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# PDGF stimulation of Müller cell proliferation: Contributions of c-JNK and the PI3K/Akt pathway

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ABSTRACT

Platelet-derived growth factor (PDGF) has a critical role in proliferative vitreoretinopathy (PVR) as a chemoattractant and mitogen for retinal pigment epithelial cells and retinal glial cells. Here, we investigated the potential effects of PDGF on the proliferation of Müller cells and the intracellular signaling pathway mediating these changes. PDGF induced Müller cell proliferation and increased phosphorylation of the PDGF receptor (PDGFR), as shown by an MTT assay and immunoprecipitation analyses. Both effects were blocked by JNJ, a PDGFR-selective tyrosine kinase inhibitor. PDGF also stimulated phosphorylation of c-JNK and Akt. PDGF-induced Müller cell proliferation was significantly reduced by pre-treatment with SP600125 and LY294002, inhibitors of c-JNK and Akt phosphorylation, respectively. Our findings collectively indicate that PDGF-stimulated Müller cell proliferation occurs via activation of the c-JNK and PI3K/Akt signaling pathways. These data provide useful information in establishing the role of Müller cells in the development of proliferative vitreoretinopathy.



## Introduction

Proliferation of Müller cells is a common feature of several diseases of the sensory retina, including proliferative vitreoretinopathy (PVR). Müller cells proliferate continuously, migrate to the retinal surface, and participate in the formation of periretinal cellular membranes in PVR [1–3]. Stimulation of insulin-like growth factor-1 (IGF-1) and/or platelet-derived growth factor (PDGF) synthesis in cultured human Müller cells causes collagen gel contraction *in vitro*, indicating their role in the formation of PVR proliferative membranes [4,5].

Platelet-derived growth factor (PDGF), a cytokine with various functions, is primarily isolated from platelets and consists of four peptides, specifically, A, B, C, and D chains (AA, AB, BB, CC, or DD dimers) [6]. PDGF-BB is the predominant isoform capable of binding both PDGFR-α and -β, thereby activating the intracellular signaling pathway. In general, PDGF facilitates cell migration, survival and proliferation throughout the body [7,8] and has a critical role in PVR as a chemoattractant and mitogen for both RPE and retinal glial cells [9]. PGDF has been identified in PVR membranes, together with other growth factors, and is believed to affect membrane development [10–12]. Upregulation of PDGF and its receptors has been described in proliferative retinal diseases [11,13]. In a rabbit model, the PDGF-α receptor has been implicated in the generation of PVR, and its inhibition attenuates experimental PVR [12,14]. In addition, a recent report has shown that transactivation of PDGF and epidermal growth factor (EGF) receptor tyrosine kinases mediates mitogenic signaling in Müller cells [15]. Ligand-bound growth factor receptors undergo autophosphorylation, resulting in the activation of multiple intracellular signaling cascades. The extensively characterized PDGF intracellular signaling cascade involves mitogen-activated protein (MAP) kinases and phosphatidylinositol 3 (PI3)-kinase/Akt, together with several other kinases [16–18].

Although increasing evidence indicates critical roles for Müller cells and PDGF in the development of PVR, the underlying mechanisms remain to be determined. In this study we demonstrated that PDGF-BB induces the proliferation of rat Müller cells at early passages, mimicking PVR conditions *in vivo*. We also examined whether PDGF uses a pro-survival signaling pathway to mediate cellular proliferation in cultured rat Müller cells.

## Materials and methods

Experimental animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

*Müller cell culture.* Four-week-old Brown Norway rats were used. Müller cells were extracted and cultured according to the

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modified method of Hicks [19]. Briefly, rats were killed by cervical dislocation, and their eyes were rapidly enucleated into Dulbecco's modified Eagle's medium (DMEM), containing 2 mM glutamine and 1:1000 penicillin/streptomycin, and stored overnight at 37 °C in the dark. Intact globes were incubated in 0.1% (w/v) trypsin at 37 °C for 60 min. Globes were placed in a Petri dish containing DMEM supplemented with 10% (v/v) fetal bovine serum (FBS). Retinae were removed and either mechanically dissociated into small aggregates using a sterile Pasteur pipette or chopped into 1 mm fragments to be seeded into 10 cm Falcon culture dishes, with six to eight tissue specimens per dish. After 2–4 days, cultures were washed extensively with medium until only strongly adherent purified flat cell populations remained in the culture dishes. Cells on the dish surfaces proliferated rapidly, becoming fully confluent within 6–8 days. Cultures were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. The culture medium was changed three times per week until cells were fully confluent. When a confluent monolayer of primary culture cells was obtained, cells were incubated with 0.05% (w/v) trypsin and 5 mM EDTA at 37 °C for 5 min and transferred to 100-mm culture dishes containing DMEM with 10% (v/v) FBS. Cells that maintained proliferative potential and uniform morphology between the second and third passages were used for further analyses.

