• Basic Research •

Pirfenidone suppresses the abnormal activation of human Müller cells after platelet-derived growth factor-BB stimulation

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Abstract

• AIM: To determine the effect of pirfenidone on the activated human Müller cells by platelet-derived growth factor-BB (PDGF-BB).

• METHODS: The primary human Müller cells were separated from retinal tissues and established the pathogenic model by stimulated with PDGF-BB. The Müller cells behaviour of normal group and the model group was measured by MTT assay, Trypan blue assay, cell migration assay, and collagen contraction assay. The expression of transforming growth factor (TGF)-β1, -β2, and pigment epithelium-derived factor (PEDF) was estimated with real-time polymerase chain reaction (PCR), Western blot and immunofluorescence analyses.

• RESULTS: A pathogenic/proliferative model of Müller cells was established by stimulating normal cultured Müller cells with 10 ng/mL PDGF-BB for 48h. After treated with 0.2 and 0.3 mg/mL pirfenidone, the proliferation, migration and collagen contraction was statistically significantly depressed in the model group compared with the normal groups. The expression levels of TGF-β1 and TGF-β2 were significantly down-regulated, while the PEDF expression was significantly up-regulated after treated with 0.2 and 0.3 mg/mL pirfenidone in the model group.

• CONCLUSION: Pirfenidone effectively suppress the proliferation, migration and collagen contraction of the human Müller cells stimulated with PDGF-BB through down- regulation of TGF-β1/TGF-β2 and up-regulation of PEDF.

• KEYWORDS: pirfenidone; Müller cells; platelet-derived growth factor-BB; transforming growth factor-β; proliferative vitreoretinopathy
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INTRODUCTION

As one type of retinal glial cells, the Müller cells play an important role in maintaining the normal function of eye retina[1]. On one hand, the active Müller cells can protect the endothelial cells and retinal neurons from cell death under stress through secreting neurotrophic factors, growth factors, and cytokines, and promote the regeneration of retina after injury. On the other hand, the out-of-control proliferation and hypertrophy of the Müller cells in response to pathogenic stimuli would lead to gliosis and produce glial scars, which may impede the normal self-repair and remodeling of retinal tissues and may further cause neurodegeneration diseases such as proliferative vitreoretinopathy (PVR).

The platelet-derived growth factor (PDGF) is a cytokine that participates in multiple physiological processes. It consists of four specific peptides (A/B/C/D chains), which can independently form AA, BB, CC, and DD dimers. It has been reported that the PDGF is essential for the disease development of PVR[2-3]. Generally, the expression of PDGF-BB in normal retinal tissues is maintained in a low level. Whereas, when it comes to the gliosis condition, the pathogenic retinal tissues tend to express and release huge amounts of PDGF-BB, most part of which is derived from the Müller cells[2]. Moreover, it has been found that PDGF can act as chemokine and mitogen in the Müller cells and the retinal pigment epithelial cells; PDGF can induce the proliferation of Müller cells *via* activation of Jun N-terminal kinase (JNK), c-JNK and protein kinase B (Akt) signaling pathways[4].

Considering the critical role of Müller cells in the pathogenic progression of proliferative retinal diseases, the development of drugs to target Müller cells would be meaningful for clinical

treatment. In a recent study, pirfenidone [5-methyl-1-phenyl-2-(1H)-pyridone] has been found to have an inhibitory effect on the proliferation, migration and collagen contraction of the human Tenon's fibroblasts[5]. In addition, pirfenidone can also inhibit the migration, differentiation, and proliferation of the human retinal pigment epithelial cells[6]. However, it still remains unclear whether pirfenidone could have a similar inhibitory effect on the Müller cell gliosis. In this study, we established a pathogenic model by using the PDGF-BB to stimulate Müller cells *in vitro*, and explored the effect of pirfenidone on the proliferation, migration and collagen contraction potential of the model cells.

SUBJECTS AND METHODS

Ethical Approval The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethnic Committees of Kunming Medical University (KMU). All the eye retina samples from the donors were obtained with prior consent for research.

Culture of the Human Müller Cells The human Müller cells were isolated and cultured. The eye retina samples isolated from the donors were washed three times in D-Hanks buffer and then digested in collagenase type I (Sigma Aldrich, USA, C0130, final concentration: 200 U/mL) at 37℃ for 4h until the tissue samples were dispersed loosely. And then the cells were centrifuged and collected at 800 rpm/min for 5min and resuspended by Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA, 11965) containing 20% fetal bovine serum (FBS; GIBCO, USA, 16000036), penicillin (100 U/mL) and streptomycin (100 U/mL). After cell counting by Trypan blue method, 2×10^4/mL viable cells were plated in 25 mL flasks at incubator (Thermo, USA, 37℃ and 5% CO_2). After 48h, the culture medium was replaced by DMEM containing 15% FBS in order to remove the unadherent cells and debris. The cell medium was changed twice per weeks, and the cells were passaged when they are 90%-100% confluent.

