Title:

Pirfenidone inhibits the expression of HSP47 in TGF-β1-stimulated human lung fibroblasts

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Running title: **Pirfenidone inhibits HSP47 in NHLF**

ABSTRACT

Pirfenidone (5-methyl-1-phenyl-2-(1*H*)-pyridone) is a novel anti-fibrotic and anti-inflammatory agent that inhibits the progression of fibrosis in animal models and patients with idiopathic pulmonary fibrosis (IPF). Heat shock protein (HSP) 47, a collagen-specific molecular chaperone, is involved in the processing and/or secretion of procollagen and plays an important role in the pathogenesis of IPF. The present study evaluated the *in vitro* effects of pirfenidone on expression of HSP47 and collagen type I in cultured normal human lung fibroblasts (NHLF). Expression levels of HSP47 and collagen type I in NHLF stimulated by transforming growth factor (TGF)-β1 were evaluated genetically, immunologically and immunocytochemically. Treatment with TGF-β1 stimulated both mRNA and protein expressions of both HSP47 and collagen type I in NHLF, and pirfenidone significantly inhibited this TGF-β1-enhanced expression in a dose-dependent manner. We concluded that the antifibrotic effect of pirfenidone may be mediated not only through direct inhibition of collagen type I expression but also at least partly through inhibition of HSP47 expression in lung fibroblasts, with a resultant reduction of collagen synthesis in lung fibrosis.

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Key Words: collagen type I, fibroblast, heat shock protein 47 (HSP47), idiopathic pulmonary fibrosis, lung fibrosis, pirfenidone

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal disorder characterized by patchy fibrotic areas with fibroblast proliferation and extracellular matrix (ECM) remodeling that results in irreversible distortion of the lung architecture(Selman et al., 2001). The underlying molecular mechanisms by which excessive collagen is deposited in the fibrotic lesions are not fully understood. Recently there is a growing body of evidence suggesting that IPF is a model of abnormal wound healing in response to alveolar epithelial injury/activation associated with the formation of patchy areas of fibroblast/myofibroblast foci, which in turn evolve into fibrosis (Selman et al., 2001). Currently available therapeutic measures for treatment of IPF are ineffective, probably because they primarily base in the concept that inflammation leads to injury and fibrosis in IPF (Selman et al., 2001). Several new antifibrotic agents which prevent directly the fibroproliferative response and alveolar epithelial damage are under development for IPF treatment.

Pirfenidone is a novel antifibrotic agent that inhibits the progression of fibrosis in experimental models of lung, kidney, and hepatic fibrosis (Di Sario et al., 2004; Hewitson et al., 2001; Iyer et al., 1998, 1999, 2000; Kakugawa et al., 2004; Kehrer and Margolin, 1997). While this drug has well-established anti-inflammatory properties including regulation of key growth factors and cytokines (Gurujeyalakshmi et al., 1999; Iyer et al., 2000; Nakazato et al., 2002; Oku et al., 2002), recent clinical trials revealed that it also exhibits therapeutic effects in patients with IPF (Azuma et al., 2005; Raghu et al., 1999). However, the exact mechanisms by which this new

compound offers protection against lung fibrosis remain unclear. We found previously using an *in vivo*, mouse model of bleomycin-induced pulmonary fibrosis that pirfenidone reduced the number of myofibroblasts, type II pneumocytes and interstitial spindle-shaped cells expressing heat shock protein (HSP)47 in fibrotic lesions (Kakugawa et al., 2004). There was also a marked reduction of fibrotic lesions in this model treated by pirfenidone, as a result of reducing the accumulation and deposition of ECM components represented by collagen within the alveolar septa (Kakugawa et al., 2004). *In vitro* studies with human leiomyoma cells (Lee et al., 1998) and rat hepatic stellate cells (Di Sario et al., 2002) suggested that the antifibrotic effects of pirfenidone might be a direct consequence of reduced mitogenesis and collagen synthesis. However, few studies into the specific effects of pirfenidone on lung fibroblasts *in vitro* have been reported.

HSP47 is a collagen-binding, stress-inducible protein localized in the endoplasmic reticulum that participates in the intracellular processing, folding, assembly, and secretion of procollagens (Ishida et al., 2006; Saga et al., 1987; Sauk et al., 1994). HSP47 is never released into the extracellular matrix, but irrespective of the tissue site and organ, induction of HSP47 expression is always noted during the process of fibrosis, particularly in and around fibrotic lesions (Abe et al., 2000; Shioshita et al., 2000). HSP47-positive cells, especially myofibroblasts, are now thought to be the main source of collagen synthesis, so these cells therefore proposed to play a central role in the synthesis, deposition, and remodelling of the ECM in pulmonary fibrosis in both human patients and animal models (Ishii et al., 2003; Kakugawa et al., 2004, 2005).

Transforming growth factor (TGF)-β is a profibrotic cytokine crucial in the development of pulmonary fibrosis (Bartram and Speer, 2004). Elevated levels of TGF-β in the lungs have been demonstrated in animal models of lung fibrosis and in humans with IPF (Broekelmann et al., 1991; Khalil et al., 1991), and overexpression of TGF-β in the lung causes severe and irreversible pulmonary fibrosis (Sime et al., 1997). TGF-β also enhances expression of HSP47 and collagen-1 in human lung fibroblasts (Yoshioka et al., 2007). In this study, we investigated the precise *in vitro* action of pirfenidone on the TGF-β1-induced expression of HSP47 and collagen in the lung using a cultured normal human lung fibroblast cell line (NHLF).

MATERIALS AND METHODS

Cells and Reagents. Normal human adult lung fibroblasts (Clonetics normal human lung fibroblasts [NHLF]) were purchased from Cambrex (Walkersville, MD). Cells were grown in fibroblast basal medium (FBM; Clonetics-BioWhittaker) supplemented with 2% foetal bovine serum, human recombinant fibroblast growth factor (1.0 µg/ml), insulin (5 mg/ml), gentamicin, and amphotericin-B at 37°C in a 5% CO_2-humidified atmosphere. All experiments were performed after 3-5 cell passages. Pirfenidone was provided by Shionogi & Co. Ltd., (Osaka, Japan). Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN).

Treatment of cells with pirfenidone and TGF-β1. To analyse the expression of HSP47 and collagen type I mRNA and protein, subcultures of NHLF were plated in 60-mm cell culture dishes (BD Falcon™, Franklin Lakes, NJ) at a density of 5 x 10^5 cells per dish. When the cells reached a confluence of approximately 70-80%, the medium was replaced by serum-free FBM. Cells were subsequently stimulated for 24, 36, and 48 h with culture medium alone (control) or with 100, 500, and 1000 µg/ml of pirfenidone with or without TGF-β1 (5 ng/ml). As determined from our previous studies, this concentration of TGF-β1 induces significant HSP47 and collagen type I production (Yoshioka et al.,2007).

Alamar Blue reduction assay. To assess the effect of pirfenidone on cell proliferation, NHLF grown to confluence in 96-well plates were separately incubated for 24 h with

100, 500, and 1000 µg/ml of pirfenidone. Cells were subsequently treated for 3 h with 10-fold diluted Alamar Blue (Serotec, Oxford, UK). Cell viability, as assessed by the reducing area of proliferating cells, was measured at 540-nm excitation and 620-nm emission. The results were compared with the nontreated control cells.