*Immunocytochemistry.* Müller cells attached to chamber slides were washed and fixed in 4% (v/v) formaldehyde for 5 min at room temperature. Cells were permeabilized by 5 min of treatment with 0.05% (v/v) Triton X-100 in phosphate-buffered saline (PBS). Cells were incubated overnight at 4 °C with the appropriate primary antibodies (Table 1), diluted 1:100 in PBS, and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA) diluted 1:100 in PBS for 2 h at room temperature in the dark. Subsequently, cells were washed three times with PBS. Cells were viewed using immunofluorescence microscopy (IX71 instrument; Olympus, Tokyo, Japan). Control experiments were simultaneously performed using PBS with anti-β3 tubulin and anti-RET-P1 (cells were negative for these markers) instead of primary antibodies being tested. Negative controls were routinely performed by incubating cells in normal buffered serum instead of primary antibody.

*Immunoprecipitation and Western blot analysis.* Total cell lysates were prepared from cultured Müller cells using lysis buffer (25 mM Hepes [pH 7.5], 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.05% [v/v] Triton X-100, 0.5 mM dithiothreitol [DTT], and 0.4 mM phenylmethylsulfonyl fluoride [PMSF]; Sigma–Aldrich, St. Louis, MO), 2 μg/ml leupeptin (Sigma–Aldrich), and 2 μg/ml aprotinin (Sigma–Aldrich). After centrifugation for 10 min at 12,000*g*, protein content was determined using a BCA assay (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard.

For PDGFR immunoprecipitation, 0.5 mg of cellular protein in 0.5 ml RIPA buffer was initially pre-cleared by incubation with protein A/G plus Agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 10 min at 4 °C, followed by centrifugation for 10 min at 14,000*g*. Monoclonal PDGFR antibody (Cell Signaling, Danvers, MA) was added to a final concentration of 1 μg/ml, and the resulting mixture was incubated overnight on a rotator at 4 °C. Immune complexes were collected by incubation with protein A/G plus Agarose for 1 h and pelleting the beads at 14,000*g*. After three more washes with RIPA buffer, immunoprecipitates were analyzed by Western blotting.

For Western blot, supernatant proteins (20 μg/lane) were subjected to SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Hybond; GE Healthcare, Piscataway, NJ). Membranes were blocked with TBST (20 mM Tris, 137 mM NaCl [pH 7.4], and 0.02% [v/v] Tween 20) containing 5% (w/v) BSA, incubated with primary antibodies diluted in TBST for 24 h at 4 °C, and washed three times with TBST.

Primary antibodies were detected by incubating the membranes with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody diluted 1:1000 in TBST for 1 h, followed by three washes in TBST. Immunoreactive proteins were visualized using chemiluminescence detection reagents (ECL; Pierce, Rockford, IL) applied to autoradiograph films. Blots were stripped and reprobed according to the manufacturer's (GE Healthcare) instructions. Anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38 MAPK, anti-phospho-Akt and anti-Akt antibodies were obtained from Cell Signaling (Danvers, MA).

*MTT (4,5-dimethyl-thiazol-2yl)-2,5-difenyltetrazolium bromide) assay.* Retinal Müller glial cells were seeded into 96-well cell culture plates. Cells were allowed to settle for 2 days in the presence or absence of PDGF-BB and inhibitors in a total volume of 200 μL per well. MTT stock solution (20 μL) (5 mg/ml in PBS; Sigma–Aldrich) was added to each well, and cells were incubated at 37 °C for 3 h. The MTT solution was discarded by gently inverting plates, and wells were filled with 200 μL dimethyl sulfoxide (DMSO). After plates were shaken vigorously for 20 min, the absorbance of each well was read using a spectrophotometric plate reader (Quant; BioTEK Instruments, Inc., Seoul, Korea), detecting emission at 560 nm.

*Statistical analysis.* Data are presented as means ± SD. Statistical comparisons between groups were performed using one-way ANOVA, followed by the LSD multiple range test of significance. Statistical significance was accepted at $P < 0.05$.