Identification of the Human Müller Cells by Immunochemical Analysis To identify the human Müller cells, the expression of the glial markers involving smooth muscle α-actin (α-SMA), glial fibrillary acidic protein (GFAP) and vimentin was respectively analyzed by immunochemical method. Briefly, about 1000 cells were cultured on 13ψ sterile cover glass overnight under normal culture condition. Next, the cover glass was washed twice by phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) for 30min. Following PBS wash, the fixed cells were stained with anti-α-SMA antibody (Sigma Aldrich, USA, A5228), anti-GFAP antibody (Sigma Aldrich, USA, G3893) and anti-vimentin (Sigma Aldrich, USA, V4630) followed by the relative secondary antibodies. Finally, the cells were restained by hematoxylin until most cell nucleus could be observed obviously under microscope.

Establishment of the Pathogenic Müller Cell Model To establish the pathogenic model, the normal Müller cells were treated with 10 ng/mL PDGF-BB (GIBCO, USA, PHG0046) for 48h and then harvested for further experiments. The cells treated with PDGF-BB were named model group.

MTT Assay After cultured in 96-well plates for 24h in the incubator, the normal and model group Müller cells were respectively treated with 0, 0.01, 0.1, 0.2, 0.3, and 1 mg/mL pirfenidone (Santa Cruz, USA, 53179-13-8, dissolved in carboxymethylcellulose solution) for 6, 12, 24, 48, and 72h. The group without any treatment (added with the same volume of DMEM/10% FBS as the pirfenidone solution) was performed as blank control, while the group treated with the drug vehicle was performed as negative control. All these groups were performed in triplicate and tested by MTT kit (Sigma Aldrich, USA, 11465007001). The absorbance of each group was examined at 570 nm light wave. The cell proliferation rate was determined by optical density (OD; treatment group)/OD (blank group). The optimal treatment concentration (IC50) was calculated for further experiments.

Trypan Blue Dyeing Assay After treatment by the optimal pirfenidone concentration at the optimal time point, the cells were harvested and stained by Trypan blue (9 drops of cell suspension with 1 drop of Trypan blue). The numbers of the vital and the dead cells were counted under microscope. The cell death rate was calculated by the number of the dead cells/the number of the total cells. The group without any treatment was performed as blank control, while the group treated with the drug vehicle was performed as negative control.

Cell Migration Assay (Wound Healing Assay) The normal and model group cells were cultured in 6-well plates. When the cells grew into 90%-100% confluent, serum was deprived for 24h. Then, the medium was removed, and 100 μL pipette tips were used to scratch vertically in each well. Next, the fall-off cells were washed by PBS twice. After that, the whole culture medium containing the optimal concentration of pirfenidone was added. The cells and the wound healing status were observed and photographed under microscope at different time points.

Collagen Contraction Assay The collagen gel was thawed and added equally into 24-well plates. After 30min sterilization under ultraviolet light, the plates were placed in the incubator for 2d. They should be washed by saline solution twice and re-sterilized under ultraviolet for 30min before use.

Both the normal and model group cells were divided into active group and inactive group (control group). The inactive groups were deprived from serum for 60h. The active groups were cultured in the whole medium. After digested by trypsin-ethylenediaminetetraacetic acid (EDTA; 0.25% trypsin, 2% EDTA), cells of the normal group and the model group were

centrifuged and suspended into 5×10^5 cells/mL. Then, the cell suspension was added by the collagen gel and mixed with the total volume of 250 μL. The cells-collagen mixture was plated into 24-well plates, followed by treatment with the optimal concentration of pirfenidone (0.2 and 0.3 mg/mL) for 7d. The groups treated with 0 mg/mL pirfenidone were performed as controls. The medium was changed every 3d using the medium containing relative concentration of pirfenidone. The status of gel contraction was observed, and the surface areas of the collagen were recorded every day. Data at day 3 and day 7 were shown.