Northern blotting. Human HSP47 and collagen-type I complementary DNAs (cDNAs) were kindly provided by the Institute for Frontier Medical Science, Kyoto University, Japan. HSP47 mRNA was detected using a cDNA probe containing a 1.5-kilobase pair (kbp) *Eco*R-I fragment of full-length human HSP47 (CBP2), whereas collagen type I mRNA was detected with a 0.6-kbp *Eco*R-V of full-length pCOLA1-I-CP-containing cDNA probe. The probes were labelled with ^{32}P using a Random Primer Labelling kit (Takara Biomedicals, Shiga, Japan). RNA was extracted from cells cultured under defined conditions for 24 h using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the method recommended by the supplier. Isolated RNAs (10 µg) were electrophoresed on a 1% agarose gel containing 1.2% formaldehyde, transferred to a nylon membrane (Hybond™-N$^+$, Amersham International, Amersham, UK), and then separately hybridized with each ^{32}P-labelled probe for 20 h at 42°C. Autoradiographed membranes were analyzed using a BAS5000 bioimage analyser (Fuji Photo Film, Japan). Relative transcription was normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe hybridization.

Western blotting. After treatment for 36 h as detailed above, cultured cells were

harvested and lysed in lysis buffer (20 mM Tris-HCl, 133 mM NaCl, 1% NP-40, 10%

glycerol, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 1 µg/ml pepstatin, and 1

µg/ml leupeptin). The supernatants obtained by centrifugation of the lysates at 15,000

x *g* for 10 min at 4°C were used as cytoplasmic extracts. Protein concentrations were

determined using a BCA assay kit (Pierce, Rockford, MI). Protein samples were

electrophoresed on 10% SDS-polyacrylamide gels. The proteins in the gels were

transferred onto nitrocellulose membranes (Pall Corporation, East Hills, NY), blocked

in Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 7.5] and 0.15 M NaCl) containing

0.05% Tween 20 (v/v) (TBST) and 5% (w/v) nonfat dry milk, and then reacted with

the mouse monoclonal anti-human HSP47 antibody (1 µg/ml) (Stressgen, Victoria,

Canada) diluted in TBST containing 5% nonfat dry milk overnight at 4°C with

constant agitation. After several washes with TBST, the membranes were incubated

with anti-mouse IgG secondary antibody (1:2000 dilution; DAKO, Glostrup,

Denmark). After several subsequent washes with TBST, proteins in the membranes

were visualized using an enhanced chemiluminescence detection kit (Amersham

Biosciences) according to the manufacturer's instructions. The intensity of HSP47 was

correlated against a mouse anti-GAPDH monoclonal antibody (1:1000 dilution)

(Chemicon International, Temecula, CA) and quantified by densitometry.

Immunocytochemistry. After 36 and 48 h incubation as noted above, cultured cells

were fixed with acetone for 10 min, and immunocytochemistry was performed using

the Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingame, CA) with

the mouse anti-human HSP47 antibody (0.25 μg/ml) and goat anti-human collagen type I polyclonal antibody (0.04 μg/ml). Irrelevant mouse or goat IgG primary antibodies were used as controls for nonspecific staining (Santa Cruz Biotechnology, Santa Cruz, CA). The staining intensity of HSP47 and collagen type I in the NHLF was scored semiquantitatively using a grading of 0 to 3 (0 = no staining; 1 = weak staining; 2=moderate staining; 3 = strong staining). Staining intensity was examined in five separate fields at × 400 magnification. Total cell count was converted into 100, and thus the maximum number was 300 in this scoring system. The results were reproducible for interobserver and intraobserver variability. Statistical analysis was applied to the representative results of one observer.

Statistical analysis. The results of northern and western blotting and Alamar Blue reduction assay were expressed as mean ± SEM. Differences between two pirfenidone-untreated groups in the presence or absence of TGF-β1 were determined by Student's *t*-test. The dose-dependent effects of pirfenidone were analyzed by Williams' test for multiple comparisons. In contrast, the cytological scores for protein expression levels were presented as the median and 10th to 90th percentile values. Mann-Whitney's U-test was used to analyze differences in the scores between two pirfenidone-untreated groups in the presence or absence of TGF-β1. The dose-dependent effects of pirfenidone on the scores were assessed by Shirley-Williams' test for multiple comparisons. A two-tailed *P* <0.05 was considered significant. Data were analyzed with STATVIEW software (ver 5.0; SAS Institute Inc, Cary, NC) in combination with Excel 2004 (ver 11.0; Microsoft, Redmond, WA).

RESULTS

Effects of pirfenidone on expression of TGF-β1-induced HSP47 and collagen type I mRNA

We first examined the effect of pirfenidone on NHLF proliferation using the Alamar Blue reduction assay. Treatment with pirfenidone tended to decrease cell proliferation of NHLF, although the change was not significant (Fig. 1). The effect of pirfenidone on basal and TGF-β1-induced expression of HSP47 and collagen type I mRNA in NHLF was analysed by northern blotting (Figs. 2 and 3). Treatment with 5 ng/ml of TGF-β1 induced a 4-fold increase in HSP47 mRNA expression in 24hours. Pirfenidone alone at 100, 500, and 1000 μg/ml had no effect on the HSP47 mRNA expression in the NHLF after the 24 h incubation. However, treatment with pirfenidone induced a dose-dependent downregulation of HSP47 mRNA in NHLF stimulated by TGF-β1, which was significant for both 500 and 1000 μg/ml dosages of pirfenidone (Fig. 2b).

Treatment with TGF-β1 also induced a 6.2-fold increase in collagen type I mRNA expression (Fig. 3b). Pirfenidone did not affect the expression of collagen type I mRNA similarly in unstimulated NHLF; however, treatment with pirfenidone also reduced mRNA levels of collagen type I in a dose-dependent manner, with significance reached with the 500 and 1000 μg/ml dosages (Fig. 3b).

Effects of pirfenidone on production of TGF-β1-induced HSP47 and collagen type I protein

Next, we analysed the effects of pirfenidone on basal and TGF-β1-induced production of HSP47 protein in NHLF by western blotting (Fig. 4). Treatment with TGF-β1 tended to increase the levels of HSP47 protein. As with the mRNA expression, pirfenidone alone had no significant effect on HSP47 protein expression in unstimulated NHLF, whereas NHLF stimulated by TGF-β1 showed a dose-dependent and significant downregulation of HSP47 protein when co-treated with pirfenidone at 500 and 1000 µg/ml.

Immunocytochemical studies using the anti-human HSP47 and collagen type I antibodies revealed some cells stained for HSP47 and collagen type I after 36 and 48 h incubation in control samples (data not shown). Treatment with TGF-β1 increased both the number of immunopositive cells and the intensity of HSP47 and collagen type I staining at 36 and 48 h (Fig. 5a for HSP 47 and Fig. 6a for collagen type I at 48 h), while treatment with pirfenidone reversed this effect and decreased the staining in a dose-dependent manner at 36 and 48 h (Fig. 5b, c, d for HSP47 and Fig. 6b, c, d for collagen type I at 48h). Data for the 36-h time-point showed a similar trend (not shown). Treatment with TGF-β1 for 48 h significantly increased the average rate of our grading score for both HSP47 (Fig. 7) and collagen type I (Fig. 8) expression (*n*=5), whereas pirfenidone co-treatment significantly reduced the average grading for both HSP47 and collagen type I dose dependently. The changes were significant for HSP47 at 500 and 1000 µg/ml of pirfenidone (Fig. 7), and at 100, 500, and 1000 µg/ml for collagen type I (Fig. 8).

Discussion

The major finding of this study was that pirfenidone directly reduced the ehnhanced production of both HSP47 and collagen type I mRNA and protein induced by TGF-β1 in human lung fibroblasts in a dose-dependent manner.

