

PDCD4 promotes inflammation/fibrosis by activating the PPAR- γ /NF- κ B pathway in mouse atrial myocytes

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Abstract. Fibrosis is the basis of structural remodeling in atrial fibrillation (AF), during which inflammation is crucial. Programmed cell death factor 4 (PDCD4) is a newly identified inflammatory gene, with unknown mechanisms of action in AF. The present study aimed to elucidate the effects of PDCD4 on the inflammation and structural remodeling of atrial myocytes. For this purpose, a PDCD4 overexpression plasmid (oePDCD4) and PDCD4 small interfering (si)RNA (siPDCD4) were used to modulate PDCD4 expression in mouse atrial myocytes (HL-1 cells). The expression of PDCD4 was detected using reverse transcription-quantitative PCR and western blot analysis. The optimal drug concentrations of peroxisome proliferator-activated receptor γ (PPAR γ) agonist (pioglitazone hydrochloride), NF- κ B inhibitor (CBL0137), PPAR γ inhibitor (GW9962) and NF- κ B agonist (betulinic acid) were screened using a Cell Counting Kit-8 assay. The levels of inflammatory factors were detected using enzyme-linked immunosorbent assays, the expression levels of fibrosis-related proteins and NF- κ B subunits were detected using western blot analysis, and the expression of phosphorylated (p-)p65/p65 was detected using immunofluorescence staining. The results revealed that PDCD4 overexpression increased the levels of fibrotic factors (collagen I, collagen III, fibronectin, α -smooth muscle actin and matrix metalloproteinase 2), pro-inflammatory cytokines (IFN- γ , IL-6, IL-17A and TNF- α) and p-p65, whereas it reduced the levels of anti-inflammatory cytokines (IL-4) in HL-1 cells. Additionally, treatment with the PPAR γ agonist and NF- κ B inhibitor reversed the levels of fibrotic-, pro-inflammatory

and anti-inflammatory factors in oePDCD4-HL-1 cells. By contrast, PDCD4 silencing exerted the opposite effects on fibrotic factors, pro-inflammatory cytokines, anti-inflammatory cytokines and p-p65. In addition, treatment with the PPAR γ inhibitor and NF- κ B agonist reversed the levels of fibrotic-, pro-inflammatory and anti-inflammatory factors in siPDCD4-HL-1 cells. In conclusion, the present study demonstrated that PDCD4 may induce inflammation and fibrosis by activating the PPAR γ /NF- κ B signaling pathway, thereby promoting the structural remodeling of atrial myocytes in AF.

Introduction

Atrial fibrillation (AF), which affects >37.5 million individuals worldwide, is the most common and persistent type of arrhythmia and AF is also the second most common cause of mortality (1,2). The main mechanism involved in AF is structural remodeling (3). AF places a burden on families and society; however, its pathogenesis remains unclear.

Previous studies have identified an association between inflammation and the occurrence/maintenance of AF (2,4). Inflammation is a protective biological response of a host to infection-induced damage; when such changes occur in the atrium, inflammation induces atrial fibrosis, which predisposes individuals to AF. Notably, fibrosis is considered the basis for the structural remodeling of the atrium (5). In addition, AF induces atrial inflammation, which activates cardiac fibroblasts to cause atrial fibrotic remodeling, thus generating a vicious cycle; that is, AF-inflammation-fibrosis (5). Fibrosis and inflammation directly or indirectly promote the structural remodeling of AF (5-7). In addition, inflammation and fibrosis not only function in the occurrence and development of AF, but also serve etiological roles in its pathogenesis.

The amino-terminal sequence which is encoded by programmed cell death factor 4 (PDCD4) is highly conserved among species and freely shuttles within and out of the nucleus. Notably, PDCD4 is enriched in the inflammatory process of AF and is associated with a high risk of developing stroke in patients with AF (8). PDCD4 regulates the pathophysiological processes of fibrosis and inflammation (9,10); the silencing or knockdown of PDCD4 has been shown to reduce inflammation in human dermal papillary cells (11), and to improve

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ventricular remodeling, oral mucosal fibrosis and liver fibrosis (12-14). However, the mechanisms by which PDCD4 affects AF, inflammation and fibrosis remain to be elucidated.

As a transcription factor in the nuclear receptor superfamily, peroxisome proliferator-activated receptor γ (PPAR γ) is deemed to be a prospective target for treating inflammatory diseases (15). The PPAR γ agonist pioglitazone hydrochloride (Pio) can reduce TNF- α expression, thereby attenuating the inflammation of bronchial occlusion following lung transplantation (16). NF- κ B is an inflammatory transcription factor, the activation of which is caused by a variety of external stimuli, such as inflammation and immune responses (17). During inflammation, the activation of PPAR γ inactivates NF- κ B by direct binding to NF- κ B p65, resulting in a diminished inflammatory response after ubiquitin degradation (18).

The present study aimed to determine whether PDCD4 mediates PPAR γ -mediated inflammation via the NF- κ B pathway, inducing the structural remodeling of mouse atrial myocytes (HL-1 cells).

Materials and methods

Cells, cell culture, transfection and treatment. Mouse atrial myocytes (HL-1; cat. no. iCell-m077; iCell Bioscience, Inc.) were grown in minimum essential medium containing 1% streptomycin/penicillin plus 10% fetal calf serum (Thermo Fisher Scientific, Inc.) in an incubator at 37°C and 5% CO₂.

Overexpression negative control plasmid pcDNA3.1 (oeNC; 2 μ g; GenScript), PDCD overexpression plasmid (oePDCD4; 2 μ g; GenScript), or small interfering (si)RNA NC (siNC; 10 nM; Guangzhou RiboBio Co., Ltd.) and PDCD4 siRNAs (siPDCD4-1, 2 and 3; 10 nM; Guangzhou RiboBio Co., Ltd.) were transfected into HL-1 cells (3 $\times$ 10⁵ cells/well) using Lipofectamine[®] 2000 (Thermo Fisher Scientific, Inc.) for 48 h at 37°C. At 48 h after transfection, cells were harvested for the subsequent experiments. The sequences of siNC, siPDCD4-1, siPDCD4-2 and siPDCD4-3 are provided in Table I.

