Estrogen inhibits TGF-β1-stimulated cardiac fibroblast differentiation and collagen synthesis by promoting Cdc42

JINGYI XU[1*], FENG WANG[2*], YUAN LI[3], YING HUANG[1], PING LI[1], YIQING ZHANG[3], GUIDONG XU[3] and KANGYUN SUN[3]

[1]Department of Central Laboratory, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou Municipal Hospital, Gusu School, Nanjing Medical University, Suzhou, Jiangsu 215008, P.R. China; [2]Department of Pharmacy, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou Municipal Hospital, Gusu School, Nanjing Medical University, Suzhou, Jiangsu 215008, P.R. China; [3]Department of Cardiology, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou Municipal Hospital, Gusu School, Nanjing Medical University, Suzhou, Jiangsu 215008, P.R. China

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Abstract. 17β-estradiol (E2) can inhibit cardiac fibrosis in female patients with heart failure (HF) and activate cell division cycle 42 (Cdc42), however it is unknown whether 17β-estradiol (E2) can ameliorate differentiation and collagen synthesis in TGF-β1-stimulated mouse cardiac fibroblasts (MCFs) by regulating cell division cycle 42 (Cdc42). The present study aimed to investigate the roles of estrogen and Cdc42 in preventing myocardial fibrosis and the underlying molecular mechanisms. An ELISA was used to measure the levels of E2 and Cdc42 in the serum of patients with heart failure (HF), and western blotting was used to measure the expression levels of Cdc42 in TGF-β1-stimulated immortalized MCFs. MCFs were transfected with a Cdc42 overexpression (OE) lentivirus or small interfering RNA (siRNA), or treated with a Cdc42 inhibitor (MLS-573151), and the function of Cdc42 was assessed by western blotting, immunofluorescence staining, reverse transcription-quantitative PCR and dual-luciferase reporter assays. Western blotting and immunofluorescence staining were performed to verify the protective effect of E2 on TGF-β1-stimulated MCFs, and the association between the protective effect and Cdc42. The results demonstrated that Cdc42 levels were increased in the serum of patients with HF and were positively correlated with the levels of E2; however, Cdc42 levels were decreased in TGF-β1-stimulated MCFs. Cdc42 inhibited MCF differentiation and collagen synthesis, as indicated by the protein expression of α-smooth muscle actin, collagen I and collagen III. Mechanistically, Cdc42 inhibited the transcription of TGF-β1 by promoting the expression of p21 (RAC1)-activated kinase 1 (Pak1)/JNK/c-Jun signaling pathway proteins and inhibiting the activity of the Tgfb1 gene promoter. In addition, E2 inhibited the differentiation and collagen synthesis of TGF-β1-stimulated MCFs, and promoted the protein expression of Pak1, JNK and c-Jun, consistent with the effects of Cdc42, whereas the effects of E2 were abolished when Cdc42 was knocked down. The aforementioned findings suggested that E2 could inhibit differentiation and collagen synthesis in TGF-β1-stimulated MCFs by regulating Cdc42 and the downstream Pak1/JNK/c-Jun signaling pathway.

Correspondence to: Dr Kangyun Sun or Dr Guidong Xu, Department of Cardiology, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou Municipal Hospital, Gusu School, Nanjing Medical University, 242 Guangji Road, Suzhou, Jiangsu 215008, P.R. China
E-mail: sunkangyun@njmu.edu.cn
E-mail: laosheng2006@163.com

*Contributed equally

Abbreviations: α-SMA, α-smooth muscle actin; Cdc42, cell division cycle 42; CON, control; E2, 17β-estradiol; HF, heart failure; MCF, mouse cardiac fibroblast; OE, overexpression; Pak1, p21 (RAC1)-activated kinase 1; qPCR, quantitative PCR; siNC, negative control for small interfering RNA

Key words: E2, cardiac fibroblast, Cdc42, HF, TGF-β1

Introduction

Myocardial fibrosis, a common pathological change in most cardiac diseases, primarily occurs through the proliferation and differentiation of myocardial fibroblasts, and the abnormal deposition of extracellular matrix (1). Myocardial fibrosis disrupts myocardial structure and leads to myocardial disorders, and electromechanical and vasomotor dysfunction, and these effects serve key roles in the pathogenesis of heart failure (HF) (2). Therefore, the prevention and treatment of myocardial fibrosis have become important goals for improving the treatment of HF (3).

A key process in myocardial fibrosis is the abnormal activation of fibroblasts (3). TGF-β1, which is the most important member of the TGF-β superfamily, is a potent fibrotic factor; its levels are increased in fibrotic heart tissue, and TGF-β1 promotes the activation of cardiac fibroblasts (3,4). Fibroblasts synthesize and secrete procollagen chains that assemble into

type I and III collagen fibers, which cross-link to form final fibers that connect cardiomyocytes, fibroblasts and vascular cells, and help maintain the structural integrity and plasticity of the heart (3). TGF-β1 activates fibroblasts via the Smad pathway or a non-Smad pathway [including the Erk/mitogen-activated protein kinase (MAPK) pathway, JNK) and MAPK) signaling and the IkB kinase pathway, PI3K-Akt pathway, and the Rho family of small GTPases] (5), and this activation stimulates fibroblast proliferation and differentiation into myofibroblasts, which highly express α-smooth muscle actin (α-SMA) (6). These myofibroblasts exhibit disordered collagen metabolism and increased extracellular matrix synthesis, and their degradation is either unaffected or decreased, which increases myocardial mechanical stiffness (6). This change in stiffness alters the contraction of cardiomyocytes, disrupts electrical connections and exacerbates tissue hypoxia (7), leading to the development of HF.

Cell division cycle 42 (Cdc42), a member of the Rho-like GTPase family, is an important regulator of actin polymerization and cytoskeletal reorganization (8,9). Cdc42 can regulate transcription factor activity, microtubule dynamics, membrane transport pathways and cell polarity (10). Furthermore, Cdc42 is an important member of the TGF-β1 non-Smad pathway (5). Cdc42 is involved in determination of the sarcomere composition of cardiomyocytes and contributes to improvements in cardiac structure after myocardial infarction (11). Cdc42 has been reported to have a protective effect on the heart. For example, myocardial-specific knockout of Cdc42 increased the cardiac hypertrophic response in mice subjected to pressure overload, and decreased JNK activity downstream of Cdc42 was the primary mechanism responsible for the increased hypertrophic response, which suggests that Cdc42 has an anti-hypertrophic effect (12). In cardiomyocytes under pathological conditions, Cdc42 is activated by GDP and GTP exchange, and activated Cdc42 cleaves its effector, p21 (RAC1)-activated kinase 1 (Pak1), to induce Pak1 activation, which regulates the activity of multiple downstream signaling pathways (13). In addition, Pak1 mediates angiotensin II-induced human atrial fibroblast differentiation via the JNK/c-Jun pathway (14).

Estrogen has various effects on the cardiovascular system and usually acts by binding estrogen receptors and activating multiple transcription factors (15). 17β-estradiol (E2) is a representative estrogen whose receptor is expressed in cardiomyocytes, endothelial cells and fibroblasts in the heart (16). E2 can activate Cdc42 in mouse embryonic stem cells and human breast cancer cells (MCF-7) (17), and regulates cytoskeletal remodeling in endometrial tissue (18). However, to the best of our knowledge, it is unknown whether E2 affects the ability of Cdc42, a key molecule in the TGF-β1 non-Smad pathway, to inhibit fibrosis. The present study aimed to clarify whether E2 could ameliorate differentiation and collagen synthesis in TGF-β1-stimulated mouse cardiac fibroblasts (MCFs) by regulating Cdc42, and to examine the mechanisms underlying these effects.