## Results and discussion

### *Primary cultures of Müller cells*

Rat Müller cells initially presented as cellular aggregates, single cells, and debris. After 2–3 days, numerous bipolar spindle-shaped cells were observed on culture dishes. As these cells proliferated, they became increasingly flattened, showing epithelioid morphology. Vigorous washing of cultures at this stage resulted in complete removal of both aggregated and loosely attached material, leaving only tightly adherent spindle-shaped and epithelioid cells on the culture substrata. The cells proliferated rapidly and, after an additional 5–6 days in culture, formed complete, even monolayers of epithelioid cells. Nearly all cells were positive for vimentin and GS, whereas none expressed β-tubulin or RET-P1 (culture day 15). Consequently, we confirmed successful cultures of rat Müller cells (Fig. 1).

**Table 1**
Antibodies used in the experiment.

| Antibodies | Type | Specificity | Source | Dilution |
|---|---|---|---|---|
| Vimentin | Mouse polyclonal | Astrocyte/Müller glia | Sigma | 1:200 |
| GS | Mouse monoclonal | RPE/Müller glia | Sigma | 1:500 |
| β-Tubulin | Mouse monoclonal | RPE | Covance | 1:500 |
| RET-P1 | Mouse monoclonal | Photoreceptor cell (Rod) | Sigma | 1:1000 |

GS, glutamine synthetase.

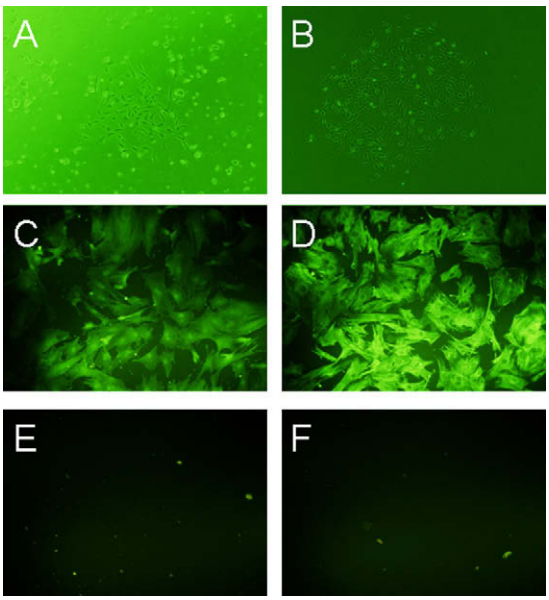


**Fig. 1.** Primary cultures of rat Müller cells examined using immunocytochemistry. (A) Phase-contrast photomicrographs of Müller cells after 5 days of culture. (B) Cells reached confluency after 7–9 days and were stained with antibodies against (C) GS, (D) vimentin, (E) Ret-P1, and (F) β-tubulin. Magnification (A, B) 40×; (C–F) 100×. Representative images from three independent experiments are shown. GS, glutamine synthetase.