The HL-1 cells in the overexpression group were further divided into four groups as follows: i) oeNC; ii) oePDCD4; iii) oePDCD4 + PPAR γ agonist (Pio; 0, 1.25, 2.5, 5, 10, 20, 40 and 80 μ M, cat. no. HY-14601; MedChemExpress); and iv) oePDCD4 + NF- κ B inhibitor (CBL0137; 0, 0.125, 0.25, 0.5, 1, 2, 4 and 8 μ M, cat. no. HY-18935A; MedChemExpress). The HL-1 cells in the silencing group were further divided into the following four groups: i) siNC; ii) siPDCD4; iii) siPDCD4 + PPAR γ inhibitor (GW9962; 0, 1.25, 2.5, 5, 10, 20, 40 and 80 μ M, cat. no. M6191-5MG; Merck KGaA); and iv) siPDCD4 + NF- κ B agonist (betulinic acid; 0, 1.25, 2.5, 5, 10, 20, 40 and 80 μ M, cat. no. HY-10529; MedChemExpress). The medium was changed every day. The cells were harvested following 48 h of transfection and 24 h of drug treatment at 37°C.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the HL-1 cells using TRIzol[®] reagent (Thermo Fisher Scientific, Inc.) and cDNA was generated using a RevertAid First Strand CDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. Subsequently, cDNA (2 μ l) was used for qPCR (20 μ l reaction volume), which was performed using 2X SYBR-Green qPCR MasterMix (Thermo Fisher Scientific, Inc.) on A 7500HT Fast

Table I. Sequences of siPDCD4-1, siPDCD4-2 and siPDCD4-3.

siRNA	Sequence, 5'-3'
siNC	UUCUCCGAACGUGUCACGU
siPDCD4-1	CCAGGAGAACUGTGUUUUAU
siPDCD4-2	GCUCCUGAGUAUGUCCAAA
siPDCD4-3	CCCACACUCAUACUCUGUU

si, small interfering; PDCD4, programmed cell death factor 4; NC, negative control.

real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling conditions were as follows: initial denaturation at 94°C for 10 min, 94°C for 20 sec, 55°C for 20 sec and 72°C for 20 sec, for a total of 40 cycles, followed by a final extension at 72°C. The mRNA expression levels of PDCD4 were normalized to GAPDH using the 2^{- $\Delta\Delta$ C_q} method (19). GAPDH served as an internal control. The sequences of the primers used are given in Table II.

Western blot analysis. Nuclear (Nu) and cytoplasmic (Cy) proteins from HL-1 cardiomyocytes were isolated using NE-PER[™] (Thermo Fisher Scientific, Inc.). Total proteins were extracted from the HL-1 cardiomyocytes utilizing RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) containing PMSF and protease inhibitor mixture (Roche Diagnostics). The protein concentration was assessed using a BCA protein concentration assay kit (Biosharp Life Sciences). The proteins (15 μ g/lane) were then separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis on 10% gels and were transferred onto polyvinylidene fluoride (PVDF) membranes (MilliporeSigma). Then the PVDF membranes were blocked by 5% non-fat milk for 2 h at room temperature. Thereafter, the PVDF membranes were incubated with primary antibodies against PDCD4 (cat. no. bs-1608R; 1:1,000; BIOSS), collagen I (cat. no. AF7001; 1:1,000; Affinity Biosciences), collagen III (cat. no. AF0136; 1:1,000; Affinity Biosciences), fibronectin (cat. no. bs-13455R; 1:1,000; BIOSS), α -smooth muscle actin (α -SMA; cat. no. bsm-33187M; 1:1,000; BIOSS), matrix metalloproteinase (MMP) 2 (cat. no. AF0577; 1:1,000; Affinity Biosciences), PPAR γ (cat. no. bs-0530R; 1:1,000; BIOSS), NF- κ B p65 (cat. no. bs-0465R; 1:1,000; BIOSS), NF- κ B phosphorylated (p-) p65 (cat. no. bs-0982R; 1:1,000; BIOSS), NF- κ B p50 (cat. no. bs-1194R; 1:1,000; BIOSS), proliferating cell nuclear antigen (PCNA; cat. no. 10205-2-AP; 1:1,000; Proteintech Group, Inc.) and GAPDH (cat. no. 60004-1-Ig; 1:1,000; Proteintech Group, Inc.) at 4°C overnight. Subsequently, the membranes were incubated with an HRP-labeled goat anti-rabbit (cat. no. A0208; 1:1,000; Beyotime Institute of Biotechnology) or goat anti-mouse secondary antibody (cat. no. A0216; 1:1,000; Beyotime Institute of Biotechnology) at room temperature for 1 h. ECL solution was then added (Beijing Dingguo Changsheng Biotechnology Co., Ltd.). Images were saved using an integrated chemiluminescence instrument (Clinx Science Instruments Co., Ltd.). PCNA served as an internal control for Nu p50, p-p65, and p65.

Table II. Primer sequences.

Gene	Forward (5'-3')	Reverse (5'-3')
PDCD4	CCTGGATGAGACCGCATTTG	GCAAAGGTCAGAAAGCAGCT
GAPDH	GGTGAAGGTCGGTGTGAACG	CTCGTCTCTGGAAGATGGTG

PDCD4, programmed cell death factor 4.

GAPDH served as an internal control for Cy p50, p-p65, p65, and the remaining proteins. Image J (Version 1.8.0; National Institutes of Health) was used for densitometry.

Cell Counting Kit-8 (CCK-8) assay. HL-1 cell viability was assessed using a CCK-8 kit (Beyotime Institute of Biotechnology). The HL-1 cells were incubated in 96-well plates for 24 h, followed by incubation with various concentrations of Pio (0, 1.25, 2.5, 5, 10, 20, 40 and 80 μ M), CBL0137 (0, 0.125, 0.25, 0.5, 1, 2, 4 and 8 μ M), GW9962 (0, 1.25, 2.5, 5, 10, 20, 40 and 80 μ M) and betulinic acid (0, 1.25, 2.5, 5, 10, 20, 40 and 80 μ M) for 24 h at 37°C. Different concentrations were selected for CBL0137 compared with the other three drugs, according to the recommendations of a previous study (20). Subsequently, CCK-8 (10 μ l, Dojindo Laboratories, Inc.) was added to each well followed by incubation in the dark for 2 h. The optical density (450 nm) was measured using an automatic microplate reader (Thermo Fisher Scientific, Inc.).

Enzyme-linked immunosorbent assays (ELISAs). The HL-1 cells were cultured until they reached the logarithmic growth phase; subsequently, the cell concentration was adjusted to 2×10^5 cells/ml. After seeding the cells in a 6-well plate (1 ml/well), the cells were transfected for 48 h and treated with the different drugs for 24 h. Thereafter, the HL-1 cells were collected, and the Mouse ELISA kits (IFN- γ , cat. no. EM007; IL-4, cat. no. EM003; IL-6, cat. no. EM004; IL-17A, cat. no. EM015; and TNF- α , cat. no. EM008; Suzhou ExCell Biotechnology Co., Ltd.) were used, according to the manufacturer's instructions.

Immunofluorescence analysis. The HL-1 cells (2×10^5 cells/well) were grown on slides, fixed in 4% paraformaldehyde for 15 min at 4°C and incubated in 5% FBS (Cytiva) at 37°C for 2 h. The slides were then incubated with primary antibodies against NF- κ B p65 (cat. no. bs-0465R; 1:1,000; BIOSS) and NF- κ B p-p65 (cat. no. bs-0982R; 1:1,000 BIOSS) at 4°C overnight. The following day, the slides were incubated with an Alexa Fluor 488-labeled Goat Anti-Rabbit IgG(H+L) secondary antibody (cat. no. A0423, 1:1,000; Beyotime Institute of Biotechnology) and an Alexa Fluor 555-labeled Donkey Anti-Rabbit IgG(H+L) secondary antibody (cat. no. A0453, 1:1,000; Beyotime Institute of Biotechnology) in the dark at room temperature for 1 h. The cells were then incubated with Hoechst 33342 (cat. no. 875756-97-1; MilliporeSigma) in the dark for 15 min at room temperature. Images were captured under a fluorescence microscope (Olympus Corporation) and semi-quantitative analyses were conducted using ImageJ v1.6 software (National Institutes of Health).

