetic retinopa-

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Gene therapy for the retina has been employed successfully in humans and animal models for the treatment of retinal dystrophies.^{1–3} The retina is attractive for gene therapy approaches because it is surgically approachable, isolated due to the presence of the blood-retina barrier and immunologically privileged. The requirements for successful gene therapy include efficient and sustained gene transfer and choice of a gene product that is capable of eliciting therapeutic efficacy.⁴ The potential value of cell-specific and regulated gene therapy for the eye has been proposed for models of AMD, photoreceptor degeneration, and retinal ischemia.^{5–8} Alterations in retinal oxygen availability can form a basis for disease-appropriate patterns of transgene expression either at early stages of oxygen deprivation due to tissue stress or damage and at later stages of disease associated with tissue ischemia and cell necrosis. Oxygen is critical for maintaining retinal function and reduction in oxygen levels serve as a trigger for pathologic effects underlying AMD and diabetic retinopathy.⁹ Hypoxia-induced changes in the retina can also serve as a trigger for activation of gene therapy vectors designed for regulating transgene expression in response to depleted oxygen levels.^{2,6} Such tight regulation of the expression from gene therapy vectors is likely to be particularly important in retinal tissue where there are numerous distinct cell types with differing abilities to tolerate stress from hypoxia or elevated reactive oxygen species.

The sensing of cellular hypoxia depends on the action of a key oxygen-dependent sensing system involving the transcription factor hypoxia inducible factor (HIF)-1, a heterodimer formed between the constitutive and ubiquitously expressed monomers HIF-1-alpha and HIF-1-beta.¹⁰ In normoxia, transcription is prevented because HIF-1-alpha is modified by hydroxylation of a proline residue and then processed for ubiquitin-mediated proteasomal degradation.¹¹ Under hypoxic conditions, however, HIF-1-alpha dimerizes with its partner HIF-1-beta and translocates to the nucleus for activation of gene transcription. Transcriptional activation by HIF-1 occurs through binding of the factor to hypoxia response elements (termed HREs) in regulatory domains of target genes.^{11,12}

Therapeutic products synthesized by hypoxia-regulated vectors have included growth factors such as bFGF and VEGF, antioxidant components, antiangiogenic factors including angiostatin and proapoptotic components such as Bax.^{13–17} The promoters of such hypoxia-regulated therapeutic vectors are designed to include a regulatory domain which incorporates multiple hypoxia responsive elements (HREs) which are known to bind the transcription factor HIF-1. We and others have reported that multimers of the HRE drive enhanced levels of gene expression relative to a single HRE.^{6,18} For further control over basal levels of expression and of inducibility, we have previously incorporated a neuronal silencing element into the promoters to prevent “leaky” gene expression under nor-

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moxic conditions.⁶ HRE containing promoters which also contain silencer elements have been found to elicit inducibility in hypoxia of greater than 50-fold.⁶

The primary focus of this study is aimed at testing the hypothesis that a hypoxia-responsive domain can be employed for activating cell-specific expression in Müller cells under conditions of hypoxic stress in the pathologic retina. A promoter containing a hypoxia responsive domain together with a cell specific promoter would be expected to retain cell specificity while also being hypoxia-inducible. In responding to hypoxia, the promoter will have potential for activation during early phase hypoxia as well as later ischemic phases noted in the progression of such disease processes as AMD and diabetic retinopathy. For developing a retinal gene therapy approach, a variety of gene products may be especially appropriate for expression in this vector system including pro-survival kinases, antioxidant enzymes that enhance Müller cell viability, and secreted factors either for preventing angiogenesis or for eliciting neuroprotection. Our HRE-regulated, retinal glial cell-specific promoter could be incorporated to investigate new therapies to treat a range of animal models of eye diseases such as inherited photoreceptor degeneration, age related macular degeneration, diabetic retinopathy, and glaucoma.

