P21-activated kinase 1 mediates angiotensin II-induced differentiation of human atrial fibroblasts via the JNK/c-Jun pathway

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Abstract. Cardiac fibrosis is a common pathophysiological condition involved in numerous types of cardiovascular disease. The renin-angiotensin system, particularly angiotensin II (AngII), serves an important role in cardiac fibrosis and remodeling. Furthermore, p21-activated kinase 1 (PAK1) is a highly conserved serine/threonine protein kinase, which is abundantly expressed in all regions of the heart. However, the role of PAK1 in AngII-mediated activation of cardiac fibroblasts remains unknown. Therefore, the present study aimed to investigate the role of PAK1 in cardiac fibroblasts and its underlying mechanisms. Human cardiac fibroblasts (HCFs) were cultured and treated with PAK1 inhibitor IPA-3 or transduced with PAK1 short hairpin (sh)RNA by lentiviral particles to silence PAK1 expression levels. Subsequently, the cell proliferation and migration abilities of the HCFs were determined. Western blot analysis was used to detect the phosphorylation status of Janus kinase (JNK) and c-Jun. A Cell Counting Kit-8 assay showed that PAK1 inhibition following treatment of HCFs with 5 μM IPA-3 or PAK1-shRNA, significantly attenuated AngII-induced proliferation of fibroblasts. In addition, wound healing and Transwell migration assays demonstrated that inhibition of PAK1 significantly inhibited AngII-induced cell migration. Finally, decreased PAK1 expression levels downregulated AngII-mediated upregulation of α-smooth muscle actin (α-SMA), collagen I, phosphorylated (p)-JNK and p-c-Jun, a downstream molecule of JNK signaling. These findings indicate that PAK1 contributes to AngII-induced proliferation, migration and transdifferentiation of HCFs via the JNK/c-Jun pathway.

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Introduction

Cardiac fibrosis is a common pathophysiological condition and a key step in the occurrence and maintenance of the majority of cardiovascular diseases, including atrial fibrillation (AF), hypertension and heart failure (1,2). Fibrosis promotes systolic and diastolic dysfunction, as well as heart rhythm disturbances. Therefore, suppression of cardiac fibrosis may impede cardiac remodeling progression and may be a therapeutic target for AF and other types of cardiovascular disease (3).

Cardiac fibrosis is a complicated process associated with myocardial infarction, pressure and/or volume overload, mechanical stress, metabolic dysfunction and aging (4-6). One of the most important cellular and molecular mechanisms of cardiac fibrosis is differentiation of fibroblasts into myofibroblasts, which is characterized by the expression of secretory and contractile proteins, such as α-smooth muscle actin (α-SMA) and matrix metalloproteinases (MMPs), and production of large amounts of extracellular matrix (ECM) components, such as collagen I and III (1,7). Activated myofibroblasts regulate matrix and cardiomyocyte remodeling. It has been reported that activation of the renin-angiotensin system (RAS) is involved in the pathogenesis of cardiac fibrosis remodeling (2,8,9). Furthermore, studies have shown that angiotensin II (AngII) increases cardiac fibroblast proliferation and migration, as well as collagen expression levels (10,11). Previous studies have suggested that a variety of cell signaling pathways, such as TGF-β and mitogen-activated protein kinases (MAPKs) pathways, are involved in the differentiation, proliferation and migration of cardiac fibroblasts (1,12-14). TGF-β-activated kinase and downstream

p38 MAPK, ERK1/2 and Janus kinase (JNK) pathways are associated with AngII- or TGF-β-mediated activation of proliferation and migration of cardiac fibroblasts (15). Therefore, the inhibition of AngII-mediated activation of myofibroblasts may be an important strategy for treating cardiac fibrosis.

P21-activated kinases (PAKs) are a group of serine/threonine protein kinases that are activated by cell division cycle 42 and Rac family small GTPase 1. The PAK family consists of six members, namely PAK1-6 (16-18). PAK1, 2 and 3 are abundantly expressed in the human heart and PAK1 is involved in actin-based cytoskeletal remodeling (19). Previous studies have shown that PAK1 contributes to TGF-β-induced cancer cell proliferation, migration and invasion (20-23); these effects have also been reported in cardiomyocytes. Therefore, it has been demonstrated that PAK1 serves a key role in protection against cardiac hypertrophy (24). However, the role of PAK1 in cardiac fibroblasts has not been fully investigated. Therefore, the present study aimed to investigate the role of PAK1 in cardiac fibroblasts and the underlying mechanisms.