Statistical analyses. The experiments were conducted three times. The results are presented as the mean $\pm$ SD. An unpaired Student's t-test or one-way analysis of variance followed by the Bonferroni post hoc test was carried out using GraphPad Prism 9 software (Dotmatics). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Transfection with oe-PDCD4-HL-1 and si-PDCD4-HL-1. The results of RT-qPCR and western blot analysis revealed that the mRNA and protein expression levels of PDCD4 were significantly higher in the oePDCD4 group than those in the oeNC group (Fig. 1A-C). By contrast, the expression levels of PDCD4 were markedly lower in the siPDCD4 groups compared with those in the siNC group (Fig. 1D-F). siPDCD4-1 exerted the most notable knockdown effect and was thus used in subsequent experiments. These findings indicated the successful transfection of oe-PDCD4 and si-PDCD4 into HL-1 cells.

Viability of HL-1 cells treated with Pio, CBL0137, GW9662 and betulinic acid. Different concentrations of Pio, CBL0137, GW9662 and betulinic acid were administered to HL-1 cells for 24 h, to screen the optimal drug concentrations using the CCK-8 assay.

Compared with in the untreated cells, HL-1 cell viability was significantly inhibited by treatment with ≥ 20 μ M Pio (Fig. 2A), ≥ 0.5 μ M CBL0137 (Fig. 2B), ≥ 20 μ M GW9662 (Fig. 2C) and ≥ 40 μ M betulinic acid (Fig. 2D). Consequently, the concentrations of the drugs used in the subsequent experiments were as follows: Pio, 10 μ M; CBL0137, 0.25 μ M; GW9662, 10 μ M; and betulinic acid, 20 μ M.

oePDCD4-induced inflammation and fibrosis are mitigated by PPAR γ agonist and NF- κ B inhibitor treatment in oePDCD4-HL-1 cells. The results of western blot analysis demonstrated that, compared with in the oeNC group, the expression levels of fibrosis-related proteins (collagen I, collagen III, fibronectin, α -SMA and MMP2) were significantly higher in the oePDCD4 group; these effects were significantly attenuated by treatment with the PPAR γ agonist (Pio) and NF- κ B inhibitor (CBL0137) (Fig. 3A and B).

ELISAs indicated that, compared with those in the oeNC group, the levels of pro-inflammatory cytokines (IFN- γ , IL-6, IL-17A and TNF- α) were significantly increased, while the levels of the anti-inflammatory factor IL-4 were markedly decreased in the oe-PDCD4 group; these effects were significantly reversed by treatment with the PPAR γ agonist (Pio) and NF- κ B inhibitor (CBL0137) (Fig. 3C).

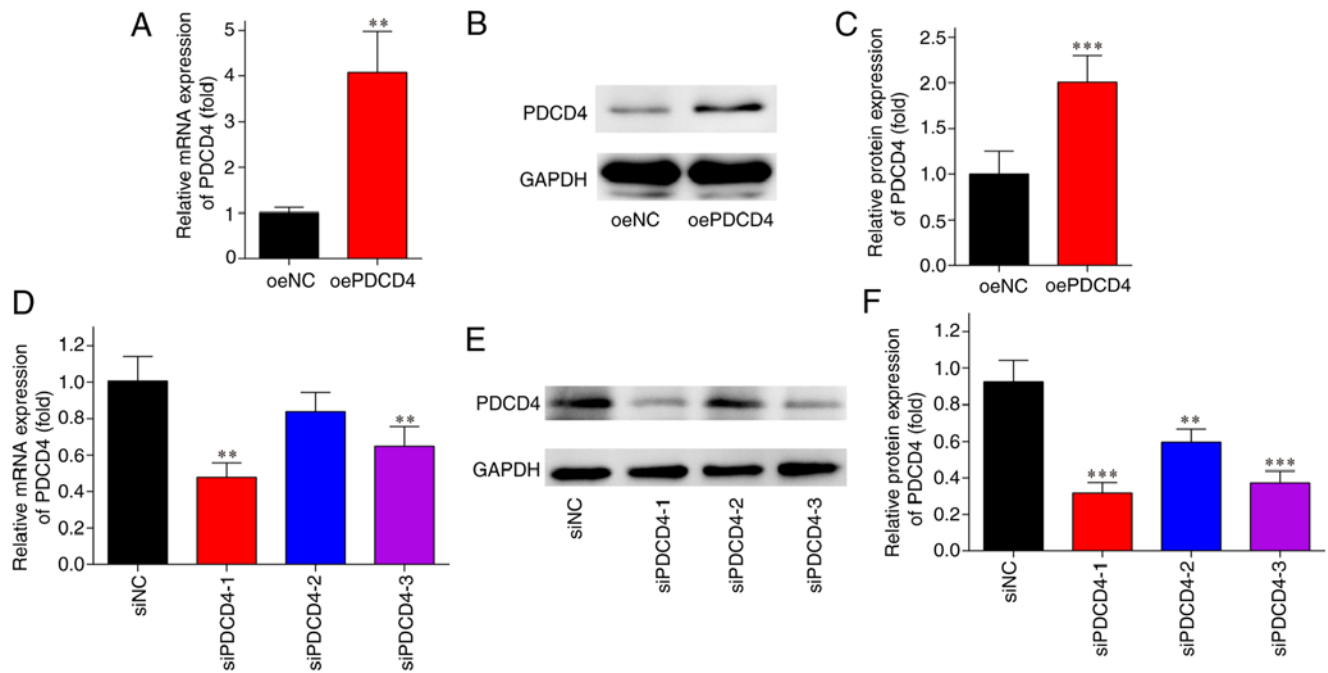


Figure 1. mRNA and protein expression levels of PDCD4 in HL-1 cells after PDCD4 overexpression and silencing. (A) Transcriptional and (B) translational analysis of PDCD4 after oePDCD4 transfection by RT-qPCR and western blotting. (C) Statistical analysis of western blotting. ** $P < 0.01$, *** $P < 0.001$ vs. oeNC. (D) Transcriptional and (E) translational analysis of PDCD4 after siPDCD4 transfection by RT-qPCR and western blotting. (F) Statistical analysis of western blotting. ** $P < 0.01$, *** $P < 0.001$ vs. siNC. $n = 3$. PDCD4, programmed cell death factor 4; RT-qPCR, reverse transcription-quantitative PCR; oe, overexpression; NC, negative control; si, small interfering.

