

Hypoxia Induces Galectin-1 Expression Via Autoinduction of Placental Growth Factor in Retinal Pigment Epithelium Cells

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PURPOSE. Galectin-1/*LGALS1*, a β -galactoside-binding protein, contributes to angiogenesis and fibrosis in various ocular diseases. Hypoxia-dependent and -independent pathways upregulate galectin-1/*LGALS1* expression in Müller glial cells. Here, we present novel findings on the galectin-1/*LGALS1* regulatory system in human retinal pigment epithelium (RPE) cells, the major cellular participant in the pathogenesis of neovascular age-related macular degeneration (nAMD).

METHODS. Human RPE cells were used to evaluate changes in gene and protein expression with real-time quantitative PCR and immunoblot analyses, respectively. The promoter and enhancer regions of *LGALS1* were analyzed by reporter assay and chromatin immunoprecipitation. Immunofluorescence analysis of nAMD patient specimens was used to confirm the in vitro findings.

RESULTS. Hypoxia induced galectin-1/*LGALS1* expression via binding of hypoxia-inducible factor 1 α (HIF-1 α) to hypoxia-responsive elements in the *LGALS1* promoter region. Blockade of vascular endothelial growth factor receptor 1 (VEGFR1) partially decreased hypoxia-induced galectin-1/*LGALS1* expression. Among several VEGFR1 ligands induced by hypoxia, placental growth factor (PlGF)/PGF alone upregulated galectin-1/*LGALS1* expression via phosphorylation of activator protein 1 (AP-1) subunits following AKT and p38 mitogen-activated protein kinase (MAPK) activation. An AP-1 site in the *LGALS1* enhancer region was required for PlGF-induced galectin-1/*LGALS1* expression in RPE cells. PlGF application upregulated PGF expression via extracellular signal-regulated kinase 1 and 2, AKT, and p38 MAPK pathways. nAMD patient specimens demonstrated co-localization of galectin-1 with HIF-1 α , PlGF, and VEGFR1 in RPE cells.

CONCLUSIONS. Our present findings implicate the significance of hypoxia as a key inducer of galectin-1/*LGALS1* in RPE cells and the autoinduction of hypoxia-induced PlGF as a vicious cycle amplifying the pathogenesis of nAMD.

Keywords: age-related macular degeneration, galectin-1, hypoxia-inducible factor-1 α , placental growth factor, retinal pigment epithelium

Age-related macular degeneration (AMD) is the leading cause of legal blindness and severe vision loss worldwide. Neovascular (exudative or wet) AMD (nAMD) is characterized by choroidal neovascularization (CNV), which develops fibrovascular proliferation beneath the macula (central area of the retina).¹ A number of studies have shown the involvement of multiple factors such as chronic inflammation in the etiology of CNV due to nAMD.² Retinal pigment epithelium (RPE) cells produce and secrete various cytokines and growth factors, thus accelerating CNV formation and subretinal fibrosis in nAMD.³ These pathogenic stimuli include a range of inflammatory, angiogenic, and fibrogenic mediators such as interleukin 6 (IL-6), vascular endothelial growth factor A (VEGF-A), and transforming growth factor β (TGF- β).^{4,5} Of these, VEGF-A is the principal mediator, and the anti-VEGF drugs ranibizumab and aflibercept have become the standard therapeutic strat-

egy for nAMD.⁶ However, a large number of patients still have poor visual prognosis during the long-term follow-up, because nAMD is a complex disorder associated with multiple molecules.

Galectin-1, a member of the β -galactoside-binding lectin family, is encoded by the *LGALS1* gene and widely expressed in a variety of cells and tissues. Galectin-1 has a wide range of biological functions, including cell proliferation and apoptosis, as well as pathological conditions such as cancer.^{7,8} Importantly, galectin-1 has been shown to play a critical role in angiogenesis via lectin-dependent binding to the N-glycans of VEGF receptor 2 (VEGFR2), thus activating its downstream signal transduction in vascular endothelial cells as a newly recognized VEGFR2 ligand.^{9,10} Our human sample and mouse model data have shown the biologic significance of galectin-1 as a key promoter for both VEGF-A-driven angiogenesis and TGF- β -driven fibrosis in the

pathogenesis of nAMD,¹¹ indicating the potential of galectin-1 as an alternative and complementary target in the treatment of anti-VEGF refractory cases with nAMD. RPE cells have been shown as the major cellular source of its production in nAMD eyes¹¹; however, the molecular mechanism still remains largely unknown. A detailed investigation into pathways for inducing the expression of galectin-1 in RPE cells is, therefore, warranted in order to improve the long-term management of nAMD.

Inadequate oxygen activates multifaceted cellular responses leading to various pathological events. Hypoxia-inducible factor 1 α (HIF-1 α) is a known regulator for increased galectin-1 expression in cancer cells,⁸ and we previously demonstrated the significant upregulation of *LGALS1* in retinal vascular endothelial cells and Müller glial cells cultured under hypoxia.^{10–13} Moreover, we have recently shown the significant role of hypoxia-responsive elements (HREs) in the *LGALS1* promoter region in hypoxia-induced galectin-1/*LGALS1* expression in Müller glial cells.¹² In addition to hypoxia, our previous studies revealed that IL-1 β induced galectin-1 expression in Müller glial cells via activator protein 1 (AP-1) activation following diabetes-associated inflammatory cascades including extracellular signal-regulated kinase (ERK) 1 and 2 and phosphatidylinositol-3 kinase (PI3K)/AKT pathways.^{13,14} In this study, we explored a novel mechanism for the upregulation of galectin-1 expression in RPE cells, which proved to be dependent on the autoinduction of hypoxia-induced placental growth factor (PlGF)/PGF.

MATERIALS AND METHODS

Cell Line and Reagents

The human RPE (hTERT-RPE1) cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM/F-12 supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA). For hypoxic exposure, cells were cultured in a gas mixture composed of 1% O₂, 5% CO₂, and 94% N₂. All reagents and antibodies are detailed in the Supplementary Materials.

Real-Time Quantitative PCR (qPCR)

Total RNA isolation was performed from cells using the SuperPrep II Cell Lysis & RT Kit for qPCR (TOYOBO, Tokyo, Japan), and real-time qPCR was performed as previously described.^{10–13} All primers are listed in Supplementary Table S1. Gene expression levels were calculated using the 2^{- $\Delta\Delta Ct$} method.

Immunoblot Analyses

Cell extracts were lysed in sodium dodecyl sulfate buffer, protease inhibitor cocktail, and phosphatase inhibitor cocktail (EMD Millipore, Temecula, CA, USA). Immunoblotting was performed as described previously.^{10–13} Signals were visualized using a SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific).

Enzyme-Linked Immunosorbent Assay

The protein levels of galectin-1 in cell lysate and PlGF in cell lysate and culture medium were determined with human galectin-1 (R&D Systems, Minneapolis, MN, USA) and human

PlGF (BioLegend, San Diego, CA, USA) ELISA kits per the manufacturer's instructions. The optical density was determined using a microplate reader (Sunrise absorbance reader; Tecan, Männedorf, Switzerland).

