

# Pirfenidone Attenuates the IL-1 $\beta$ -Induced Hyaluronic Acid Increase in Orbital Fibroblasts From Patients With Thyroid-Associated Ophthalmopathy

Seung Ah Chung,<sup>1</sup> Bo Kyung Jeon,<sup>2</sup> Youn-Hee Choi,<sup>2</sup> Keum Ok Back,<sup>1</sup> Jong Bok Lee,<sup>3</sup> and Kounghoon Kook<sup>1</sup>

<sup>1</sup>Department of Ophthalmology, Ajou University School of Medicine, Suwon, Korea

<sup>2</sup>Department of Physiology, Tissue Injury Defense Research Center, Ewha Womans University School of Medicine, Seoul, Korea

<sup>3</sup>The Institute of Vision Research, Department of Ophthalmology, Yonsei University College of Medicine, Seoul, Korea

Correspondence: Kounghoon Kook, Department of Ophthalmology, Ajou University School of Medicine, San 5, Woncheon-dong, Yeongtong-gu, Suwon 443-721, Korea; drkook@ajou.ac.kr

SAC and BKJ contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: December 11, 2013

Accepted: March 3, 2014

Citation: Chung SA, Jeon BK, Choi YH, Back KO, Lee JB, Kook KH. Pirfenidone attenuates the IL-1 $\beta$ -induced hyaluronic acid increase in orbital fibroblasts from patients with thyroid-associated ophthalmopathy. *Invest Ophthalmol Vis Sci*. 2014;55:2276-2283. DOI:10.1167/iov.13-13759

**PURPOSE.** This study aimed to investigate the effect of pirfenidone on the IL-1 $\beta$ -induced hyaluronic acid (HA) increase in orbital fibroblasts from patients with thyroid-associated ophthalmopathy (TAO).

**METHODS.** Primary cultured orbital fibroblasts were obtained from patients with TAO, and the excreted levels of HA from IL-1 $\beta$ -treated cells with or without pirfenidone were measured. The effect of pirfenidone on IL-1 $\beta$ -induced hyaluronic acid synthase (HAS) expression was evaluated. The relevance of the mitogen-activated protein kinase (MAPK)-mediated signaling pathway in IL-1 $\beta$ -induced HAS expression was assessed using specific inhibitors to p38, extracellular signal-regulated kinase (ERK), or c-Jun N-terminal kinase (JNK). The phosphorylation level of each MAPK in IL-1 $\beta$ -treated cells with or without pirfenidone and the level of AP-1 DNA binding were measured. The inhibitory potency of pirfenidone on HA production was evaluated using dexamethasone as a reference agent.

**RESULTS.** Pirfenidone strongly attenuated the IL-1 $\beta$ -induced HA release in a dose-dependent manner. The IL-1 $\beta$ -induced HAS expression was decreased significantly following cotreatment with pirfenidone at the mRNA and protein levels. The production of mRNAs was halted by cotreatment with inhibitors of ERK and p38, but not by inhibitors of JNK. The IL-1 $\beta$ -induced ERK and p38 phosphorylation, and AP-1 DNA binding were attenuated in the presence of pirfenidone. Pirfenidone showed greater potency than dexamethasone in inhibiting increases in IL-1 $\beta$ -induced HA.

**CONCLUSIONS.** Pirfenidone attenuates the IL-1 $\beta$ -induced HA production in orbital fibroblasts from patients with TAO, at least in part, through suppression of the MAPK-mediated HAS expression. These results support the potential use of pirfenidone for treatment of patients with TAO.

**Keywords:** hyaluronic acid, hyaluronic acid synthase, IL-1 $\beta$ , orbital fibroblast, mitogen-activated protein kinase, pirfenidone, thyroid-associated ophthalmopathy

Thyroid-associated ophthalmopathy (TAO), one of the extrathyroidal manifestations of Graves' disease, is an autoimmune component that remains a vexing clinical problem that includes proptosis, eyelid retraction, limitation of ocular motion, and even visual loss due to compressive optic neuropathy.<sup>1,2</sup> The hallmarks of TAO include the volumetric expansion of orbital tissues, such as orbital fat and extraocular muscles, in the limited space of the bony orbit.<sup>2-4</sup> Remodeling of these orbital tissues in TAO mainly consists of an accumulation of the glycosaminoglycan, hyaluronic acid (HA).<sup>1-4</sup> Because of its important rheological property, which includes a profound hydrophilic nature and extreme molecular bulk when hydrated,<sup>3</sup> the extracellular accumulations of HA result in edematous changes in corresponding tissues, which currently is believed to underlie much of the volumetric expansion noted in patients with TAO. Therefore, an effective suppression of HA increase could be a therapeutic target to halt the pathologic progression in TAO.<sup>5</sup>

Orbital fibroblasts, which are abundant in orbital connective tissues embedded in fatty compartments, are an important source of HA.<sup>6-8</sup> Orbital fibroblasts have been shown to enhance HA synthesis when activated by pro-inflammatory cytokines, such as IL-1 $\beta$  and TGF- $\beta$ .<sup>5,9,10</sup> In addition, the magnitude of enhanced HA synthesis was greater in orbital than dermal fibroblasts.<sup>8,11</sup> Dexamethasone attenuates IL-1 $\beta$ -induced increases of HA levels in orbital fibroblasts *in vitro*<sup>5</sup>; clinically, glucocorticoids still are indicated as the first-line treatment for suppression of the disease activity regardless of their possible multiple side effects.<sup>12</sup> Unfortunately, a pathogenic mechanism-based therapeutic agent for clinical use with few side effects remains elusive.

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is a small molecule that exhibits novel antifibrotic effects not only in several experimental disease models, such as pulmonary fibrosis<sup>13</sup> and liver cirrhosis,<sup>14</sup> but also in clinical trials of idiopathic pulmonary fibrosis.<sup>15</sup> Regarding TAO, we have

reported previously that pirfenidone attenuates IL-1 $\beta$ -induced tissue inhibitors of metalloproteinase (TIMP)-1/collagen levels in orbital fibroblasts without significant toxicity at the concentrations used.<sup>16</sup> In addition to its antifibrotic effect, pirfenidone has been found to possess anti-inflammatory and antioxidant properties.<sup>17,18</sup> Additionally, we reported recently that pirfenidone attenuates IL-1 $\beta$ -induced cyclooxygenase (COX)-2/prostaglandin (PG)E<sub>2</sub> levels,<sup>19</sup> suggesting the efficacy of this particular drug in attenuating the pathologic conditions of TAO.

In this study, we showed that pirfenidone effectively attenuates the IL-1 $\beta$ -induced HA increase in orbital fibroblasts in patients with TAO by suppressing hyaluronic acid synthase (HAS) expression. Moreover, this inhibitory effect is, at least in part, mediated by attenuating the activation of mitogen-activated protein kinases (MAPKs). These data suggested the possibility of future clinical use of pirfenidone in the treatment of TAO.