Materials and methods

Chemicals and reagents. IPA-3, a direct non-ATP-competitive PAK1 inhibitor, was purchased from MedChemExpress (cat. no. HY-15663). AngII was obtained from Sigma-Aldrich (cat. no. 4474-91-3; Merck KGaA).

RNA interference. The short hairpin (sh)RNA lentiviral particle package for PAK1 interference (PAK1-shRNA) was purchased from Shanghai Jikai Gene Chemical Technology Co., Ltd. Human cardiac fibroblasts (HCFs) were cultured to 60-70% confluence and then transduced with shRNA package ($1x10^6$ cells transduced with 5 μl lentivirus) according to the manufacturer's instructions. Following transduction for 48-72 h, the cells were used for subsequent experiments. The knocked-down mRNA and protein levels of PAK1 were determined using reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis, respectively. The target sequence of PAK1-shRNA was 5'-CCGCTTGCTTCAAACATCAAA-3'. The sequence of negative control (NC)-shRNA (scrambled control) was 5'-TTCTCCGAACGTGTCACGT-3'.

Cell culture. Adult HCFs and fibroblast medium-2 were purchased from ScienCell Research Laboratories, Inc. (cat. no. 6320; sciencellonline.com/human-cardiac-fibroblasts-juvenile-atrial.html). Cell passage did not exceed 7-10 generations.

Cell migration assay. Fibroblast migration ability was measured using wound healing and Transwell migration assays. Briefly, fibroblasts were cultured to 60-70% confluence in 3.5-cm diameter dishes. Cells were starved of serum for 12 h, then divided into the following groups: i) Control; ii) AngII; iii) IPA-3; and iv) AngII + IPA-3 or i) NC; ii) AngII; iii) PAK1-shRNA; and iv) AngII + PAK1-shRNA for wound healing and Transwell migration assay. The multiplicity of infection of PAK1-shRNA package by lentiviral particles in the present study was difficult to determine because human adult cardiac fibroblasts can proliferate. In order to ensure the efficiency of virus interference, confluence of the cells was decreased for PAK1-shRNA silencing. In order to determine the wound area, a thin scratch (wound) was made in the central area of the dish using 1-ml pipette tips. Following washing with fibroblast medium-2 to remove detached cells, fibroblasts were cultured with fresh medium with 5% fetal bovine serum. Images of the scratch wounds were captured with a light microscope (magnification, x4) at 0 and 24 h, and the wound width was calculated using the following formula: (T0 - T24/T0) x 100%, where T0 and T24 indicated the width of the wound area at the start time (0 h) and end point (24 h), respectively. For the Transwell migration assay, fibroblasts (density, $3x10^4$) were rehydrated in 100 μl fibroblast medium-2 supplemented with 0.1% bovine serum albumin. Following digestion and resuspension in serum-free fibroblast medium-2, fibroblasts were placed into a Costar Transwell chamber with 8.0-μm pore size membrane (Corning, Inc.). Fibroblasts were seeded into 6-well plates containing 200 μl fibroblast medium-2 supplemented with 5% fetal bovine serum at 37˚C with 5% CO_2 for 12 h. Subsequently, fibroblasts on the inner side of the membrane were removed with a cotton bud; migrated cells on the outer side of the membrane were stained with 0.5% crystal violet for 10 min at room temperature, then observed with a light microscope (magnification, x4) and counted manually.

Cell proliferation assay. CCK-8 kit was used to analyze cell proliferation in the present study (25). HCFs (density, $1x10^4$ cells/well) were cultured in 96-well plates and grown to 50% confluence at 37˚C with 5% CO_2. Subsequently, the cells were cultured in serum-free fibroblast medium-2 for 12 h followed by treatment with 1 μM AngII in the presence or absence of 5 μM IPA-3 for 24 h at 37˚C with 5% CO_2. Following incubation for 24 h, the cells were treated with 10 μl CCK-8 solution (Nanjing Jiangcheng Bioengineering Institute) at 37˚C with 5% CO_2 for 4 h according to the manufacturer's instructions. Finally, the optical density of each well at a wavelength of 450 nm was measured using a Spectramax M5 microplate reader (Molecular Devices, LLC).