Reporter Assays

The luciferase reporter experiments were performed using the Dual-Luciferase Reporter Assays System (Promega Corporation, Madison, WI, USA). RPE cells were transfected with the pGL4 vector containing one of three fragments spanning nucleotides -500 bp to +67 bp from the *LGALS1* transcription start site (promoter region; pGal), +450 bp to +1750 bp (enhancer region; pGal+AP-1), and AP-1 site (TGACTCA)-mutated enhancer region (pGal Δ AP-1), as previously described.^{14,15} Mutation in two HRE sites in the *LGALS1* promoter region (CACGC to CAAAAC, positions at -441 bp to -437 bp and -427 bp to -423 bp)^{8,12} was synthesized and sequenced by Integrated DNA Technologies (Coralville, IA, USA), and subcloned into the pGL4 vector (pGal Δ HRE). The pRL-CMV *Renilla* luciferase plasmid (Promega) was used as internal control. Cells were transfected with plasmid DNA using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific) following the manufacturer's protocols.

Chromatin Immunoprecipitation (ChIP)-qPCR

Assays were performed using the SimpleChIP Enzymatic Immunoprecipitation Chromatin IP Kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's protocols. After chromatin was immunoprecipitated with antibodies, immunoprecipitates were evaluated by real-time qPCR using the primers specific for the previously described HRE sites in the *LGALS1* promoter region^{8,12} and AP-1-binding site in the *LGALS1* enhancer region,^{14–16} together with 2% input DNA as reference samples. All primers are listed in Supplementary Table S1. Real-time qPCR was performed as described previously.¹² ChIP-qPCR signals were calculated as percentage of input.

Immunofluorescence Microscopy

nAMD patient specimens were obtained in our clinic by enucleation due to suspected melanoma from an 82-year-old male with massive subretinal and vitreous hemorrhages secondary to CNV. This study was approved by the ethics committee of Hokkaido University Hospital, and written informed consent was obtained from the patient after an explanation of our research use. Immunofluorescence analyses were performed as described previously.¹¹

Statistical Analysis

All the results are expressed as the mean \pm SEM. Student's *t*-test was used for statistical comparison between groups, and one-way analysis of variance (ANOVA) followed by the Tukey-Kramer method as a post hoc test was used for multiple comparison procedures. Differences were considered statistically significant at *P* < 0.05.

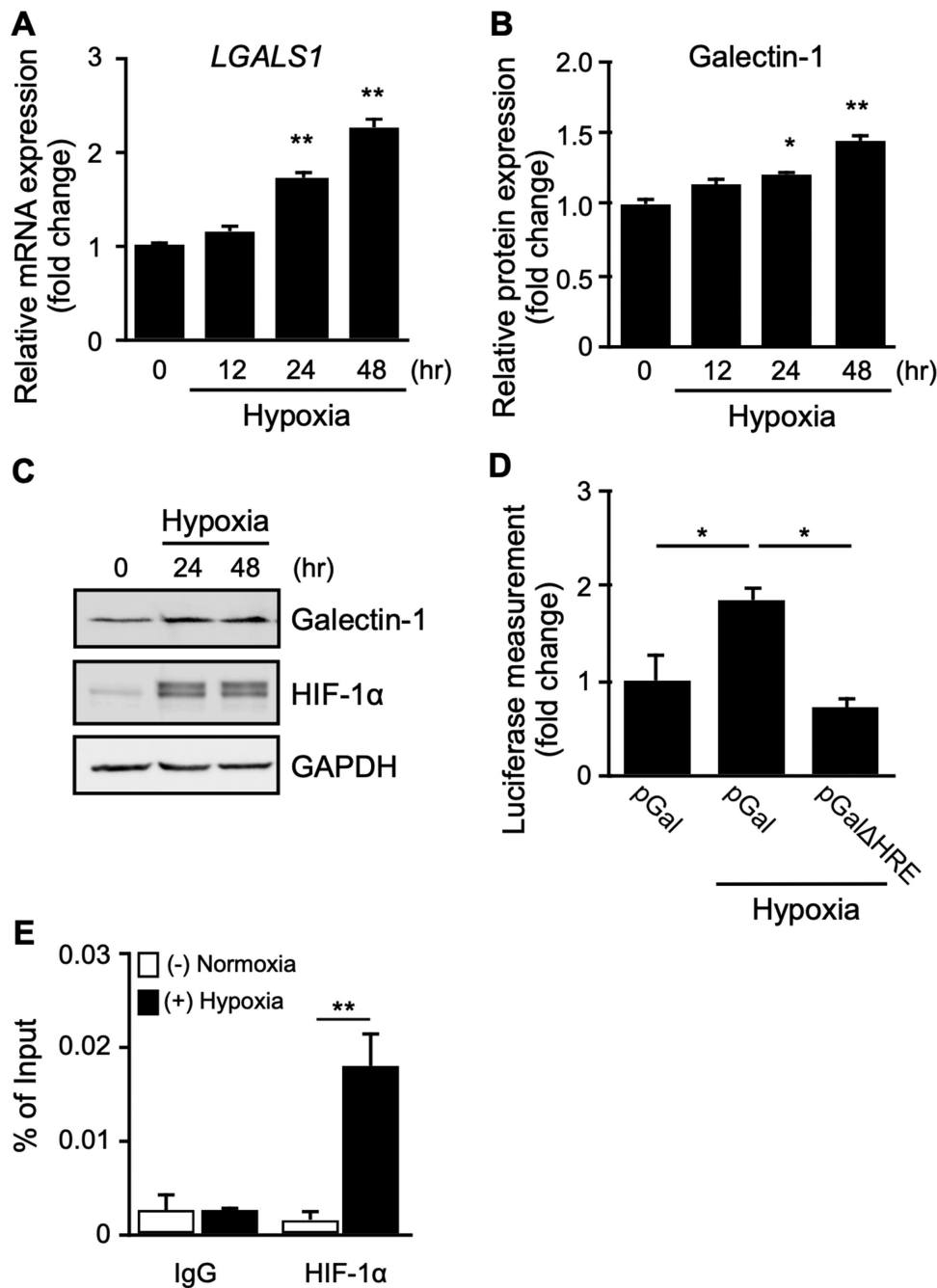


FIGURE 1. Involvement of HIF-1 α in hypoxia-induced galectin-1/*LGALS1* expression in RPE cells. (A–C) *LGALS1* mRNA expression levels and galectin-1 and HIF-1 α protein levels were analyzed by real-time qPCR (A), ELISA (B), and immunoblotting (C) in hypoxia (1% O₂)-stimulated RPE cells. (D) Human *LGALS1* promoter (–500 to +67, pGal) and HRE mutant (pGal Δ HRE) constructs were transfected into RPE cells together with control reporter plasmid pRL-CMV. Transfected RPE cells were cultured in hypoxia for 24 hours and assayed for luciferase activities. (E) RPE cells were cultured in hypoxia for 1 hour before harvest of samples. Binding of HIF-1 α to HREs in the *LGALS1* promoter region was analyzed by ChIP-qPCR. * $P < 0.05$, ** $P < 0.01$; $n = 6$ –8 per group.