## MATERIALS AND METHODS

### Reagents

Pirfenidone and BSA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin/EDTA were purchased from Caisson (North Logan, UT, USA). Recombinant human IL-1 $\beta$  was purchased from PeproTech (Rocky Hill, NJ, USA), and TRIzol was obtained from Invitrogen (Carlsbad, CA, USA). The nuclear extraction kit was obtained from Thermo Scientific (Rockford, IL, USA). Anti-human HAS1, HAS2, and HAS3 rabbit antibodies were purchased from Abcam (Cambridge, UK). Antibodies against p38, phosphorylated (p)-p38, p44/42, p-p44/42, and actin were purchased from Cell Signaling Technology (Beverly, MA, USA). The HA-ELISA kit was obtained from Echelon Biosciences (Salt Lake City, UT, USA). The inhibitors of MAPKs; that is, SB 203580 (p38 MAPK [p38]), PD 98059 (MAPK kinase 1 [MEK1]), and SP 600125 (c-Jun N-terminal kinase [JNK]/stress activated protein kinase [SAPK]), were purchased from Sigma-Aldrich.

### Cell Culture

Human orbital fibroblasts were cultivated from orbital fatty connective tissue obtained as surgical waste during decompression surgery in patients with TAO as described previously.<sup>16</sup> The protocol for obtaining orbital tissue was approved by the Institutional Review Board of Ajou University Hospital and followed the tenets of the Declaration of Helsinki. Written informed consent was obtained from all donors. Five different orbital fibroblast culture strains were obtained from each patient with TAO. All patients with TAO had experienced at least 6 months of inactive disease status with a euthyroid condition before the decompression surgery. The patient characteristics are listed in the Table. Cell cultures were maintained in a humidified 5% CO<sub>2</sub> incubator at 37°C in DMEM containing 10% FBS and antibiotics. Once a fibroblast monolayer was obtained, cultures were serially passaged after gentle treatment with trypsin/EDTA. The medium was changed every 3 days, and cells at passages 3 to 8 were used for experiments.

### ELISA Results

The HA levels in cell culture supernatants of orbital fibroblasts were measured in cells seeded in six-well culture plates at a final concentration of  $5 \times 10^5$ /well. After stabilization for 48

TABLE. Characteristics of Patients With TAO

	Patients With TAO, n = 5
Mean age, y (range)	51.8 (41~64)
Sex, male/female	1/4
Smoking, yes	0
Treatment for Graves' disease	
Radioiodine therapy	0
Antithyroid drug	5
Surgery	0
Treatment for TAO	
Surgery	5
Prednisone	2
Radiation	0
Clinical activity score (range)	1 (0~2)
TSH-receptor antibodies	5
Euthyroid	5

TSH, thyroid-stimulating hormone.

hours, orbital fibroblasts were treated with 10 ng/mL IL-1 $\beta$  and 1, 5, 10 mM pirfenidone or 100 nM dexamethasone in fresh DMEM containing 1% charcoal-filtered FBS for 48 hours. Supernatants from the cell cultures were collected, and the concentrations of HA therein were determined using a competitive binding HA-ELISA kit according to the manufacturer's instructions. Briefly, after adding 100  $\mu$ L of standards and samples into the corresponding wells, 150 and 100  $\mu$ L of diluent were added to the blank and zero HA control wells, respectively. Working detector (50  $\mu$ L) was added to all wells except the blank, and the plate was covered and incubated for 1 hour at 37°C. Then, 100  $\mu$ L of controls and samples were transferred to the corresponding wells of the HA-ELISA plate. After incubation for 30 minutes at 4°C, the solution was discarded, the wells were washed, and 100  $\mu$ L of working enzyme suspension were added to each well. After incubation for 30 minutes at 37°C followed by washes, 100  $\mu$ L of working substrate solution were added to each well, and the plate was incubated in the dark at room temperature for 30 minutes. Absorbance was measured spectrophotometrically at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The concentration of HA in each sample was determined by reference to a standard curve generated with known amounts of HA.

### RT-PCR Studies

Orbital fibroblasts ( $5 \times 10^5$ /well) were seeded in six-well cell culture plates and treated with 10 ng/mL IL-1 $\beta$  in the presence or absence of pirfenidone or inhibitors for MAPKs (SP 600125, SB 203580, PD 98059). Total RNA was extracted using TRIzol reagent at each designated time point, and cDNA was generated from RNA (1  $\mu$ g) using the SuperScript First-Strand Synthesis System (Invitrogen). The PCR was performed using 1- $\mu$ l cDNA, 0.25 mM dNTP, 1 U of *Pfu* DNA polymerase, and 10 pmole of the primer pair, with a thermal cycler. The specific primer sequences were as follows: *HAS1* forward, 5'-TGT GTA TCC TGC ATC AGC GGT-3' and reverse, 5'-CTG GAG GTG TAC TTG GTA GCA TAA CC-3'; *HAS2* forward, 5'-GTG TTA TAC ATG TCG AGT TTA CTT CC-3' and reverse, 5'-GTC ATA TTG TTG TCC CTT CTT CCG C-3'; *HAS3* forward, 5'-GGT ACC ATC AGA AGT TCC TAG GCA GC-3' and reverse, 5'-GAG GAG AAT GTT CCA GAT GCG-3'; and  $\beta$ -actin forward, 5'-CCA AGG CCA ACC GCG AGA AGA TGA C-3' and reverse, 5'-AGG GTA CAT GGT GGT GCC GCC AGA C-3'. The PCR cycles were composed of one cycle of 95°C for 5

minutes, 30 cycles of 45 seconds at 94°C, 45 seconds at 55°C, and 45 seconds at 72°C. The reaction was terminated at 72°C for 5 minutes and quenched at 4°C. Band densities were quantified using the ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA). The measured intensities were corrected to the  $\beta$ -actin level in each lane and subjected to statistical analyses.

### Western Blot Analysis

Orbital fibroblasts ( $5 \times 10^5$ /well) were cultured and treated as described for ELISA. After treating fibroblasts for the designated incubation times, cells were scraped into a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Gendepot, Barker, TX, USA), and centrifuged at 14,171g for 20 minutes at 4°C. Proteins were separated by SDS-PAGE on 10% (wt/vol) gels and transferred to nitrocellulose membranes (Schleicher & Schuell BioScience, Keene, NH, USA). Each membrane was blocked by incubation at room temperature for 1 hour with Tris-buffered saline (TBS, pH 7.4) containing 5% (wt/vol) skim milk or BSA, and then was incubated at 4°C overnight with the primary antibody. After three washes with TBST (TBS containing 0.1% [vol/vol] Tween-20), membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies at room temperature for 1 hour. The immunoreactive proteins were detected by incubation with enhanced chemiluminescence blotting detection agent (Pierce Biotechnology, Rockford, IL) using the LAS Imaging System (Fujifilm, Tokyo, Japan). The band densities were quantified using the ImageJ software (NIH). The measured intensities were corrected to the actin level in each lane and subjected to statistical analyses.