In animal models of pulmonary fibrosis induced by bleomycin (Iyer et al., 1998, 1999, 2000) and cyclophosphamide (Kehrer and Margolin, 1997), pirfenidone demonstrated both anti-inflammatory and anti-fibrotic activities. In these *in vivo* studies, treatment with pirfenidone clearly reduced the histological and biochemical signs of lung fibrosis including hydroxyproline content. It also decreased the pulmonary protein levels and gene expression of some fibrogenic cytokines such as TGF-β1(Iyer et al., 2000) and platelet-derived growth factor (PDGF) (Gurujeyalakshmi et al., 1999). In the murine endotoxin shock model (Oku et al., 2002) and a murine macrophage cell line(Nakazato et al., 2002), pirfenidone potently inhibited the production of proinflammatory cytokines such as TNF-α, which mediates the enhanced fibroblast proliferative response, and enhanced the production of anti-inflammatory cytokines like interleukin-10. In an *in vitro* study using NHLF, this drug was also effective in inhibiting cell proliferation (Dosanjh et al., 1998). Together, these findings indicated the strong potential of pirfenidone as a novel, broad-spectrum antifibrotic agent. However, in an *ex-vivo* rat ureteric obstruction model, pirfenidone did not inhibit collagen synthesis in renal fibroblasts(Hewitson et al., 2001), and induced an attenuated fibrotic reaction at the pulmonary level without significant impact on hydroxyproline synthesis in the cyclophosphamide- induced

mouse model of lung fibrosis(Kehrer and Margolin, 1997). Therefore, the precise *in vitro* molecular effects of this agent, particularly on collagen synthesis, need to be elucidated. Here, we clearly demonstrated that pirfenidone directly inhibits the synthesis of collagen in human lung fibroblasts at both the protein and mRNA levels *in vitro*, which is in agreement with other *in vitro* studies using rat hepatic stellate cells(Di Sario et al., 2002) and leiomyoma cells(Lee et al., 1998). Together, these findings confirm the potential action of pirfenidone at the cellular level.

The present study also showed that pirfenidone inhibits the synthesis of HSP47 in NHLF at both the mRNA and protein levels. HSP47, a collagen-binding, stress-inducible protein, acts specifically as a collagen-specific molecular chaperone in the intracellular processing of procollagen (Ishida et al., 2006; Saga et al., 1987; Sauk et al., 1994). We reported previously that HSP47-positive fibroblasts were increased in fibrotic lesions of IPF (Kakugawa et al., 2005), fibrotic transplanted kidney (Abe et al., 2000), and peritoneal sclerosis(Shioshita et al., 2000). We also demonstrated an increase in HSP47 mRNA and protein in a murine bleomycin-induced pulmonary lung fibrosis model (Ishii et al., 2003; Kakugawa et al., 2004). HSP47 is localized predominantly in $\alpha-$SMA-positive myofibroblasts in lung interstitium and the relative amounts of HSP47 mRNA in lung correlate significantly with hydroxyproline content (Ishii et al., 2003; Kakugawa et al., 2004). In surgical biopsy specimens from human patients, expression of HSP47 in lung fibroblasts was significantly higher in idiopathic usual interstitial pneumonia (UIP) patients than in those with collagen vascular disease-associated UIP(Kakugawa et al., 2005, which has the better prognosis of the two conditions(Flaherty et al., 2003); a similar expression

pattern was reported for procollagen type I(Kakugawa et al., 2005). These findings suggested that an increased expression of HSP47 underlies the accumulation and deposition of ECM seen in pulmonary fibrosis and that this might correlate with prognosis. Our report that pirfenidone treatment suppressed the increased expression of HSP47 in the bleomycin-induced lung fibrosis model in mice(Kakugawa et al., 2004) suggested that this drug might actually change the fibroblast phenotype. Here, we demonstrated a similar inhibitory effect of pirfenidone on HSP47 in NHLF at both the protein and mRNA levels, confirming that the *in vivo* effect of pirfenidone on HSP47 represented a direct effect on lung fibroblasts. In this context, our immunocytochemical study for collagen type I has shown that pirfenidone co-treatment significantly reduced the average grading at 100 μg/ml, while this agent significantly inhibited TGF-β1-enhanced HSP47 and collagen type I mRNA expression at 500 μg/ml. This suggests that pirfenidone may act as an anti-fibrotic agent by directly inhibiting both HSP47 and collagen type I mRNA expression with a resultant reduction of collagen synthesis in lung fibroblasts. In support of this proposal, inhibition of HSP47 by antisense oligodeoxynucleotides markedly suppressed the production of collagen in 3T6 cells(Sauk et al., 1994), experimental proliferative glomerulonephritis, and peritoneal fibrosis(Nishino et al., 2003; Sunamoto et al., 1998), implicating HSP47 as a promising target for the treatment of fibrotic diseases including IPF. This study thus identified pirfenidone as the first agent able to control HSP47 expression.

  Finally, our previous studies showed that regenerated type II pneumocytes in UIP patients and the mouse model start expressing type I procollagen through the

induction of HSP47 (Ishii et al., 2003; Kakugawa et al., 2004, 2005), suggesting that in addition to fibroblasts, type II pneumocytes also contribute to lung fibrosis. Pirfenidone also suppressed the bleomycin-induced increases in HSP47-positive type II pneumocytes in the mouse model (Kakugawa et al., 2004). Therefore, further *in vitro* studies using type II pneumocytes would be valuable for defining the mode of action for pirfenidone in lung fibrosis.

Acknowledgement

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FIGURE LEGENDS

Figure 1. Effects of pirfenidone on viability and proliferation of NHLF measured by the Alamar Blue reduction assay. NHLF were separately incubated for 24 h with 0 (control) to 1000 μg/ml of pirfenidone. The growth levels were compared with nontreated control cells and resulting ratio is shown as the percentage change from control values. Data are mean ± SEM of three independent experiments. Pirfenidone tended to decrease NHLF cell proliferation, although the change was not significant.

Figure 2. Effects of pirfenidone on HSP47 mRNA expression in NHLF. Northern blots (a) showing mRNA expression of HSP47 in NHLF after 24 h incubation with medium alone (control), 100, 500 and 1000 μg/ml of pirfenidone co-incubated with 5 ng/ml of TGF-β1. The densities of the mRNA bands were normalized against the GAPDH mRNA band and shown as a ratio (b). Values are mean ± SEM of four independent experiments.

Figure 3. Effects of pirfenidone on collagen type I mRNA expression in NHLF. Northern blots (a) showing mRNA expression of collagen type I in NHLF after 24 h incubation with medium alone (control), 100, 500 and 1000 μg/ml of pirfenidone co-incubated with 5 ng/ml of TGF-β1. The densities of the mRNA bands were normalized against the GAPDH mRNA band and shown as a ratio (b). Values are mean ± SEM of four independent experiments.

Figure 4. Effects of pirfenidone on HSP47 protein production in NHLF. Western blotting (a) for HSP47 showing protein production after 24 h incubation with medium alone (control), 100, 500, or 1000 µg/ml of pirfenidone co-incubated with 5 ng/ml of TGF-β1. T The densities of the protein bands were normalized against GAPDH and expressed as a ratio (b). Values are mean ± SEM of four independent experiments.

Figure 5. Immunocytochemistry of HSP47 expression in NHLF stimulated with TGF-β1 (5 ng/ml) after 48 h incubation with 0 (a), 100 (b), 500 (c), and 1000 µg/ml (d) of pirfenidone. A marked increase in HSP47 immunostaining was noted in NHLF treated with TGF-β1 (5 ng/ml) alone (a), while co-treatment with pirfenidone decreased the expression of HSP47 in a dose-dependent manner. Original magnification: ×400.

Figure 6. Immunocytochemistry of collagen type I expression in NHLF stimulated with TGF-β1 (5 ng/ml) after 48 h incubation with 0 (a), 100 (b), 500 (c), and 1000 µg/ml (d) of pirfenidone. A marked increase in collagen type I immunostaining was noted in NHLF treated with TGF-β1 (5 ng/ml) alone (a). Co-treatment with pirfenidone decreased the expression of collagen type I in a dose-dependent manner. Original magnification: ×400.