Glial fibrillary acidic protein (GFAP) is the major intermediate filament in astrocytes and other glial cells, including non-myelinating Schwann cells and Müller cells (the major glial element in the retina).^{19–21} Expression of GFAP serves as a marker of developmental processes as well as an indicator of gliosis in response to injury.^{21–23} Elevated levels of GFAP expression in retinal glial cells have been observed in light-induced retinal damage and in a number of eye diseases including diabetic retinopathy and AMD. Identification of the upstream regulatory sequences of the GFAP gene has been valuable for achieving glial cell-specific transgene expression either in transgenic mice or when employing gene transfer vectors. The domain from bp –2163 to +47 of the human GFAP gene has been found to drive astrocyte-specific expression whereas an internally truncated –2163 GFAP promoter lacking bp –1488 to –132 loses its cell specificity. However a short gfaABC1D promoter consisting of enhancer domains plus a proximal promoter domain was found to retain astrocyte-specific expression in the brain, while demonstrating at least a twofold higher activity than –2163 GFAP.²⁴ In transgenic mice, the astrocyte-specific GfaABC1D provides more regional specificity in the brain than other GFAP promoter domains.²⁴

In this investigation, both *in vitro* and *in vivo* studies were used to confirm the applicability of these regulatory domains for gene therapy strategies. The complete promoter was incorporated into a recombinant self complementary AAV (scAAV) containing the HRE-GFAP promoter driving GFP. Primary cultures of Müller cells were transfected to demonstrate a lack of expression of the GFP transgene in normoxia and high level transgene expression in hypoxia. For *in vivo* studies, the murine model of oxygen-induced retinopathy (OIR) is widely used to study neovascularization.²⁵ In this model, postnatal day 7 (P7) mouse pups are exposed to high oxygen until P12 then returned to room air for another 5 days. During the initial high oxygen phase, normal retinal blood vessels regress. During the regression phase, retinal cells produce several HIF-1-mediated proangiogenic factors. This leads to development of abnormal neovascularization in the retina by P17.²⁶ The expression of HIF-1 in P17 hypoxic retina is 31-fold greater than in P17 normoxic retina.²⁷ The OIR model is used to study hypoxia-regulated reporter gene expression in the mouse retina.⁵ As our promoter requires HIF-1 for its induction, we chose the OIR model to evaluate the activity of our regulated promoter for *in vivo* studies.

METHODS

Cell Cultures and Hypoxia Treatment

Cells employed were the human Müller cell line M10-M1 (gift from Astrid Limb, University College, London), the human retinal epithelial cell line ARPE-19 (American Type Culture Collection, Manassas, VA), the human embryonic kidney cell line HEK 293 (Stratagene, La Jolla, CA) the glioma cell line C6 (gift from Ajay Verma, Uniform Health Services University, Bethesda, MD), and the mouse hippocampal neuronal cell line HT22 (gift from David R. Schubert, The Salk Institute, San Diego, CA). Cells were cultured using previously described methods and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Rat primary astrocytes were cultured from 1-day-old pups by standard procedures.

All procedures involving mice were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic research and approved by the Animal Care and Use Committee at Florida Atlantic University. Primary cultures of Müller cells were obtained from P1 mice according to previously described methods with minor modifications. Briefly retinas from P5–P6 mice were dissected and dissociated with activated papain (Worthington Biochemical Corp, Lakewood, NJ). After dissociation and centrifugation cells were resuspended and plated on cell culture flasks for experimental treatment and analysis.

Cultures were exposed to hypoxic conditions at 1% oxygen as previously described⁶ using an anaerobic chamber. Oxygen levels were monitored continuously with an oxygen electrode (Engineered Systems & Designs, Newark, DE).

Construction of Hypoxia-Inducible Promoter (Reg-GFAP)

The GfaABC1D (681 bp) domain (a gift from Michael Brenner, University of Alabama) which contains subfragments of the GFAP promoter was previously reported to drive astrocyte-specific gene expression in transgenic mice²⁴ and the promoter subdomains of GfaABC1D were previously described by Lee et al., 2008.²⁴ pGL3-HRSE-GFAP-Luc (Fig. 1) was constructed in the pGL3 (Promega, Madison, WI) plasmid backbone by incorporation of the following promoter domains: a silencing region (HRSE; provided by Keith Webster, University of Miami Miller School of Medicine,⁶); six copies of the hypoxia response element (6XHRE) of the phosphoglycerate kinase gene as previously described⁶; and GfaABC1D (please see above). This regulated plasmid, pGL3-HRSE-GFAP-Luc was given the name “Reg-GFAP”.