### Nuclear Extracts (NEs) and Electrophoretic Mobility Shift Assay (EMSA)

The EMSA was performed with 1  $\mu$ g of NEs as described previously.<sup>20</sup> The NEs from cells treated with IL-1 $\beta$  for 30 minutes were incubated with the AP-1 sequence (5'-AGT TGA GGG GAC TTT CCC AGG C-3'; SC-2505; Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was end-labeled with [<sup>32</sup>P]ATP for 30 minutes. A 100 molar excess of cold oligonucleotide was added to the NEs for 30 minutes before adding labeled probe for the competition experiments. Bound and free DNA were then resolved by 5% PAGE and exposed to film for autoradiography.

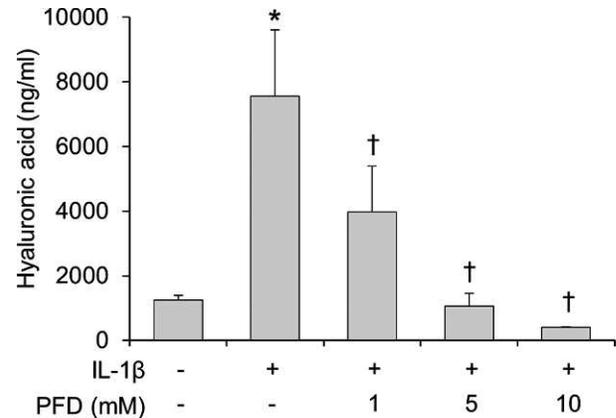
### Statistical Analysis

Data were analyzed using the SPSS software version 15.0 (SPSS, Inc., Chicago, IL, USA). The *t*-test was used to analyze the inhibitory effects of pirfenidone on the IL-1 $\beta$ -induced increase in HA and HAS levels. For comparison of inhibitory effects between pirfenidone and dexamethasone, the *t*-test was performed. *P* values of less than 0.05 were considered to indicate statistical significance.

## RESULTS

### Pirfenidone Attenuates the IL-1 $\beta$ -Induced HA Increase in Orbital Fibroblasts

Orbital fibroblasts from patients with TAO express relatively high levels of HA in response to IL-1 $\beta$  stimulation.<sup>5,8</sup> Thus,

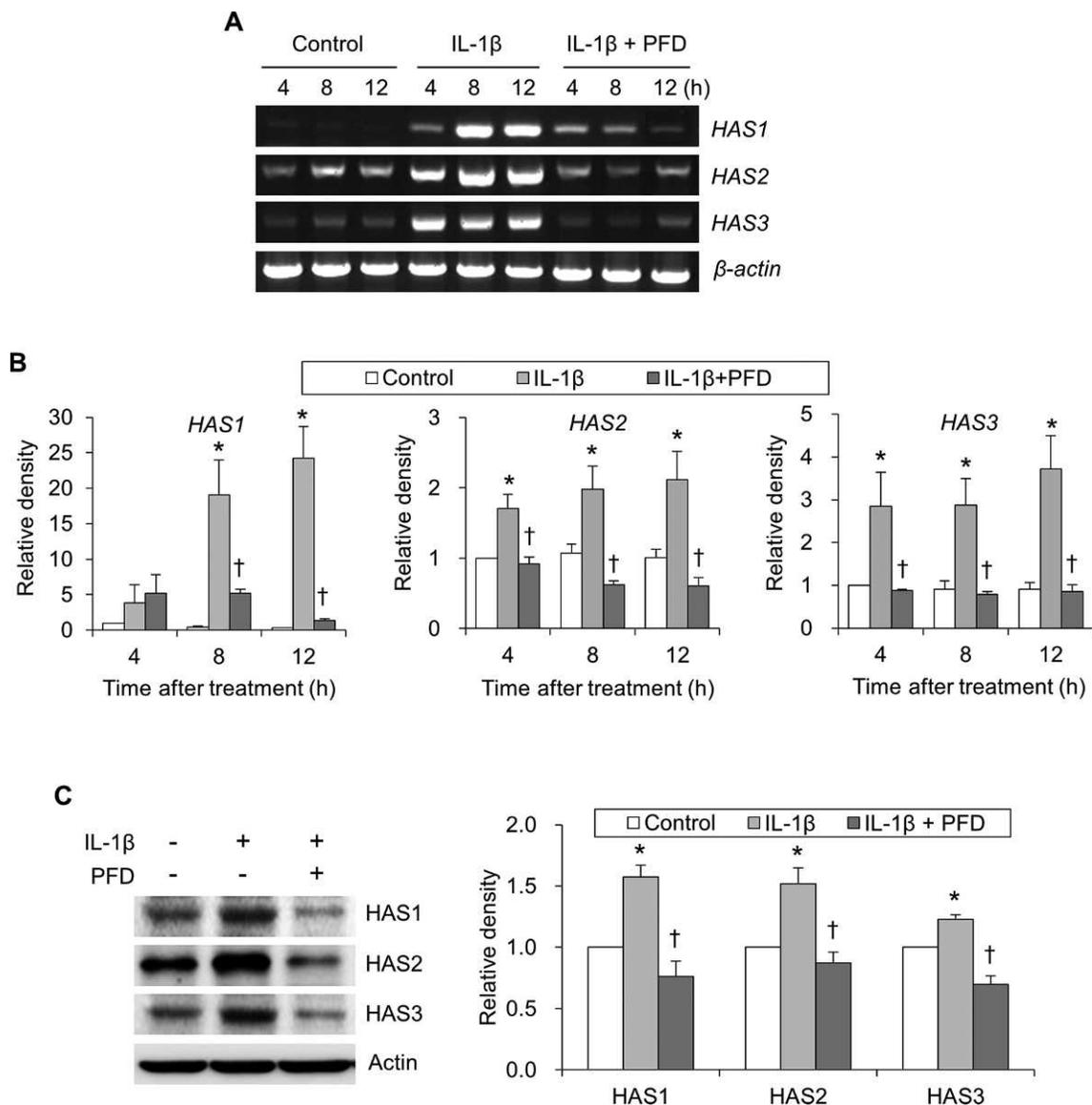


**FIGURE 1.** Effect of pirfenidone (PFD) on the IL-1 $\beta$ -induced HA increase in orbital fibroblasts. Primary cultures of orbital fibroblasts from patients with TAO were cotreated with 10 ng/mL of IL-1 $\beta$  and various concentrations of PFD (1, 5, or 10 mM) for 48 hours. The HA levels in culture supernatants were measured by ELISA. Data are expressed as means  $\pm$  SD of quadruplicate determinations (\**P* < 0.05 versus untreated control, †*P* < 0.05 versus IL-1 $\beta$ -treated cells).