Immunofluorescence Examination The cells of the normal the model groups were cultured on the cover glass and treated with 0, 0.2, and 0.3 mg/mL pirfenidone, respectively. Then, the glass were fixed in 4% PFA and stained by anti-transforming growth factor-β1 (anti-TGF-β1) antibody (Boster, USA, A04630), anti-transforming growth factor-β2 (anti-TGF-β2) antibody (Boster, USA, A00892) and anti-pigment epithelium-derived factor (PEDF) antibody (Abcam, ab233120). Anti-rabbit IgG antibody with fluorescent dye was used as secondary antibody. DAPI was used to stain the nucleus. Cells of different groups were observed and the fluorescent images were recorded by confocal microscopy (Leica TCS SP5, Leica Microsystems, Exton, PA, USA).

Quantitative Analysis of Cytokines and Growth Factors by Real-time Polymerase Chain Reaction The total mRNA was extracted using the TRIzol-chloroform method (TRIzol, Invitrogen, USA, 15596026). The cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, USA, K1622). The primers for the human TGF-β1 (NM_000660.4), TGF-β2 (NM_000660.4), PEDF (NM_002615.5) and β-actin (NM_002615.5) gene were designed by Primer 5.0 software.

Primer sequences are as followed: TGF-β1, forward: 5'-ACAATTCCTGGCGATACCTCAGCA-3', reverse: 5'-CGCTAAGGCGAAAGCCCTCAATTT-3' [polymerase chain reaction (PCR) product 158 bp]; TGF-β2, forward: 5'-TGCAGAGAGATGTTTGCACCATGC-3', reverse: 5'-CGCTAAGGCGAAAGCCCTCAATTT-3' (PCR product 212 bp); PEDF, forward: 5'-TGATGTC GGACCCTAAGGCTGTTT-3', reverse: 5'-ATGAATGAAC TCGGAGGTGAGGCT-3' (PCR product 193 bp); β-actin, forward: 5'-AGTTGCGTTACACCCTTTCTTG-3', reverse: 5'-TCACCTTCACCGTTCCAGTTT-3' (PCR product 402 bp).

The real-time PCR was performed using Maxima® SYBR Green/ROX qPCR Master Mix (2×) (Thermo Scientific Fermentas, USA, K0221). The absorbance was monitored by ABI Prism 7300.

Western Blotting Briefly, the cells of the pirfenidone treated groups and the control group were harvested and lysed in 1×SDS loading buffer, and boiled at 97℃ in a water bath for 10min. The samples were then resolved and separated in 10% SDS-PAGE and then transferred electronically into PVDF membrane. The expression of TGF-β1, TGF-β2, PEDF, and β-actin was respectively tested by anti-TGF-β1 antibody (Boster, USA, A04630), anti-TGF-β2 antibody (Boster, USA, A00892), anti-PEDF antibody (Abcam, USA, ab233120) and anti-β-actin antibody (Invitrogen, USA, PA1183). Anti-rabbit IgG antibody (Invitrogen, USA, A11034) was used as secondary antibody.

Statistical Analysis All experiments were conduct in triplicate. Quantitiy One (Bio-Rad) and Graphpad Prism 6.0 software were used to analyze the data. All data were shown as mean±standard deviation. One-way analysis of variance (ANOVA) test was used to compare the difference between the groups. $P<0.05$ stands for having a significant statistical difference.

RESULTS

Müller Cell Culture and Identification As shown in Figure 1A, the Müller cells present spindle shape and dense granules in the cytoplasm. It has been reported that the Müller cells express ubiquitous level of GFAP, high level of intermediate filament vimentin, and low level of α-SMA[7]. To determine the Müller cells used in our following study, we accordingly detected the expression of these markers by means of immunochemical analysis. As expected, it was observed that the α-SMA expression is nearly negative, while the GFAP and vimentin have strong expression in the cells (Figure 1B), suggesting that the cells we obtained are indeed Müller cells.

Pathogenic Müller Cell Model Establishment To further establish a pathogenic/proliferative model of Müller cells, we treated the normal Müller cells with 10 ng/mL PDGF-BB for 48h (model group). The Müller cells without treatment was set as control (normal group). In our observation, there is no evident difference in the cell morphology between the normal group and the model group, but the proliferation rate of the model group is significantly higher than the normal group ($P<0.0001$), similar to the pathogenic gliosis as reported in previous studies[4].