RESULTS

Involvement of HIF-1 α in Hypoxia-Induced Galectin-1/*LGALS1* Expression in RPE Cells

We recently revealed the significant upregulation of galectin-1 in RPE cells of mice with laser-induced CNV.¹¹ HIF-1 α was reported to be expressed in RPE cells in CNV tissue samples collected from patients with nAMD.¹⁷ We and others demon-

strated the hypoxic stimulation of galectin-1/*LGALS1* expression in various cell species.^{8,10,12,15} Consistent with previous reports, RPE cells demonstrated time-dependent responsiveness to hypoxia in *LGALS1* transcripts (Fig. 1A) and products (Figs. 1B, 1C). Moreover, immunoblot analysis confirmed that hypoxia led to a significant upregulation of HIF-1 α protein expression in RPE cells (Fig. 1C). HIF-1 α , an oxygen-dependent transcriptional activator, induces the expression of its transcriptional targets via binding to HREs.¹⁸ HIF-1 α

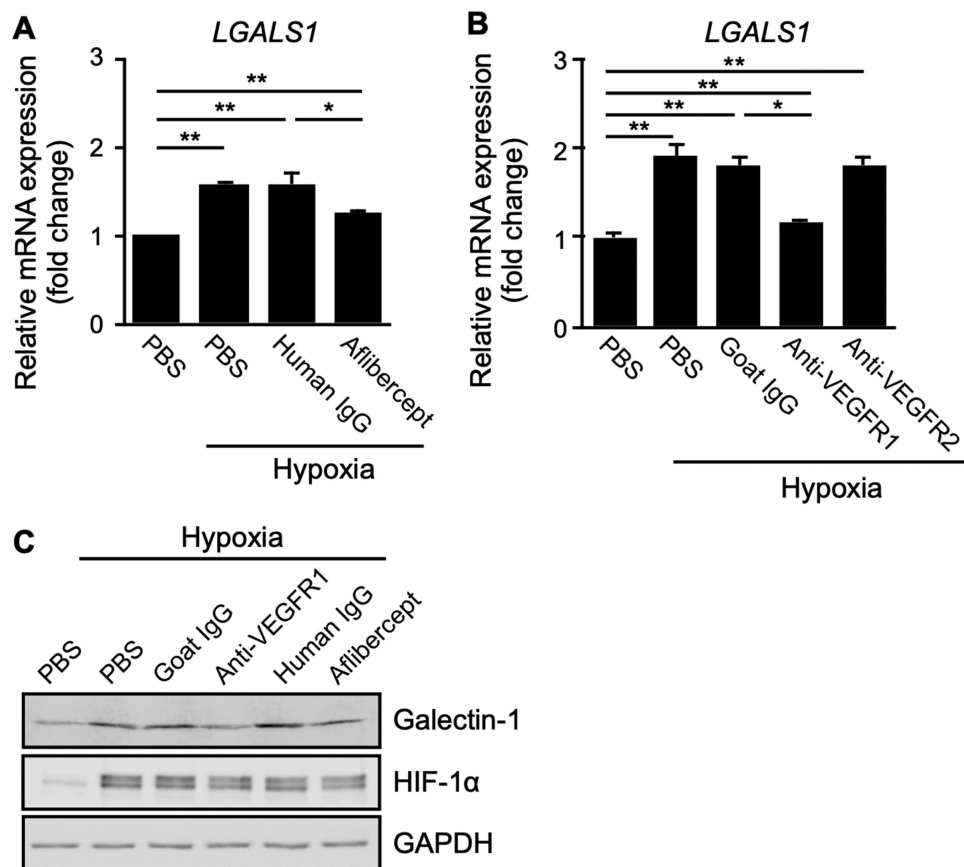


FIGURE 2. Involvement of VEGFR1 in hypoxia-induced galectin-1/*LGALS1* expression in RPE cells. **(A)** RPE cells were pretreated with aflibercept (250 $\mu\text{g}/\text{mL}$) and human normal IgG (250 $\mu\text{g}/\text{mL}$) for 30 minutes before culture in hypoxia for 24 hours, and *LGALS1* gene expression levels were analyzed. **(B)** RPE cells were pretreated with neutralizing antibodies against VEGFR1 (10 $\mu\text{g}/\text{mL}$) and VEGFR2 (10 $\mu\text{g}/\text{mL}$) and goat normal IgG (10 $\mu\text{g}/\text{mL}$) for 30 minutes before culture in hypoxia for 24 hours, and *LGALS1* gene expression levels were analyzed. **(C)** RPE cells were pretreated with anti-VEGFR1 antibody, aflibercept, and normal IgGs for 30 minutes before culture in hypoxia for 24 hours, and galectin-1 and HIF-1 α protein levels were analyzed. * $P < 0.05$, ** $P < 0.01$; $n = 6$ per group.

upregulates *LGALS1* mRNA in human cancer and Müller glial cells via binding to HREs in the *LGALS1* promoter region, located from 441 bp to 423 bp upstream of the transcriptional start site of the *LGALS1* gene.^{8,12} To study the involvement of HREs in the expression of galectin-1/*LGALS1* in RPE cells under hypoxia, luciferase reporter plasmids driven by *LGALS1* promoter (pGal) that contains HREs were transfected into RPE cells, and the luciferase activity was measured. *LGALS1* promoter-luciferase was induced by hypoxia, and the HRE-mutated construct (pGal Δ HRE) exhibited significantly lower luciferase activity (Fig. 1D). Additionally, ChIP-qPCR clarified that binding of HIF-1 α to HREs in the *LGALS1* promoter region was significantly increased under hypoxia (Fig. 1E), suggesting the contribution of HREs in the *LGALS1* promoter region to hypoxia-induced galectin-1/*LGALS1* expression in RPE cells.

Involvement of VEGFR1 in Hypoxia-Induced Galectin-1/*LGALS1* Expression in RPE Cells

Given that aflibercept, a chimeric VEGFR1/VEGFR2-based decoy receptor,¹⁹ has the ability to bind several VEGFR ligands VEGF-A, VEGF-B, PIGF, and galectin-1,^{10,20} at least some of which are induced by hypoxia, we hypothesized that blockade of such multiple cytokines affects

galectin-1 expression under hypoxia. Interestingly, hypoxia-induced *LGALS1* mRNA levels were partially but significantly decreased by aflibercept (fold change = 1.26; $P < 0.05$) compared to human normal IgG (fold change = 1.60) (Fig. 2A). Next, in order to determine which VEGFR ligands contributed to *LGALS1* mRNA expression, we carried out blocking experiments. The upregulated *LGALS1* mRNA expression was suppressed by pretreatment with anti-VEGFR1 neutralizing antibody (fold change = 1.16; $P < 0.05$), but not with anti-VEGFR2 neutralizing antibody (fold change = 1.81) or normal IgG (fold change = 1.81) (Fig. 2B). We also confirmed the effects of VEGFR1 neutralization, as well as aflibercept, on galectin-1, but not HIF-1 α , protein expression under hypoxia (Fig. 2C), suggesting the significant contribution of VEGFR1 ligand(s) to galectin-1/*LGALS1* expression under hypoxia.