Figure 7. Grading score for HSP47 immunostaining of NHLF after TGF-β1 (0 and 5 ng/ml) and pirfenidone (0, 100, 500, 1000 µg/ml) co-treatment for 48 h. Treatment

with TGF-β1 significantly increased the average rate of the grading score. Treatment

with pirfenidone significantly decreased this average rate in a dose-dependent manner.

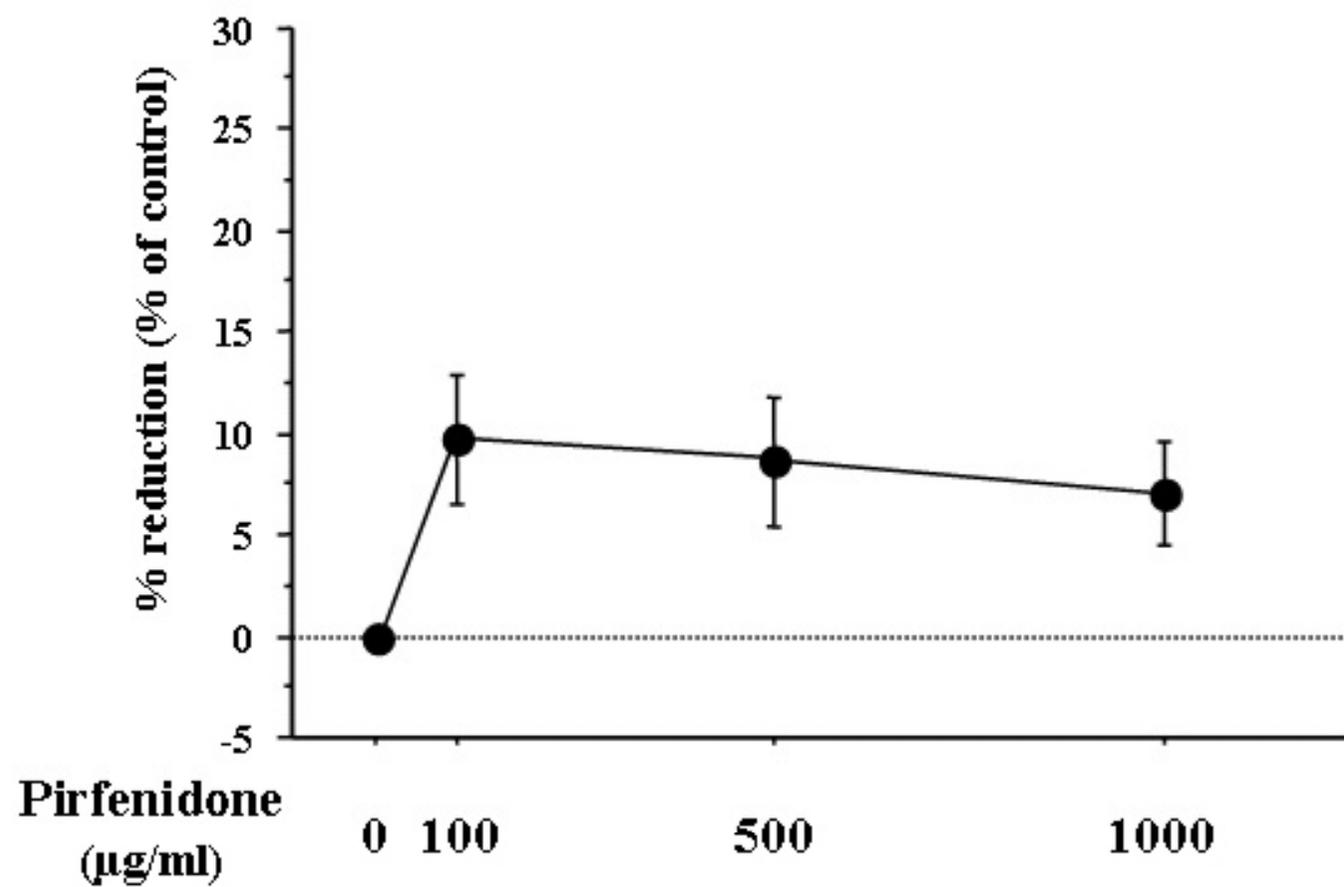Values are mean ± SEM of five independent experiments.

Figure 8. Grading score of collagen type I immunostaining of NHLF after TGF-β1 (0,

5 ng/ml) and pirfenidone (0, 100, 500, 1000 μg/ml) co-treatment for 48 h. Treatment

with TGF-β1 increased significantly the average rate of the grading score. Treatment