Inhibitory Effect of Pirfenidone on Pathogenic Müller Cell Model To determine whether pirfenidone has inhibitory effect on Müller cells, we treated the cells of the normal group and the model group with gradient concentrations of pirfenidone (0, 0.01, 0.2, 0.3 and 1 mg/mL) respectively for 6, 12, 24, 48 and 72h. The cell viability of different groups was examined by MTT assay. Our results showed that pirfenidone does not have obvious inhibitory effect on the normal groups, but significantly suppress the viability of the model group cells

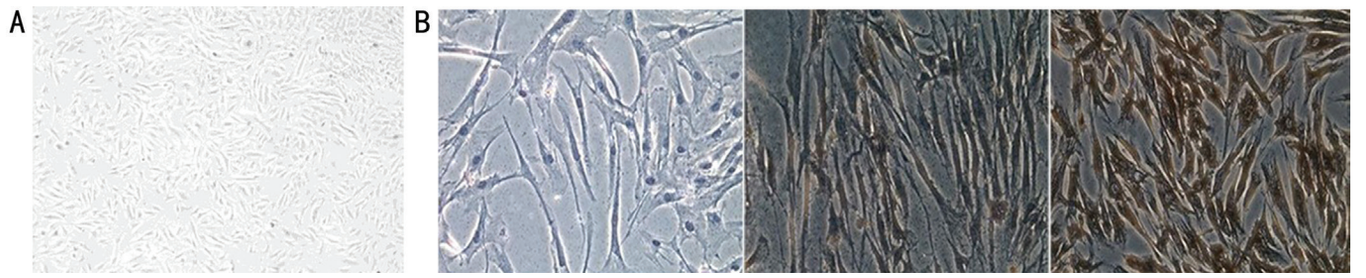

Figure 1 The human Müller cells were identified according to the expression of α-SMA, GFAP and vimentin A: The morphology of the Müller cells were observed in white light visual field (10×); B: The expression of α-SMA, GFAP and vimentin in the Müller cells was detected by immunochemistry, and photographed in white light visual field (20×).