Materials and methods

Clinical sample collection and ELISA. A total of 23 patients with HF aged 50-80 years who were hospitalized at the Cardiovascular Center of The Affiliated Suzhou Hospital of Nanjing Medical University, Gusu School (Suzhou, China) between March 2022 and May 2022 were enrolled in the present study. The inclusion criteria were based on the Chinese Guidelines for the Diagnosis and Treatment of Heart Failure 2018 (19). Briefly, the inclusion criteria were as follows: i) Dyspnea, fatigue and fluid retention; ii) left ventricular ejection fraction <40%; and iii) elevated natriuretic peptide with a left ventricular ejection fraction of ≥40% and at least one of the following: i) Left ventricular hypertrophy and/or left atrial enlargement; and ii) abnormal cardiac diastolic function. Patients with acute infection, serious primary disease, trauma, pregnancy, cancer, drug use or surgery in the month before blood collection were excluded. During the same period, 21 volunteers who underwent routine physical examination but did not have heart disease, and these subjects were similar to patients in terms of age, sex ratio and body mass index at the Suzhou Physical Examination Center (The Affiliated Suzhou Hospital of Nanjing Medical University) were recruited as the control group. The present study was approved by the Ethics Committees of Affiliated Suzhou Hospital of Nanjing Medical University, Gusu School (Suzhou, China; approval no. K-2021-GSKY20210202). Blood samples (2 ml) were collected from patients and controls using procoagulant blood collection vessels, and the sera were collected after centrifugation at 3,000 x g for 5 min at 4˚C. The serum levels of Cdc42 and E2 were measured using human Cdc42 ELISA kit (cat. no. YX-76511. Sinobestbio, Co., Ltd.) and the human 17β-E2 ELISA kit (cat. no. YK90121. Sinobestbio, Co., Ltd.) according to the manufacturer's instructions, and the results were obtained using a Varioskan Lux multifunctional microplate reader (Thermo Fisher Scientific, Inc.) by measuring the absorbance at a wavelength of 425 nm.

Cell culture and TGF-β1 treatment. Immortalized MCFs (subsequently referred to as MCFs in the present study) purchased from Shanghai Yilei Information Technology Co., Ltd. were cultured at 37˚C with 5% CO_2 in DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 100 U/ml streptomycin, 100 U/ml penicillin and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). After the MCFs were cultured for 24 h and had grown to 50-70% confluence, they were treated with 20 ng/ml TGF-β1 (PeproTech, Inc.) for 0, 12, 24, 48 or 72 h at 37˚C. Finally, the cultured cells were collected, and the protein expression levels of α-SMA, collagen I, collagen III and Cdc42 were examined via western blotting.

Transfection of MCFs with Cdc42 overexpression (OE) lentivirus or small interfering RNA (siRNA) targeting Cdc42. MCFs were divided into the negative control (NC)-OE, Cdc42-OE, negative control for siRNA (siNC), TGF-β1 + siNC, siRNA of Cdc42 (siCdc42)-1 and siCdc42-2 groups. For the NC-OE and Cdc42-OE groups, the pGC-FU-3FLAG-CBh-gcGFP-IRES-puromycin vector (Shanghai Genechem Co., Ltd.) was used for the OE experiment, and for the NC-OE group, an empty vector with no sequence inserted at the multiple cloning site (Shanghai Genechem Co., Ltd.) was used for lentiviral transfection. Cell lines stably transfected with lentivirus were generated according to the manufacturer's instructions. Briefly, the

lentivirus ($1x10^8$ TU/ml) was added to cells that had grown to 20-40% confluence in 6-well plates. After 16 h of infection at 37˚C, the medium was replaced with fresh complete medium, and stable cell lines were selected with 10 μg/ml puromycin at 72 h after infection and maintained with 2 μg/ml puromycin. The generated cell lines stably transfected with lentivirus were frozen for later use or cultured with fresh complete medium for 24 h for subsequent experiments. For the siNC, TGF-β1 + siNC, siCdc42-1 and siCdc42-2 groups, MCFs were cultured in 6-well plates for 24 h grown to 50-70% confluence and transfected with 7.5 pmol siRNA according to the manufacturer's instructions (Shanghai GenePharma, Co., Ltd..). Briefly, serum-free medium was divided into two parts, and siRNA was added to one half, while Lipofectamine® 3000 reagent (Thermo Fisher Scientific, Inc.) was added to the other half. After 5 min, the two halves were mixed. The mixture was allowed to rest for 20 min before being added to cells at 50 to 70%. After 24 h of transfection at 37˚C, the medium was replaced with fresh medium, and 20 ng/ml TGF-β1 was added to the TGF-β1 + siNC group, which was subsequently incubated for another 24 h at 37˚C. The following siRNA sequences were used: siRNA-1 sense, 5'-GCUAAGUUAUCC ACAGACATT-3' and antisense, 5'-UGUCUGUGGAUAACU UAGCTT-3'; siRNA-2 sense, 5'-GCUUGUUGGGACCCA AAUUTT-3' and antisense, 5'-AAUUUGGGUCCCAAC AAGCTT-3'; and non-targeted siRNA control sense, 5'-UUC UCCGAACGUGUCACGUTT-3' and antisense, 5'-ACG UGACACGUUCGGAGAATT-3'. *Tgfb1* mRNA expression levels were detected by quantitative PCR (qPCR), and the protein expression levels of α-SMA, collagen I and III, Pak1, JNK, c-Jun and TGF-β1 were detected by western blotting. Immunofluorescence staining was used to observe α-SMA expression. The activity of the *Tgfb1* promoter (-1,799/+55) (20) was evaluated using a dual-luciferase reporter assay.

Treatment of MCFs with the Cdc42 inhibitor MLS-573151. After MCFs were cultured for 24 h, they were divided into untreated control, DMSO and MLS-573151-treated groups. The MCFs were treated with vehicle (0.04% DMSO) or 10 μM MLS-573151 for 24 h at 37˚C, and the protein expression of total and phosphorylated (p-)Cdc42, Pak1, JNK, c-Jun and TGF-β1 was detected by western blotting.

Treatment of TGF-β1-stimulated MCFs with E2 and transfection with siCdc42. After MCFs were cultured for 24 h, they were divided into the TGF-β1 + control, TGF-β1 + NC and TGF-β1 + E2-treated (5, 10 and 50 nM) groups. The MCFs were pretreated with 20 ng/ml TGF-β1 for 6 h at 37˚C, after which vehicle (0.04% DMSO) or E2 (5 or 10 or 50 nM, MedChemExpress Co., Ltd.) was added for another 48 h at 37˚C, and the protein expression levels of α-SMA, collagen I, collagen III and Cdc42 were determined via western blotting. Subsequently, the cells were divided into the control, TGF-β1, TGF-β1 + E2 and TGF-β1 + E2 + siCdc42-1 groups. The MCFs were pretreated with 20 ng/ml TGF-β1 for 6 h at 37˚C, and then 10 nM E2 was added for another 48 h at 37˚C. MCFs in the TGF-β1 + E2 + siCdc42 group were transfected with siCdc42-1 (Shanghai GenePharma Co., Ltd.) according to the manufacturer's instructions as aforementioned with addition of E2, and MCFs in the remaining groups were transfected with siNC, as aforementioned. The protein expression levels of α-SMA, collagen I, collagen III, Pak1, JNK and c-Jun were detected by western blotting. Immunofluorescence staining was used to observe α-SMA expression.