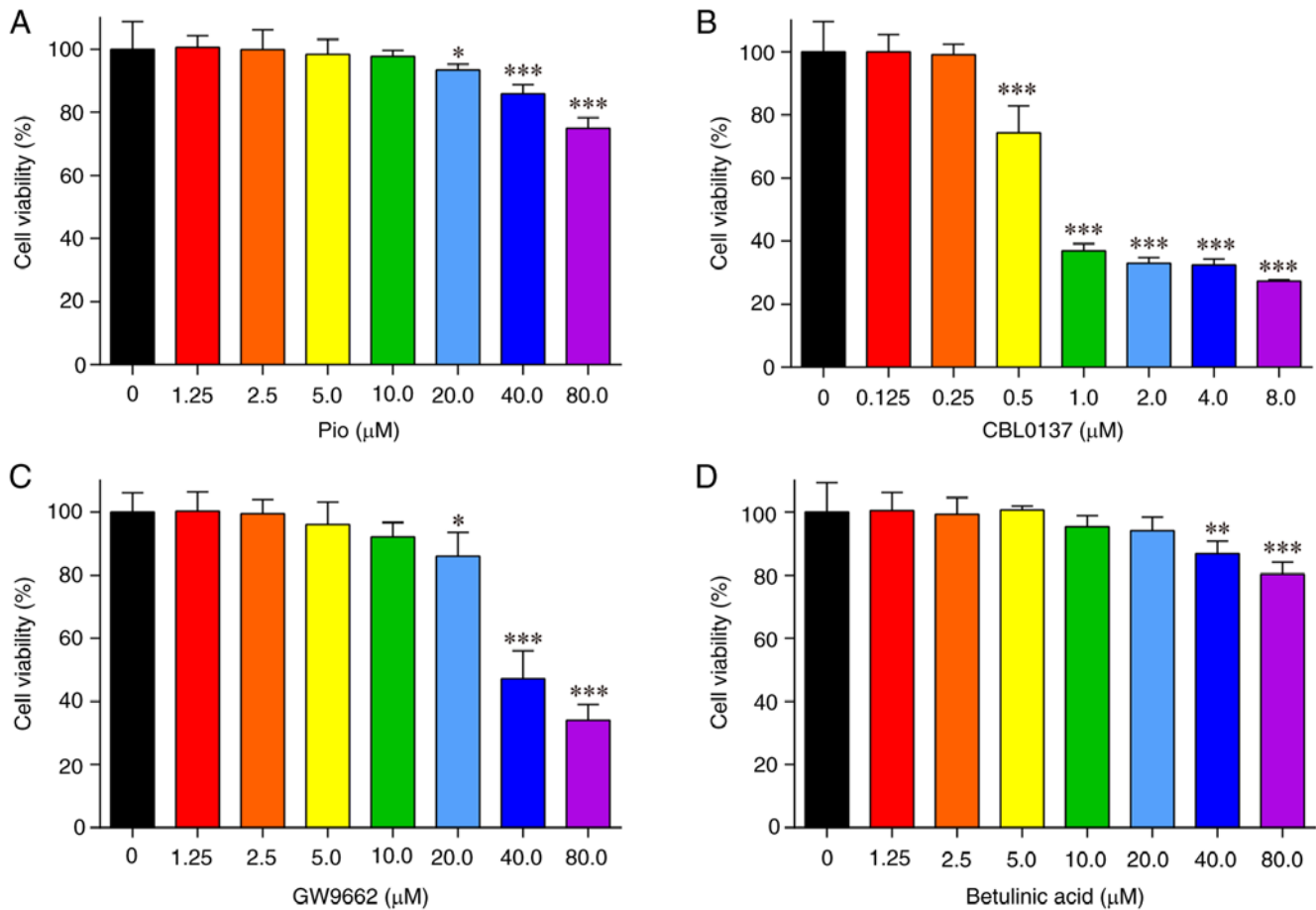


Figure 2. HL-1 cell viability after treatment with different drugs at different concentrations. The optimal dosing concentrations of (A) Pio, (B) CBL0137, (C) GW9662 and (D) betulinic acid were selected based on the results of the Cell Counting Kit-8 assay. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. 0 μM . $n = 4$. Pio, pioglitazone hydrochloride.

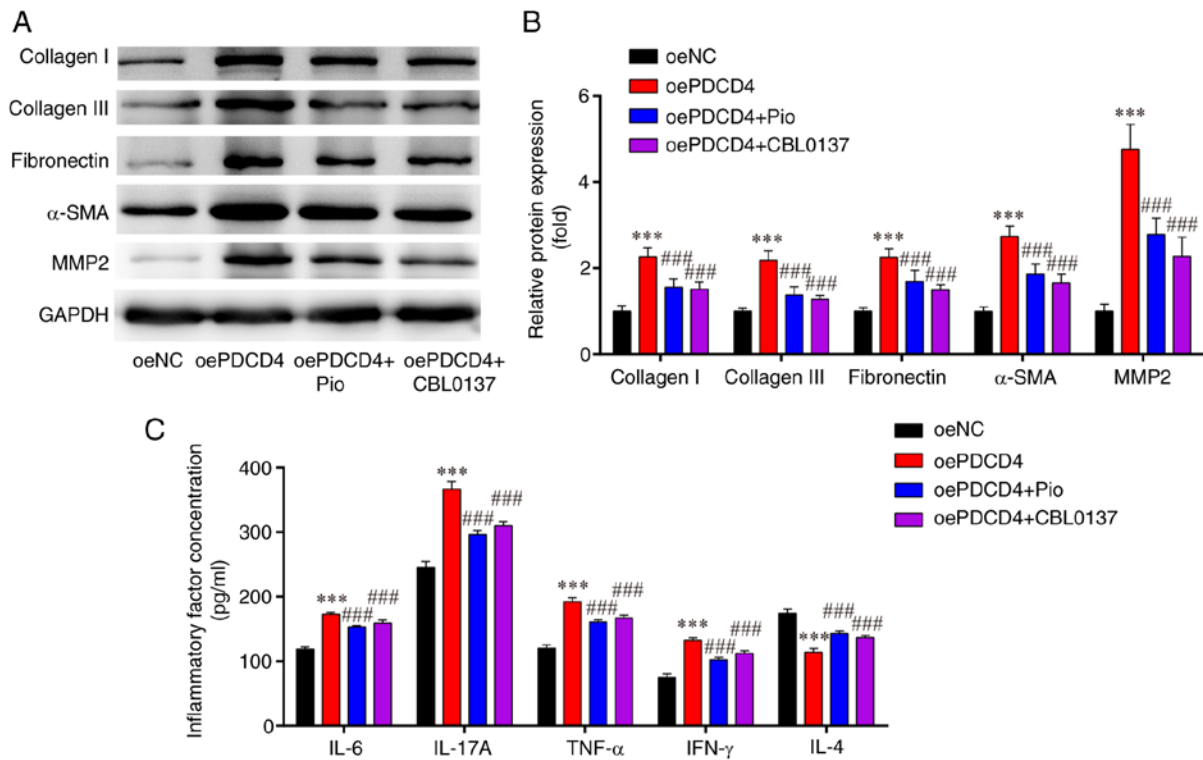


Figure 3. Levels of fibrosis-associated proteins and inflammatory-related cytokines in oePDCD4-HL-1 cells. (A) Western blotting indicated that the upregulated protein expression levels of collagen I, collagen III, fibronectin, α-SMA and MMP2 in oe-PDCD4-HL-1 cells were attenuated by Pio and CBL0137. (B) Statistical analysis of western blotting. (C) Enzyme-linked immunosorbent assays showed that the upregulated protein concentrations of IL-6, IL-17A, IFN-γ and TNF-α, and downregulated protein concentration of IL-4, in oe-PDCD4-HL-1 cells were reversed by Pio and CBL0137. ***P<0.001 vs. oeNC; ###P<0.001 vs. oe-PDCD4. n=3. oe, overexpression; PDCD4, programmed cell death factor 4; α-SMA, α-smooth muscle actin; MMP, matrix metalloproteinase; Pio, pioglitazone hydrochloride; NC, negative control.