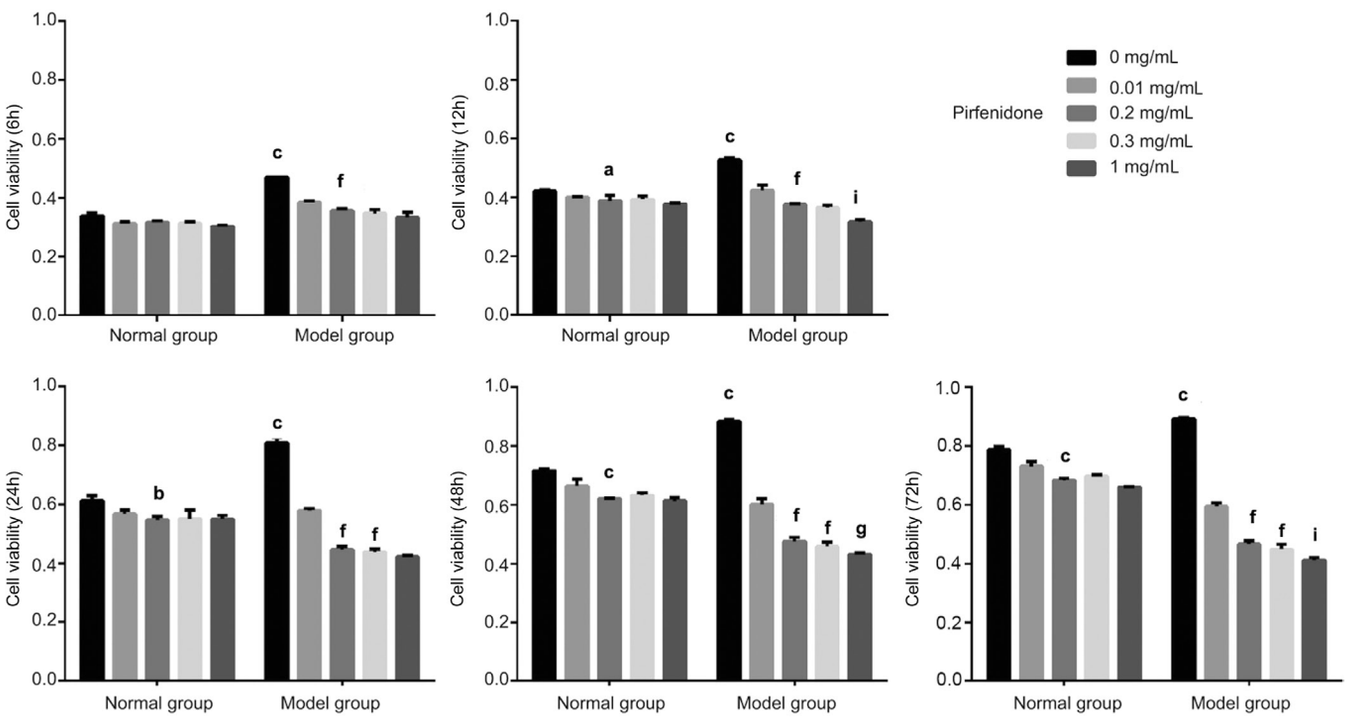

Figure 2 The pirfenidone shows stronger inhibitory effect on the model group than the normal group The cells of normal group and model group were respectively treated with 0, 0.01, 0.1, 0.2, 0.3, 0.5 and 1 mg/mL pirfenidone for 6, 12, 24, 48, and 72h. The cell viability was determined by MTT assay. [a]P<0.05 *vs* normal+0 mg/mL pirfenidone group, [b]P<0.01 *vs* normal+0 mg/mL pirfenidone group, [c]P<0.0001 *vs* normal+0 mg/mL pirfenidone group; [d]P<0.05 *vs* model+0 mg/mL pirfenidone group, [e]P<0.01 *vs* model+0 mg/mL pirfenidone group, [f]P<0.0001 *vs* model+0 mg/mL pirfenidone group; [g]P<0.05 *vs* model+0.2 mg/mL pirfenidone group, [h]P<0.01 *vs* model+0.2 mg/mL pirfenidone group, [i]P<0.0001 *vs* model+0.2 mg/mL pirfenidone group.

(Figure 2). The 0.2 and 0.3 mg/mL dosages of pirfenidone at 24h after treatment have shown prominent inhibitory effect on the model group with significant difference compared with 0 mg/mL dosage (P<0.0001; Figure 2), and this effect does not change obviously over time (24h compared with 48h and 72h after treatment; Figure 2). Thus, we determined the optimal condition of pirfenidone treatment for the next functional verification (0.2 and 0.3 mg/mL for 24h).

Toxicology of Pirfenidone We further used the Trypan blue assay to determine the cell death rates of the normal group and the model group after treatment with 0, 0.2, and 0.3 mg/mL dosages of pirfenidone, respectively. Consistent with the MTT assay, pirfenidone shows a more significant effect on the cell death of the model group than the normal group (model group+0.2 mg/mL pirfenidone *vs* model group+0 mg/mL pirfenidone, P<0.05; model group+0.3 mg/mL pirfenidone *vs* model group+0 mg/mL pirfenidone, P<0.0001, Figure 3A). In comparison, the treatment of pirfenidone shows no significant difference with the non-treatment samples in cell death rate of the normal group (Figure 3A).

Effect of Pirfenidone on Cell Collagen Contraction and Migration Pirfenidone shows a more significant inhibition on the cell collagen contraction capability at both day 3 and day 7 (model group+0.2 mg/mL pirfenidone *vs* model group+0 mg/mL pirfenidone, P<0.01 at day 3 and P<0.0001 at day 7; model group+0.3 mg/mL pirfenidone *vs* model group+