Treatment of TGF-β1-stimulated MCFs with E2 and transfection with Cdc42-OE. After the two stable MCF lines, NC-OE and Cdc42-OE, were cultured for 24 h, the cells were divided into the control, TGF-β1, TGF-β1 + Cdc42-OE and TGF-β1 + E2 groups. The cells in the TGF-β1 + Cdc42-OE group were the Cdc42-OE cells, and the cells in the other groups were the NC-OE cells. The MCFs were pretreated with 20 ng/ml TGF-β1 for 6 h at 37˚C, and 10 nM E2 was added for another 48 h at 37˚C. The protein expression levels of α-SMA, collagen I, collagen III, Cdc42, Pak1, JNK and c-Jun were detected by western blotting. Immunofluorescence staining was used to observe α-SMA expression.

Immunofluorescence staining. Cell slides were added to 24-well plates before cell culture. After the MCFs had grown to 20-30% confluence, the medium containing the treated cells in the plate was discarded, and the cells were fixed with 4% paraformaldehyde at room temperature for 10 min, permeabilized with 0.1% Triton X-100 at room temperature for 10 min and blocked with 1% BSA (Beyotime Institute of Biotechnology) at room temperature for 45 min. An α-SMA antibody (1:200; cat. no. CY5295. Shanghai Abways Biotechnology Co., Ltd.) was added to the glass slide and the cells were incubated at 4˚C overnight in the dark. The CoraLite488-conjugated goat anti-rabbit IgG(H+L) (1:500; cat. no. SA00013-2. Proteintech Group, Inc.) was added to each well for incubation at room temperature for 60 min. The secondary antibodies were discarded, DAPI was added at room temperature for 20 min and the cells were washed. The glass slide in each well was removed and sealed with an anti-fluorescence quenching sealing solution. A confocal microscope (Carl Zeiss AG) was used to capture images of the cells. ImageJ software v1.8.0 (National Institutes of Health) was used to measure and analyze the mean fluorescence intensity.

Dual-luciferase reporter assay. MCFs were seeded into a 12-well plate and cultured for 24 h to 50-70% confluence. Subsequently, the cells were divided into the siNC + pGL3-basic + thymidine kinase (TK)-*Renilla*, siCdc42 + pGL3-basic + TK-*Renilla*, siNC + pGL3-TGF-β1 promoter + TK-*Renilla* and siCdc42-1 + pGL3-TGF-β1 promoter + TK-*Renilla* (Guangzhou RiboBio Co., Ltd.) groups. Briefly, 7.5 pmol siNC (sense, 5'-UUCUCC GAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACAC GUUCGGAGAATT-3'. Shanghai GenePharma Co., Ltd.) or siCdc42-1 (sense, 5'-GCUAAGUUAUCCACAGACATT-3' and antisense, 5'-UGUCUGUGGAUAACUUAGCTT-3'. Shanghai GenePharma Co., Ltd.), 0.5 μg reporter plasmid pGL3-Basic (Guangzhou RiboBio Co., Ltd.) or the plasmid pGL3-TGF-β1 (-1,799/+55) (Guangzhou RiboBio Co., Ltd.) (20) and 0.05 μg control *Renilla* luciferase plasmid TK-*Renilla* (Guangzhou RiboBio Co., Ltd.) were simultaneously transfected using Lipofectamine® 3000 reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cells were harvested 24 h after transfection. Luciferase activity was determined using a dual-luciferase reporter assay system

(cat. no. E1910; Promega Corporation). Luciferase activity was normalized to the *Renilla* luciferase activity.

Reverse transcription-qPCR. After total RNA was extracted from the cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.), the RNA concentration and purity were measured using a NanoDrop Life spectrophotometer (Thermo Fisher Scientific, Inc.). RNA (1,000 ng) was reverse transcribed into cDNA using PrimeScript™ RT Master Mix (Takara Bio, Inc.) at 37˚C for 15 min and 85˚C for 5 sec. qPCR was performed with TB Green® Premix Ex Taq™ II (Takara Bio, Inc.) according to the manufacturer's instructions. The PCR program was as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The program was run using the LightCycler® 480 II real-time PCR detection system (Roche Diagnostics) to detect *Tgfb1* mRNA levels. The following primer sequences (Genewiz, Inc.) were used: TGF-β1 forward, 5'-CAACTTCTG TCTGGGACCCT-3' and reverse, 5'-TAGTAGACGATGG GCAGTGG-3'; and GAPDH forward, 5'-AGGTCGGTGTGA ACGGATTTG-3' and reverse, 5'-TGTAGACCATGTAGT TGAGGTCA-3'. All measurements were performed in triplicate. Relative expression data were analyzed using the $2^{-\Delta\Delta Cq}$ method (21) and were normalized to the expression levels of GAPDH.

Western blotting. After total protein was extracted from MCFs using RIPA buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitors and quantified by BCA assay, protein samples (30 μg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis, and the proteins were transferred to polyvinylidene difluoride membranes, which were blocked with 5% skimmed milk at room temperature for 1 h and then incubated with primary antibodies at 4˚C overnight. Primary antibodies against α-SMA (1:1,000; cat. no. CY5295. Shanghai Abways Biotechnology Co., Ltd.), collagen I (1:1,000; cat. no. A1352. ABclonal Biotech Co., Ltd.), Cdc42 (1:1,000; cat. no. 10155-1-AP. Proteintech Group, Inc.), p-Cdc42 (1:1,000; cat.no. YP1620. ImmunoWay Biotechnology Company), TGF-β1 (1:2,000; cat. no. 21898-1-AP. Proteintech Group, Inc.), Pak1 (1:3,000; cat. no. 21401-1-AP. Proteintech Group, Inc.), JNK (1:1,000; cat. no. CY5490. Shanghai Abways Biotechnology Co., Ltd.), c-Jun (1:1,000; cat. no. CY5290; Shanghai Abways Biotechnology Co., Ltd.) and mouse collagen III (1:1,000; cat. no. YM3123. ImmunoWay Biotechnology Company), GAPDH (1:5,000; cat. no. 60004-1-Ig. Proteintech Group, Inc.) were used. The next day, the membranes were washed three times with TBS-0.1% Tween-20 wash buffer for 10 min each. The sections were incubated with goat anti-mouse IgG (H+L) HRP (1:20,000; cat. no. 70-GAM007. MultiSciences (Lianke) Biotech Co., Ltd) or goat anti-rabbit IgG (H+L) HRP (1:20,000; cat. no. 70-GAR007. MultiSciences (Lianke)Biotech Co., Ltd) secondary antibodies for 1 h at room temperature. After the membranes were washed with TBS-0.1% Tween-20 wash buffer, protein expression levels were detected with an automatic Tanon 5200 Multi chemiluminescence image analyzer using Tanon™ High-sig ECL Western Blotting Substrate (Tanon Science and Technology Co., Ltd.). The gray areas were measured and analyzed using ImageJ software v1.8.0 (National Institutes of Health).

Statistical analysis. SPSS 21.0 statistical software (IBM Corp.) was used for statistical analysis, GraphPad Prism 8 (Dotmatics) was used for graph preparation. All data are presented as the mean ± SD of ≥2 repeats. Shapiro-Wilk test was used to evaluate the normality of the data. A standard unpaired Student's t-test was used to compare two groups of normally distributed quantitative data, Mann-Whitney U test was used to compare two groups of non-normally distributed quantitative data, and the Pearson χ^2 test, Fisher's exact test or Yates' correction was used to compare two groups of non-quantitative data. Comparisons of multiple groups of normally distributed quantitative data were performed using one-way ANOVA followed by Bonferroni post hoc correction. Spearman's analysis was used to assess linear correlations between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cdc42 and E2 levels are increased in the serum of patients with HF. Serum was isolated from 23 patients with HF and 21 controls for analysis. The baseline characteristics of the patients and controls are shown in Table I. There were no significant differences in terms of the baseline characteristics between the patients with HF and the controls.