Screening of Hypoxia-Induced VEGFR1 Ligands and Induction of Galectin-1/*LGALS1* in RPE Cells Stimulated Exclusively by PIGF

Based on our results showing the possible involvement of VEGFR1 ligand(s) in galectin-1/*LGALS1* expression under hypoxia (Fig. 2), we checked which VEGFR1 ligands (i.e., VEGF-A, VEGF-B, and PIGF) are induced in hypoxic RPE

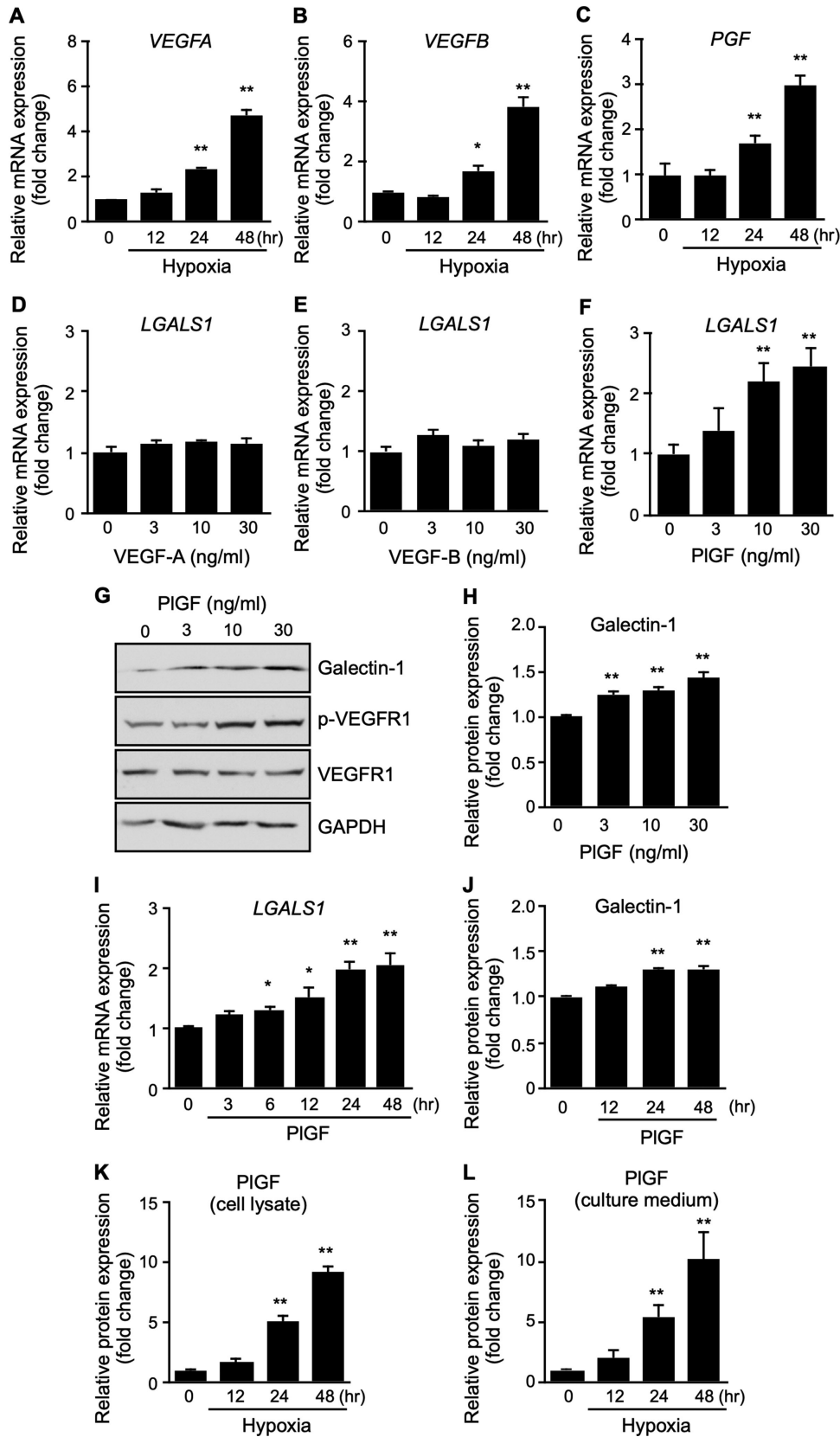


FIGURE 3. Screening of hypoxia-induced VEGFR1 ligands and induction of galectin-1/*LGALS1* in RPE cells stimulated exclusively by PIGF. (A–C) *VEGFA* (A), *VEGFB* (B), and *PGF* (C) mRNA expression levels were analyzed by real-time qPCR in hypoxia-stimulated RPE cells. (D–F) RPE cells were treated with VEGF-A (3–30 ng/mL) (D), VEGF-B (3–30 ng/mL) (E), and PIGF (3–30 ng/mL) (F) for 24 hours, and *LGALS1*

mRNA levels were analyzed. (G, H) RPE cells were treated with PIGF (3–30 ng/mL) for 24 hours, and galectin-1 protein levels were analyzed by immunoblotting (G) and ELISA (H), together with phosphorylated and total forms of VEGFR1 by immunoblotting (G). (I, J) RPE cells were treated with or without 10 ng/mL PIGF. Real-time qPCR (I) and ELISA (J) for the time course of expression levels of *LGALS1*/galectin-1. (K, L) PIGF protein levels were analyzed in both RPE cell lysate (K) and culture medium (L) by ELISA under hypoxic conditions. * $P < 0.05$, ** $P < 0.01$; $n = 6$ per group.

cells. Hypoxia upregulated *VEGFA*, *VEGFB*, and *PGF* mRNA expression in RPE cells (Figs. 3A–3C). To investigate which can induce galectin-1/*LGALS1* expression in RPE cells, we stimulated RPE cells with these hypoxia-induced cytokines. Application of PIGF, but not VEGF-A or VEGF-B, to RPE cells elevated *LGALS1* mRNA (Figs. 3D–3F) and protein levels (Figs. 3G, 3I) in a dose-dependent manner. Given that VEGF-B and PIGF are high-affinity VEGFR1-specific ligands, this observation could be explained at least partly by their structural difference in potency to induce VEGFR1 downstream signaling.²¹ Consistently, the phosphorylated levels of VEGFR1 were also augmented following PIGF stimulation in a dose-dependent manner (Fig. 3G). Moreover, the upregulation of galectin-1/*LGALS1* expression was observed in a time-dependent manner, as well (Figs. 3I, 3J). To ensure the validity of these experiments with PIGF application (Figs. 3F–3J), we confirmed PIGF protein production in hypoxic RPE cells (Fig. 3K) and concurrent secretion into the culture medium (Fig. 3L) in a time-dependent manner.

In parallel, we denied the possibility of the VEGFR2 ligand galectin-1, which is also induced by hypoxia and trapped by aflibercept, as another inducer for *LGALS1* expression (Supplementary Fig. S1A). Recently, we reported that stimulation with IL-1 β to human Müller glial cells led to the significant upregulation of galectin-1/*LGALS1* expression.¹³ However, the expression of *LGALS1* did not change after IL-1 β application to RPE cells (Supplementary Fig. S1B) or PIGF application to Müller glial cells (Supplementary Fig. S1C), underscoring the currently observed cell- and cytokine-specific induction of galectin-1/*LGALS1* expression after stimulation with PIGF to human RPE cells.