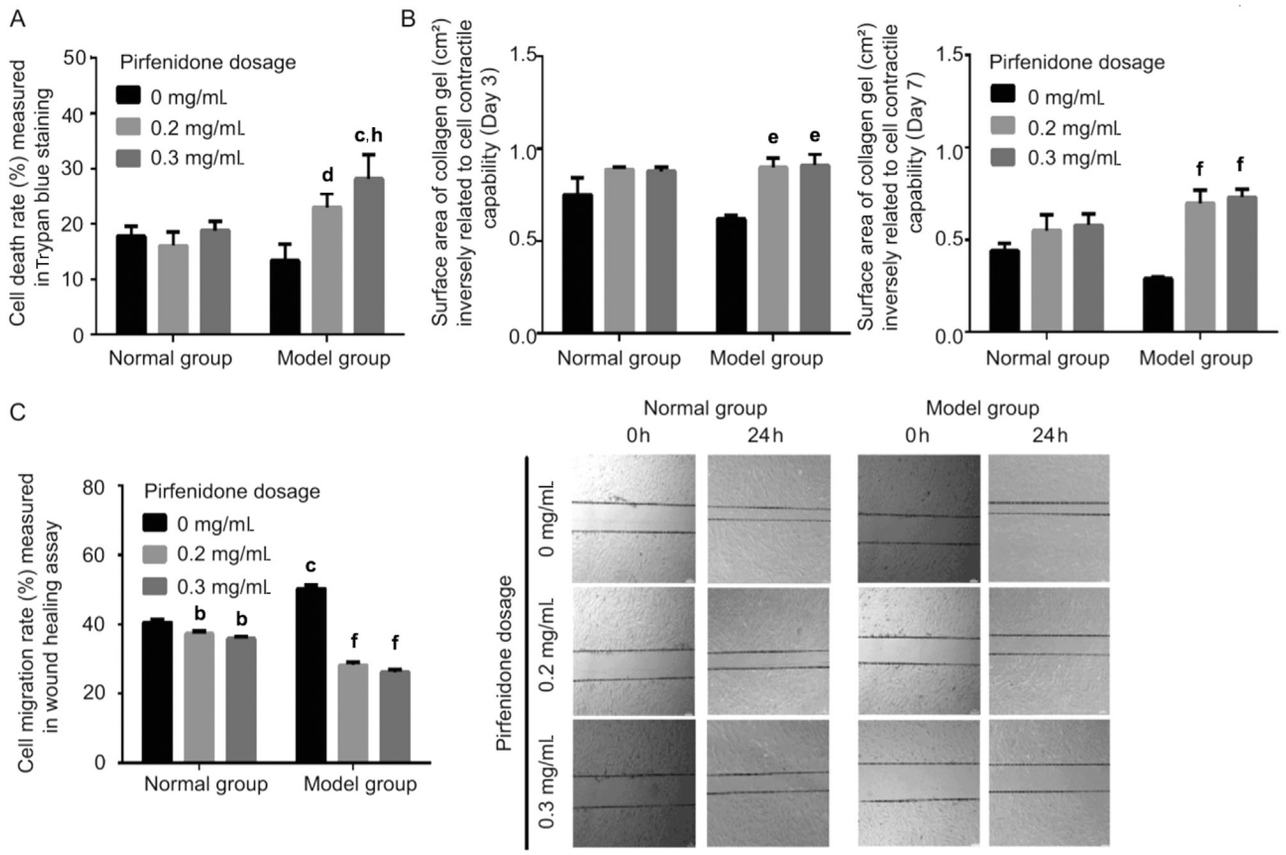

Figure 3 The pirfenidone shows stronger inhibitory effect on the potential of cell contraction and migration of the model group than the normal group The cells of the normal group and the model group were respectively treated with 0, 0.2, 0.3 mg/mL pirfenidone. A: The cell death rate was determined by Trypan blue method; B: The cell contraction potential, which is inverse to the surface area of the collagen gel, was determined by collagen gel-based cell contraction assay; C: The cell migration potential was determined by wound healing assay. [a]P<0.05 *vs* normal+0 mg/mL pirfenidone group, [b]P<0.01 *vs* normal+0 mg/mL pirfenidone group, [c]P<0.0001 *vs* normal+0 mg/mL pirfenidone group; [d]P<0.05 *vs* model+0 mg/mL pirfenidone group, [e]P<0.01 *vs* model+0 mg/mL pirfenidone group, [f]P<0.0001 *vs* model+0 mg/mL pirfenidone group; [g]P<0.05 *vs* model+0.2 mg/mL pirfenidone group, [h]P<0.01 *vs* model+0.2 mg/mL pirfenidone group, [i]P<0.0001 *vs* model+0.2 mg/mL pirfenidone group.

0 mg/mL pirfenidone, P<0.01 at day 3 and P<0.0001 at day 7, Figure 3B). In comparison, the treatment of pirfenidone shows no significant difference with the non-treatment samples in the collagen contraction capability of the normal group (Figure 3B). In addition, we further examined the effect of pirfenidone on the cell migration of the normal group and the model group by would healing assay. It shows that pirfenidone has a more prominent inhibitory effect on the cell migration potential of the model group compared with the normal group (Figure 3C).

Effect of Pirfenidone on TGF-β1/ β2 and PEDF Expression in Pathogenic Müller Cell Model It has been previously reported that TGF-β can induce the fibrosis and migration of the Müller cells[7], while PEDF has neuroprotective function and angioinhibitory effect on retina[8]. To further determine whether the inhibitory effect of pirfenidone acts through regulation of these important signaling factors, we firstly analyzed the mRNA levels of TGF-β1, TGF-β2 and PEDF by real-time PCR. It shows that in the model groups the mRNA levels of TGF-β1 and TGF-β2 are significantly down-regulated in the samples treated with pirfenidone compared with those without any treatment (P<0.0001), while in the normal group the difference is not significant (Figure 4A). However, in the model group the mRNA levels of PEDF in the samples treated with pirfenidone are significantly up-regulated compared with the samples without any treatment (P<0.0001), while in the normal group the difference is not significant (Figure 4A).

We further determined the protein level change of these factors by Western blotting and immunofluorescence (Figure 4B, 4C). Consistent with the change of the mRNA levels, we found that the expression levels of TGF-β1 and TGF-β2 are obviously down-regulated; while the expression levels of PEDF are up-regulated after treatment with pirfenidone in the model group Müller cells (Figure 4B, 4C).