we initially examined the effects of pirfenidone on IL-1 $\beta$ -induced HA expression in TAO orbital fibroblasts. Cells were cotreated with various concentrations of pirfenidone and 10 ng/mL of IL-1 $\beta$  for 48 hours, and the HA protein levels in culture media were measured by ELISA. Guided by our previous study showing an antifibrotic effect of pirfenidone in human orbital fibroblasts without significant toxicity,<sup>16</sup> we used pirfenidone at concentrations up to 10 mM. The IL-1 $\beta$  induced a 6-fold increase in HA protein levels compared to that reached in untreated controls, and pirfenidone significantly reduced the IL-1 $\beta$ -induced HA release in a dose-dependent manner. As shown in Figure 1, pirfenidone attenuated the IL-1 $\beta$ -induced HA increase at 1, 5, and 10 mM, decreasing HA concentrations to 52%, 14%, and 5% of the values obtained by IL-1 $\beta$  stimulation, respectively. When treated with 10 mM pirfenidone, HA production was decreased dramatically below the untreated control level, even in the presence of IL-1 $\beta$ .

### Pirfenidone Attenuates the IL-1 $\beta$ -Induced HAS Expression in Orbital Fibroblasts

The HA synthesis in mammals is regulated by three specific HAS enzymes that are encoded by individual genes: *HAS1*, *HAS2*, and *HAS3*.<sup>21,22</sup> We investigated whether pirfenidone influences the expression of these *HAS* genes induced by IL-1 $\beta$  in TAO orbital fibroblasts. Orbital fibroblasts were cotreated with 10 ng/mL IL-1 $\beta$  and 10 mM pirfenidone for 4, 8, or 12 hours, and *HAS* mRNA levels were measured by RT-PCR. Responding to IL-1 $\beta$  treatment, mRNA expression of each *HAS* was enhanced significantly in a time-dependent manner, while pirfenidone diminished this inducing effect of IL-1 $\beta$  (Fig. 2A). In the case of *HAS1*, mRNA expression was increased up to 25-fold greater than control. The *HAS2* and *HAS3* mRNA levels also were enhanced by IL-1 $\beta$ , but to a lesser extent than *HAS1*. Pirfenidone significantly attenuated the IL-1 $\beta$ -induced *HAS1* mRNA levels at 8 and 12 hours (73% and 94% reduction of the IL-1 $\beta$ -induced levels, respectively) and those of *HAS2* and *HAS3* at 4, 8, and 12 hours (46%, 69%, and 71% reduction of the IL-1 $\beta$ -induced levels for *HAS2*, 69%, 73%, and 77% reduction of the IL-1 $\beta$ -induced levels for *HAS3*, respectively, Fig. 2B). This inhibitory effect of pirfenidone on the IL-1 $\beta$ -induced HAS increase was verified by Western blot (Fig. 2C).



**FIGURE 2.** Effect of PFD on the IL-1 $\beta$ -induced HAS expressions in orbital fibroblasts. (A) Orbital fibroblasts from patients with TAO were cotreated with 10 ng/mL of IL-1 $\beta$  and 10 mM of PFD for the indicated times (4, 8, or 12 hours), and RT-PCR analysis of three types of *HAS* (*HAS1*, *HAS2*, and *HAS3*) mRNA levels was performed. Representative bands from independent experiments using five strains of TAO cells are shown. (B) Relative signal intensities of HAS mRNA from the results of (A) are shown. The measured intensities of HAS mRNA were corrected for their respective  $\beta$ -actin signals and analyzed (*bar graphs*, mean  $\pm$  SD; \* $P$  < 0.05 versus untreated controls; † $P$  < 0.05 versus IL-1 $\beta$ -treated cells). (C) Western blotting analysis was performed to verify HAS protein levels in cell culture supernatants of orbital fibroblasts. Cells were cotreated with 10 ng/mL of IL-1 $\beta$  and 10 mM of PFD for 24 hours. Representative blots (*left*) and relative signal intensities of HAS protein (*right*) from independent experiments using five strains of TAO cells are shown. The measured intensities of HAS protein were corrected for their respective actin signals and analyzed (*bar graph*, mean  $\pm$  SD; \* $P$  < 0.05 versus untreated controls; † $P$  < 0.05 versus IL-1 $\beta$ -treated cells).

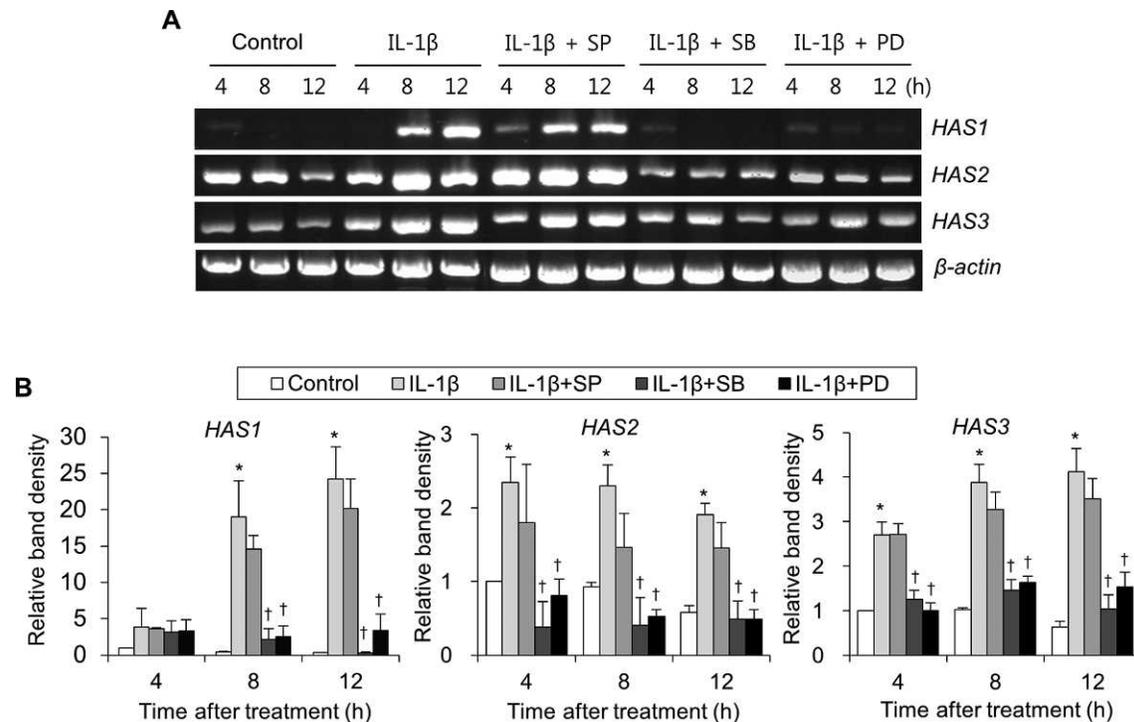
### IL-1 $\beta$ Induces HAS Expression Through the MAPK-Mediated Signaling Pathway in Orbital Fibroblasts