Transfection and Dual Luciferase Assay

Cell lines and primary Müller cells were transfected with liposome reagents (Lipofectamine 2000 and Lipofectamine LTX; Invitrogen,

FIGURE 1. Schematic diagram of vectors used in this study. (A) Conserved domains of GFAP promoter (686 bp) were incorporated into pGL3-based vector driving the Luciferase (Luc) reporter gene, (B) hypoxia-responsive and aerobically silenced elements (HRSE) and 6XHRE regulatory elements are incorporated into the 5' end of pGL3-GFAP-Luc vector, (C) GFAP promoter was ligated to self-complementary AAV plasmid vector by replacing the CMV promoter, and (D) regulated element including GFAP promoter has been ligated to self-complementary AAV plasmid vector by replacement of CMV promoter.

Carlsbad, CA) with the exception of primary astrocytes which are known to require specially optimized transfection reagents (Haces A, personal communication, 2011), and for this reason these cells were transfected (NovaFECTOR; VennNova, Pompano Beach, FL). To ensure comparable transfection efficiencies with different cell types, a strongly expressing promoter plasmid (CMV-Luciferase) was employed for comparison to "test" promoter plasmids in all plasmid transfection experiments. For an internal control in each experimental condition, TK renilla plasmid was cotransfected with the plasmid of interest. Data on promoter activities were obtained by the dual luciferase assay as previously described.⁶

Self-Complementary AAV

For production of AAV and cellular transduction, standard methods were employed.⁶ scAAV plasmid was a gift from D. McCarty (Ohio State University, Columbus, OH). Promoter cassettes were amplified by PCR and inserted into the scAAV plasmid. scAAV serotype-2 viruses were produced at the Gene Therapy Vector Core, University of North Carolina (Chapel Hill, NC) and titers were determined by standard dot blot analysis. Primary Müller cells were transduced with scAAV 6 days before subjecting the cells to hypoxic conditions.

Intravitreal Injection and Oxygen Induced Retinopathy (OIR) Model

The OIR model was generated by standard methods.²⁵ Briefly, P7 pups were anesthetized with ketamine/xylazine and subjected to intravitreal injection of scAAV. P7 pups with their mothers were then either maintained at normal room air (21% oxygen) or exposed to 75% O₂ for 5 days in a hypoxia chamber. Pups at P12 were moved to normoxic conditions in room air for a further 5 days and euthanized at P17.

Flat Mount

P17 pups were anesthetized using ketamine/xylazine and intracardial perfusion performed using Tomato lectin^{28,29} and conjugated (Dylight595; Vector Laboratory, Burlingame, CA) to label retinal endothelial cells. Eyes were enucleated, the cornea and lens removed and the eye cups fixed with 4% paraformaldehyde (PFA) for an hour. Retina flat mounts were prepared after removing the retinal pigment epithelial (RPE) cell layer.²⁶ Fluorescent images of the flat mount retina were obtained using an inverted fluorescence microscope (Nikon, Melville, NY).

Immunohistochemistry

For analysis of GFP expression retinal sections were labeled with primary rabbit anti-GFP IgG (Cat # A11122; Invitrogen) and dylight-488

conjugated goat anti-rabbit secondary antibodies (Vector Laboratory). Images were obtained using a confocal microscope (Nikon Eclipse).

Statistical Analysis

Promoter activity levels were expressed as luciferase activity ratios relative to control Renilla luciferase activity as described previously.⁶ Data are expressed as mean $\pm$ SEM. Significance was determined using ANOVA. Differences between conditions were regarded as significant if $P < 0.05$.

RESULTS

Cell-Specific Expression of GFAP Promoter

High levels of expression of the GFAP promoter were observed in mouse primary Müller cells and rat primary astrocytes; firefly luciferase activity of GFAP promoter was measured as 28 ± 10 -fold ($n = 8$, $P < 0.001$) and 30 ± 1.6 -fold ($n = 6$, $P < 0.05$) respectively (Figs. 2A, 2B) compared with control TK renilla activity. In MIO-M1 cells, the GFAP promoter activity was measured as 0.75 ± 0.15 -fold ($n = 9$, $P < 0.001$) and minimal expression occurred in HEK cells (0.16 ± 0.01 , $n = 8$, $P < 0.001$) (Fig. 2C). Although MIO-M1 cells exhibited relatively low levels of transfection, there was clear cell specificity in MIO-M1 cells relative to HEK cells. GFAP promoter also shows basal or minimal activity in C6, HT22, and ARPE-19 cells (data not shown). Firefly luciferase activity of pGL3-CMV-Luc plasmid was used as a control to compare activities of test promoters irrespective of transfection efficiencies.