DISCUSSION

PVR is a leading cause of vision loss following retinal detachment, retinal reattachment surgery, and ocular trauma. The pathogenesis of PVR is currently understood as migration and proliferation of retinal pigment epithelium and Müller cells

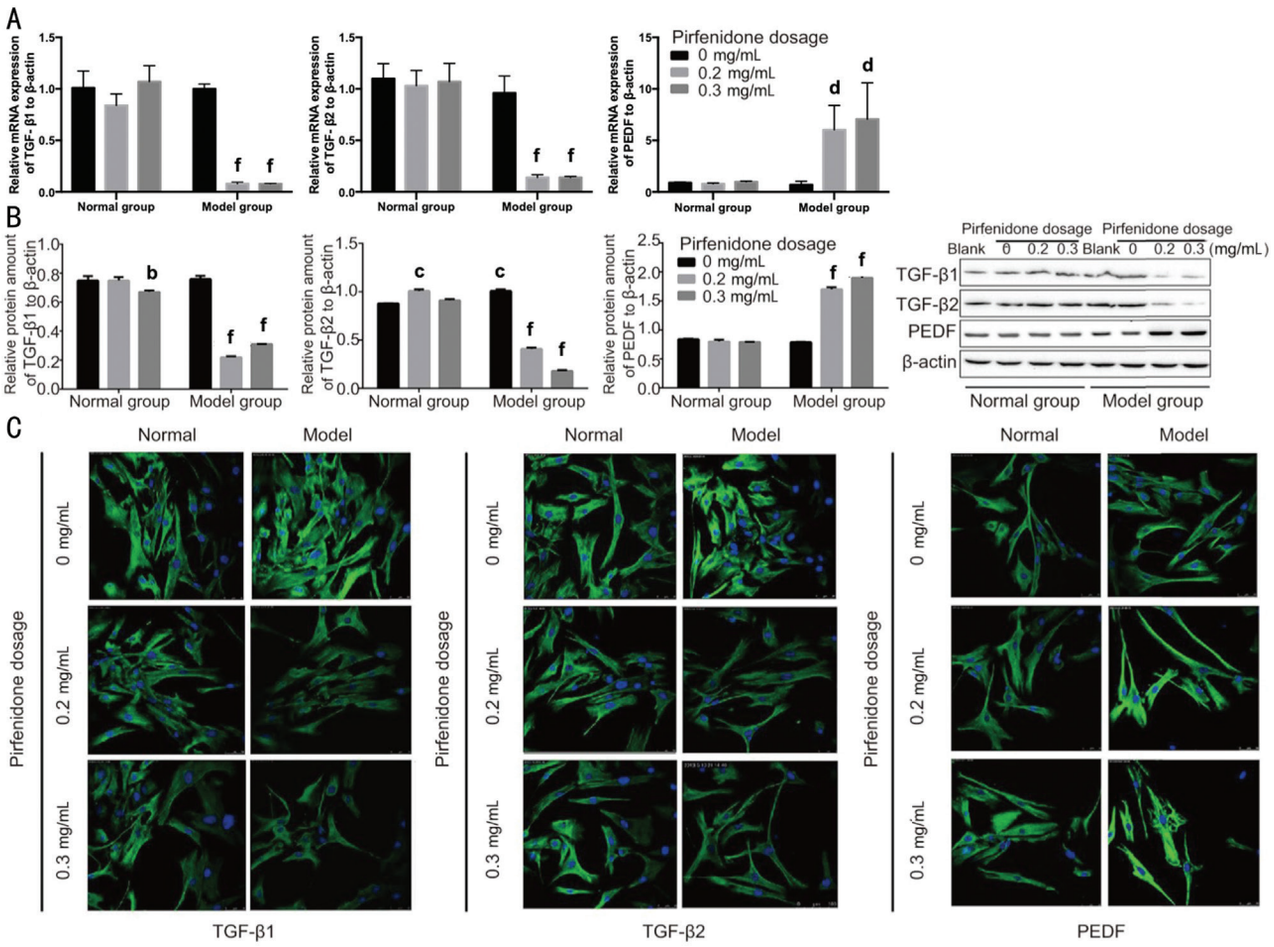

Figure 4 The expression levels of TGF-β1, TGF-β2 and PEDF are obviously regulated after treatment by pirfenidone in the model group