The IL-1 $\beta$  induces a number of genes in orbital fibroblasts. Induction of several of these is mediated through the activation of MAPK pathways.<sup>23,24</sup> In addition, a previous study reported that HA synthesis in dermal fibroblasts is mediated by the MAPK signaling pathway.<sup>25</sup> Therefore, we examined the contribution of MAPKs to IL-1 $\beta$ -induced HAS expression in orbital fibroblasts using specific inhibitors of three types of MAPKs: SB203580 for p38, PD98059 for extracellular signal-regulated kinase (ERK), and SP 600125 for JNK. The levels of *HAS* mRNA in cells cotreated with IL-1 $\beta$ , and each specific

inhibitor for 4, 8, and 12 hours were measured by RT-PCR. As shown in Figure 3, inhibitors of ERK or p38 successfully abrogated the IL-1 $\beta$ -induced enhancement of all three *HAS* subtypes, while the JNK inhibitor did not yield a significant reduction in *HAS* expression levels compared to the other inhibitors.

### Pirfenidone Attenuates the IL-1 $\beta$ -Induced Activation of the MAPK-Mediated Signaling Pathway in Orbital Fibroblasts

We next evaluated whether pirfenidone affects the IL-1 $\beta$ -induced activation of the MAPK-mediated signaling pathway,



**FIGURE 3.** Involvement of MAPK-mediated signaling pathway in IL-1 $\beta$ -induced *HAS* expression in orbital fibroblasts. (A) Orbital fibroblasts from patients with TAO were cotreated with 10 ng/mL of IL-1 $\beta$  and 20  $\mu$ M of each specific inhibitor for MAPK-mediated signaling pathway (SP600125 [SP], a JNK inhibitor; SB203580 [SB], a p38 inhibitor; PD98059 [PD], a ERK inhibitor) for the indicated times (4, 8, or 12 hours) and RT-PCR analysis for three types of *HAS* mRNA was performed. Representative bands from independent experiments using five strains of TAO cells are shown. (B) Signal intensities of *HAS* from the results of (A) are shown. The measured intensities of *HAS* mRNA were corrected for their respective  $\beta$ -actin signals and analyzed (*bar graphs*, mean  $\pm$  SD; \* $P$  < 0.05 versus untreated control; † $P$  < 0.05 versus IL-1 $\beta$ -treated cells).

particularly of p38 and ERK, in orbital fibroblasts. We performed Western blot analyses for phosphorylated and total levels of p38 and ERK protein. As shown in Figures 4A and 4B, IL-1 $\beta$  strikingly induced the phosphorylation of p38 at 5 minutes, and cotreatment with pirfenidone suppressed IL-1 $\beta$ -induced phosphorylation of p38, while the decreased level at 10 minutes did not show significance. In the case of ERK, the IL-1 $\beta$ -induced increase in phosphorylation was observed at 10 minutes, and pirfenidone attenuated it. Although the increased phosphorylation of ERK was not observed at 5 minutes of treatment with IL-1 $\beta$ , pirfenidone decreased the level of ERK phosphorylation much below that of the untreated control. Pirfenidone or IL-1 $\beta$  itself showed no effect on the total levels of p38 or ERK. Since the p38 and ERK signaling pathways have been shown to affect AP-1 activity, which is a downstream target of MAPK, we performed EMSA to investigate the effect of pirfenidone on AP-1 binding to the consensus oligonucleotide in the presence of IL-1 $\beta$ . Responding to 10 ng/mL of IL-1 $\beta$  treatment, increased DNA binding of the AP-1 consensus oligonucleotide was observed in NEs from two independent TAO orbital fibroblasts (Fig. 4C, lanes 3 and 7), and the binding was reduced in cells cotreated with IL-1 $\beta$  and pirfenidone comparable to each control level (lanes 5 and 9). Competition with excess unlabeled AP-1 oligonucleotide abrogated the complex formation (lane 10), which confirmed a specific interaction between AP-1 and the AP-1 consensus oligonucleotide.

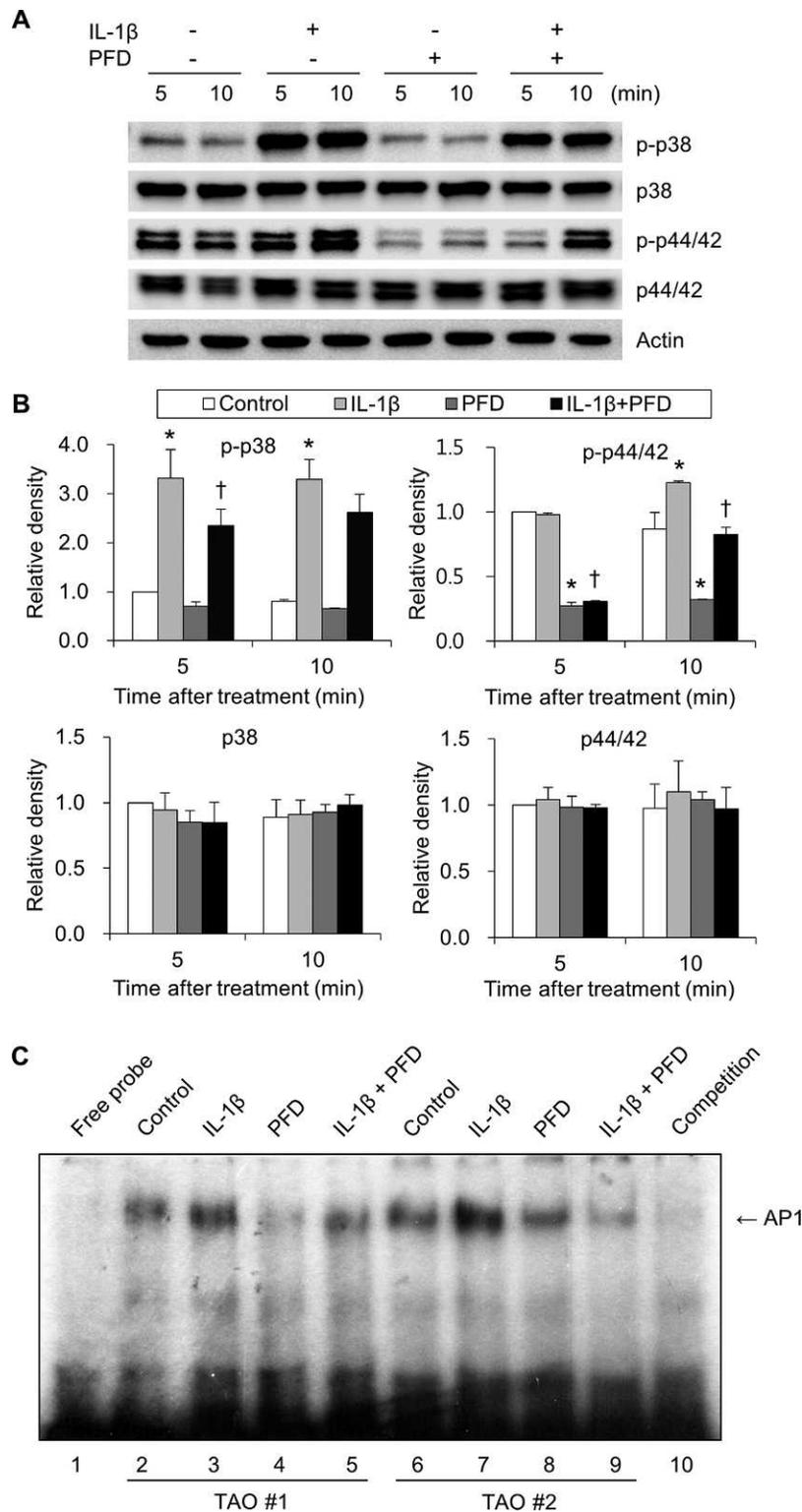
### Pirfenidone Has Greater Potency Than Dexamethasone in Attenuating the IL-1 $\beta$ -Induced HA Increase in Orbital Fibroblasts