Regulated GFAP Promoter (Reg-GFAP) (pGL3-HRSE-6XHRE-GFAP-Luc)

Aerobic Silencing. The luciferase constructs bearing the GFAP promoter or the regulated GFAP promoter were transfected to mouse primary Müller cells and the human MIO-M1 cell line. The transfected cells were cultured in 21% oxygen. The suppressive action of the HRSE in normoxia was confirmed by dual luciferase assay data showing that the regulated GFAP construct was silenced by 82% in mouse primary Müller cells (Fig. 3A) and by 21% in MIO-M1 in comparison with unregulated GFAP promoter (Fig. 3B).

Hypoxic Induction and Cell Specificity. HRE elements in the regulated promoter successfully responded to hypoxia resulting in dramatically elevated induction of luciferase activity. As shown by dual luciferase assay data in Figure 4, the regulated GFAP promoter was induced in hypoxic mouse pri-

FIGURE 2. Müller cell specificity of GFAP promoter. GFAP promoter driving the luciferase gene was transfected into MIO-M1, HEK cells, primary mouse Müller cell, and rat primary astrocytes. The promoter shows the maximal luciferase activity in (A) primary mouse Müller cells (28.6 ± 10 , $n = 8$, $P > 0.05$) and in (B) rat primary astrocytes (30 ± 1.6 , $n = 6$, $P > 0.05$) compared with TK Renilla activity. (C) GFAP promoter restricts expression of reporter gene to MIO-M1 cells (0.75 ± 0.15 , $n = 9$, $P < 0.001$) with basal expression in HEK cells (0.16 ± 0.01 , $n = 8$, $P < 0.001$). (Note: The abscissa in C has been reduced compared with A and B reflecting the low level of transfection in MIO-M1 cells).

FIGURE 3. Conditional silencing of regulated GFAP promoter. After 40-hour exposure to normoxia, the activity of the reg-GFAP promoter in comparison with the GFAP promoter was significantly reduced by 21% in (A) MIO-M1 and by 82% in (B) mouse primary Müller cells.

mary Müller cells approximately 12-fold (29.5 ± 0.6 vs. 2.4 ± 0.3 , $n = 8$, $P > 0.05$) and in hypoxic MIO-M1 cells approximately 16-fold (9.5 ± 0.9 vs. 0.6 ± 0.03 , $n = 9$, $P < 0.001$). Hypoxic induction of the regulated promoter was observed in Müller cells but not in the following cell lines: kidney HEK, glioma C6, hippocampal neuronal HT22, and retinal pigment epithelial ARPE-19 (Fig. 4).

Silencing and Hypoxia-Induced Expression of GFP in scAAV-reg-GFAP Infected Cells

The GFAP and regulated promoters were successfully cloned into scAAV packaging plasmids and scAAV2/2 vectors were produced for both plasmids. On infection in reduced serum media, the vectors transduced mouse primary Müller cells. AAV2 transduced cells were exposed to hypoxia for another 6 days and GFP expression analyzed. Primary Müller cells infected with AAV2-GFAP-GFP express GFP in both normoxia (Figs. 5A, 5C) and hypoxia (Figs. 5B, 5D), whereas AAV2-Reg-GFAP-GFP transduced cells expressed GFP only under hypoxic conditions (Figs. 5F, 5H). (Note: GFP expression in normoxia [Figs. 5E, 5G] was below detection limits under standard microscopic conditions.)

FIGURE 4. Hypoxia induction and cell specificity of regulated promoter. There was approximately a 12-fold induction of the regulated GFAP promoter (29.5 ± 0.6 , $n = 8$, $P > 0.05$) in mouse primary Müller cells after 40 hours of hypoxia. Similarly in MIO-M1, the regulated GFAP vector shows approximately 16-fold induction (9.5 ± 0.9 , $n = 9$, $P < 0.001$) greater than in normoxia. Basal or minimal expression of the promoter was evident in different cell lines such as HEK, C6, HT22, and ARPE-19.