The levels of Cdc42 and E2 in the serum of patients with HF and the controls were detected to examine the association between Cdc42 and estrogen in HF. The serum expression levels of Cdc42 and E2 were higher in patients with HF than in the control group (Table I; Fig. 1A and B), and the serum expression levels of Cdc42 and E2 were positively correlated in the patients with HF, with a correlation coefficient of $r=0.4456$ (Table I; Fig. 1C). These findings revealed links between HF and elevated Cdc42 protein levels, and between E2 and Cdc42 protein levels.

Effects of Cdc42 on MCF differentiation and collagen synthesis. The results in Fig. 2A-C demonstrate that the protein expression levels of α-SMA, collagen I and collagen III were increased after MCFs were stimulated with 20 ng/ml TGF-β1 for 24 h; however, the protein expression levels of Cdc42 were decreased. These data suggested that TGF-β1 could stimulate MCF proliferation and differentiation into myofibroblasts, which highly express α-SMA, and these myofibroblasts could in turn cause collagen metabolism disorders and increase expression of collagen I and collagen III. Furthermore, the decrease in Cdc42 expression indicated that, with the activation of MCFs, Cdc42 expression was regulated by TGF-β1 and negatively associated with the activation level of MCFs.

To investigate the effect of Cdc42 on MCFs, Cdc42-OE lentivirus or siRNA was used to induce or reduce Cdc42 expression, respectively. The expression levels of α-SMA, collagen I and collagen III were reduced after Cdc42 OE (Fig. 2D and E). Furthermore, the knockdown of Cdc42 expression increased the expression levels of α-SMA, collagen I and collagen III, which was consistent with the stimulant effect of TGF-β1 (Fig. 2D and F). Immunofluorescence staining also confirmed that Cdc42-OE lentivirus transfection decreased α-SMA protein expression, while siCdc42 transfection increased α-SMA protein expression (Fig. 2G-I). These data

Table I. Baseline characteristics and serological test results of the study subjects.

Variable	CON (n=21)	HF (n=23)	P-value
Baseline characteristics			
Age, years	62.90±9.10	66.52±13.94	0.319[a]
Male (%)	11 (52.4)	12 (52.2)	0.989[b]
Body surface area, m^2	1.72±1.38	1.79±0.26	0.288[a]
Body mass index, kg/m^2	24.27±3.33	25.66±5.38	0.297[c]
Smoking history, n (%)	4 (19.0)	7 (30.4)	0.494[d]
Drinking history, n (%)	5 (23.8)	5 (21.7)	>0.999[e]
Co-morbidities, n (%)			
Diabetes mellitus	3 (14.3)	9 (39.1)	0.065[b]
Hypertension	11 (52.4)	18 (78.3)	0.070[b]
Dyslipidemia	1 (4.8)	5 (21.7)	0.230[e]
Serological test results			
Cdc42 in the serum, ng/ml	6.66±1.31	7.60±1.27	0.019[a,f]
E2 in the serum, pmol/l	37.63±6.29	42.60±3.92	0.005[c,g]

[a]Standard Student's t-test. [b]Pearson χ^2 test. [c]Mann-Whitney U test. [d]Fisher's exact test; [e]Yates' correction. [f]P<0.05. [g]P<0.01. Cdc42, cell division cycle 42; CON, control; E2, 17β-estradiol; HF, heart failure. Data are expressed as the mean ± SD.

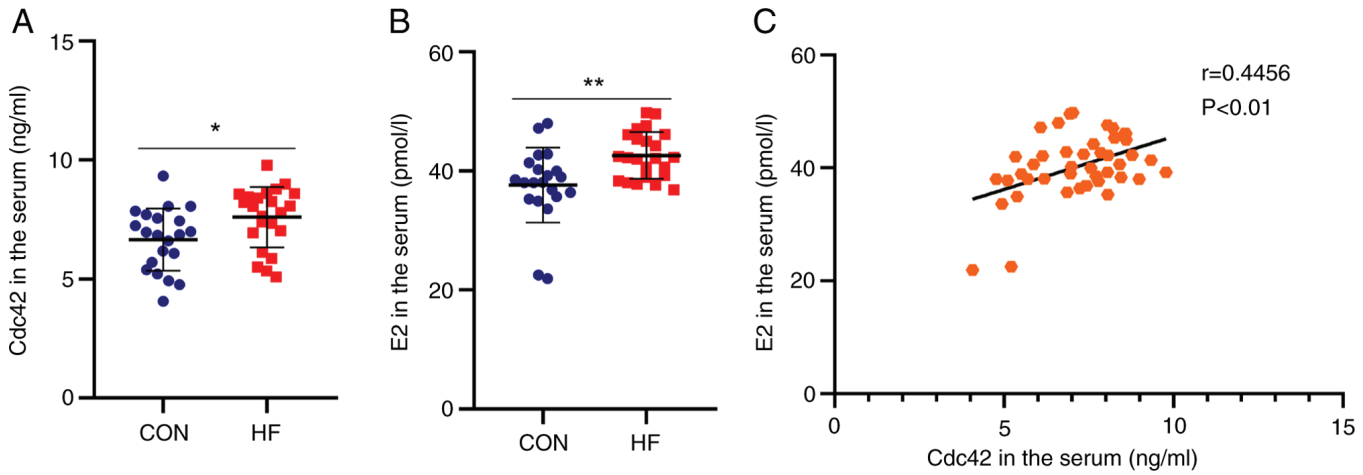

Figure 1. Cdc42 and E2 levels are increased in patients with HF. (A) Serum Cdc42 levels in patients with HF (n=23) and healthy controls (n=21) were determined using an ELISA. (B) Serum E2 levels were determined in patients with HF (n=23) and healthy controls (n=21) using an ELISA. (C) Correlation analysis of serum Cdc42 and E2 levels in patients with HF (n=23). The data are presented as the mean ± SD. P<0.05 was considered to indicate a statistically significant difference. *P<0.05, **P<0.01. Cdc42, cell division cycle 42; CON, control; E2, 17β-estradiol; HF, heart failure.

suggested that Cdc42 could inhibit MCF differentiation and collagen synthesis, and that decreased Cdc42 expression could promote fibrosis.

Effects of Cdc42 on the Pak1/JNK/c-Jun signaling pathway in MCFs. To investigate whether Cdc42 inhibited the autocrine activity of TGF-β1, the present study examined TGF-β1 mRNA levels. The results showed that TGF-β1 transcript levels were decreased when Cdc42 was overexpressed, and TGF-β1 transcript levels were increased when Cdc42 was knocked down by siCdc42-1 (Fig. 3A and B). The dual-luciferase reporter assay demonstrated that activity of the *Tgfb1* gene promoter was significantly enhanced after siCdc42-1 transfection of MCFs, indicating that Cdc42 knockdown promoted the activity of the *Tgfb1* gene promoter (Fig. 3C). As shown in Fig. 3D-F, the expression levels of Pak1, JNK and c-Jun were higher and the expression level of TGF-β1 was lower in the Cdc42 OE group than those in the NC group. The inhibition of Cdc42 expression decreased the expression levels of Pak1, JNK and c-Jun, and increased the expression level of TGF-β1. (Fig. 3D-F). These data suggested that Cdc42 might exert its antifibrotic effects by regulating the Pak1/JNK/c-Jun signaling pathway and subsequently inhibiting the autocrine activity of TGF-β1.