RT-qPCR. Total RNA from cells was isolated with TRIzol (Qiagen China Co., Ltd.) and reverse transcribed into cDNA using the ReverTra Ace qPCR RT Master Mix (Toyobo Life Science) as follows: 37˚C for 15 min; 50˚C for 5 min; and 98˚C for 5 min. Subsequently, the expression levels of PAK1 in PAK1-shRNA-treated cells were quantified by qPCR using SYBR Green (Qiagen China Co., Ltd.) as follows: 95˚C for 10 sec; 52˚C for 10 sec; and 72˚C for 10 sec for 40 cycles. The following primer pairs were used: PAK1: Forward, 5'-TCC GCCAGATGCTTTGACCC-3' and reverse, 5'-AGCCTCCA GCCAAGTATTCC-3'; and GAPDH: Forward, 5'-GTGGACC TGACCTGCCGTCT-3' and reverse, 5'-GGAGGAGTGGG TGTCGCTGT-3'. The expression levels of PAK1 were normalized to those of GAPDH and calculated using the $2^{-\Delta\Delta Cq}$ method (26).

Western blot analysis. In order to determine the protein expression levels, cells were lysed with ice-cold RIPA buffer (Beyotime Institute of Biotechnology) supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc.), and the protein concentration was measured via

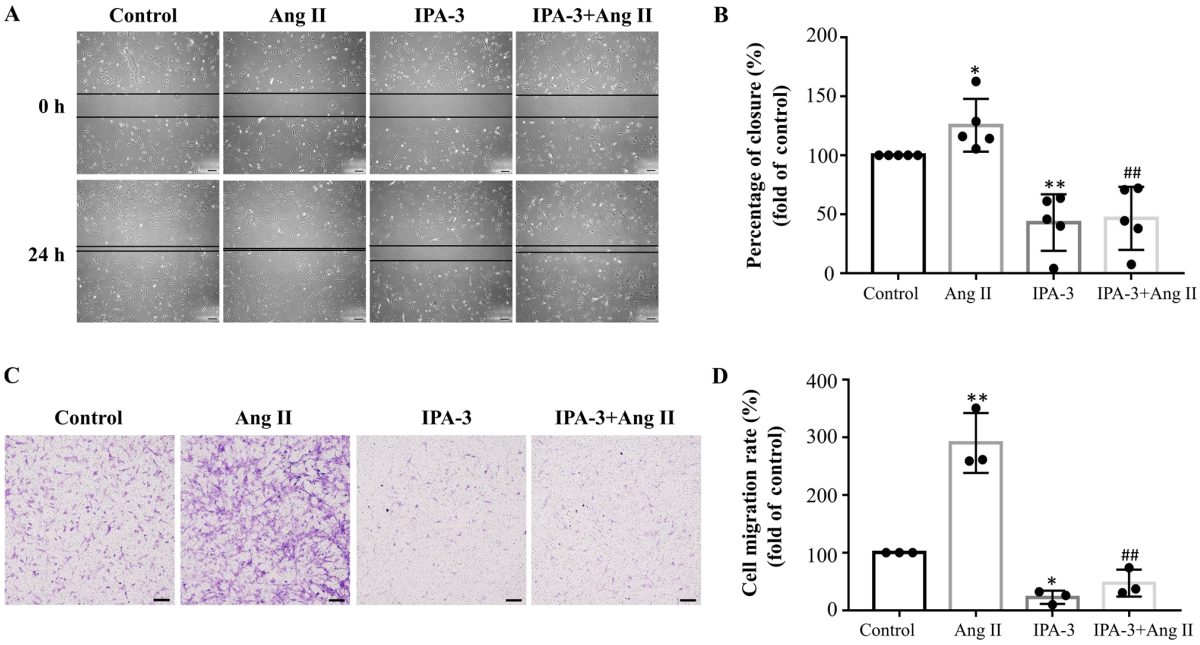

Figure 1. PAK1 inhibition with IPA-3 attenuates AngII-induced migration of HCFs. (A) Representative images of wound healing assays showing that IPA-3 (5 μM) attenuated AngII-induced migration ability of HCFs (scale bar, 200 μm). (B) Wound closure rate as % of wound area at 24 h vs. original wound area (0 h). (C) Representative images from Transwell migration assays showing that treatment of HCFs with 5 μM IPA-3 decreased AngII-induced cell migration ability (scale bar, 200 μm). (D) Cell migration rate in the different groups (%). Data shown are mean fold change ± SEM. *P<0.05, **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. AngII. PAK1, p21-activated kinase 1; AngII, angiotensin II; HCFs, human cardiac fibroblasts.