In Vivo Silencing and Hypoxia-Induced Expression of GFP

Flat mount retinas from P17 mice demonstrate normal vascular pattern, and the abnormal vascular pattern and ischemic region present in the OIR model (Figs. 6A and 6B, respectively). scAAV-GFAP-GFP injected eyes demonstrated GFP induction in Müller cells and astrocytes in P17 retinas of mice exposed to either room air (controls; Fig. 6C) or high oxygen (Fig. 6D). In contrast, there was no GFP expression in the retinas of scAAV-reg-GFAP vector-injected eyes of mice kept at room air (Fig. 6E), thus confirming that the promoter is silenced in normoxia. Prominent induction of GFP expression was found almost exclusively in Müller cells in hypoxic regions of retinas of P17 mice in the OIR model (Fig. 6F). GFP was clearly localized in the Müller cell cytosol extending from the outer retina to the foot processes along the inner limiting membrane (ILM). The possibility that astrocytes along the ILM expressed GFP cannot be precluded; however, if so, the level is clearly reduced in comparison with the unregulated promoter. These results confirm hypoxia inducibility and cell specificity of the regulated promoter in vivo.

DISCUSSION

An important concern in the design of gene therapy vectors is that the foreign gene product should be expressed only in the location it is needed: in diseased tissue but not in normal tissue and at the appropriate time. While unregulated expression from gene therapy vectors could be of some value, inappropriate expression of transgene products in unaffected tissue has the potential to be harmful.^{4,30} Furthermore levels of the therapeutic protein may need to be optimized, especially if the protein is intracellular.^{15,16} The ability of a particular promoter to control the bioavailability of a therapeutic gene product will depend on its strength when activated in a specific cell type. A key finding of this study is that the hypoxia regulated GFAP promoter was induced both in Müller cell cultures and in the in vivo OIR model. The application of a cell-specific, hypoxia-regulated gene therapy may be especially valuable in the eye where oxygen availability is modified as part of the pathology of such diseases as AMD and diabetic retinopathy.

HIF-1 Responsive Promoters for Retinal Gene Therapy

Local tissue hypoxia³¹ and inflammation^{32–35} are associated with retinal pathologies leading to neovascularization^{36–39} and are possibly linked to photoreceptor loss in RCS rats.⁴⁰ Hypoxic induction of exogenous gene products was achieved in

FIGURE 5. GFP expression in transduced mouse primary Müller cells. Images of sAAV2-GFAP-GFP and sAAV-reg-GFAP-GFP transduced primary Müller cells under normoxia and hypoxia. GFP was expressed by the sAAV-GFAP-GFP vector in transduced mouse primary Müller cells under (A) normoxic and (B) hypoxic conditions. GFP expression was inactive in normoxia (E) and induced in hypoxic Müller cells transduced with sAAV2-reg-GFAP-GFP vector (F). (A, B, E, F) Fluorescent images; (C, D, G, H) phase images.

retina using vectors with non-cell-specific HRE promoters⁵; because these earlier HRE promoters also lacked suppression in normoxia, both normoxic and hypoxic regions were treated. It is important to note that additional stimuli apart from hypoxia have been shown to induce HIF-1 activity. Indeed reactive oxygen species activated HIF-1 via oxidation responsive domains on the HIF-1 protein.⁴¹ Furthermore, other inflammatory factors including interleukin1 beta (IL-1beta) and tumor necrosis factor (TNF)-alpha and TNF-beta have been found to elicit responses through activation of HIF-1.^{42–45}

Oxygen Levels in the Retina

Incorporating cell-specific promoters will be important in development of hypoxia-regulated gene therapy for the retina because oxygen concentrations and oxygen requirements within different layers of the retina vary remarkably. The soma of Müller cells extend from the deep retinal capillary bed (where oxygen tension in rat was approximately 52 mm Hg or

approximately 8% oxygen) through a “hypoxic” region in inner retina of approximately 10 mm Hg (approximately 1.5% oxygen), to the inner limiting membrane where its end feet would reside in approximately 73 mm Hg (approximately 11% oxygen).⁴⁶ In studies from Linsenmeier’s group⁴⁷ it was shown that O₂ concentrations at the level of photoreceptor inner segments was very low in dark-adapted retina and slightly higher in light-adapted retina. Hypoxia at this location is likely to be accompanied by hypoxia at the outermost Müller cell processes. The cellular sensing of hypoxia will depend on inputs from different parts of the Müller cell but may also be affected by localized hypoxic stimuli. Furthermore, the biological outcomes or actions of effector responses may not be restricted to specific parts of the cell but may be more general. Because of the regional variations in levels of hypoxia in retinal tissue, cell-specific HRE vectors may demonstrate dynamic fluctuations in topographical expression patterns across the retina. An implication from our data is that the degree of activation of our promoter is likely to be influenced by the extent to which the Müller cell cytoplasm is exposed to hypoxic conditions. Furthermore the Müller cell may experience relative hypoxia where there is an imbalance between the amount of oxygen needed compared with the amount of oxygen being delivered to the tissue. In the OIR model, on entering ambient oxygen conditions, the mouse retina will experience a state of ischemia and relative hypoxia.²⁷