To further demonstrate that Cdc42 might be involved in regulating the Pak1/JNK/c-Jun signaling pathway, MLS-573151 (22), an inhibitor of Cdc42, was used to reduce

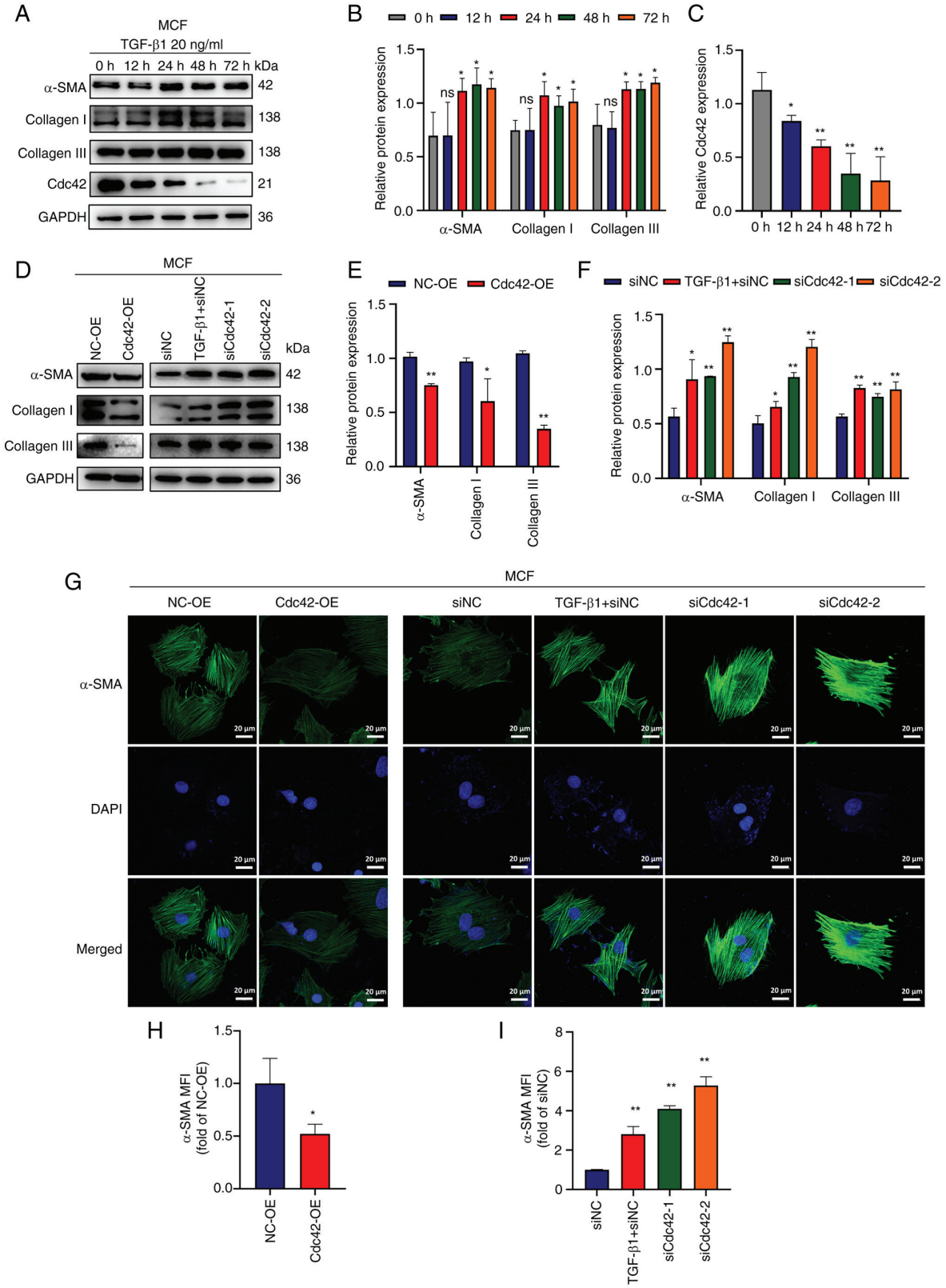

Figure 2. Cdc42 inhibits α-SMA expression and collagen synthesis. (A) Representative immunoblots of α-SMA, collagen I, collagen III, Cdc42 and GAPDH in MCFs stimulated with 20 ng/ml TGF-β1. (B) Quantification analysis of α-SMA, collagen I and collagen III and (C) Quantification analysis of Cdc42 protein. *P<0.05 and **P<0.01 vs. 0 h. (repeats, n=2; replicates, n=3). (D) MCFs were divided into the NC-OE, Cdc42-OE, siNC, TGF-β1 + siNC, siCdc42-1 and siCdc42-2 groups, and representative immunoblots showing changes in α-SMA, collagen I, collagen III and GAPDH levels in the cells are shown. (E) Quantification analysis of each protein level of NC-OE and Cdc42-OE groups. *P<0.05 and **P<0.01 vs. NC-OE. (F) Quantification analysis of each protein level of siNC, TGF-β1 + siNC, siCdc42-1 and siCdc42-2 groups. *P<0.05 and **P<0.01 vs. siNC. (repeats, n=2; replicates, n=3). (G) MCFs were divided into the NC-OE, Cdc42-OE, siNC, TGF-β1 + siNC, siCdc42-1 and siCdc42-2 groups for immunofluorescence staining of α-SMA Scale bar, 20 μm. (H) Quantification analysis of α -SMA immunofluorescence intensity of NC-OE and Cdc42-OE groups. *P<0.05 vs. NC-OE. (I) Quantification analysis of α-SMA immunofluorescence intensity of siNC, TGF-β1 + siNC, siCdc42-1 and siCdc42-2 groups. **P<0.01 vs. siNC. (n=3). The data are presented as the mean ± SD. α-SMA, α-smooth muscle actin; Cdc42, cell division cycle 42; MCF, mouse cardiac fibroblast; MFI, mean fluorescence intensity; NC, negative control; ns, not significant; OE, overexpression; siCdc42, small interfering RNA targeting Cdc42; siNC, negative control small interfering RNA.