the bicinchoninic acid method. Cell lysate (30 μg) was resolved by 5% SDS-PAGE and transferred onto PVDF membranes. Following blocking for 2 h with 5% non-fat dry milk in TBS-T [20.00 mM Tris (pH 8.0), 150.00 mM NaCl, 0.05% Tween-20] at room temperature, membranes were incubated with primary antibodies against α-SMA, collagen I (both 1:2,000; cat. nos. GB11044 and GB11022, respectively; both Servicebio, Inc.); JNK, phosphorylated (p)-JNK; ERK, p-ERK; p38, p-p38 (all 1:1,000; cat. nos. 9252, 9255, 4696, 4370, 8690 and 4511, respectively; all Cell Signaling Technology, Inc.) and GAPDH (1:5,000; cat. no. G9295; Sigma-Aldrich; Merck KGaA) at 4˚C overnight. The PVDF membrane was then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG and secondary antibodies (both 1:2,000; cat. nos. 7076 and 7074, respectively; both Cell Signaling Technology, Inc.) at room temperature for 1 h and treated with Chemiluminescent HRP Substrate (cat. no. WBKLS0500; EMD Millipore). Images were captured and immunoreactive bands were quantified using Quantity One software v4.6.6 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are expressed as the mean ± SEM. Results were analyzed by one-way ANOVA followed by Tukey's posthoc comparison. P<0.05 was considered to indicate a statistically significant difference.

Results

IPA-3-mediated inhibition of PAK1 attenuates AngII-induced migration and transdifferentiation of HCFs. The effect of PAK1 on AngII-induced migration of HCFs was first investigated. Growth and migration of HCFs were decreased using a PAK1 inhibitor, IPA-3 (concentration, 5 μM) or PAK1-shRNA, respectively (Figs. 1 and 2). Exposure of HCFs to 1 μM AngII for 24 h increased their migration ability, as indicated by Transwell and wound healing migration assays (Fig. 1A and C). Compared with the control group, treatment of HCFs with AngII significantly increased wound closure (P<0.05; n=5) and cell migration rate (P<0.01; n=3; Fig. 1B and D). Furthermore, co-treatment of AngII-treated HCFs with 5 μM IPA-3 decreased wound closure (P<0.01, n=5) and relative cell migration rate (P<0.01; n=3) compared with the AngII-treated group (Fig. 1B and D).

Collagen I and α-SMA were used as markers of transdifferentiation of fibroblasts into myofibroblasts. Treatment of HCFs with AngII significantly upregulated α-SMA (P<0.05, n=4) and collagen I (P<0.01, n=3) expression levels, whereas co-treatment with IPA-3 downregulated AngII-mediated upregulation of both proteins (P<0.01; Fig. 3A-C). AngII also significantly increased the expression levels of PAK1 in fibroblasts (Fig. 3D and E). Finally, CCK-8 assay revealed that IPA-3 significantly inhibited AngII-induced cell proliferation (P<0.05, n=8; Fig. 3F).

PAK1 knockdown attenuates AngII-induced migration and transdifferentiation of HCFs. Subsequently, the effect of PAK1-knockdown (PAK1-shRNA) on AngII-induced migration and transdifferentiation of HCFs was investigated. Interference rate of PAK1-shRNA is presented in Fig. 4A-C. PAK1-shRNA significantly decreased the mRNA (Fig. 4A) and protein levels (Fig. 4B and C) of PAK1 in HCFs. Similar results were observed between HCFs exposed to IPA-3 and HCFs transduced with PAK1-shRNA. Transwell and wound healing migration assays and western blot analysis demonstrated that PAK1 knockdown significantly inhibited AngII-induced wound closure (Fig. 2A and B) and cell