An Optimized Hypoxia-Inducible and Retinal Glial Cell-Specific Promoter

A hypoxia-regulated, non-cell-specific promoter (HRE and CMV) has been used previously by Ali and colleagues.⁵ The regulatory domain described in our research has two properties that should offer an advantage over the CMV or HRE promoter. First, Müller cell specificity of the promoter has important implications because the Müller cell traverses the width of the retina thus it experiences a distinct temporal pattern of hypoxia exposure. The radial distribution of the Müller cell suggests it is in a position to be affected by hypoxia resulting from damage in any part of the vascular bed, and would thus offer highly specific, very local responses to hypoxia. A second major advantage of our vector is the low basal levels of expression in normoxia.

GFAP Expression in Retina

Prolonged ischemia is known to promote Müller cell activation.^{48–50} It is not certain whether the time course for the activated phenotype is identical with that of induction of hypoxia signaling. In the event of hypoxia or oxidative stress, our HRE-driven GFAP promoter is designed to provide finely tuned transcriptional activation that can be distinct from the GFAP induction associated with Müller cell activation. Interestingly, exposure of the established Müller cell line rMC-1 to hypoxia for 24 hours elicited a decrease in GFAP expression relative to normoxic conditions.⁵¹ As Müller cells express low levels of GFAP in nondiseased retina, it is possible that our regulated promoter will be activated by brief transient hypoxic signals in the absence of endogenous GFAP induction.²¹ By contrast, astrocytes in the nondiseased retina express GFAP constitutively. Hence, using our vector, foreign gene expression in astrocytes in the retina would be silenced in normoxia and activated in hypoxia. One of our future directions is aimed at regulating therapeutic transgene expression by targeting astrocytes under hypoxic conditions either in retina or in other diseases involving hypoxia such as in the ischemic brain.

FIGURE 6. Retinal flat mounts and Müller cell restricted GFP expression. Retinal flat mounts prepared from P17 mice raised in room air (A) or exposed to hyperoxia for 5 days (B) and perfused with Tomato lectin. Central vascular occlusion (*dashed line*) and increased vascular tufts (*arrows*) occurred in the OIR model. Injection of the unregulated scAAV-GFAP-GFP resulted in GFP expression in Müller cells and astrocytes in both room air control (C) and oxygen exposed retinas (D). The regulated construct scAAV-reg-GFAP-GFP was silent in room air-exposed retinas (E) but in hypoxic retinas, GFP expression was evident in the soma as well as cell processes of Müller cells. There was less evidence of GFP expression in astrocytes of hypoxic retinas expressing the regulated construct (F) than in hypoxic or normoxic retinas expressing the unregulated construct (C, D). INL, inner nuclear layer; ONL, outer nuclear layer; GCL, ganglion cell layer.

The ABC1D GFAP Promoter Plus the HRE Domain: Cell Specificity and Hypoxia Inducibility

A previous study by Brenner and colleagues²⁴ identified a segment from -1488 to -1434 in their ABC1D GFAP promoter that contains brain region-specific elements and another segment from -1443 to -1399 required for silencing expression in neurons. By combining specific enhancer domains from the full length (-2163) GFAP promoter these investigators generated a highly active promoter containing enhancer domains A, B, C1, and D taken from -1757 to -1488 (AB), -1488 to -1256 (C1), and -132 to -56 (D) plus the proximal

promoter region. For our hypoxia-regulated GFAP promoter construct, we employed a promoter sequence consisting of a hybrid of the four enhancer segments A, B, C1, and D. Brenner and colleagues also showed in transgenic mice that gfaABC1D generally drove beta galactosidase expression at a stronger level than was obtained in -2163 GFAP-lacZ mice.