In light of a previous *in vitro* study showing that dexamethasone attenuates IL-1 $\beta$ -induced increases in HA levels in orbital

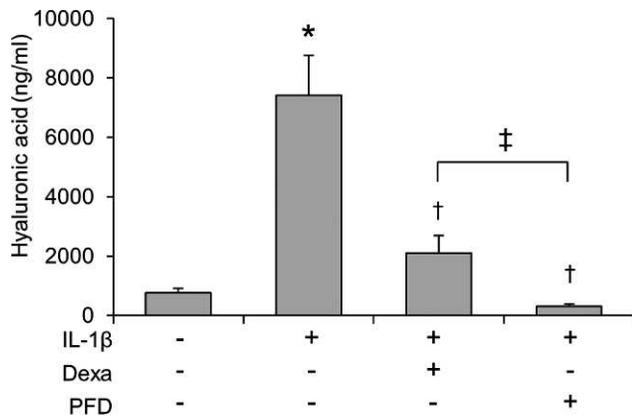
fibroblasts,<sup>5</sup> we compared the inhibitory effect of pirfenidone on HA expression to that of dexamethasone. After cotreating orbital fibroblasts with 10 ng/mL IL-1 $\beta$  and 10 mM pirfenidone or 100 nM dexamethasone for 48 hours, HA levels in the culture media were measured by ELISA. As shown in Figure 5, the inhibitory effect of pirfenidone on the IL-1 $\beta$ -induced HA increase was significantly greater than that of dexamethasone (96% and 72% reduction of the IL-1 $\beta$ -induced levels, respectively).

## DISCUSSION

Our data demonstrated that pirfenidone attenuated IL-1 $\beta$ -induced HA production in primary cultured orbital fibroblasts from patients with TAO by interfering with the MAPK-mediated *HAS* expression. The pathology of TAO is characterized by the volumetric expansions of extraocular muscles and orbital fat within the limited bony orbital space, which result in congestion and finally organ dysfunction.<sup>1-4</sup> At the heart of this tissue remodeling is the disordered accumulation of the glycosaminoglycan, HA.<sup>2-4</sup> Because HA-accumulated tissues can expand by hydration due to its remarkable avidity for water, the abnormal HA accumulation in orbital tissues is thought to be the major cause of edematous changes in orbital tissues noted in patients with TAO.<sup>3,26</sup> In addition, since HA lacks a core protein, protein synthesis is not required, and the production of HA proceeds rapidly.<sup>26</sup> Of note, orbital fibroblasts express extraordinary levels of HA in an anatomic site-selective manner with exaggerated responses to pro-inflammatory cytokines, such as IL-1 $\beta$ .<sup>5,7</sup> Therefore, effective attenuation of this exaggerated HA production in orbital



**FIGURE 4.** Effect of PFD on the IL-1 $\beta$ -induced MAPK-mediated signaling pathway. (A) Orbital fibroblasts from patients with TAO were treated with 10 ng/mL of IL-1 $\beta$  in the presence or absence of 10 mM of PFD for the indicated times (5 or 10 minutes) and Western blottings were performed for phosphorylated p38 (p-p38) or phosphorylated p44/42 (p-p44/42). Representative bands from independent experiments using five strains of cells are shown. (B) Relative signal intensities from the results of (A) are shown. The measured intensities of p-p38, p-p44/42, p38, and p44/42 were corrected for their respective actin signals and analyzed (*bar graphs*, mean  $\pm$  SD; \* $P$  < 0.05 versus untreated control; † $P$  < 0.05 versus IL-1 $\beta$ -treated cells). (C) Orbital fibroblasts from a patient with TAO were treated with 10 ng/mL IL-1 $\beta$  in the presence or absence of 10 mM PFD for 30 minutes. Then, nuclear extracts were obtained and the levels of AP-1 DNA binding were evaluated by EMSA. Representative blots from three independent experiments are shown.



**FIGURE 5.** Comparison of the efficacy of PFD and dexamethasone (Dexa) in decreasing HA protein levels. Orbital fibroblasts from patients with TAO were treated with 10 ng/mL of IL-1 $\beta$  in the presence or absence of 10 mM PFD or 100 nM Dexa for 48 hours. Then, HA protein levels in the culture media were measured by ELISA. Data are expressed as means  $\pm$  SD of four independent replicates ( $^*P < 0.05$  versus untreated control;  $^\dagger P < 0.05$  versus IL-1 $\beta$ -treated cells;  $^\ddagger P < 0.05$  between PFD and Dexa).

fibroblasts could be a promising target for controlling the major pathogenetic mechanism of TAO.