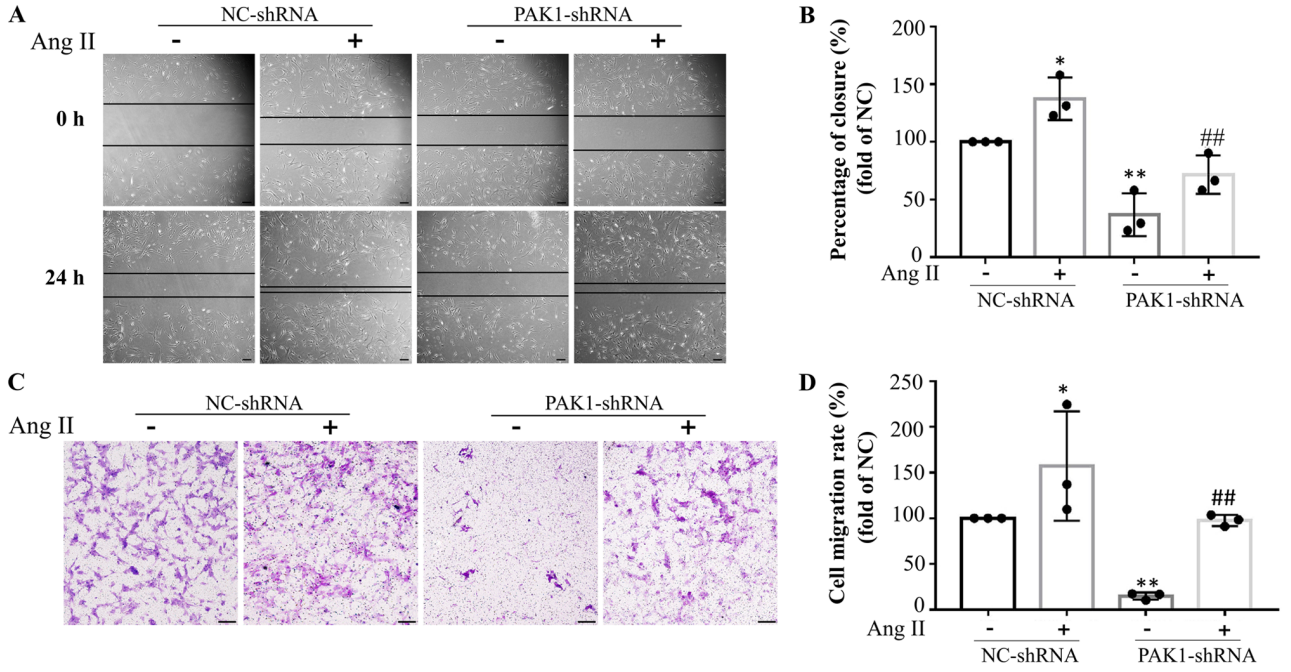

Figure 2. PAK1 knockdown alleviates AngII-induced migration of HCFs. (A) Representative images of wound healing assays showing that PAK1 knockdown with PAK1-shRNA attenuated AngII-induced migration of HCFs (scale bar, 200 μm). (B) Wound closure rate as % of wound area at 24 h vs. original wound area (0 h). (C) Representative images from Transwell migration assays showing that treatment of HCFs with PAK1-shRNA decreased AngII-induced cell migration ability (scale bar, 200 μm). (D) Cell migration rate in different groups. Data are presented as the mean fold change ± SEM. *P<0.05, **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. Ang II. PAK1, p21-activated kinase 1; AngII, angiotensin II; HCFs, human cardiac fibroblasts; sh, short hairpin; NC, negative control.