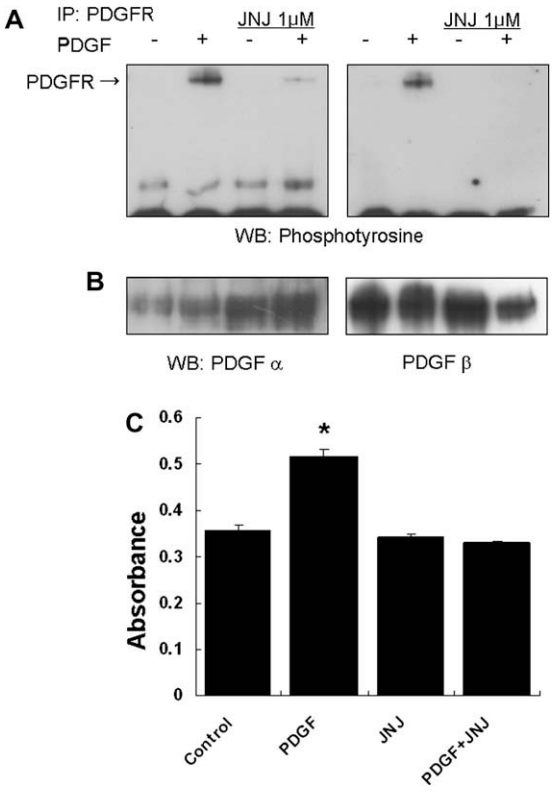


**Fig. 2.** PDGF induces rat Müller cell proliferation through PDGFR tyrosine phosphorylation. Immunoprecipitation analysis of PDGFR tyrosine phosphorylation. Müller cell suspension cultures were pre-incubated for 1 h in the presence or absence of 10 ng/ml PDGF. Cell extracts were immunoprecipitated with a PDGFR-specific antibody, and processed for Western blotting. (A) Anti-phosphotyrosine blot. (B) Anti-PDGFR blot. The Western blot in (A) was stripped and reprobed with an anti-PDGFR antibody. Representative images from three independent experiments are shown. (C) Cell proliferation was assessed using the MTT assay, with treatment of Müller cell suspension cultures with PDGF in the presence or absence of JNJ (1 μM). Data are the mean ± SD of results from three independent experiments. * indicates $P < 0.05$, compared to control.

*PDGF stimulation of PDGFR tyrosine phosphorylation and cell proliferation*

Extracts from suspension cultures exposed to PDGF-BB (10 ng/ml) were subjected to immunoprecipitation analysis. As shown in Fig. 2, an antibody specific for the receptor precipitated a single tyrosine-phosphorylated protein (Fig. 2A), which was also labeled with anti-PDGFR in Western blot assays (Fig. 2B). Treatment of Müller cell cultures with PDGF resulted in increased tyrosine phosphate content of the precipitated protein compared to that from non-stimulated cells. As shown in Fig. 2C, the MTT cell proliferation assay showed that exogenous PDGF-BB significantly increased the absorbance of treated cells, indicating increased proliferation. To assess whether this increase was dependent on protein tyrosine phosphorylation, an MTT assay was performed in the presence or absence of the selective PDGFR tyrosine kinase inhibitor JNJ. JNJ (1 μM) completely blocked the effects of PDGF on proliferation, as shown in Fig. 2C. The inhibitor (1 μM) additionally induced a substantial decrease in PDGFR tyrosine phosphorylation in immunoprecipitation and Western blot analyses (Fig. 2A, fourth lane).

*C-JNK signaling and PDGF-stimulated Müller cell proliferation*

The MAPK pathway is activated after PDGF stimulation in other cell systems [20,21]. Therefore, we examined the role of MAPK, including c-JNK, in PDGF-stimulated Müller cell proliferation. To determine the activation states of ERK1/2, JNK, and p38, we assessed phosphorylation after PDGF-BB (10 ng/ml) treatment using Western blot analysis. Staining with phospho-ERK1/2, JNK, and p38 was significantly increased following treatment with PDGF-BB (Fig. 3A). Pre-treatment of cells with inhibitors such as U0126 (MEK1/2 phosphorylation inhibitor), SP600125 (JNK phosphorylation inhibitor) or SB203580 (p38 phosphorylation inhibitor) effectively blocked PDGF-induced ERK1/2, JNK, and p38 phosphorylation. Increases in cell proliferation by PDGF, however, were significantly attenuated by SP600125, indicating that JNK is a downstream mediator of the proliferation-promoting effects of PDGF (Fig. 3B). The increase in JNK phosphorylation was also blocked by pre-treatment with JNJ, a PDGFR inhibitor (Fig. 3C).

*PI3K/Akt signaling and PDGF-stimulated Müller cell proliferation*

We also examined the role of PI3K/Akt in PDGF-stimulated Müller cell proliferation using LY294002, a selective inhibitor of the catalytic subunit of PI3K. Data from Western blot analyses to determine the phosphorylation state of Akt, one of the major downstream targets of the PI3K pathway, are shown in Fig. 4A. Staining with the phospho-Akt antibody was significantly increased after PDGF-BB (10 ng/ml) treatment, and pre-treatment of cells with LY294002 effectively blocked PDGF-induced Akt phosphorylation. LY294002 was also used to evaluate PI3K as a downstream mediator of the proliferation-promoting effects of PDGF. Increases in cell proliferation with PDGF were significantly attenuated in the presence of 50 μM LY204002 (Fig. 4B). Increases in Akt phosphorylation were also blocked by pre-treatment with JNJ, a PDGFR inhibitor (Fig. 4C).