Normoxic Silencing and Cell-Specific Hypoxic Induction

Our data confirmed the cell specificity of the ABC1D region of the GFAP promoter in Müller cells. While Brenner et al.²⁴

reported that ABC1D was astrocyte-specific, they also demonstrated that hybrid enhancers containing subregions of the GFAP promoter were more active than the full length (–2163) GFAP promoter because inhibitory domains were removed. By employing our hypoxia-regulated GFAP promoter we were able to achieve low level expression under silenced normoxic conditions in Müller cells while ensuring high level transcriptional induction in hypoxia. The OIR model was used to test HRE-driven GFP expression in mouse retina.⁵ P17 Retinas from the OIR model differed from normal retinas in terms of development of retinal vasculature. High oxygen exposure (during P7 to P12) caused regression of normal retinal vasculature; on return to room air the regions of the retina now lacking sufficient vascular supply become hypoxic, eliciting expression of proangiogenic factors and neovascularization. The retinal flat mounts (Fig. 6A) confirmed the central avascular zone and peripheral neovascular tufts reported in the literature.^{25–27} Induction of GFP expression was demonstrated in Müller cell P17 oxygen-exposed retinas that had been transfected with our regulated vector, while GFP expression was silenced in P17 retina from mice raised in room air. Our data confirm the cell specificity and hypoxia inducibility of the regulated promoter both in primary Müller cell cultures and in the in vivo OIR model. Although GFP expression was localized predominantly in retinal Müller cells from in P17 OIR mice, the possibility of GFP expression in some retinal astrocytes near the border of the inner limiting membrane cannot be excluded.

Use of Vector in Retinal Diseases

Diabetic Retinopathy. Our hypoxia-regulated, retinal glial cell-specific vector is likely to be applicable to a range of diseases. GFAP induction in diabetic retinas has been reported in Müller cells of diabetic rats, and in astrocytes, but not Müller cells, of diabetic mice.⁵² Induction of GFAP in Müller cells in rats correlated with proapoptotic changes in neurons observed after 2 to 5 months of experimental diabetes.⁵² The HRE-dependent regulation in our promoter is likely to cause its activation characteristics to be different in astrocytes and Müller cells. The astrocytes may experience different levels of hypoxia than the Müller cells and therefore the response in these two cell types in retina could be different both in timing and in levels of activation. Moreover our promoter might be activated under oxidative conditions known to stimulate apoptosis in the diabetic retina.^{53–55} In diabetes the inner retinal vessels are lost. It is likely that in diabetes, astrocytes in the inner retina will experience equivalent or more severe hypoxia than Müller cells that span the width of the retina.

AMD. In AMD our hypoxia-regulated, retinal glial cell-specific gene therapy would be applicable for induction of neurotrophic factors which are known to be highly effective in limiting the death of photoreceptor cells.^{56,57} AMD is characterized by both oxidative conditions and VEGF-dependent neovascularization induced by inflammatory and/or hypoxic cellular microenvironments.^{58,59} Agents blocking VEGF function and neurotrophic interventions are important strategies for targeting AMD. Neurotrophic factors that already show promise in models of AMD include FGF, brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CNTF).^{60,61} For example photoreceptors in animal models have been successfully protected by delivery of CNTF by transgene insertion into encapsulated cells and by direct gene transfer.^{62–64} Activation of the promoter by the oxidative/inflammatory environment contributing to geographic atrophy in dry AMD would provide an efficient means to deliver neurotrophic therapy only to the pathologic regions of the retina. Moreover, the fact that our promoter is activated by the conditions contributing to pathology provides a foundation for prophylactic neuroprotective gene therapy for both dry and wet AMD.^{65–67} Such an ap-

proach would represent a more effective and potentially less expensive therapy for management of AMD.

Conclusion

In summary, we have described construction of a novel promoter consisting of GFAP regulatory sequences and a hypoxia responsive domain that is activated in Müller cells. Specifically, the promoter was activated in Müller cells, silenced in normoxia, and induced > 15-fold in hypoxia. The promoter retained its cell specificity and induction in hypoxia when incorporated into a self-complementary AAV2 vector and tested in transfected cells and the in vivo OIR model. We propose that the retinal glial cell-specific, hypoxia-inducible promoter would be applicable to models of gene therapy for a number of retinal diseases including diabetic retinopathy and AMD.

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