Requirement of PI3K/AKT- and p38 MAPK-Dependent AP-1 Activity in PIGF/VEGFR1-Induced Galectin-1/*LGALS1* Expression in RPE Cells

Galectin-1/*LGALS1* mRNA and protein levels in PIGF-stimulated RPE cells were significantly suppressed by pretreatment with anti-VEGFR1 neutralizing antibody, as well as aflibercept, compared to normal IgGs (Figs. 4A, 4B), indicating the significance of the PIGF/VEGFR1 axis. To determine its downstream intracellular signaling pathways, we employed specific inhibitors. PIGF-induced *LGALS1* mRNA levels were significantly reversed by inhibitors for PI3K and p38 mitogen-activated protein kinase (MAPK), but not the others (Fig. 4C). Additionally, we confirmed the impact of PI3K/AKT and p38 MAPK pathways in protein levels (Fig. 4D), suggesting the PIGF/VEGFR1 downstream regulation via PI3K/AKT and p38 MAPK pathways in RPE cells.

To investigate the detailed regulatory mechanism of PIGF-induced *LGALS1* gene expression, we analyzed the *LGALS1* promoter and enhancer regions. Because the 0.5-kbp upstream region from the transcription start site of *LGALS1* promoter plays a critical role in *LGALS1* gene expression,¹⁵ we first checked a luciferase vector driven by

LGALS1 promoter (pGal). However, there was no significant increase in luciferase activity after PIGF stimulation (Fig. 4E). Recently, we showed that IL-1 β -induced *LGALS1* expression in human Müller glial cells was mediated by an AP-1 site located in the *LGALS1* enhancer region.¹⁴ The construct including the enhancer region (pGal+AP-1) was shown to enhance PIGF-untreated luciferase activity as compared to the construct without the enhancer region (pGal). Moreover, PIGF administration significantly increased luciferase activity in RPE cells transfected with the enhancer-containing construct (pGal+AP-1). Additionally, the construct lacking an AP-1 site (pGal Δ AP-1) exhibited significantly lower luciferase activity (Fig. 4E), indicating that the AP-1 site in the *LGALS1* enhancer region was essential for PIGF-induced galectin-1/*LGALS1* expression in RPE cells. Supporting these findings, ChIP-qPCR revealed that binding of activating transcription factor 2 (ATF2) and c-Jun, but not c-Fos, to the AP-1 site in the *LGALS1* enhancer region significantly increased after stimulation with PIGF (Fig. 4F). Moreover, administration of PIGF to RPE cells increased the phosphorylated levels of ATF2 and c-Jun, both of which were reduced by inhibition of VEGFR1 (Fig. 4G). These results suggest the facilitatory role of PI3K/AKT- and p38 MAPK-mediated AP-1 pathways in PIGF/VEGFR1-induced galectin-1/*LGALS1* expression in RPE cells.

Requirement of VEGFR1-Mediated ERK1/2, PI3K/AKT, and p38 MAPK Pathways in PIGF/*PGF* Autoinduction in RPE Cells

VEGF family proteins including PIGF have been shown to promote cell growth, survival, and cycle^{22–24} and their own expression^{25–27} via autocrine signaling. Next, we investigated whether administration of PIGF induces *PGF* gene expression in RPE cells. PIGF stimulation of RPE cells significantly increased *PGF* mRNA levels in a time-dependent manner (Fig. 5A). PIGF-induced *PGF* mRNA expression was suppressed by pretreatment with anti-VEGFR1 neutralizing antibody and aflibercept, as compared to normal IgGs (Fig. 5B). Pretreatment with inhibitors for ERK1/2, PI3K, and p38 MAPK, but not the others, effectively blocked PIGF-induced *PGF* expression (Fig. 5C). We also confirmed the impact of VEGFR1 inhibition on the protein levels of phosphorylated ERK1/2, AKT, and p38 MAPK (Fig. 5D). These findings imply the establishment of the vicious cycle of PIGF autoinduction via VEGFR1-mediated ERK1/2, PI3K/AKT, and p38 MAPK pathways in RPE cells.

Co-Localization of Galectin-1 with HIF-1 α , PIGF, and VEGFR1 in RPE Cells in nAMD Patient Specimens

Previously, we demonstrated galectin-1 localization in RPE cells in nAMD patient samples.¹¹ To confirm our in vitro findings, we performed immunofluorescence for nAMD patient tissue specimens. Double-staining experiments demonstrated galectin-1 immunoreactivity in RPE65-positive