In the present study, we investigated the effects of PDGF-BB on cultured rat Müller cells. The addition of PDGF-BB (10 ng/ml) to Müller cells induced a significant increase in PDGFR tyrosine phosphorylation, which was blocked by the PDGFR-selective inhibitor JNJ. Inhibitors suppressing receptor tyrosine autophosphorylation also blocked the effects of PDGF on Müller cell proliferation, indicating that PDGFR autophosphorylation is essential for the effects

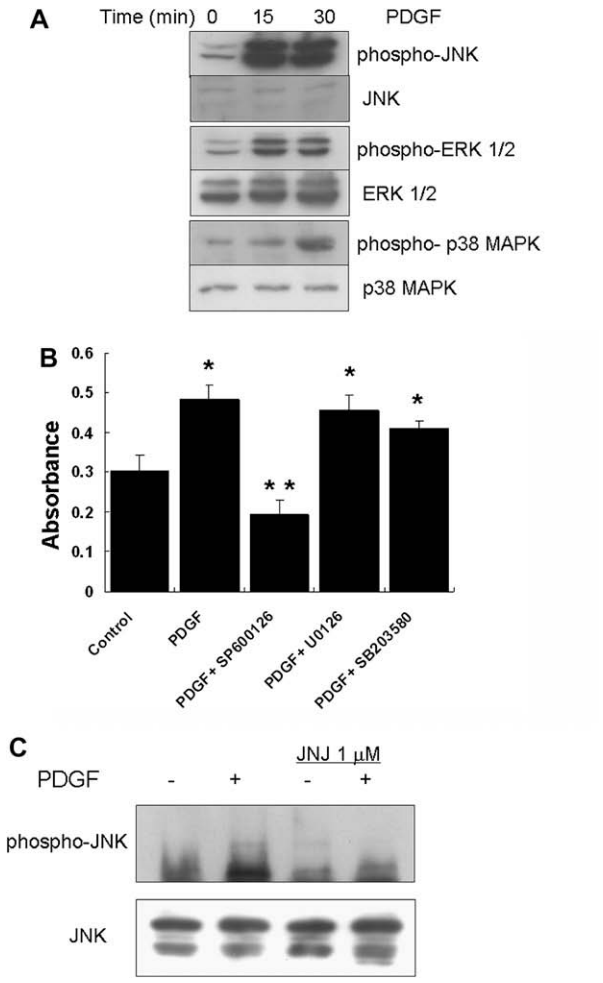


**Fig. 3.** Effects of JNK inhibition on PDGF-induced Müller cell proliferation. (A) JNK, ERK1/2, p38 kinase phosphorylation. Suspension cultures were left untreated or treated with 10 ng/ml PDGF for 15–30 min. (B) Cell proliferation. Cultures were left untreated or treated with 10 ng/ml PDGF in the presence or absence of SP 600125 (20 μM), U0126 (25 μM), or SB203580 (10 μM). Cell proliferation was assessed at 48 h using the MTT assay. Data are the mean ± SD of results from three independent experiments. * indicates $P < 0.05$, compared to control. ** indicates a significant effect of the blocker ($P < 0.05$). (C) JNK phosphorylation was assessed with Müller cell suspension cultures treated with PDGF in the presence or absence of JNJ (1 μM). Representative images from three independent experiments are shown.