Synthesis of HA occurs at the cell surface and involves the activation of three synthetases, HAS1, 2, and 3.<sup>21,22</sup> Each isoform is encoded by a separate gene localizing to a different human chromosome, and is associated with a characteristic pattern of tissue distribution and chain length of product.<sup>21,22</sup> In a study of unfettered HA production in fibroblast-like synoviocytes of rheumatoid arthritis,<sup>27</sup> *HAS2* and *HAS3* were expressed constitutively, while the level of *HAS1* mRNA was very low or undetectable. Although all three enzymes are known to be expressed in orbital fibroblasts from patients with TAO in response to IL-1 $\beta$ ,<sup>5</sup> little is known about the differences in regulation and biological functions of each HAS isoform in TAO. However, in agreement with a previous report by Kaback and Smith,<sup>5</sup> our study revealed that orbital fibroblasts of TAO expressed much lower levels of *HAS1* mRNA under basal culture conditions than those of *HAS2* and *HAS3*. Of note, when activated by IL-1 $\beta$ , the relative expression of *HAS1* mRNA was greater than those of *HAS2* and *HAS3*. As a result, the observed inhibitory effect of pirfenidone on IL-1 $\beta$ -induced *HAS1* mRNA was more prominent than those of *HAS2* and *HAS3*. However, this induction of *HAS1* by IL-1 $\beta$  occurred at later time points when compared to *HAS2* and *HAS3*. These differences in inducibility observed in our studies suggested that each HAS isoform may have a distinct role in the inflammatory response of orbital fibroblasts with regard to magnitude and timing.

In our study, IL-1 $\beta$ -induced *HAS* mRNA production in orbital fibroblasts appears to be mediated through the MAPK-mediated pathway involving ERK or p38. These observations are in line with previous reports on IL-1 $\beta$ -induced signaling pathways in the upregulation of prostaglandin E2 synthesis and induction of IL-6 expression in orbital fibroblasts.<sup>23,24</sup> In our system, we showed that the JNK-dependent pathway was not related to IL-1 $\beta$ -induced *HAS* mRNA expression, which is in disagreement with a previous report showing the utilization of the JNK-dependent pathway during palmitate-induced IL-6 expression in orbital fibroblasts.<sup>28</sup> In studies using other cell types, such as human osteoarthritic fibroblast-like synoviocytes, IL-1 $\beta$ -induced *HAS* expression was mediated by the JNK-dependent pathway as well as ERK and p-38.<sup>29</sup> However, in human intestinal mesenchymal cells, *HAS2* expression by IL-1 $\beta$

is mediated by ERK and p38, but not by JNK.<sup>30</sup> These discrepancies may be due to the different characteristics of the particular stimulants and cell types used.

Pirfenidone is a novel agent that has shown anti-inflammatory and antifibrotic potential in animal models and clinical trials.<sup>31-33</sup> It exerts its downregulating effects on a series of cytokines, including TGF- $\beta$ , connective tissue growth factor, platelet-derived growth factors, TNF- $\alpha$ , IFN- $\gamma$ , intercellular adhesion molecule-1, IL-6, and IL-1.<sup>32,33</sup> Regarding TAO, we reported previously that pirfenidone exerts antifibrotic and anti-inflammatory effects in orbital fibroblasts by inhibiting TIMP-1 and COX-2 expression without causing significant cytotoxicity. In addition, pirfenidone showed superior potency compared to that of dexamethasone.<sup>16,19</sup> Herein, we revealed an additional effect of pirfenidone at a concentration that is corresponding to the ones used in our previous reports<sup>16,19</sup>: the attenuation of IL-1 $\beta$ -induced HA production through the inhibition of MAPK-mediated *HAS* expression. However, this inhibitory effect of pirfenidone on MAPK activity, particularly on p38, was relatively incomplete when compared to its effect on HA production. These results suggested the existence of other pharmacologic mechanisms of pirfenidone in attenuating the IL-1 $\beta$ -induced HA production in orbital fibroblasts, in addition to the inhibition of the MAPK signaling pathway shown here. In human umbilical vein endothelial cells, IL-1 $\beta$  induces *HAS2* transcription via the nuclear factor (NF)- $\kappa$ B-dependent pathway.<sup>34</sup> Taken together with our previous report of the inhibitory effect of pirfenidone on IL-1 $\beta$ -induced activation of NF- $\kappa$ B signaling pathway in orbital fibroblasts,<sup>19</sup> this pharmacologic action mechanism might be applicable in our present results.

In this study, we demonstrated that pirfenidone showed a superior potency in inhibiting IL-1 $\beta$ -induced HA production in orbital fibroblasts compared to dexamethasone. In contrast with the well-known side effects of corticosteroids, oral pirfenidone was generally well tolerated with few side effects in several large-scale phase III clinical trials,<sup>33</sup> indicating that pirfenidone could be safer than high-dose glucocorticoids.<sup>32,33</sup> The most frequent side effects include photosensitivity rash, nausea, dyspepsia, and dizziness, which appear to be dose-related and typically resolve completely once the drug is withdrawn.<sup>32,33</sup> Further research and clinical studies are necessary to ensure the safety of pirfenidone treatment and to ascertain the optimum doses for treatment of TAO.

In summary, our results provided initial evidence that pirfenidone has inhibitory effects on IL-1 $\beta$ -induced HA production by attenuating *HAS* expression in orbital fibroblasts from patients with TAO. These results suggested that pirfenidone may be a promising candidate for future use as therapeutic agent in TAO.

### Acknowledgments

Supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (NRF-2011-0013586 [KHK]), and a grant from Ajou University School of Medicine (M-2012-C0460-00035 [SAC]). The authors alone are responsible for the content and writing of the paper.

Disclosure: S.A. Chung, None; B.K. Jeon, None; Y.-H. Choi, None; K.O. Back, None; J.B. Lee, None; K.H. Kook, None

### References

- Naik VM, Naik MN, Goldberg RA, Smith TJ, Douglas RS. Immunopathogenesis of thyroid eye disease: emerging paradigms. *Surv Ophthalmol*. 2010;55:215-226.