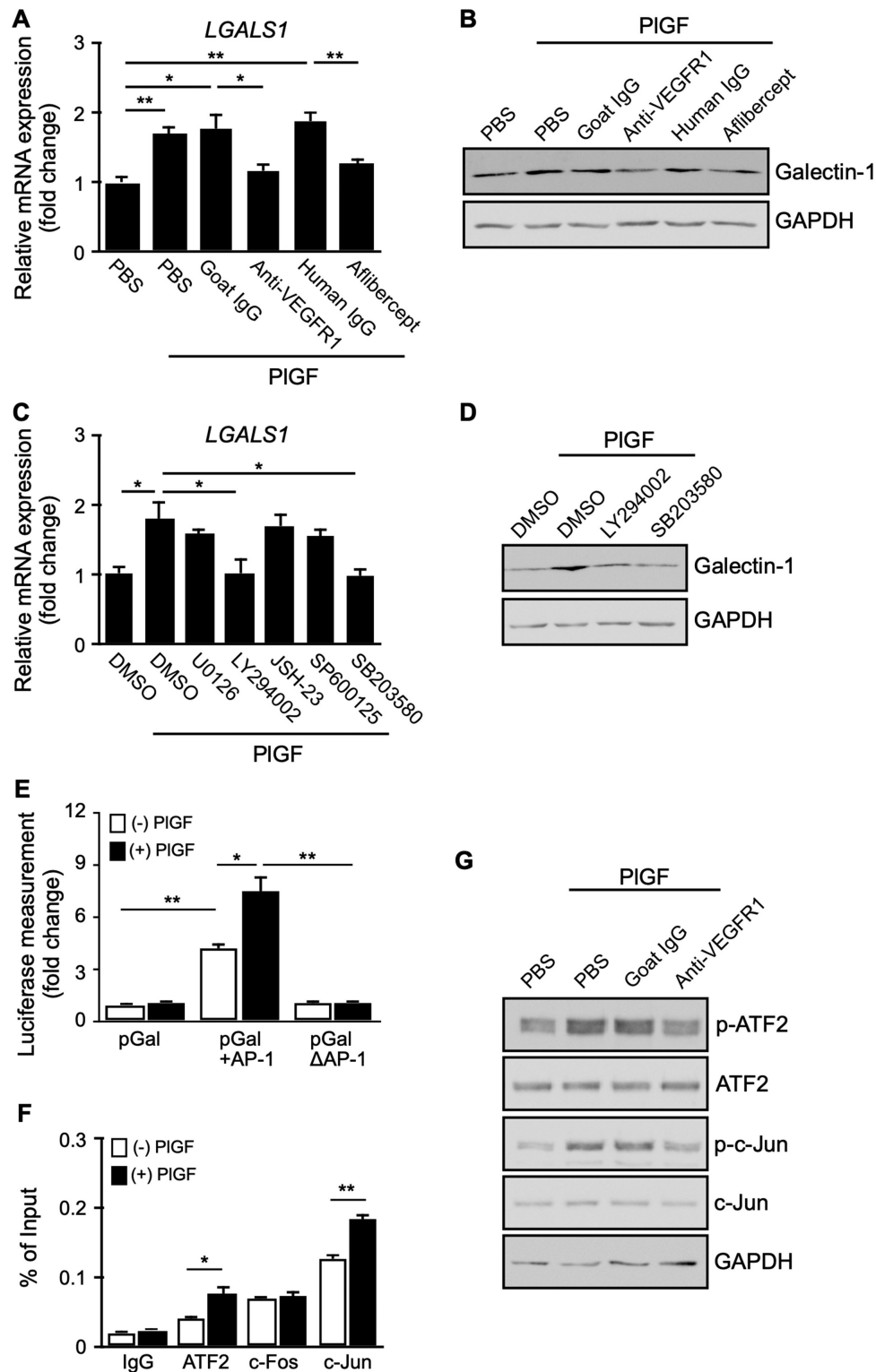


FIGURE 4. Requirement of PI3K/AKT- and p38 MAPK-dependent AP-1 activity in PIGF/VEGFR1-induced galectin-1/*LGALS1* expression in RPE cells. (**A**, **B**) RPE cells were pretreated with anti-VEGFR1 neutralizing antibody (10 μ g/mL), aflibercept (250 μ g/mL), and control normal IgG (10 μ g/mL for goat, 250 μ g/mL for human) for 30 minutes followed by stimulation with PIGF (10 ng/mL) for 24 hours. Galectin-1/*LGALS1* mRNA (**A**) and protein (**B**) expression levels were analyzed. (**C**, **D**) RPE cells were pretreated with each inhibitor (U0126, ERK1/2; LY294002, PI3K; JSH-23, nuclear factor [NF]- κ B; SP600125, c-Jun N-terminal kinase [JNK]; SB203580, p38 MAPK) at 10 μ M for 30 minutes before application with PIGF (10 ng/mL) for 24 hours. Galectin-1/*LGALS1* mRNA (**C**) and protein (**D**) expression levels were analyzed. (**E**) RPE cells were transfected with the *LGALS1* promoter (pGal), the promoter with AP-1-dependent *LGALS1* enhancer (pGal+AP-1), or the promoter with AP-1 site-mutated enhancer (pGal Δ AP-1). Transfected RPE cells were stimulated with PIGF (10 ng/mL) for 24 hours and assayed for luciferase activity. Representative luciferase activities were normalized to *Renilla* luciferase activity. (**F**) RPE cells were stimulated with PIGF (10 ng/mL) for 1 hour before harvest of samples. Binding of ATF2, c-Fos, and c-Jun to the AP-1 site in the *LGALS1* enhancer region was analyzed by ChIP-qPCR. (**G**) RPE cells were pretreated with anti-VEGFR1 neutralizing antibody (10 μ g/mL) for 30 minutes before stimulation with PIGF (10 ng/mL) for 1 hour, and protein levels of phosphorylated and total forms of AP-1 subunits were analyzed. * $P < 0.05$, ** $P < 0.01$; $n = 4$ –6 per group.

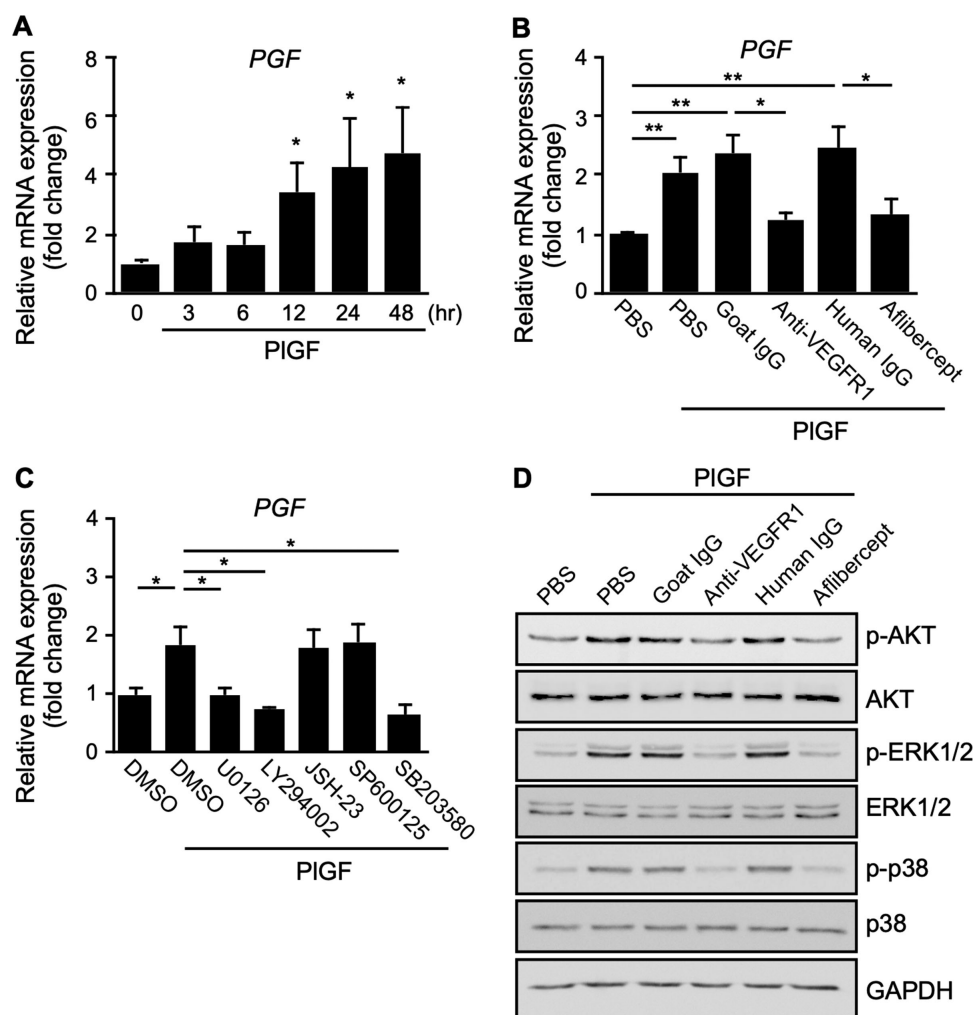


FIGURE 5. Requirement of VEGFR1-mediated ERK1/2, PI3K/AKT, and p38 MAPK pathways in PIGF/PGF autoinduction in RPE cells. **(A)** RPE cells were treated with or without 10 ng/mL PIGF. Real-time qPCR analyses were carried out for the time course of expression levels of *PGF*. **(B)** RPE cells were pretreated with anti-VEGFR1 neutralizing antibody (10 μ g/mL), aflibercept (250 μ g/mL), and control normal IgG (10 μ g/mL for goat, 250 μ g/mL for human) for 30 minutes followed by stimulation with PIGF (10 ng/mL) for 24 hours, and *PGF* gene expression levels were analyzed. **(C)** RPE cells were pretreated with each inhibitor (U0126, ERK1/2; LY294002, PI3K; JSH-23, NF- κ B; SP600125, JNK; SB203580, p38 MAPK) at 10 μ M for 30 minutes before application with PIGF (10 ng/mL) for 24 hours, and *PGF* gene expression levels were analyzed. **(D)** RPE cells were pretreated with anti-VEGFR1 neutralizing antibody (10 μ g/mL), aflibercept (250 μ g/mL), and control normal IgG (10 μ g/mL for goat, 250 μ g/mL for human) for 30 minutes followed by stimulation with PIGF (10 ng/mL) for 24 hours. Protein levels of phosphorylated and total forms of AKT, ERK1/2, and p38 were analyzed. * $P < 0.05$, ** $P < 0.01$; $n = 6$ per group.