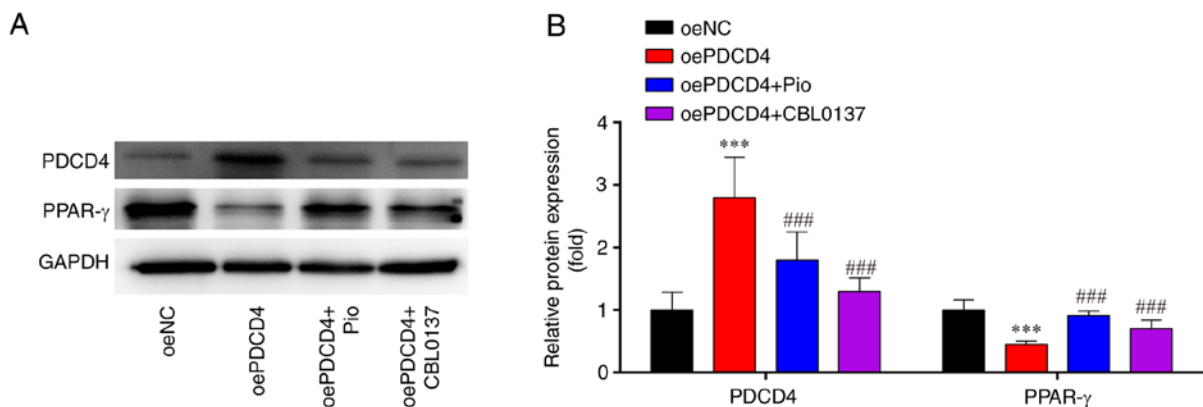


Figure 4. Expression of PPARγ and PDCD4 in oePDCD4-HL-1 cells. (A) Western blotting indicated that the downregulated protein expression levels of PPARγ and upregulated protein expression levels of PDCD4 in oe-PDCD4-HL-1 cells were attenuated by Pio and CBL0137. (B) Statistical analysis of western blotting. ***P<0.001 vs. oeNC; ###P<0.001 vs. oe-PDCD4. n=3. PPARγ, peroxisome proliferator-activated receptor γ; PDCD4, programmed cell death factor 4; oe, overexpression; Pio, pioglitazone hydrochloride; NC, negative control.

These findings demonstrated that the overexpression of PDCD4 may induce fibrosis and inflammation in HL-1 cells, and these effects could be reversed by a PPARγ agonist and NF-κB inhibitor.

oePDCD4-induced activation of p65 is mitigated by PPARγ agonist and NF-κB inhibitor treatment in oePDCD4-HL-1 cells. Western blot analysis demonstrated that, compared with those in the oeNC group, the expression levels of PDCD4 were

markedly higher, whereas the expression levels of PPARγ were significantly lower in the oePDCD4 group; these effects were significantly reversed by treatment with the PPARγ agonist (Pio) and NF-κB inhibitor (CBL0137; Fig. 4A and B).

In addition, western blot analysis demonstrated that, compared with those in the oeNC group, the Nu p-p65/p65 ratio and p-p50/PCNA ratio were increased in the oePDCD4 group, and these effects were partially reversed by treatment with the PPARγ agonist (Pio) and NF-κB inhibitor (CBL0137)