with pirfenidone significantly decreased this average rate in a dose-dependent manner.
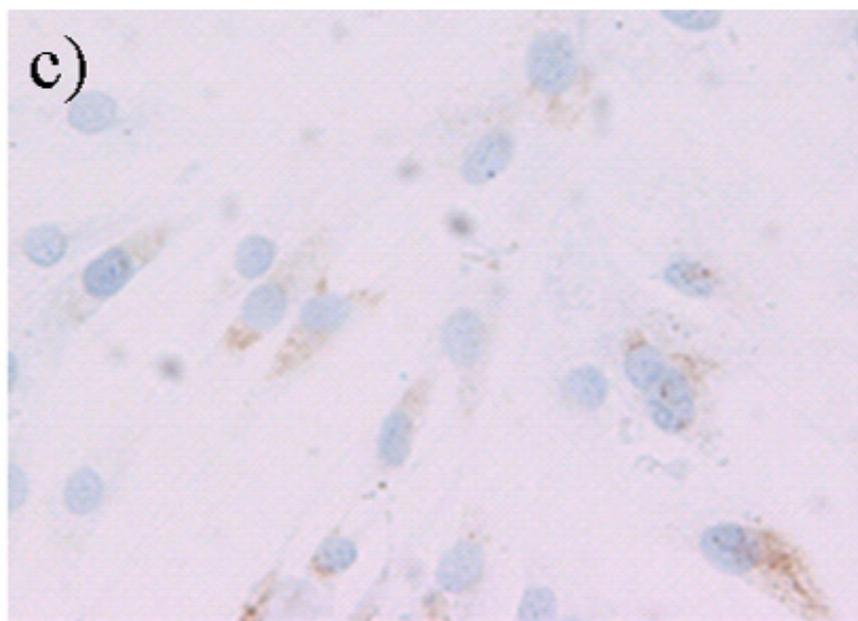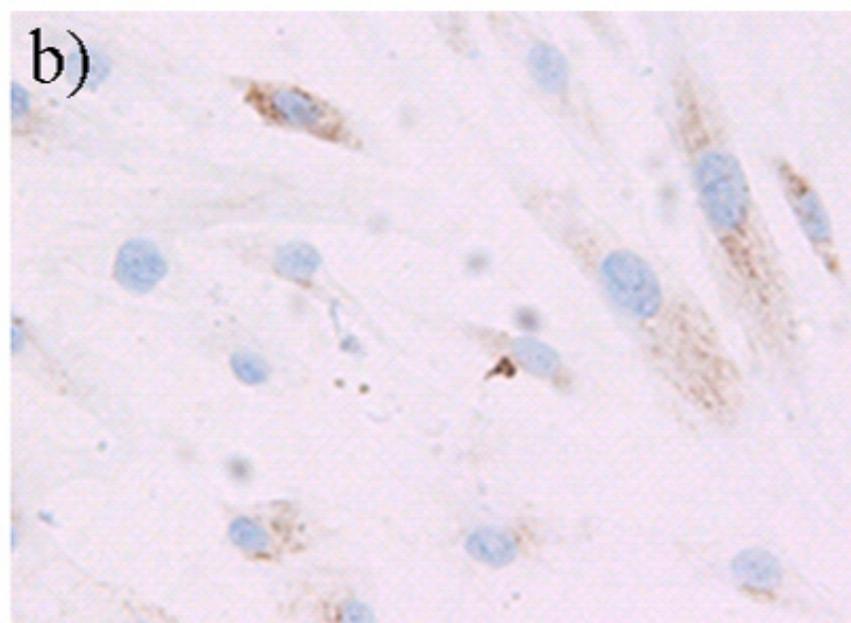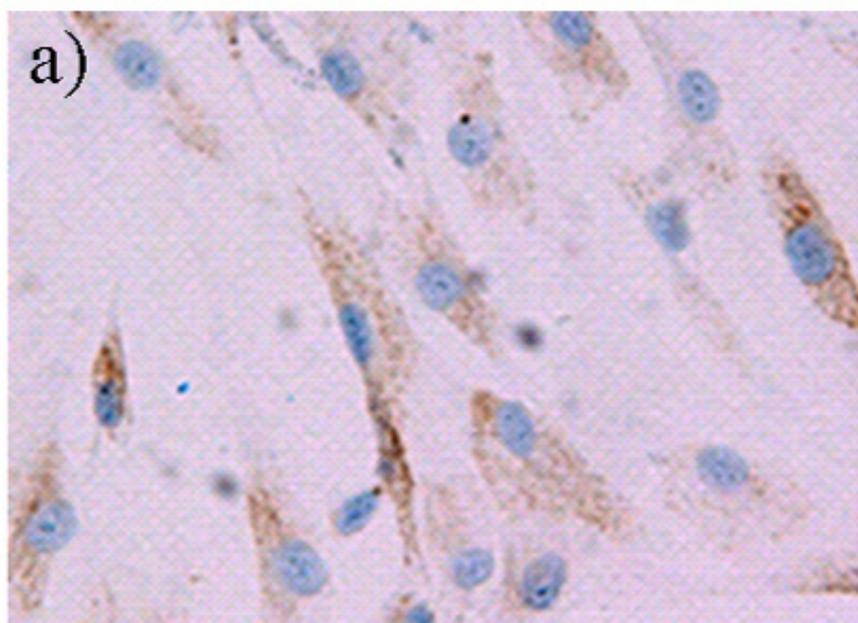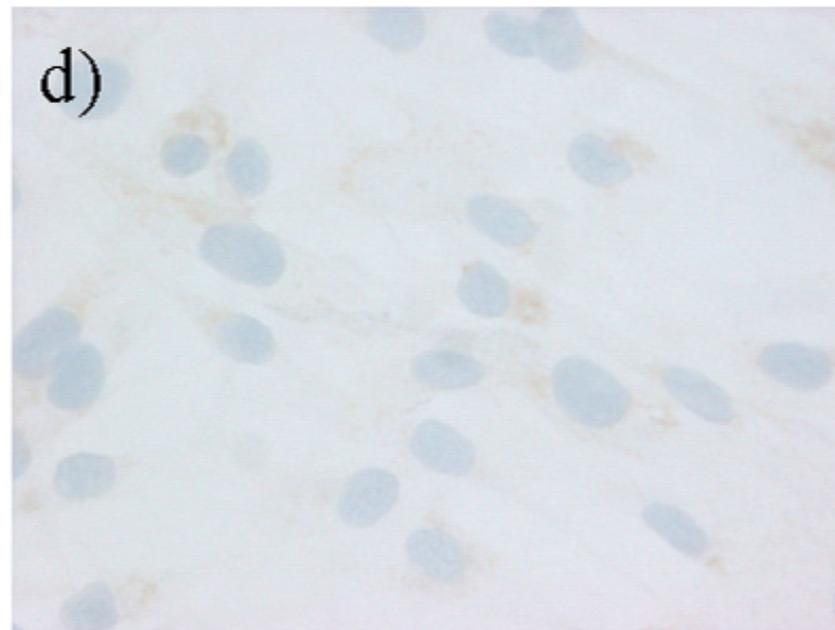
Values are mean ± SEM of five independent experiments.

Figure 1, Nakayama S et al.

Figure 2, Nakayama S et al.,
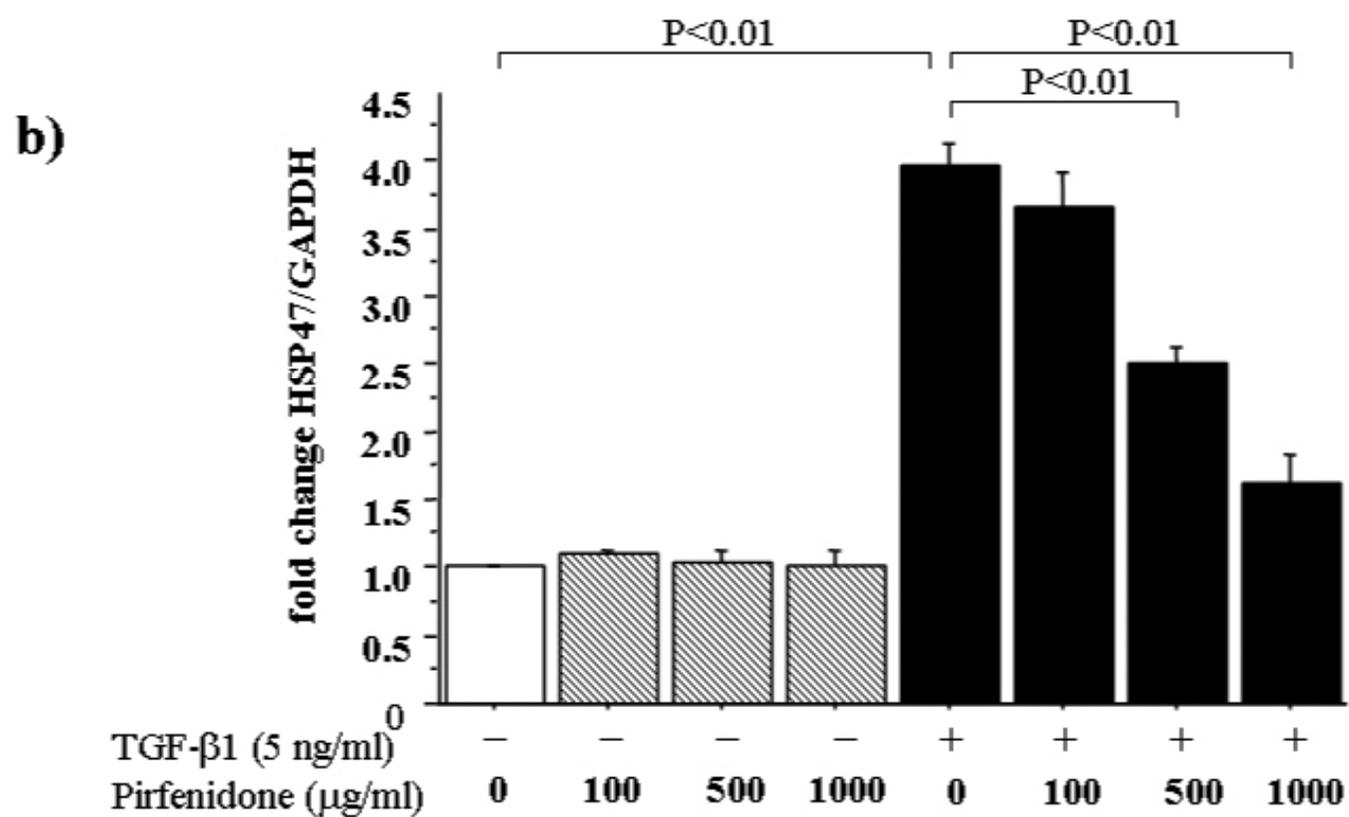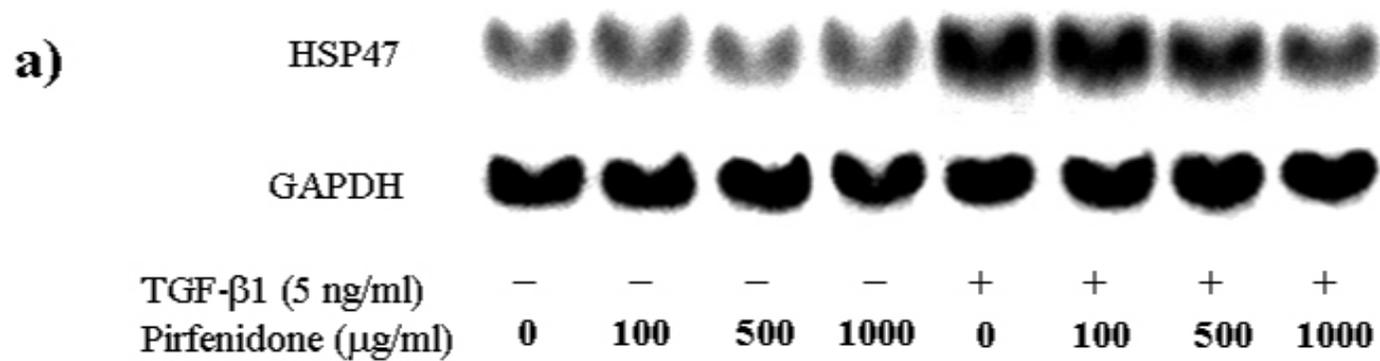
a)