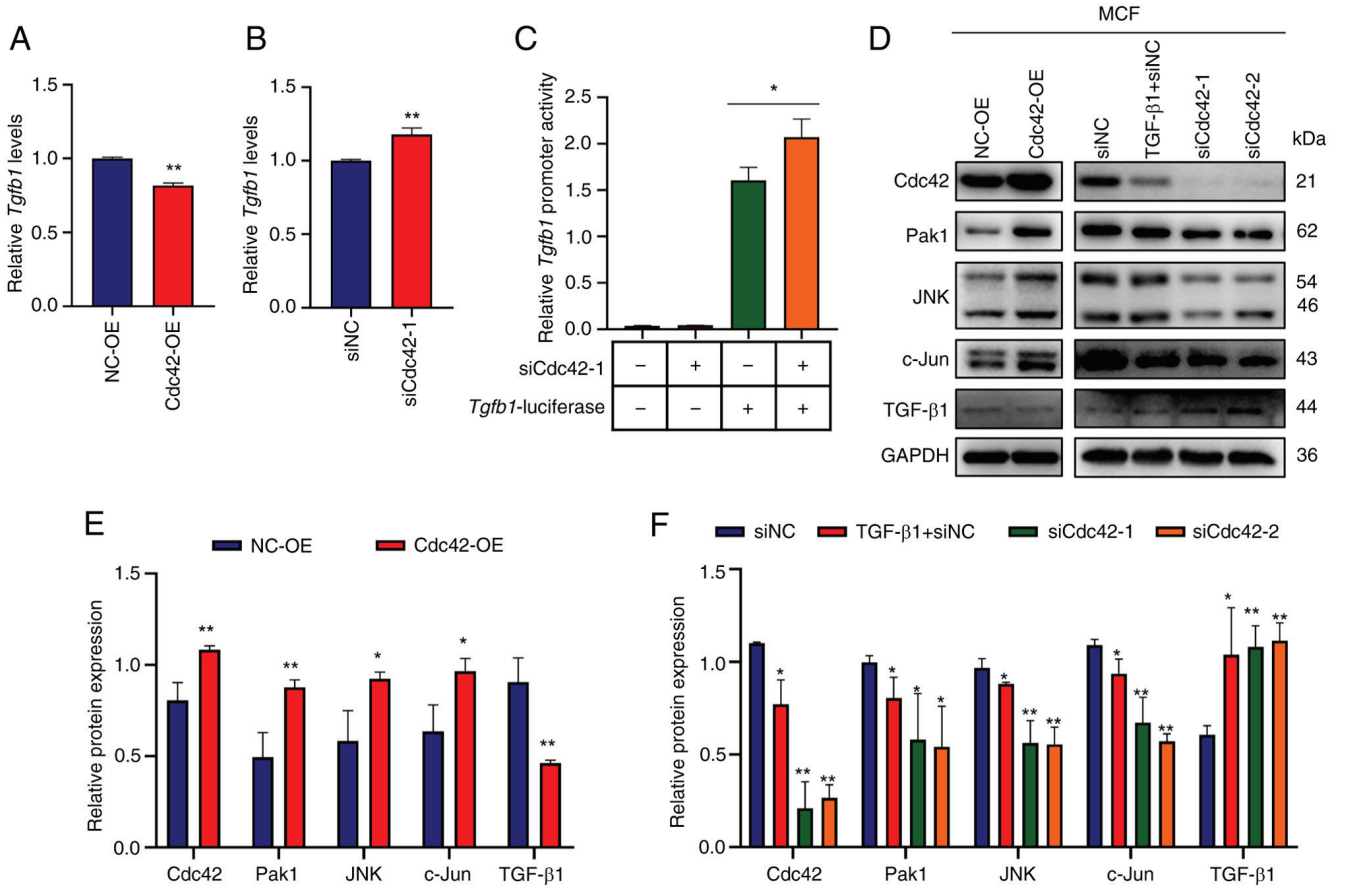

Figure 3. Cdc42 regulates the Pak1/JNK/c-Jun signaling pathway and inhibits TGF-β1 autocrine signaling. (A) Relative expression levels of *Tgfb1* mRNA in MCFs with or without Cdc42 overexpression. **P<0.01 vs. NC-OE. (n=4). (B) Relative expression levels of *Tgfb1* mRNA in MCFs with or without Cdc42 knockdown. **P<0.01 vs. siNC. (n=4). (C) MCFs were transfected with the *Tgfb1* luciferase plasmid and siCdc42 for 24 h, after which a dual-luciferase reporter assay was performed. *P<0.05 vs. *Tgfb1*-luciferase + siNC. (n=3). (D) MCFs were divided into the NC-OE, Cdc42-OE, siNC, TGF-β1 + siNC, siCdc42-1 and siCdc42-2 groups, and representative immunoblots showing changes in Cdc42, Pak1, JNK, c-Jun, TGF-β1 and GAPDH expression in the cells are presented. (E) Quantification analysis of each protein level of NC-OE and Cdc42-OE groups. *P<0.05 and **P<0.01 vs. NC-OE. (F) Quantification of each protein level of siNC, TGF-β1 + siNC, siCdc42-1 and siCdc42-2 groups. *P<0.05 and **P<0.01 vs. siNC (repeats, n=2; replicates, n=3). TGF-β1 + siNC group received TGF-β1 treatment. This group was used as a control for the siCdc42-1 and siCdc42-2 groups to observe whether Cdc42 silencing had the same effect as TGF-β1 treatment. The data are presented as the mean ± SD. P<0.05 was considered to indicate a statistically significant difference. Cdc42, cell division cycle 42; Luc, luciferase; MCF, mouse cardiac fibroblast; NC, negative control; OE, overexpression; Pak1, p21 (RAC1)-activated kinase 1; siCdc42, small interfering RNA targeting Cdc42; siNC, negative control small interfering RNA.

Cdc42 activity in MCFs. The phosphorylation of serine 71 in Cdc42 is known to negatively regulate Cdc42 activity, and thus, the increased expression of p-Cdc42 indicates a decrease in active (GTP-bound) Cdc42 (23). After Cdc42 activity was decreased, the expression levels of Pak1, JNK and c-Jun were decreased, and the expression level of TGF-β1 was increased (Fig. S1A and B), consistent with the effect of Cdc42 knockdown.

Effects of E2 and Cdc42 on TGF-β1-stimulated MCFs. Fig. 4A-C shows that the protein expression levels of α-SMA, collagen I and collagen III were decreased and the protein expression levels of Cdc42 were increased compared with NC group after treatment of TGF-β1-stimulated MCFs with 5, 10 and 50 nM E2 for 24 h. Furthermore, the protein expression levels of Pak1, JNK and c-Jun were increased compared with those in the TGF-β1-stimulated group after treatment of TGF-β1-stimulated MCFs with 10 nM E2 for 24 h. However, the effect of E2 on the expression of those proteins in TGF-β1-stimulated MCFs was abolished after siCdc42 transfection (Fig. 4D-F). Immunofluorescence staining also confirmed that siCdc42 abolished the E2-mediated reversal of changes in α-SMA protein expression following TGF-β1 treatment (Fig. 4G and H). Given these results, we hypothesized that the ameliorative effects of E2 were mediated by an increase in Cdc42. Fig. 5A-C shows that similar effects were observed in the Cdc42-OE-treated group and the E2-treated group of TGF-β1-stimulated MCFs, namely, decreased expression of α-SMA, collagen I and collagen III and increased expression of Pak1, JNK and c-Jun compared with the TGF-β1-stimulated group. Immunofluorescence staining also confirmed that Cdc42 OE inhibited α-SMA protein expression, which was consistent with the effect of E2-treated group (Fig. 5D and E). The aforementioned results indicated that Cdc42 could exert ameliorative effects similar to those of E2.

Discussion

Myocardial fibrosis is the manifestation of an adaptive response of the heart to pressure overload, which can lead to