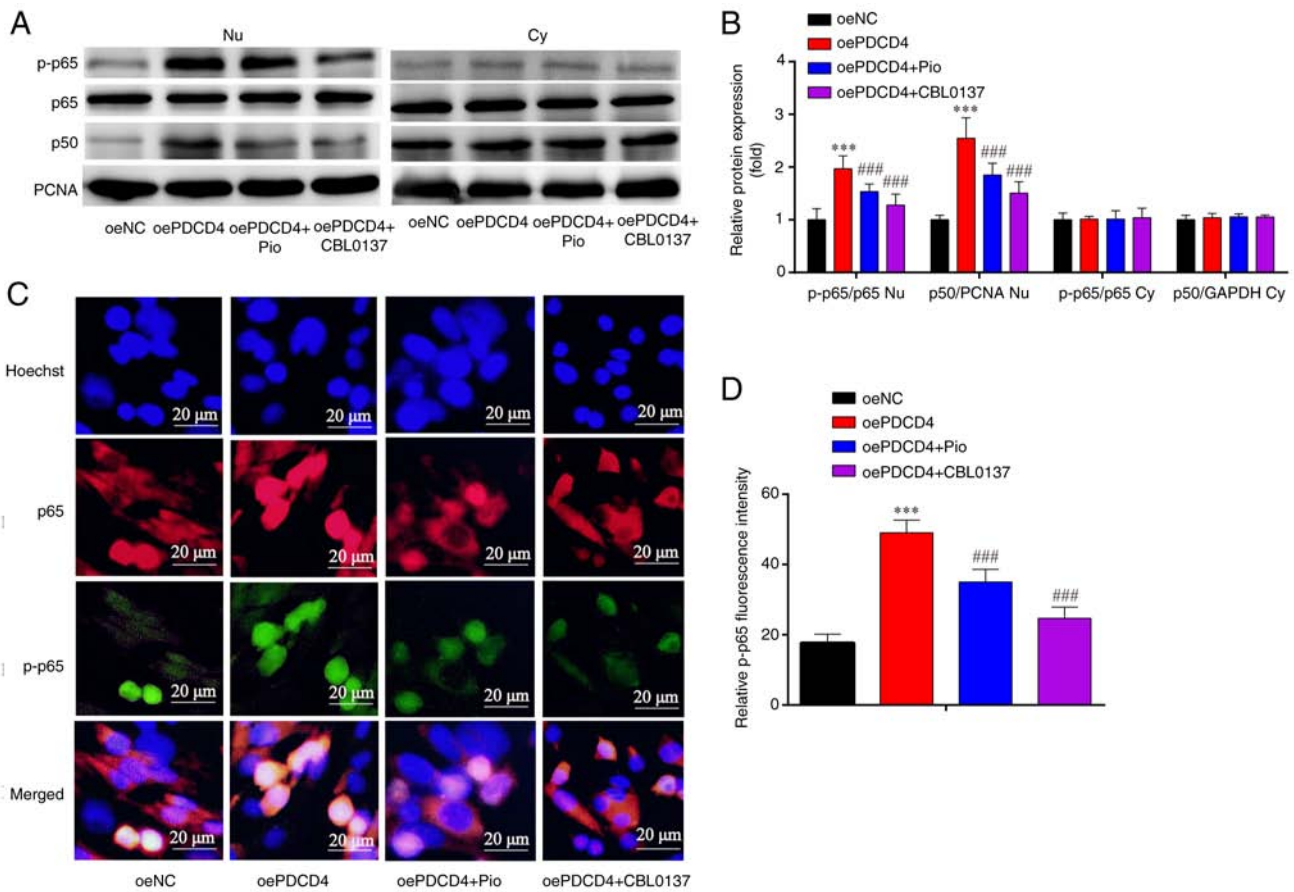


Figure 5. Expression of NF- κ B subunits in oePDCD4-HL-1. (A) Western blotting indicated that, compared with in the oeNC group, the Nu p-p65/p65 ratio and Nu p-p50/PCNA ratio were increased in the oePDCD4 group, and these findings were partially reversed by Pio and CBL0137. However, there was no significant change in Cy p-p65/p65 ratio and Cy p-p50/GAPDH ratio among the four groups. (B) Statistical analysis of western blotting. (C) Immunofluorescence analysis showed that the increased protein expression of p-p65 in oe-PDCD4-HL-1 cells was attenuated by Pio and CBL0137. (D) Statistical analysis of fluorescence intensity. *** $P < 0.001$ vs. oeNC; ### $P < 0.001$ vs. oe-PDCD4. $n = 3$. Magnification, $\times 1,000$. oe, overexpression; PDCD4, programmed cell death factor 4; NC, negative control; p-, phosphorylated; PCNA, proliferating cell nuclear antigen; Pio, pioglitazone hydrochloride; Nu, nuclear; Cy, cytoplasmic; NC, negative control.

(Fig. 5A and B). However, there was no significant change in the Cy p-p65/p65 ratio or p-p50/GAPDH ratio among the four groups (Fig. 5A and B).

The results of immunofluorescence analysis revealed that, compared with in the oeNC group, the expression of p-p65 was increased in the oePDCD4 group; this effect was partially reversed by treatment with the PPAR γ agonist (Pio) and NF- κ B inhibitor (CBL0137) (Fig. 5C and D). These findings suggested that the overexpression of PDCD4 activated NF- κ B p65 in HL-1 cells, and these effects were reversed by the PPAR γ agonist and NF- κ B inhibitor.

siPDCD4-induced mitigation of fibrosis and inflammation are aggravated by PPAR γ inhibitor and NF- κ B agonist treatment in siPDCD4-HL-1 cells. Western blot analysis demonstrated that, compared with those in the siNC group, the expression levels of fibrosis-related proteins (collagen I, collagen III, fibronectin, α -SMA and MMP2) were significantly lower in the siPDCD4 group; these effects were partially reversed by treatment with the PPAR γ inhibitor (GW9662) and NF- κ B agonist (betulinic acid) (Fig. 6A and B).

The results of ELISAs indicated that, compared with those in the siNC group, the levels of pro-inflammatory cytokines (IFN- γ , IL-6, IL-17A and TNF- α) were significantly lower,

while the levels of the anti-inflammatory factor IL-4 were markedly higher in the siPDCD4 group; these effects were partially reversed by the PPAR γ inhibitor (GW9662) and NF- κ B agonist (betulinic acid) (Fig. 6C). These results indicated that the silencing of PDCD4 may attenuate fibrosis and inflammatory injury responses in HL-1 cells, and these effects could be reversed by a PPAR γ inhibitor and NF- κ B agonist.

siPDCD4-induced inactivation of p65 is reversed by PPAR γ inhibitor and NF- κ B agonist treatment in siPDCD4-HL-1 cells. Western blot analysis demonstrated that, compared with those in the siNC group, the expression levels of PDCD4 were significantly lower, whereas PPAR- γ expression was significantly higher in the siPDCD4 group; these effects were partially reversed by treatment with the PPAR γ inhibitor (GW9662) and NF- κ B agonist (betulinic acid) (Fig. 7A and B).

The results of western blot analysis also demonstrated that, compared with those in the siNC group, the Nu p-p65/p65 ratio and p-p50/PCNA ratio were decreased in the siPDCD4 group; these effects were partially reversed by treatment with the PPAR γ inhibitor (GW9662) and NF- κ B agonist (betulinic acid) (Fig. 8A and B). However, there was no significant change in the Cy p-p65/p65 ratio and p-p50/GAPDH ratio among the four groups (Fig. 8A and B).

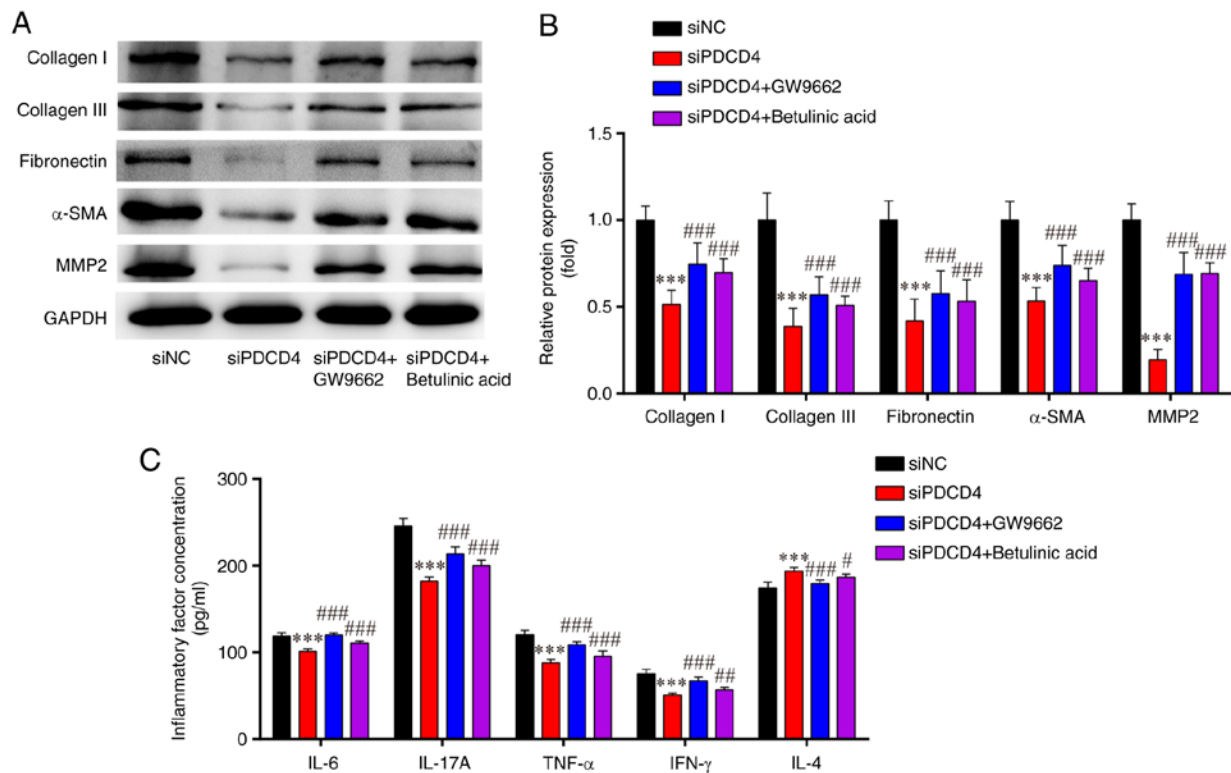


Figure 6. Levels of fibrosis-associated proteins and inflammatory-related cytokines in siPDCD4-HL-1 cells. (A) Western blotting indicated that the downregulated protein expression levels of collagen I, collagen III, fibronectin, α -SMA and MMP2 in si-PDCD4-HL-1 cells were attenuated by GW9662 and betulinic acid. (B) Statistical analysis of western blotting. (C) Enzyme-linked immunosorbent assays showed that the downregulated protein concentrations of IL-6, IL-17A, IFN- γ and TNF- α , and upregulated protein concentration of IL-4, in si-PDCD4-HL-1 cells were reversed by GW9662 and betulinic acid. *** $P < 0.001$ vs. siNC; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs. si-PDCD4. n=3. si, small interfering; PDCD4, programmed cell death factor 4; α -SMA, α -smooth muscle actin; MMP, matrix metalloproteinase; NC, negative control.