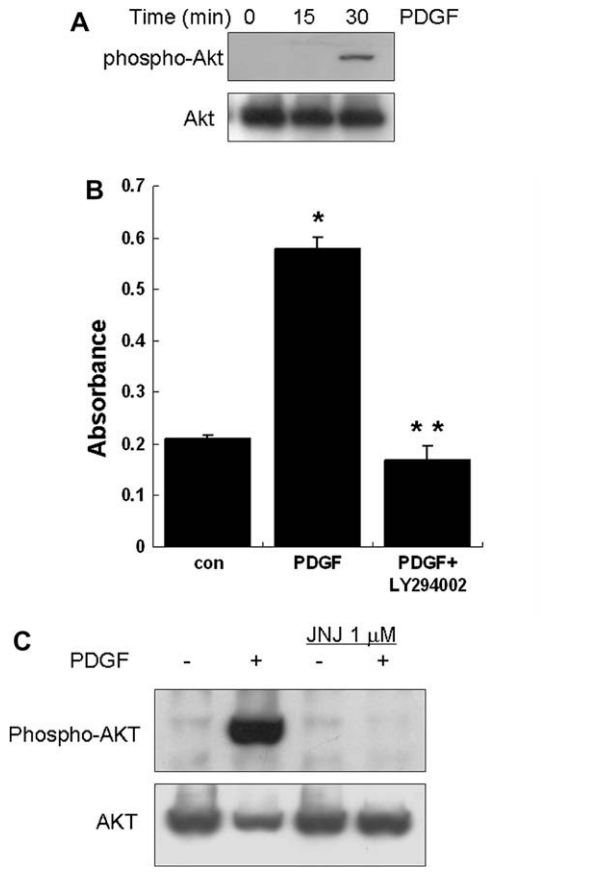


**Fig. 4.** Effects of PI3K/Akt inhibition on PDGF-induced Müller cell proliferation. (A) Akt phosphorylation. Suspension cultures were left untreated or treated with 10 ng/ml PDGF for 15–30 min. (B) Cell proliferation. Cultures were left untreated or treated with 10 ng/ml PDGF in the presence or absence of LY294002 (50 μM). Cell proliferation was assessed at 48 h using the MTT assay. Data are the mean ± SD of results from three independent experiments. * indicates $P < 0.05$, compared to control. ** indicates a significant effect of the blocker ($P < 0.05$). (C) Akt phosphorylation was assessed using Müller cell suspension cultures treated with PDGF in the presence or absence of JNJ (1 μM). Representative images from three independent experiments are shown.

of growth factors on cell proliferation. In previous reports PDGF has been identified as a major mitogen and potent inducer of growth and motility in several cell types, including fibroblasts, endothelial cells and smooth muscle cells [21–23]. Our data confirm these findings and further indicate that PDGF-BB stimulates the proliferation of Müller cells, which are the main glial cells of the retina.

To determine the intracellular signaling mechanisms responsible for the effects of PDGF on Müller cell proliferation, Western blot analyses and MTT assays were performed using specific inhibitors for intracellular signaling kinases. Our results demonstrate that both JNK and PI3K/Akt signaling pathways contribute to increased Müller cell proliferation in response to PDGF. PDGF treatment resulted in the phosphorylation of both JNK and Akt, an established target of PI3K-generated signals. Pharmacological inhibition of JNK and Akt phosphorylation antagonized PDGF-stimulated Müller cell proliferation. Proliferation was not inhibited by U0126 and SB203580, however, indicating no specific roles for ERK1/2 and p38 MAPK in Müller cell proliferation. In contrast to our observations, previous studies have shown that ERK is involved in proliferation stimulated by growth factors such as PDGF, whereas JNK is involved in stress-induced apoptosis and extension of lifespan under conditions of oxidative stress [24–27]. In addition, Milenkovic and colleagues demonstrated that PDGF-induced mitogenic effects of guinea pig Müller cells are mediated by ERK1/2 and PI3K, but not by p38 MAPK [15]. Although these results have some similarities to those of the present study in that the PI3K pathway, but not p38 MAPK, is involved in PDGF-induced Müller cell proliferation, there is a discrepancy regarding the role of ERK1/2. This could be attributed to species differences, given that primary cultures of Müller cells were obtained from guinea pigs in the experiments by Milenkovic et al., whereas Brown Norway rats were used in the current experiments [15]. On the other hand, Kang et al. demonstrated that PDGF-induced proliferation of mesenchymal stem cells is mediated by JNK, but not p38 MAPK or ERK1/2 [28]. Results from several studies focusing on the neural stem cell properties of Müller cells are supportive of this finding by Kang et al., which is consistent with the findings of the present study [29–31]. Moreover, increasing evidence indicates that JNK has a key role in survival and proliferation in a variety of cell types [28,32,33]. Overexpression of a dominant-negative JNK mutant and pharmacological inhibition of JNK blocks PDGF-induced proliferation in smooth muscle cells [16,34]. These results support our conclusion that both JNK and PI3K/Akt have crucial roles in PDGF-induced Müller cell proliferation. The PI3K/Akt pathway also mediates PDGF-induced cell

proliferation in various cell types [21,25,26,28]. Further studies are necessary to investigate the specific roles of these signaling pathways in PDGF-induced Müller cell proliferation.

PDGF-stimulated Müller cell proliferation through JNK and Akt signaling pathways was blocked by JNJ, a PDGFR-selective tyrosine kinase inhibitor, indicating that PDGFR autophosphorylation is necessary to mediate intracellular signaling. To our knowledge this is the first report to show that JNK phosphorylation is activated by PDGF and mediates the PDGF-induced proliferation of Müller cells. These results increase our understanding of the role and mechanism of action of Müller cells in the development of PVR in clinical settings and indicate that JNK and PI3K/Akt are target molecules that regulate the proliferation of Müller cells.

In conclusion, PDGF induces the proliferation of rat Müller cells *in vitro*. Both JNK and PI3K/Akt signaling pathways are essential for Müller cell proliferation in response to PDGF. These findings provide useful information regarding the role of Müller cells in PVR development.

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