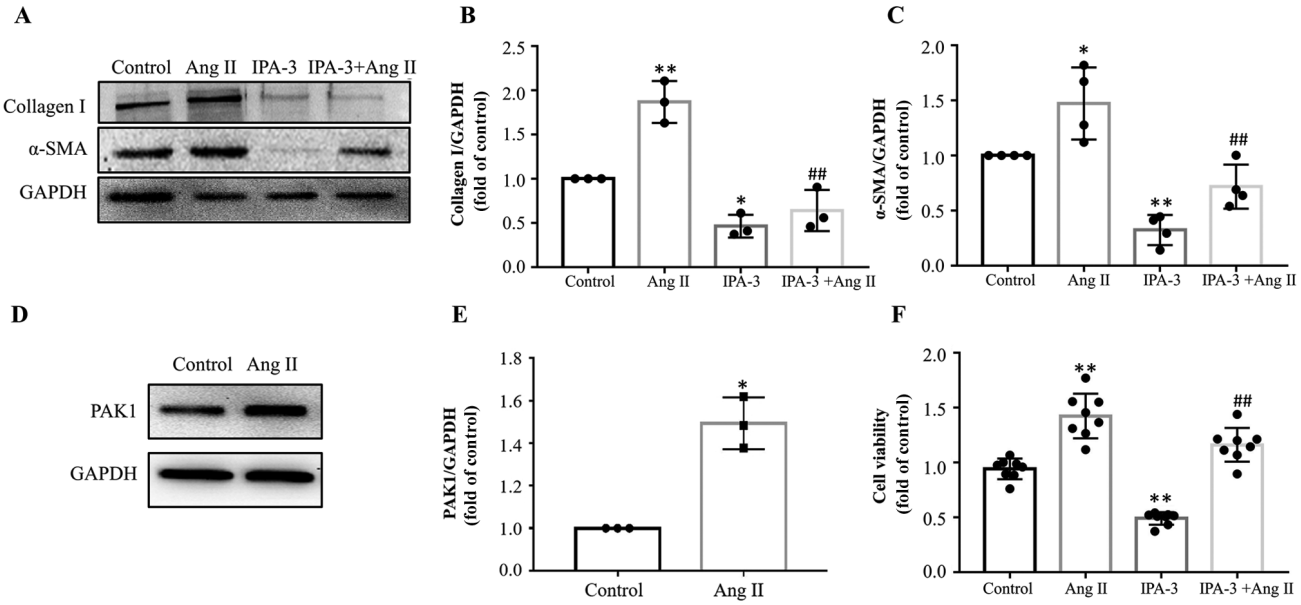

Figure 3. IPA-3 inhibits AngII-induced transdifferentiation and proliferation of HCFs. (A) Representative images of western blot assays and (B and C) histograms, showing that IPA-3 inhibits AngII-mediated upregulation of (B) collagen I and (C) α-SMA. (D) Representative images of western blot assays and (E) histogram showing that AngII increased the expression levels of PAK1 in HCFs. (F) Cell proliferation measured by Cell Counting Kit-8 assay showing that IPA-3 inhibited AngII-induced cell proliferation. Data are presented as the mean fold change ± SEM. *P<0.05, **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. AngII. AngII, angiotensin II; HCFs, human cardiac fibroblasts; α-SMA, α smooth muscle actin; PAK1, p21-activated kinase 1.

migration (Fig. 2C and D) and PAK1 knockdown significantly (Fig. 4A-C) decreased the expression of collagen I and α-SMA (Fig. 4D-F), respectively.

JNK/c-Jun pathway is involved in the effects of PAK1 on HCFs. Furthermore, the underlying signaling pathway associated with the effect of PAK1 on fibroblasts was investigated. The results showed that the JNK/c-Jun pathway is involved in the inhibitory effect of PAK1 on AngII-induced migration and transdifferentiation of HCFs (Fig. 5). Treatment of HCFs with AngII increased phosphorylation of JNK and its downstream molecule c-Jun, whereas PAK1 inhibition with