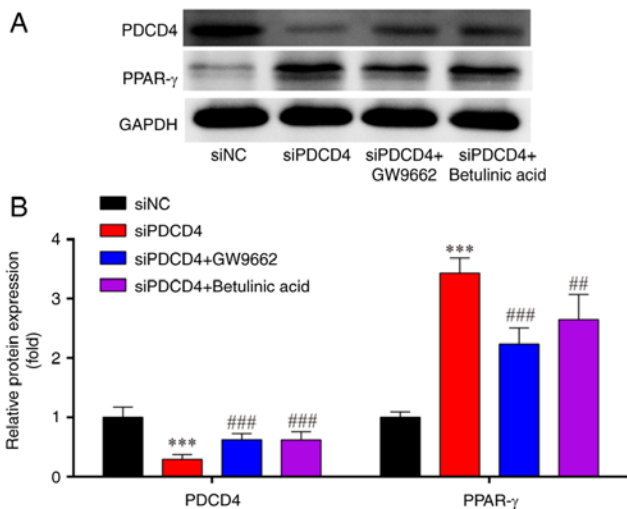


Figure 7. Expression of PPAR γ and PDCD4 in siPDCD4-HL-1. (A) Western blotting indicated that the upregulated protein expression levels of PPAR γ and downregulated protein expression levels of PDCD4 in si-PDCD4-HL-1 cells were attenuated by GW9662 and betulinic acid. (B) Statistical analysis of western blotting. *** $P < 0.001$ vs. siNC; ** $P < 0.01$, *** $P < 0.001$ vs. si-PDCD4. n=3. PPAR γ , peroxisome proliferator-activated receptor γ ; PDCD4, programmed cell death factor 4; si, small interfering; NC, negative control.

Immunofluorescence analysis revealed that, compared with that in the siNC group, p-p65 expression was decreased in the siPDCD4 group; this effect was partially reversed by

treatment with the PPAR γ inhibitor (GW9662) and NF- κ B agonist (betulinic acid) (Fig. 8C and D). These findings suggested that the silencing of PDCD4 reduced the levels of p-p65, and these effects were reversed by PPAR γ inhibitor and NF- κ B agonist in HL-1 cells.

Discussion

In patients with paroxysmal AF, AF is positively associated with IL-6 and TNF- α expression (2). In addition, patients with AF who received 12-month catheter ablation were shown to have higher plasma levels of IFN- γ , IL-6 and TNF- α (21). TNF- α , an endogenous mediator of inflammation, induces the transcription of MMPs (22). Notably, an increased level of inflammatory factors, which promotes the expression of related fibrotic proteins, leads to the development of AF (23). The fibrotic process and inflammatory process influence each other, forming a vicious cycle, which eventually promotes structural remodeling in AF.

PDCD4 is involved in the formation of coronary atherosclerosis by upregulating the transcription of IL-6 (24). The downregulation of PDCD4 has been reported to attenuate myocardial collagen deposition and fibrotic remodeling in mice and rats (25,26). IFN- γ , IL-4, IL-6, IL-17A and TNF- α are common markers of inflammatory injury in myocardial cells (5,27). In the present study, the levels of fibrotic proteins (α -SMA, collagen I, collagen III, fibronectin and MMP2) and pro-inflammatory factors (TNF- α , IL-6, IL-17A and IFN- γ)