collagen type I

GAPDH

TGF-β1 (5 ng/ml)	–	–	–	–	+	+	+	+
Pirfenidone (μg/ml)	0	100	500	1000	0	100	500	1000

b)

TGF-β1 (5 ng/ml)	–	–	–	–	+	+	+	+
Pirfenidone (μg/ml)	0	100	500	1000	0	100	500	1000

a)

HSP47							
GAPDH							

TGF-β1 (5 ng/ml) − − − − + + + +
Pirfenidone (μg/ml) 0 100 500 1000 0 100 500 1000

b)

TGF-β1 (5 ng/ml) − − − − + + + +
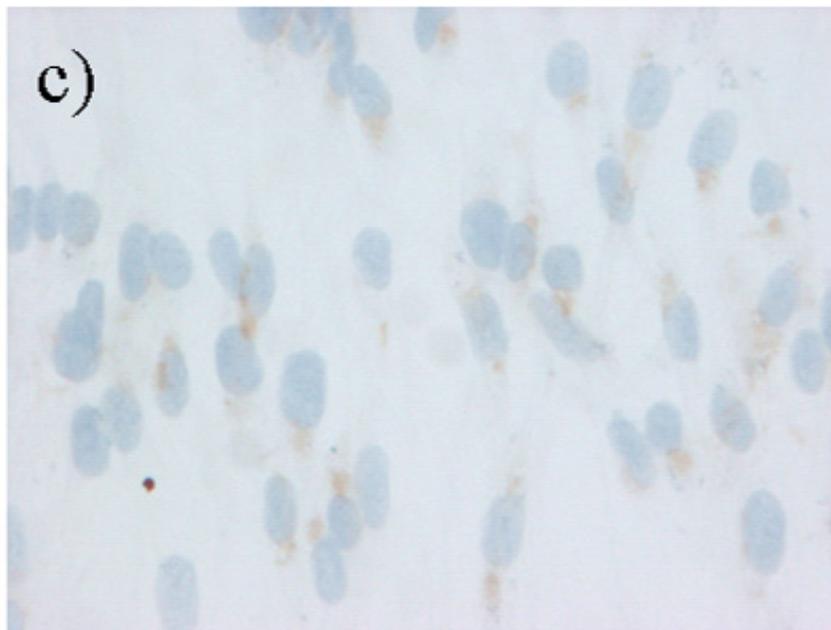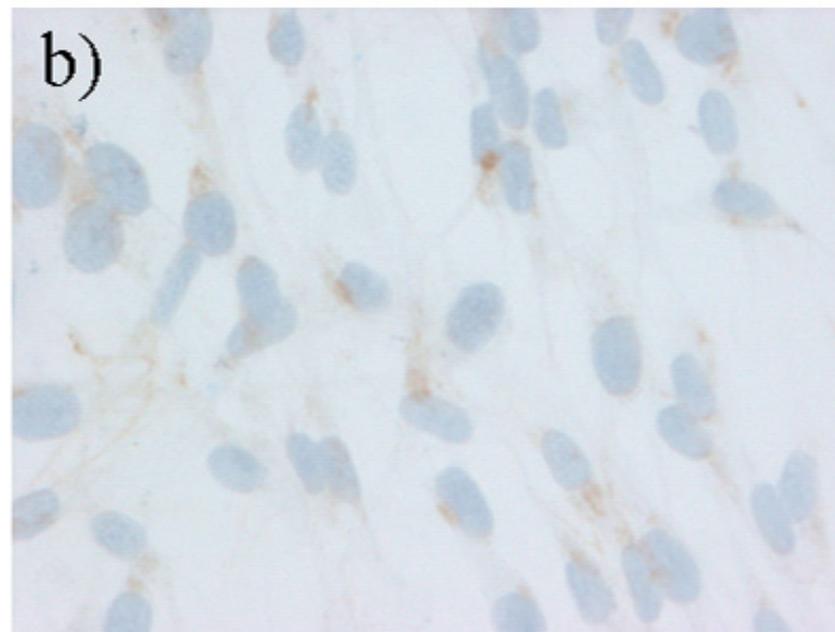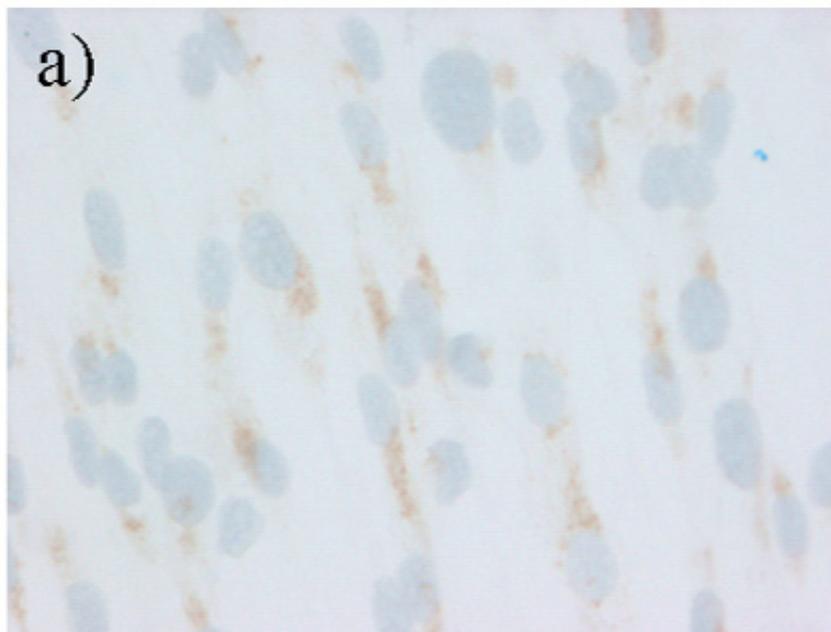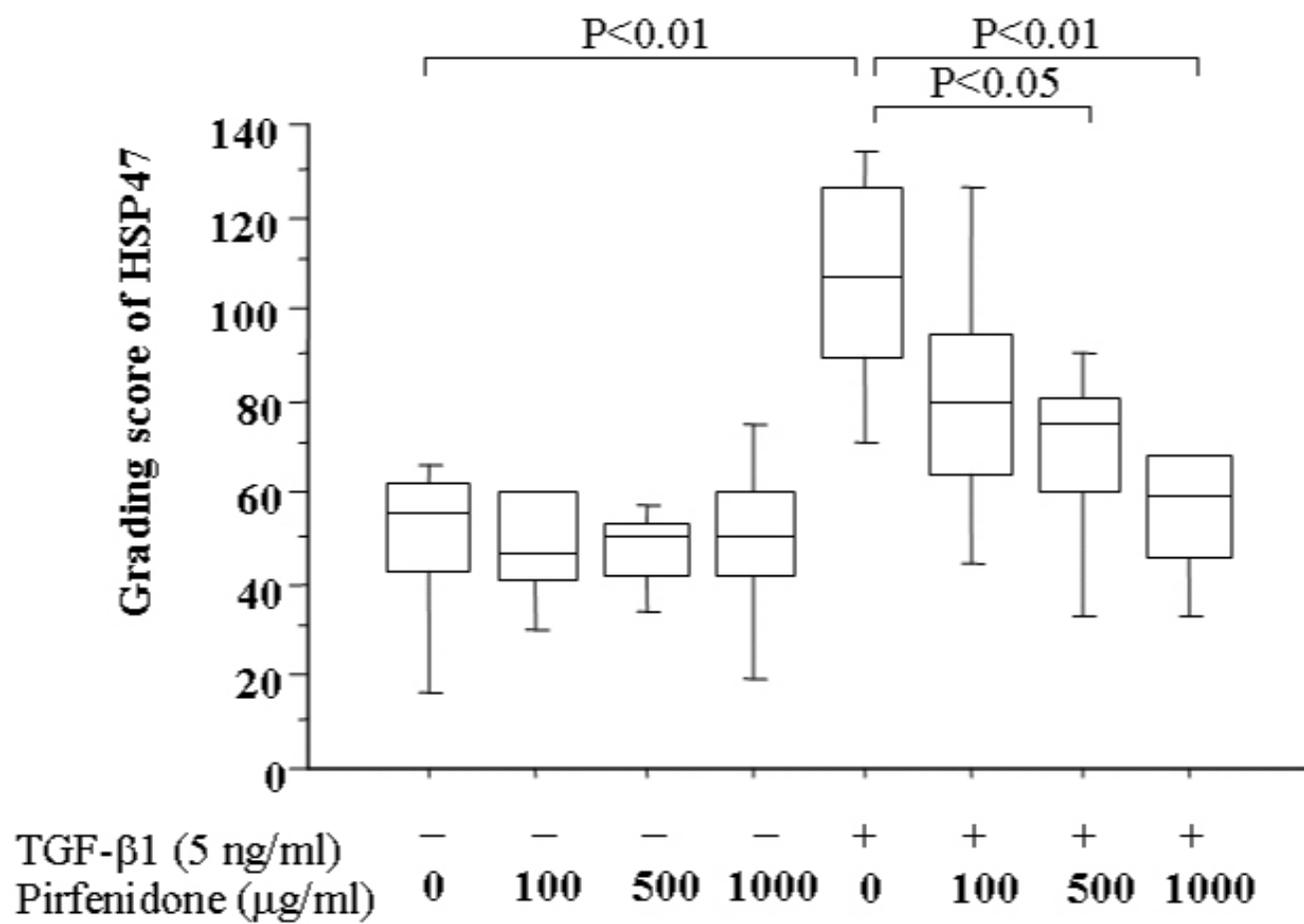Pirfenidone (μg/ml) 0 100 500 1000 0 100 500 1000