A: The cells of the normal group and the model group were respectively treated by 0, 0.2 and 0.3 mg/mL pirfenidone. The relative mRNA levels of TGF-β1, TGF-β2 and PEDF were determined by real-time PCR. Results are shown as indicated. The expression levels of TGF-β1, TGF-β2 and PEDF under the indicated treatment of pirfenidone were detected by Western blotting (B) and immunofluorescence (C). Green fluorescence, for the indicated proteins; blue fluorescence for the nucleus. [a]P<0.05 *vs* normal+0 mg/mL pirfenidone group, [b]P<0.01 *vs* normal+0 mg/mL pirfenidone group, [c]P<0.0001 *vs* normal+0 mg/mL pirfenidone group; [d]P<0.05 *vs* model+0 mg/mL pirfenidone group, [e]P<0.01 *vs* model+0 mg/mL pirfenidone group, [f]P<0.0001 *vs* model+0 mg/mL pirfenidone group; [g]P<0.05 *vs* model+0.2 mg/mL pirfenidone group, [h]P<0.01 *vs* model+0.2 mg/mL pirfenidone group, [i]P<0.0001 *vs* model+0.2 mg/mL pirfenidone group.

along with synthesis of extracellular matrix proteins[9-10]. Since PVR is a complex disease involving cell proliferation, growth, spreading, and contractility, no effective pharmacological treatment has been found to date[11].

Pirfenidone, as an anti-fibrotic agent, has been found the prevention effect of PVR in a post-traumatic PVR animal model[12]. However, the molecular mechanism of pirfenidone in suppressing PVR formation is not clear. And the effect of pirfenidone on human Müller cells was still unknown. In this study, we used the Müller cells stimulated with PDGF-BB, a pathogenic gliosis model, to answer this question for the first time. We treated the Müller cells of normal group and model group with pirfenidone respectively, and revealed that pirfenidone can significantly suppress the proliferation, migration and collagen contraction potential of the model group Müller cells.

In previous reports, PDGF has been found as a mitogen and potent inducer of growth and motility in several cell types, including fibroblasts, endothelial cells and smooth muscle cells[13]. The PDGF plays a critical role in the development of proliferative retinopathies[14-16]. It can promote the proliferation and chemotaxis of the Müller cells[4], which is similar to the gliosis pathological condition. Considering this, we used PDGF-BB to stimulate the normal Müller cells to establish the pathological model. In our experiments, we stimulated the Müller cells with PDGF-BB for 48h and removed it for the following treatment of pirfenidone. Despite the lack of the exogenous PDGF-BB, the endogenous PDGF produced by Müller cells themselves may maintain the stimuli effect according to the previous report[2], but the self-feedback loop still needs further verification. In the previous studies, pirfenidone can inhibit the proliferation, migration and collagen

contraction of the human Tenon's fibroblasts, and also inhibit the migration, differentiation, and proliferation of the human retinal pigment epithelial cells[5-6]. The inhibitory effects are similar to the observation of the model group Müller cells in our study.

Furtherly, we explored the mechanism of this effect on the model group cells. According to the previous reports, TGF-β can induce the fibrosis and migration of the Müller cells[7,17]. Also, the TGF-β1 derived from the Müller cells themselves contributes to the inhibitory effect on the proliferation of retinal endothelial cells, which is one of the morphologies of PVR[18]. In addition, TGF-β1 can up-regulate the expression of α-SMA stress fibers in retinal Müller cells and fibroblasts that has a cell-specific effect on intracellular collagen expression[19]. All of these above suggest that TGF-β has an important role in the pathogenesis of PVR. On the other hand, PEDF has neuroprotective function and angioinhibitory effect on retina[8,20]. Evidences indicated that sustained PEDF expression not only can help to protect the retinal pigment epithelium cells by creating an anti-angiogenic environment under hypothermia[21], but also can improve the mitochondrial function in retinal pigment epithelium cells during oxidative stress[22-25]. Thus, the down-regulation of TGF-β1/TGF-β2 and the down-regulation of PEDF may contribute to the inhibitory effect of pirfenidone on the model group cells. Previous study demonstrated that PDGF induced the proliferation of rat Müller cell through activating JNK and PI3K/Akt signaling pathways[4]. It suggested that other signaling factors could also participate in the effect of pirfenidone on the model group Müller cells. Further investigation into the signaling pathway of pirfenidone is still needed in the future.

In conclusion, we found that pirfenidone can effectively suppress the proliferation, migration and collagen contraction of the human Müller cells stimulated with PDGF-BB through down-regulation of TGF-β1/TGF-β2 and upregulation of PEDF. Pirfenidone would be a potential candidate drug to target the pathogenic Müller cells in the proliferative retinopathies.

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