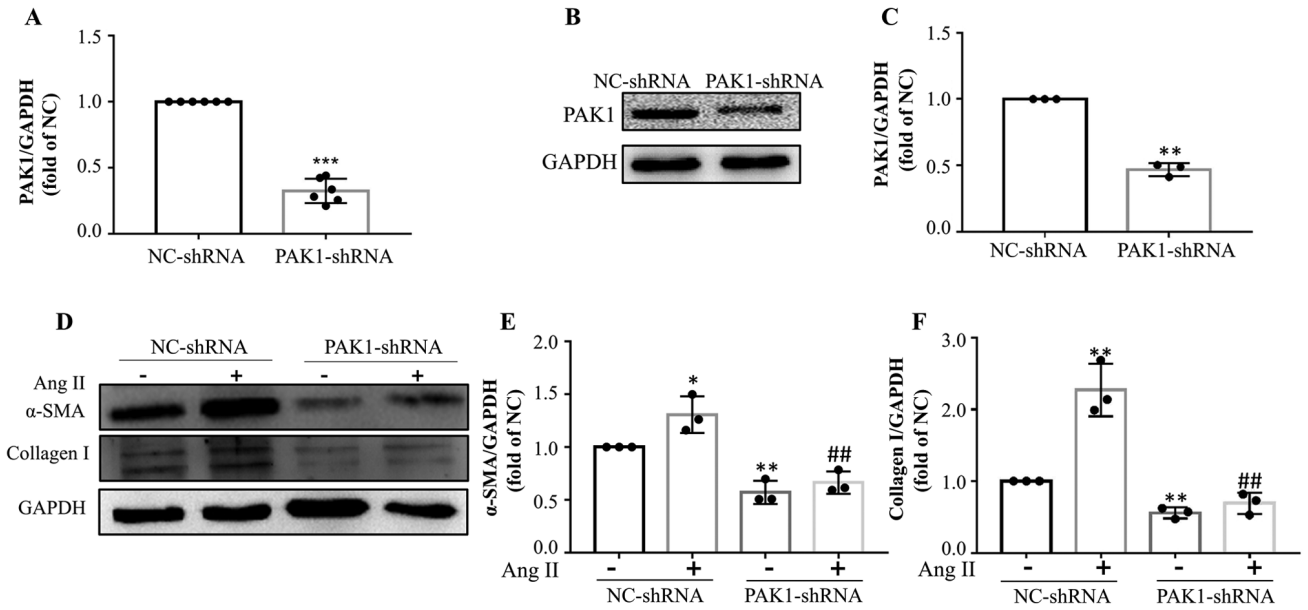

Figure 4. PAK1 knockdown inhibits Ang II-induced transdifferentiation of HCFs. (A) PAK-shRNA decreased mRNA expression levels of PAK1. (B and C) Western blot and histogram showing that PAK1 knockdown decreased the protein expression levels of PAK1. (D) Representative images of western blot assays and (E and F) histograms are presented, showing that PAK1-knockdown inhibited AngII-mediated upregulation of (E) α-SMA and (F) collagen I. Data are presented as the mean fold change ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. NC; $^{\#\#}$P<0.01 vs. AngII. PAK1, p21-activated kinase 1; AngII, angiotensin II; HCFs, human cardiac fibroblasts; sh, short hairpin; α-SMA, α smooth muscle actin; NC, negative control.