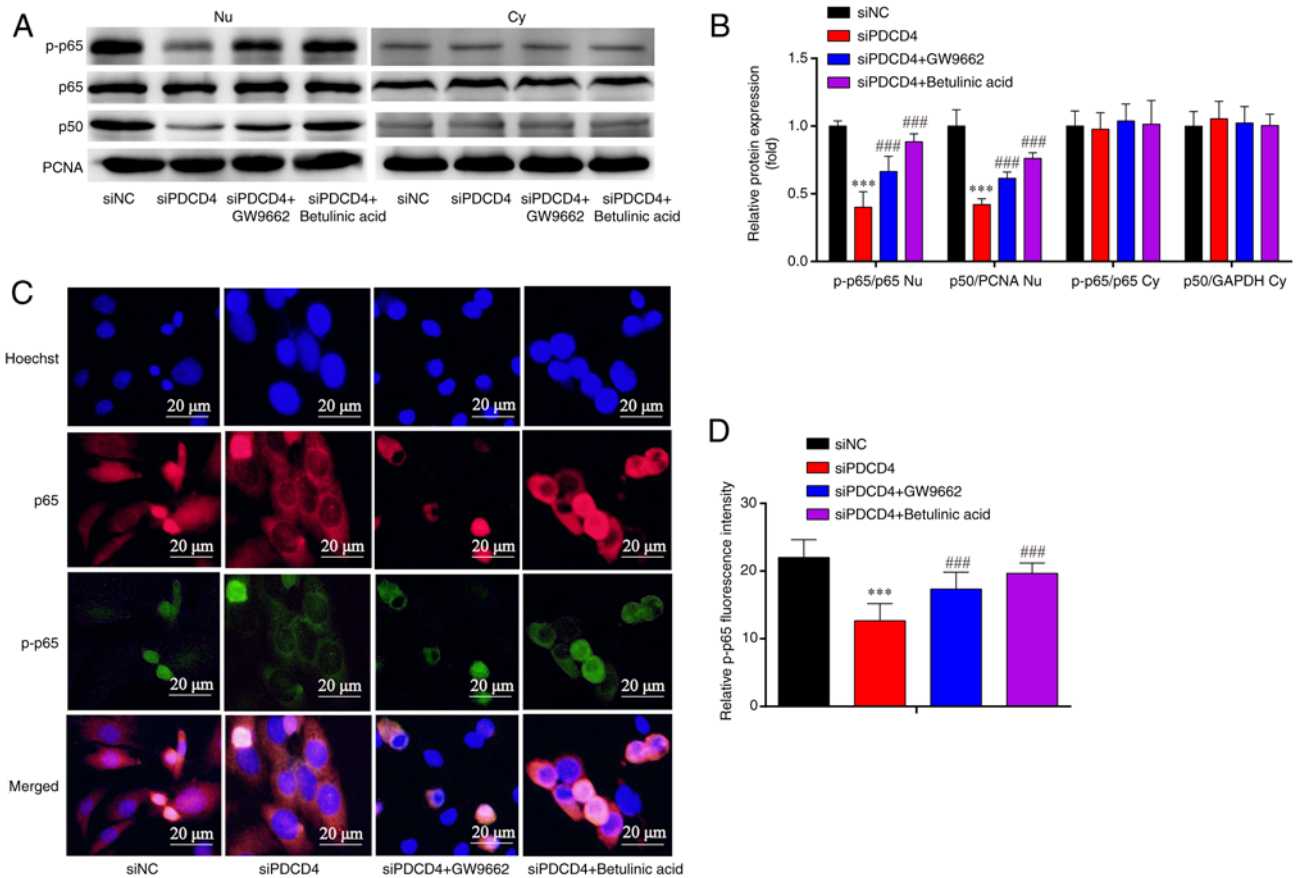


Figure 8. Expression of NF- κ B subunits in siPDCD4-HL-1. (A) Western blotting showed that, compared with in the siNC group, the Nu p-p65/p65 ratio and Nu p-p50/PCNA ratio were decreased in the siPDCD4 group, and the results were partially reversed by GW9662 and betulinic acid. However, there was no significant change in Cy p-p65/p65 ratio and Cy p-p50/GAPDH ratio among the four groups. (B) Statistical analysis of western blotting. (C) Immunofluorescence analysis showed that the decreased protein expression of p-p65 in si-PDCD4-HL-1 cells was attenuated by GW9662 and betulinic acid. (D) Statistical analysis of fluorescence intensity. Magnification, $\times 1,000$. *** $P < 0.001$ vs. siNC; ### $P < 0.001$ vs. si-PDCD4. n=3. si, small interfering; PDCD4, programmed cell death factor 4; NC, negative control; p-, phosphorylated; PCNA, proliferating cell nuclear antigen; Nu, nuclear; Cy, cytoplasmic.

were increased, whereas the levels of the anti-inflammatory factor IL-4 were decreased in response to the overexpression of PDCD4. By contrast, silencing PDCD4 exerted the opposite effects. Therefore, it was hypothesized that PDCD4 may possess a crucial function in AF structural remodeling by regulating inflammation and fibrosis. However, the mechanisms through which PDCD4 carries out its functions in inflammation and fibrosis remain unclear.

PDCD4 has been shown to regulate fibrotic expression by PPAR γ (28). The upregulation of PPAR- γ exerts its antifibrotic effects on liver fibrosis and lung fibrosis by reducing the collagen I content in veins, and exerts its anti-inflammatory effects by decreasing IL-17A and IFN- γ expression (29-32). However, it is unclear whether PDCD4 regulates inflammation and fibrosis in HL-1 cells via PPAR γ . Therefore, oePDCD4-HL-1 cells were treated with a PPAR γ agonist and siPDCD4-HL-1 cells were treated with a PPAR γ inhibitor. Notably, there was a decrease in factors associated with inflammatory damage and fibrosis in oePDCD4-HL-1 cells following treatment with the PPAR γ agonist; by contrast, there was an increase in factors associated with inflammatory damage and fibrosis in siPDCD4-HL-1 cells following treatment with the PPAR γ inhibitor. These findings indicated that PDCD4 regulates inflammation and fibrosis in HL-1 cells

via the PPAR γ pathway and thus serves a role in structural remodeling in AF.

PDCD4 can trigger local inflammation by activating NF- κ B (11), which promotes the transcription and translation of inflammatory cytokines (11,33), and induces inflammation and fibrosis in myocardial cells in mouse atria (33,34). However, whether PDCD4 affects fibrosis and inflammation in HL-1 cells via the NF- κ B pathway remains unclear. Therefore, in the present study, oePDCD4-HL-1 cells were treated with an NF- κ B inhibitor, whereas siPDCD4-HL-1 cells were treated with an NF- κ B agonist. There was a decrease in factors associated with inflammatory damage and fibrosis in oePDCD4-HL-1 cells following treatment with the NF- κ B inhibitor; By contrast, there was an increase in factors associated with inflammatory damage and fibrosis in the siPDCD4-HL-1 cells following treatment with the NF- κ B agonist, suggesting that PDCD4 may regulate fibrosis and inflammation in HL-1 cells via the NF- κ B pathway and is crucial for AF structural remodeling. Furthermore, the silencing of PDCD4 has been shown to improve left ventricular structural remodeling in rabbits with type 2 diabetes (12). PDCD4 inhibition has also been shown to decrease the expression of pro-inflammatory cytokines (IL-1 β , IL-6 and TNF- α) and increase the expression of the anti-inflammatory factor IL-10 by inactivating NF- κ B,

thus attenuating inflammatory injury in macrophage-mediated inflammation, atherosclerosis and lipopolysaccharide-induced injury (35-37). However, it is unclear whether the function of PDCD4 was mediated by NF- κ B in HL-1 cells.

NF- κ B is a key molecule in inflammatory signaling pathways; once activated, p65 and p50 heterodimers in the cytoplasm are translocated to the nucleus, where they are responsible for the transcription of target genes (38). In the present study, immunofluorescence was performed and the results indicated that p-p65 expression was increased in the oePDCD4 group, whereas this effect was attenuated by treatment with the NF- κ B inhibitor. In addition, p-p65 expression was decreased in the siPDCD4 group and this effect was reversed by treatment with the NF- κ B agonist, indicating that PDCD4 may regulate inflammation and fibrosis in HL-1 cells by activating p65. These findings are consistent with those of previous studies; for example, in a previous study, the function of PPAR γ in inflammation was reported to be inactivation of NF- κ B (18). Furthermore, PPAR- γ upregulation has been shown to inactivate NF- κ B p65 and exert its antifibrotic effect by reducing type I collagen in veins (29,30), and a PPAR γ agonist (Pio) can decrease the NF- κ B level in the left atrium in rabbits (39).

The present study revealed the following: i) In HL-1 cells, the overexpression of PDCD4 promoted the secretion of pro-inflammatory cytokines and fibrotic factors, whereas it inhibited the secretion of anti-inflammatory factors; however, silencing of PDCD4 exerted the opposite effects. ii) In oePDCD4-HL-1 cells, PPAR γ activation and NF- κ B inhibition attenuated the levels of factors associated with inflammatory injury and fibrosis; however, in siPDCD4-HL-1 cells, PPAR γ inhibition and NF- κ B activation aggravated the levels of factors associated with inflammatory injury and fibrosis. iii) By PPAR γ indirectly or directly phosphorylating NF- κ B p65, PDCD4 may regulate the expression of inflammation- and fibrosis-related factors, possibly by inducing the nuclear import of p-p65.

However, several limitations remain in the present study. The *in vitro* experimental repeats should be increased. The clinical safety and feasibility of PDCD4 still require further animal experiments and clinical studies. Knockdown of PDCD4 under pathological conditions needs to be performed to further demonstrate its role in inflammation and fibrosis, which are under investigation in our ongoing work.

In conclusion, the present study demonstrated that PDCD4 may promote inflammatory damage and fibrosis by activating the PPAR γ /NF- κ B pathway, which could induce the structural remodeling of HL-1 cells. These findings provide a novel basic understanding of AF and proposed PDCD4 as a potential therapeutic target for AF structural remodeling. However, the clinical safety and feasibility of PDCD4 require further animal experiments and clinical studies.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LY, YY, JW and MW conceived the project, conducted the experiments and analyzed the data. ZB, MZ, XW and YZ conducted the experiments and analyzed the data. MW drafted the manuscript. LY, YY, JW and MW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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