2. Kazim M, Goldberg RA, Smith TJ. Insights into the pathogenesis of thyroid-associated orbitopathy: evolving rationale for therapy. *Arch Ophthalmol*. 2002;120:380-386.
3. Smith TJ, Bahn RS, Gorman CA. Connective tissue, glycosaminoglycans, and diseases of the thyroid. *Endocr Rev*. 1989;10:366-391.
4. Hufnagel TJ, Hickey WF, Cobbs WH, Jakobiec FA, Iwamoto T, Eagle RC. Immunohistochemical and ultrastructural studies on the exenterated orbital tissues of a patient with Graves' disease. *Ophthalmology*. 1984;91:1411-1419.
5. Kaback LA, Smith TJ. Expression of hyaluronan synthase messenger ribonucleic acids and their induction by interleukin-1beta in human orbital fibroblasts: potential insight into the molecular pathogenesis of thyroid-associated ophthalmopathy. *J Clin Endocrinol Metab*. 1999;84:4079-4084.
6. Smith TJ. Novel aspects of orbital fibroblast pathology. *J Endocrinol Invest*. 2004;27:246-253.
7. Smith TJ. Orbital fibroblasts exhibit a novel pattern of responses to proinflammatory cytokines: potential basis for the pathogenesis of thyroid-associated ophthalmopathy. *Thyroid*. 2002;12:197-203.
8. Smith TJ, Wang HS, Evans CH. Leukoregulin is a potent inducer of hyaluronan synthesis in cultured human orbital fibroblasts. *Am J Physiol*. 1995;268:C382-C388.
9. Wong YK, Tang KT, Wu JC, Hwang JJ, Wang HS. Stimulation of hyaluronan synthesis by interleukin-1beta involves activation of protein kinase C betaII in fibroblasts from patients with Graves' ophthalmopathy. *J Cell Biochem*. 2001;82:58-67.
10. Wang HS, Tung WH, Tang KT, et al. TGF-beta induced hyaluronan synthesis in orbital fibroblasts involves protein kinase C betaII activation in vitro. *J Cell Biochem*. 2005;95:256-267.
11. Smith TJ, Hoa N. Immunoglobulins from patients with Graves' disease induce hyaluronan synthesis in their orbital fibroblasts through the self-antigen, insulin-like growth factor-I receptor. *J Clin Endocrinol Metab*. 2004;89:5076-5080.
12. Bartalena L, Baldeschi L, Dickinson A, et al. Consensus statement of the European Group on Graves' orbitopathy (EUGOGO) on management of GO. *Eur J Endocrinol*. 2008;158:273-285.
13. Kehrler JP, Margolin SB. Pirfenidone diminishes cyclophosphamide-induced lung fibrosis in mice. *Toxicol Lett*. 1997;90:125-132.
14. Di Sario A, Bendia E, Macarri G, et al. The anti-fibrotic effect of pirfenidone in rat liver fibrosis is mediated by downregulation of procollagen alpha1(I), TIMP-1 and MMP-2. *Dig Liver Dis*. 2004;36:744-751.
15. Noble PW, Albera C, Bradford WZ, et al. Pirfenidone in patients with idiopathic pulmonary fibrosis (CAPACITY): two randomised trials. *Lancet*. 2011;377:1760-1769.
16. Kim H, Choi YH, Park SJ, et al. Antifibrotic effect of pirfenidone on orbital fibroblasts of patients with thyroid-associated ophthalmopathy by decreasing TIMP-1 and collagen levels. *Invest Ophthalmol Vis Sci*. 2010;51:3061-3066.
17. Spond J, Case N, Chapman RW, et al. Inhibition of experimental acute pulmonary inflammation by pirfenidone. *Pulm Pharmacol Ther*. 2003;16:207-214.
18. Nakanishi H, Kaibori M, Teshima S, et al. Pirfenidone inhibits the induction of iNOS stimulated by interleukin-1beta at a step of NF-kappaB DNA binding in hepatocytes. *J Hepatol*. 2004;41:730-736.
19. Choi YH, Back KO, Kim HJ, Lee SY, Kook KH. Pirfenidone attenuates IL-1beta-induced COX-2 and PGE2 production in orbital fibroblasts through suppression of NF-kappaB activity. *Exp Eye Res*. 2013;113:1-8.
20. Choi YH, Bernardi R, Pandolfi PP, Benveniste EN. The promyelocytic leukemia protein functions as a negative regulator of IFN-gamma signaling. *Proc Natl Acad Sci U S A*. 2006;103:18715-18720.
21. Weigel PH, Hascall VC, Tammi M. Hyaluronan synthases. *J Biol Chem*. 1997;272:13997-14000.
22. Itano N, Sawai T, Yoshida M, et al. Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. *J Biol Chem*. 1999;274:25085-25092.
23. Chen B, Tsui S, Smith TJ. IL-1 beta induces IL-6 expression in human orbital fibroblasts: identification of an anatomic-site specific phenotypic attribute relevant to thyroid-associated ophthalmopathy. *J Immunol*. 2005;175:1310-1319.
24. Han R, Tsui S, Smith TJ. Up-regulation of prostaglandin E2 synthesis by interleukin-1beta in human orbital fibroblasts involves coordinate induction of prostaglandin-endoperoxide H synthase-2 and glutathione-dependent prostaglandin E2 synthase expression. *J Biol Chem*. 2002;277:16355-16364.
25. Li L, Asteriou T, Bernert B, Heldin CH, Heldin P. Growth factor regulation of hyaluronan synthesis and degradation in human dermal fibroblasts: importance of hyaluronan for the mitogenic response of PDGF-BB. *Biochem J*. 2007;404:327-336.
26. de la Motte CA. Hyaluronan in intestinal homeostasis and inflammation: implications for fibrosis. *Am J Physiol Gastrointest Liver Physiol*. 2011;301:G945-G949.
27. Stuhlmeier KM. Effects of leflunomide on hyaluronan synthases (HAS): NF-kappa B-independent suppression of IL-1-induced HAS1 transcription by leflunomide. *J Immunol*. 2005;174:7376-7382.
28. Paik JS, Cho WK, Oh EH, Lee SB, Yang SW. Palmitate induced secretion of IL-6 and MCP-1 in orbital fibroblasts derived from patients with thyroid-associated ophthalmopathy. *Mol Vis*. 2012;18:1467-1477.
29. David-Raoudi M, Deschrevel B, Leclercq S, Galera P, Boumediene K, Pujol JP. Chondroitin sulfate increases hyaluronan production by human synoviocytes through differential regulation of hyaluronan synthases: role of p38 and Akt. *Arthritis Rheum*. 2009;60:760-770.
30. Ducale AE, Ward SI, Dechert T, Yager DR. Regulation of hyaluronan synthase-2 expression in human intestinal mesenchymal cells: mechanisms of interleukin-1beta-mediated induction. *Am J Physiol Gastrointest Liver Physiol*. 2005;289:G462-G470.
31. Azuma A. Pirfenidone treatment of idiopathic pulmonary fibrosis. *Thorax*. 2012;67:107-114.
32. Carter NJ. Pirfenidone: in idiopathic pulmonary fibrosis. *Drugs*. 2011;71:1721-1732.
33. Richeldi L, du Bois RM. Pirfenidone in idiopathic pulmonary fibrosis: the CAPACITY program. *Expert Rev Respir Med*. 2011;5:473-481.
34. Vignetti D, Genasetti A, Karousou E, et al. Proinflammatory cytokines induce hyaluronan synthesis and monocyte adhesion in human endothelial cells through hyaluronan synthase 2 (HAS2) and the nuclear factor-kappaB (NF-kappaB) pathway. *J Biol Chem*. 2010;285:24639-24645.