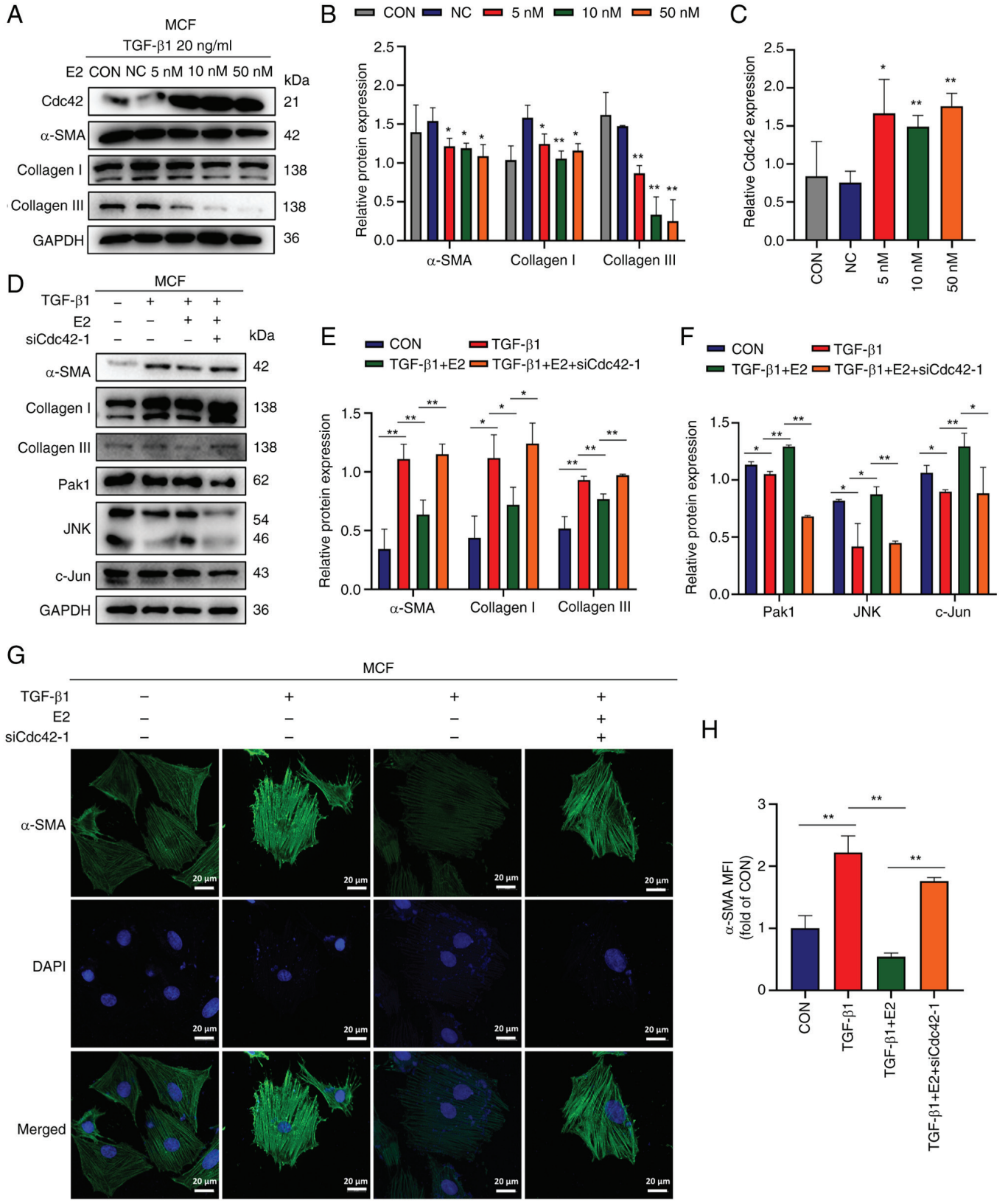

Figure 4. siCdc42 abolishes the E2-mediated reversal of changes in TGF-β1-stimulated MCFs. (A) After MCFs were stimulated with 20 ng/ml TGF-β1 for 6 h, E2 was applied at increasing concentrations for 48 h. The CON group was not treated, and the NC group was treated with vehicle (0.04% DMSO). Representative immunoblots showing changes in Cdc42, α-SMA, collagen I, collagen III and GAPDH are shown. (B) Quantification of α-SMA, collagen I and collagen III and (C) Cdc42 protein. *P<0.05 and **P<0.01 vs. NC. (D) MCFs were divided into the CON, TGF-β1, TGF-β1 + E2 and TGF-β1 + E2 + siCdc42 groups, and representative immunoblots showing changes in α-SMA, collagen I, collagen III, Pak1, JNK, c-Jun and GAPDH expression in the cells are shown. (E,F) Quantification analysis of each protein (repeats, n=2; replicates, n=3). (G) MCFs were divided into the CON, TGF-β1, TGF-β1 + E2 and TGF-β1 + E2 + siCdc42 groups, and immunofluorescence staining of α-SMA was performed. Scale bar, 20 μm. (H) Quantification of α-SMA immunofluorescence intensity in panel G. (n=3). The data are presented as the mean ± SD. P<0.05 was considered to indicate a statistically significant difference. *P<0.05, **P<0.01. α-SMA, α-smooth muscle actin; Cdc42, cell division cycle 42; CON, control; E2, 17β-estradiol; MCF, mouse cardiac fibroblast; MFI, mean fluorescence intensity; NC, negative control; Pak1, p21 (RAC1)-activated kinase 1; siCdc42, small interfering RNA targeting Cdc42.