RPE cells (Figs. 6A–6C) and its co-localization with HIF-1 α (Figs. 6D–6F), PIGF (Figs. 6G–6I), and VEGFR1 (Figs. 6J–6L). These results suggest the involvement of HIF-1 α and PIGF-VEGFR1 signaling in galectin-1 expression in RPE cells in the pathogenesis of nAMD. Regarding galectin-1 localization in the entire retinal layers, its immunoreactivity was ubiquitously detected in the ganglion cell layer, inner and outer nuclear layers, outer plexiform layer, and photoreceptor layer, in addition to RPE and choroidal vasculature, both of which exhibited the prominent expression of galectin-1 (Supplementary Fig. S2A). The broad distribution of galectin-1 signals was comparable with our previous data on the mouse retina.¹¹ There was no apparent differences in its expression pattern or intensity between central and peripheral areas of the retina (Supplementary Figs. S2A, S2B).

DISCUSSION

The RPE, a monolayer between the choriocapillaris (choroidal capillary layer) and photoreceptor outer segments, plays important roles in highly specialized metabolic and transport systems essential for the homeostasis of the neural retina. Chronic inflammation, oxidative damage, and impaired cholesterol metabolism adjacent to and/or within RPE cells have been implicated in the development of AMD.² The current study identified two distinct induction pathways for RPE expression of galectin-1, which contributes to both angiogenic and fibrogenic processes in nAMD.¹¹ As illustrated in the overall schema of our data (Fig. 7), galectin-1/*LGALS1* expression in RPE cells was mediated via both HIF-1 α and AP-1 pathways, the latter of which was amplified by the autoinduction of hypoxia-induced PIGF.

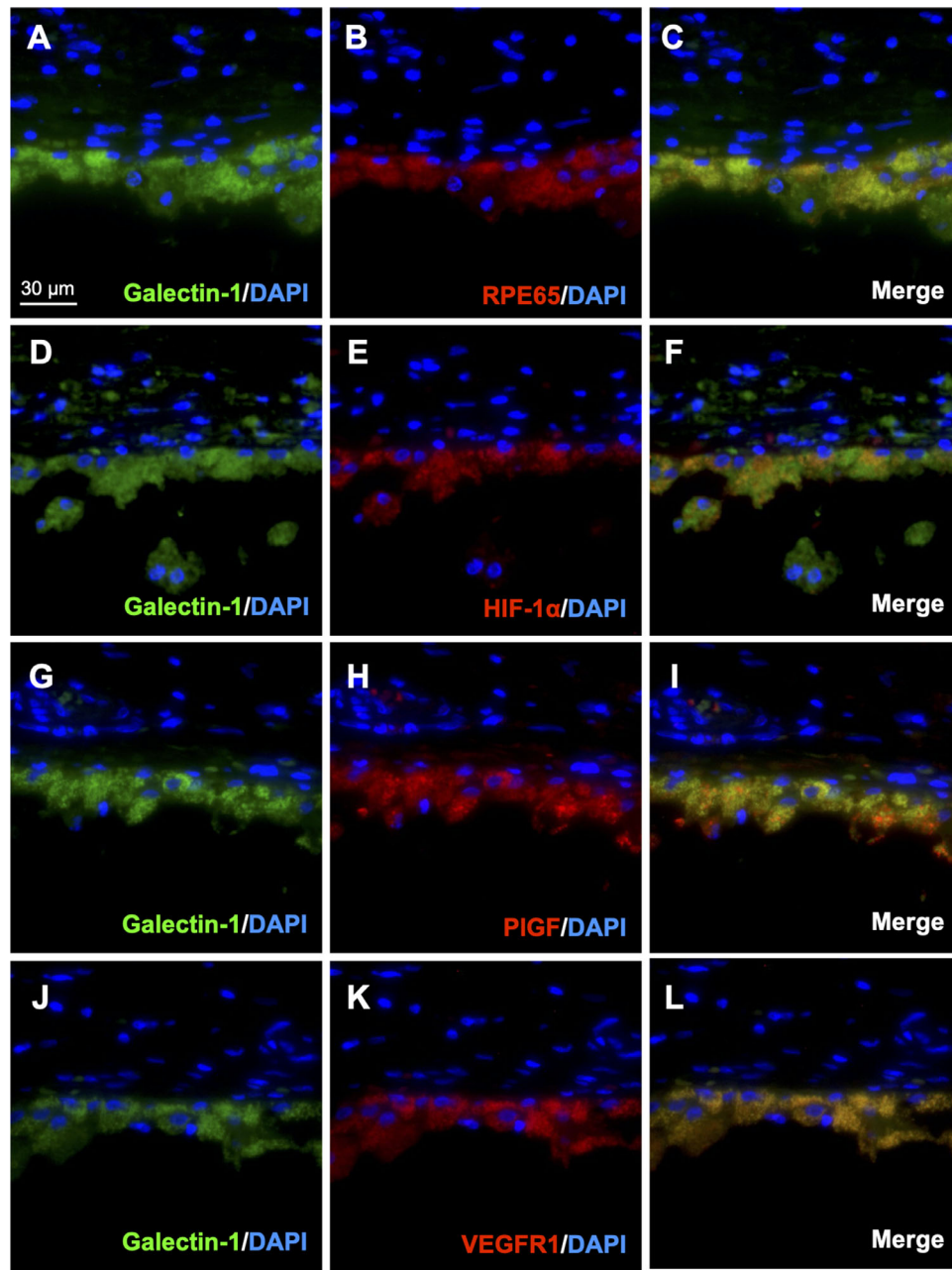


FIGURE 6. Co-localization of galectin-1 with HIF-1 α , PlGF, and VEGFR1 in RPE cells in nAMD patient specimens. (A–L) Double labeling of galectin-1 (green), RPE65 (red), and DAPI (blue) (A–C); galectin-1 (green), HIF-1 α (red), and DAPI (blue) (D–F); galectin-1 (green), PlGF (red), and DAPI (blue) (G–I); galectin-1 (green), VEGFR1 (red), and DAPI (blue) (J–L) in the nAMD patient specimens. Scale bar: 30 μ m.