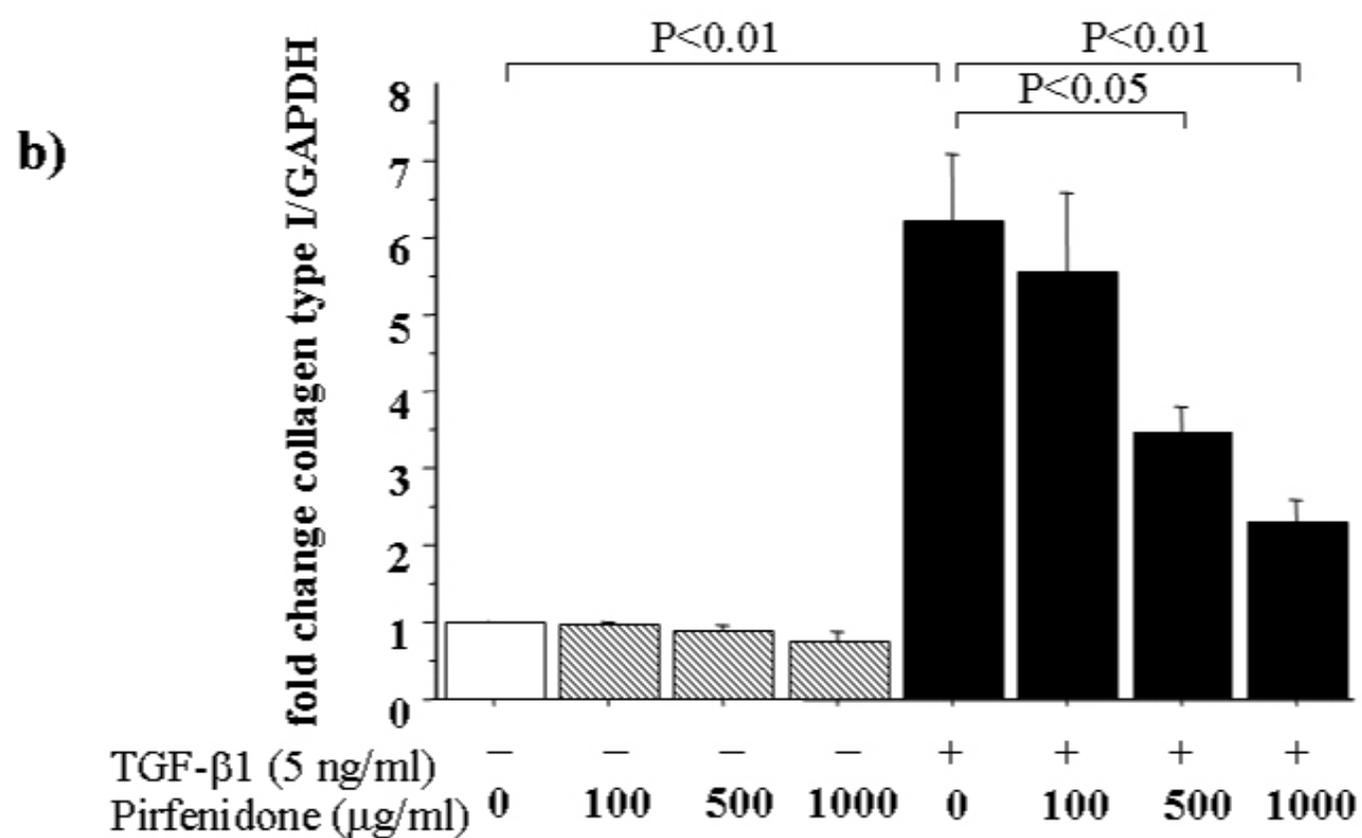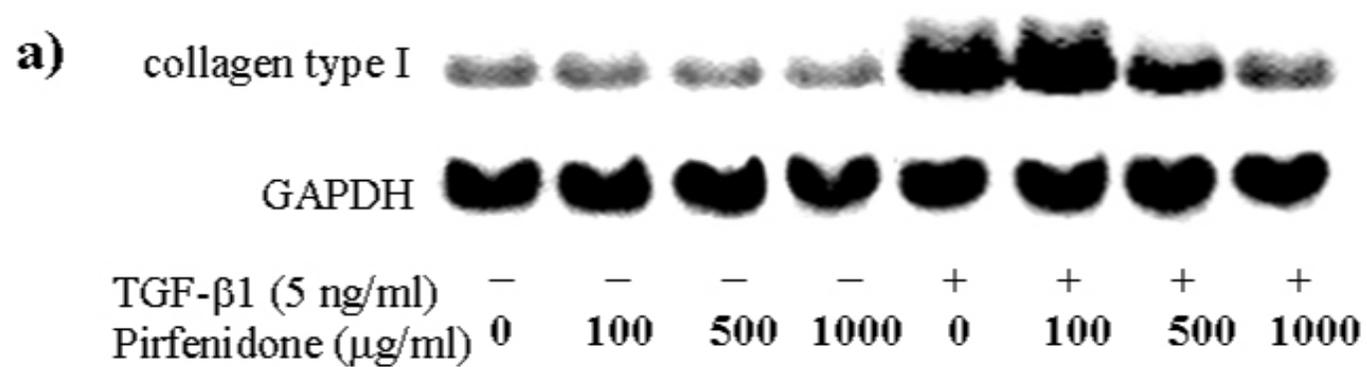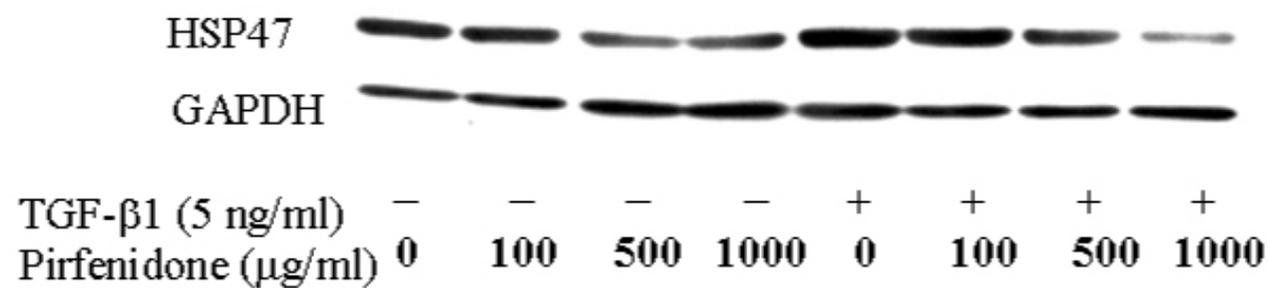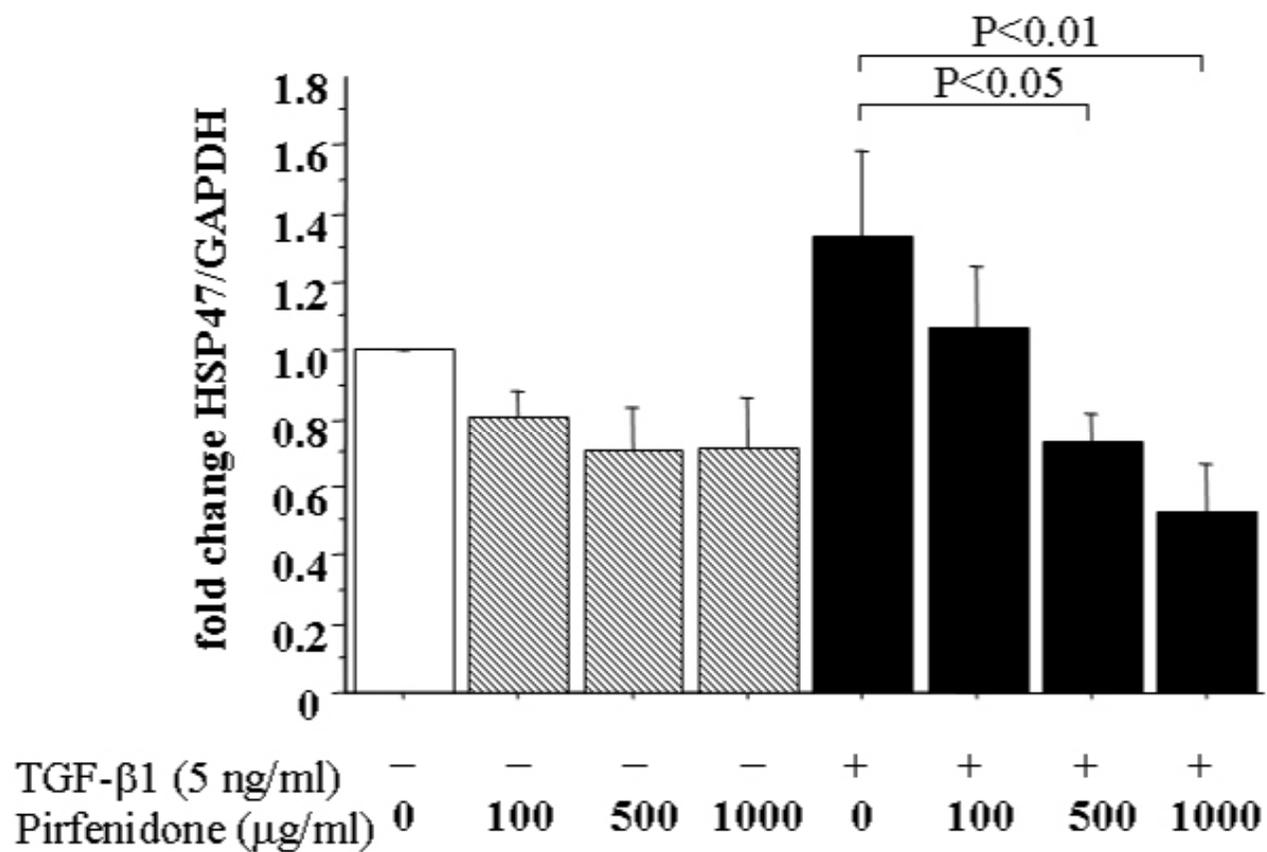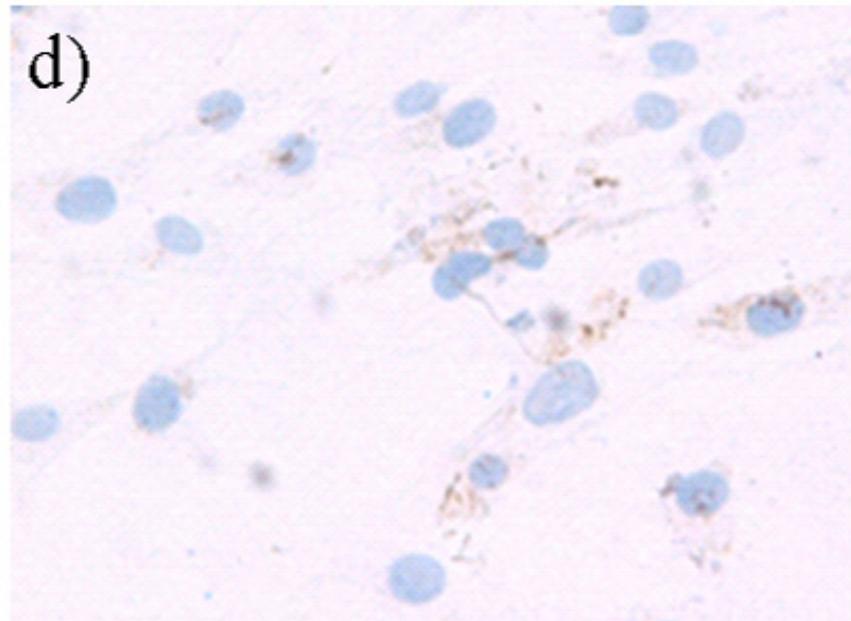
Figure 7, Nakayama S et al.,

Figure 8, Nakayama S et al.,