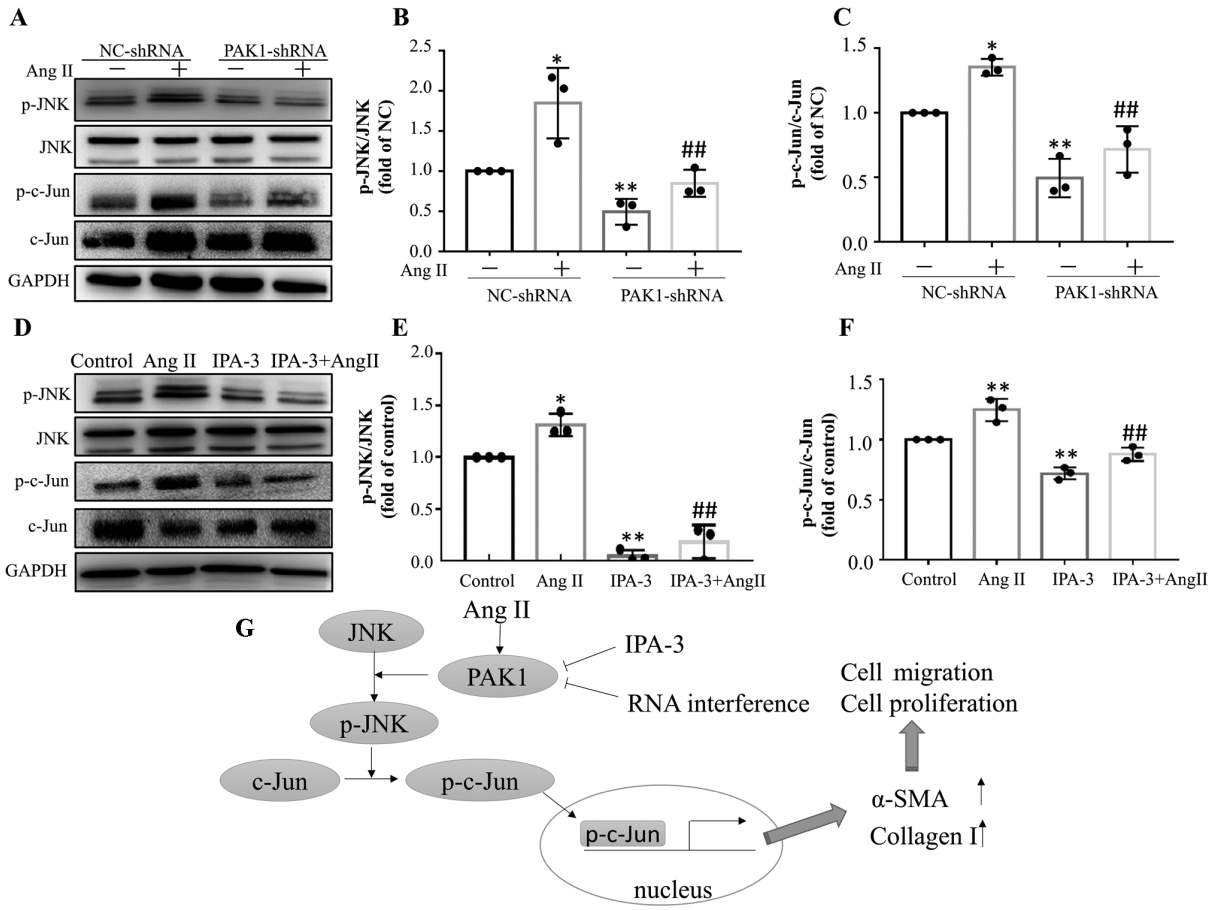

Figure 5. JNK/c-Jun signaling pathway mediates the effects of PAK1 in HCFs. (A) Representative images of western blot assays and histograms showing that transduction of HCFs with PAK1-shRNA attenuated AngII-induced phosphorylation of (B) JNK and (C) c-Jun. *P<0.05, **P<0.01 vs. NC. $^{\#\#}$P<0.01 vs. AngII. (D) Representative images of western blot assays and histograms showing that PAK1 inhibition with IPA-3 (5 μM) attenuated AngII-induced phosphorylation of (E) JNK and (F) c-Jun. *P<0.05, **P<0.01 vs. control. $^{\#\#}$P<0.01 vs. AngII. (G) Mechanism underlying the effect of PAK1 on the migration and transdifferentiation of HCFs. Data are presented as the mean fold change ± SEM. JNK, Janus kinase; PAK1, p21-activated kinase 1; HCF, human cardiac fibroblast; sh, short hairpin; AngII, angiotensin II; NC, negative control; p, phosphorylated; α-SMA, α smooth muscle actin.

IPA-3 (Fig. 5D-F) or PAK1-shRNA (Fig. 5A-C) significantly attenuated the effect of AngII. Knockdown of PAK1 with PAK1-shRNA significantly decreased AngII-induced phosphorylation of JNK and c-Jun (Fig. 5A-C). Similarly, IPA-3 significantly decreased AngII-induced phosphorylation of JNK and c-Jun (Fig. 5D-F).

Discussion

Activation of myofibroblasts is a key step in cardiac fibrosis (1,6,27). The present study showed that p21-activated kinase 1 (PAK1) contributed to angiotensin II (AngII)-mediated activation of myofibroblasts, whereas its inhibition significantly attenuated the effects of AngII on human cardiac fibroblasts (HCFs) (Fig. 5G).

Activation of the renin-angiotensin system (RAS), particularly AngII, is involved in numerous types of cardiovascular disease by promoting cardiac fibroblast differentiation into myofibroblasts (28). Therefore, in the present study AngII-activated myofibroblasts were used to investigate the underlying mechanism of PAK1 in HCFs.

The activation of myofibroblasts is associated with overexpression of α-smooth muscle actin (α-SMA) and collagen I and II (29). Consistent with previous studies, the present study demonstrated that treatment of HCFs with AngII significantly upregulated α-SMA and collagen I expression levels (8,30). Furthermore, inhibition/downregulation of PAK1 activity using IPA-3 inhibitor or shRNA interference, respectively, attenuated AngII-mediated overexpression of α-SMA and collagen I. These findings indicate that PAK1 may be involved in AngII-mediated differentiation of HCFs. The present study also found that treatment with IPA-3 or PAK1-shRNA in the absence of AngII inhibited the migration and proliferation of fibroblasts. These data indicated that PAK1 was involved in the basic properties of fibroblasts, such as proliferation and migration.

The activation of myofibroblasts is characterized by increased cell migration and proliferation ability. Activation of RAS or upregulation of AngII increase cardiac fibroblast migration and proliferation (31). Herein, Transwell and wound healing migration assays also confirmed the effect of AngII on the migration ability of HCFs. Additionally, inhibition or downregulation of PAK1 significantly decreased AngII-induced migration and proliferation of HCFs.

It has been documented that numerous signaling pathways are involved in AngII-mediated activation of myofibroblasts, such as the TGFβ1/Smad and MAPK pathways (14,32). However, the mechanism underlying the effect of PAK1 in AngII-activated HCFs remains unknown. c-Jun is an important downstream molecule of the JNK pathway and promotes the proliferation of fibroblasts (33). The results of the present study demonstrated that the JNK/c-Jun pathway mediated the effects of PAK1 on AngII-induced myofibroblasts.

To the best of our knowledge, the present study is the first to demonstrate that PAK1 contributes to AngII-mediated activation of myofibroblasts via the JNK/c-Jun pathway (Fig. 5G). The effect of PAK1 on fibroblasts was different from that observed in cardiomyocytes in previous study (20,34). Therefore, it was hypothesized that PAK1 downregulation may decrease the AngII-induced differentiation, proliferation and migration of myofibroblasts, thus providing a novel approach in preventing cardiac fibrosis. In the present study, the role of PAK1 was investigated only *in vitro* using an HCF cell line; therefore, *in vivo* studies using a PAK1-knockout mouse model should be performed to further investigate the role of PAK1 in myofibroblasts.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XT, YZ and ZF designed the experiment. YZ, YX, TL, and PZ performed the experiments. XT, YZ, YX and TC performed the data analysis. XT, YZ and ZF wrote the manuscript. All authors read and approved the final 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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