The secretion of various inflammatory and angiogenic cytokines such as IL-6, PlGF, and VEGF-A from RPE cells is increased under hypoxia,^{28–30} contributing to CNV development in the pathogenesis of nAMD.³ Given that the retina consumes an exceptionally high amount of energy and that the highest blood flow in the body is supplied to the macula via the choriocapillaris fenestration,^{31,32} a decrease in choroidal blood flow can cause outer retinal hypoxia and dysfunction. Previous reports indicated the disturbance of macular choroidal blood flow in patients with CNV due to nAMD,³³ as well as the positive correlation between reduced choroidal perfusion and CNV development in nAMD patients.³⁴ Indeed, RPE cells were shown to

exhibit HIF-1 α immunoreactivity in surgically removed CNV tissues from patients with nAMD.¹⁷ These findings indicate that hypoxia is involved in the pathogenesis of nAMD. HIF-1 α protein is continually synthesized but rapidly degraded under normoxia, whereas reduced oxygen concentration results in its stabilization, translocation to the nucleus, binding with HREs, and promotion of target gene transcription. We recently demonstrated that binding of HIF-1 α to two HREs in the *LGALS1* promoter region was required for hypoxia-induced expression of galectin-1 in human Müller glial cells.¹² In our current results, this was also true with RPE cells, suggesting that hypoxia contributes to the pathogenesis of nAMD via galectin-1 induction in RPE cells.

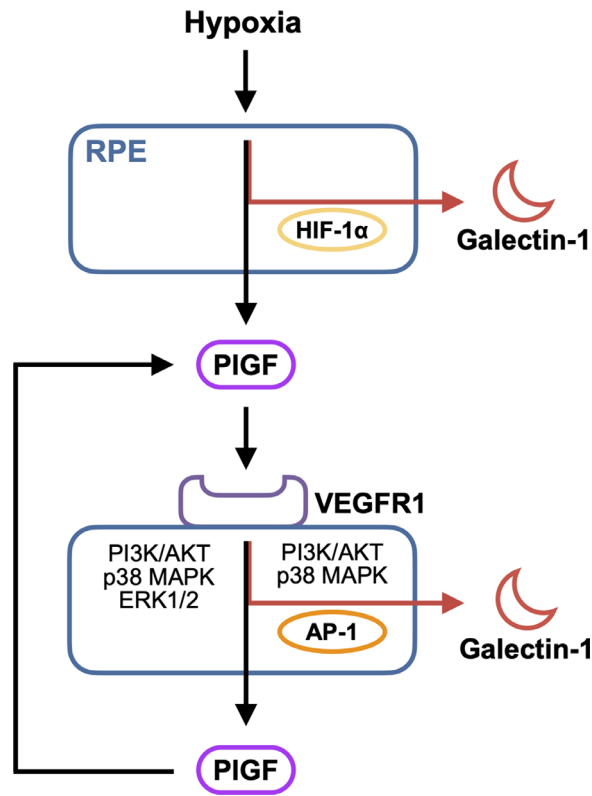


FIGURE 7. Schema showing hypoxia-induced galectin-1 expression amplified by VEGFR1-mediated autoinduction of hypoxia-induced PIGF in RPE cells. Our current data demonstrated the significance of hypoxia as a key mediator of galectin-1 expression enhanced further by VEGFR1-mediated autoinduction of hypoxia-induced PIGF in RPE cells.

PIGF, a member of the VEGF family, has proven to be associated with pathological angiogenesis in ocular diseases such as nAMD and proliferative diabetic retinopathy.^{35,36} Although its expression is low but constitutive under physiological conditions, PIGF is expressed in various tissues including the heart, lung, and adipose tissue. Previous studies revealed that PIGF was produced by RPE cells under pathological conditions,^{25,29} and that PIGF protein levels increased in the aqueous humor of nAMD patients.³⁷ Increased PIGF selectively binds to VEGFR1, which evokes various transcriptional factors such as AP-1 via the phosphorylation of intracellular signaling molecules (e.g., PI3K/AKT, p38 MAPK, ERK1/2), leading to cell survival and migration.^{38–40} AP-1 subunit proteins including ATF, c-Fos, and c-Jun form homo/heterodimers and function as transcriptional factors via binding to the specific promoter and/or enhancer regions of target genes,⁴¹ thus playing important roles in the physiological and pathological processes.^{42,43} In the current study, we revealed that PIGF-induced galectin-1/*LGALS1* expression in RPE cells was caused by PI3K/AKT- and p38 MAPK-mediated phosphorylation of AP-1 components ATF2 and c-Jun. We recently demonstrated that IL-1 β promoted galectin-1/*LGALS1* expression through the phosphorylation of PI3K/AKT and ERK1/2, followed by AP-1 binding to the AP-1 site in the *LGALS1* enhancer region in Müller glial cells.¹⁴ Interestingly, *LGALS1* expression was not altered by IL-1 β application to RPE cells (Supplementary Fig. S1B) or PIGF application to Müller glial cells (Supplemen-

tary Fig. S1C), underlining the specific cytokine-cell combinations (IL-1 β to Müller cells and PIGF to RPE cells) for galectin-1 induction via AP-1 signaling.

RPE cells derived from patient eyes with CNV due to nAMD were shown to exhibit higher VEGFR1 immunoreactivity than those of normal eyes.⁴⁴ The autocrine mechanism of PIGF has been observed in various cell species positive for VEGFR1,^{23,25,27} whereas PIGF autoinduction, in which PIGF induces its own expression in an autocrine manner, has not been fully documented in the literature. Importantly, we revealed that PIGF enhanced *PGF* mRNA expression via VEGFR1 in RPE cells, together with the detailed molecular mechanism of PIGF autoinduction through multiple pathways. Moreover, consistent with previous reports,^{29,45} we also demonstrated the enhancement of PIGF/*PGF* expression in RPE cells under hypoxia. HIF-1 α binding to HRES in the second intron of the *PGF* gene was required for hypoxic induction of PIGF in human umbilical vein endothelial cells.⁴⁶ Taken together, PIGF expression was shown to be dually regulated by hypoxia and parallelly induced PIGF itself, thus forming the vicious cycle of PIGF autoinduction in hypoxic RPE cells. PIGF activates and attracts VEGFR1-bearing macrophages,⁴⁷ and recruited macrophages generate various growth factors such as TGF- β and VEGF-A.⁴⁸ TGF- β superfamily members were shown to potently induce PIGF expression in RPE cells.²⁵ These findings, in concert with our present data on PIGF autoinduction, highlight the autocrine and paracrine regulation of PIGF in RPE cells as the amplifier of angiogenic activity. Indeed, the genetic ablation of PIGF in mice led to significant reduction in the development of laser-induced CNV.⁴⁹ Accordingly, inhibition of overproduced PIGF is theorized to suppress not only the vicious cycle of PIGF autoinduction but also galectin-1 production in RPE cells, thus reducing pathological angiogenesis and fibrosis.

This study has some limitations related especially to human tissue samples. We did not check healthy cadaver eyes to compare with the immunostaining pattern in our nAMD patient specimens. The RPE cells strongly positive for galectin-1 were similarly distributed at both central and peripheral areas (Supplementary Fig. S2), possibly due to pathogenic expansion of molecular stimuli throughout the entire globe of this particular case with massive subretinal and vitreous hemorrhages. Although our recent data revealed galectin-1 localization in migrated RPE cells within CNV tissue from the same eye,¹¹ the present study demonstrated tissue co-localization of molecules in residing RPE cells adjacent to CNV area only, which was indeed from the lack of a CNV site due to our repeated use for research.

In summary, our present findings implicate the significance of hypoxia as a key mediator of galectin-1/*LGALS1* expression in RPE cells and autoinduction of hypoxia-induced PIGF as a pathogenic cue to amplify the pathogenesis of nAMD. Future studies are necessary to further verify the potential validity of inhibiting galectin-1 and/or PIGF in patients with nAMD.

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