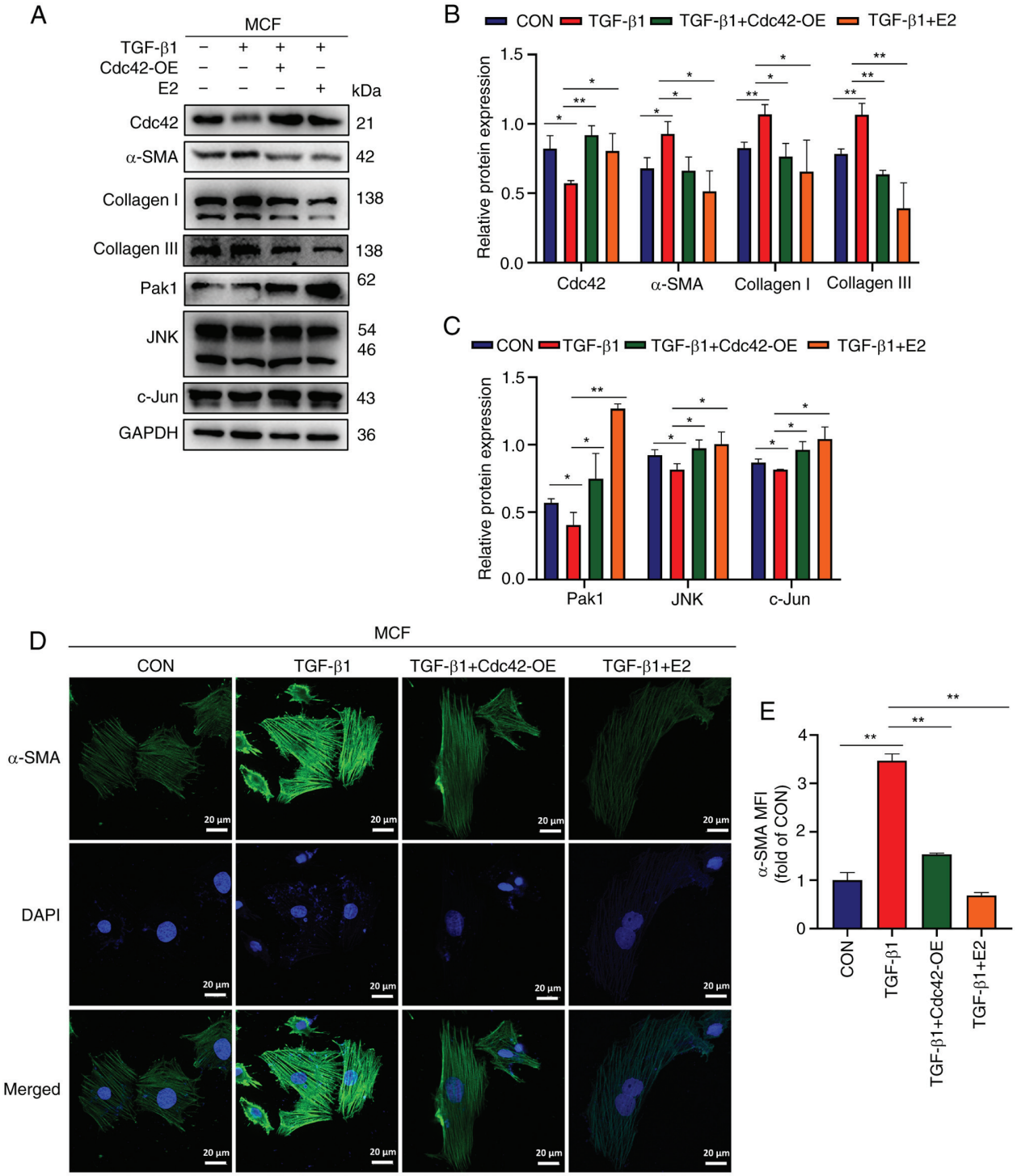

Figure 5. Cdc42-OE exerts ameliorative effects on TGF-β1-stimulated MCFs similar to those of E2. (A) MCFs were divided into the CON, TGF-β1, TGF-β1 + Cdc42-OE and TGF-β1 + E2 groups, and representative immunoblots showing changes in Cdc42, α-SMA, collagen I, collagen III, Pak1, JNK, c-Jun and GAPDH expression in the cells are shown. (B) Quantification of Cdc42, α-SMA, collagen I and collagen and (C) Pak1, JNK and c-Jun proteins. (repeats, n=2; replicates, n=3). (D) MCFs were divided into the CON, TGF-β1, TGF-β1 + Cdc42-OE and TGF-β1 + E2 groups, and immunofluorescence staining of α-SMA was performed. Scale bar, 20 μm. (E) Quantification analysis of α-SMA immunofluorescence intensity in panel D. (n=3). The data are presented as the mean ± SD. P<0.05 was considered to indicate a statistically significant difference. *P<0.05, **P<0.01. α-SMA, α-smooth muscle actin; Cdc42, cell division cycle 42; CON, control; E2, 17β-estradiol; MCF, mouse cardiac fibroblast; MFI, mean fluorescence intensity; OE, overexpression; Pak1, p21 (RAC1)-activated kinase 1.

HF (1). Cdc42 can inhibit the development of various types of tissue fibrosis, including progressive pulmonary fibrosis and renal interstitial fibrosis, and epithelial-mesenchymal transition, which is associated with fibrosis (24-26). Cdc42 is involved in the determination of the sarcomere composition of cardiomyocytes and helps improve cardiac structure after myocardial infarction (11). Previous data have indicated that myocardial-specific Cdc42 knockout increased the myocardial hypertrophic response in mice subjected to stress overload (12). To determine the functional role of Cdc42 in

myocardial fibrosis, the present study first determined the levels of Cdc42 in the serum of patients with HF and controls. Control subjects who underwent routine physical examination but did not have heart disease were selected, and these subjects were similar to the patients in the experimental group in terms of age, sex and body mass index (27). Although the levels of Cdc42 in the serum of patients with HF have not been reported in the literature, it has been reported that Cdc42 is not associated with age, sex, body mass index, hypertension or hyperlipidemia in patients with coronary heart disease, but that it is associated with diabetes mellitus (28). Therefore, diabetes mellitus was also selected as a control variable for the control group, and the incidence of diabetes mellitus in the control population was not significantly different from that in the patients with HF. The serum expression levels of Cdc42 were higher in patients with HF than in the control group. Using gene OE and silencing, it was revealed that Cdc42 attenuated TGF-β1-induced MCFs differentiation and collagen synthesis. These results indicated that Cdc42 might exert a protective effect in patients with HF by inhibiting myocardial fibrosis.

Pak1 is an effector of Cdc42 that serves an important role in a variety of pathological responses, including myocardial hypertrophy (13). A previous study has demonstrated that Cdc42 inhibits myocardial hypertrophy by activating JNK (12), which promotes c-Jun expression and inhibits the activation of the *Tgfb1* promoter (20). Cdc42 also serves an important role in regulating transcription factor activity (10). In the present study, in TGF-β1-stimulated MCFs, the protein expression levels of Cdc42, Pak1, JNK and c-Jun were decreased. In MCFs, Cdc42 OE could induce the expression of Pak1, JNK and c-Jun, and Cdc42 knockdown or inhibiting Cdc42 activity had the opposite effect. The results of the present study demonstrated that Cdc42 could decrease the expression of TGF-β1 at the transcriptional and translational levels. These results indicated that Cdc42 might inhibit TGF-β1-induced differentiation and collagen synthesis by regulating the Pak1/JNK/c-Jun signaling pathway, inhibiting TGF-β1 synthesis and preventing TGF-β1 from exerting profibrotic effects via autocrine signaling.

Previous studies have demonstrated that estrogen has a broad protective effect against cardiac diseases, such as dilated cardiomyopathy, cardiac hypertrophy and atherosclerosis (29-31). An epidemiological study has have demonstrated that premenopausal female patients have a lower risk of cardiovascular disease than male patients of the same age; however, after menopause, the risk in female patients is equal to or greater than that in male patients (32). Clinical data suggest the presence of sex-dependent differences in myocardial fibrosis under stress overload due to aortic stenosis, and the expression of collagen is greater in male than that in female patients (33). In females, estrogen is mainly secreted by the ovaries, while in males, estrogen is formed by the aromatization of androgens and cannot be synthesized in the heart (34,35); therefore, myocardial fibroblasts receive only exogenous estrogen via receptors (16). The results of the present study demonstrated that the levels of E2 in the serum of patients with HF were increased and that E2 reduced TGF-β1-stimulated MCF differentiation and collagen synthesis, suggesting that E2 might exert a protective effect in patients with HF. Although the cardioprotective effects of estrogen have been widely reported, hormone replacement therapy was less effective than anticipated in preventing cardiovascular disease in clinical trials initiated by the Women's Health Initiative (36,37). Furthermore, the use of hormone replacement therapy requires considerations, such as age, initiation time, treatment duration, dosage, route of administration and the presence or absence of progestin (38). Therefore, the use of E2 in the form of hormone replacement therapy in humans has been controversial, and the associated major controversies include the inconsistent efficacy of hormone replacement therapy and the risk of thrombotic events, cancer and arrhythmia (39). Therefore, it is essential to clarify the mechanism by which estrogen inhibits HF and myocardial fibrosis, and to identify its endogenous targets. Understanding these issues may provide a novel strategy for the treatment of this disease.

Inhibiting the overactivation of the TGF-β1 pathway is an important way to improve myocardial interstitial fibrosis in patients with HF, and estrogen inhibits the TGF-β1 pathway (16,33). The present data support these results. In TGF-β1-stimulated MCFs, E2 could induce Cdc42 expression, and promoted Pak1, JNK and c-Jun expression. However, the aforementioned effects of E2 in TGF-β1-stimulated MCFs were abolished after Cdc42 knockdown. The serum expression levels of Cdc42 and E2 in patients with HF were positively correlated, with a correlation coefficient of r=0.4456. Based on these data, it was concluded that E2 inhibits TGF-β1-stimulated MCF differentiation and collagen synthesis by inducing Cdc42 expression and subsequently modulating the Pak1/JNK/c-Jun signaling pathway. These results revealed that Cdc42 is an endogenous inhibitor of TGF-β1 activity in myocardial fibrosis and might be an effective target for the treatment of HF that avoids the side effects of hormone replacement therapy in the clinic as aforementioned.

In conclusion, in the present study, E2 ameliorated differentiation and collagen synthesis in TGF-β1-stimulated MCFs, potentially by upregulating Cdc42 protein expression and the subsequent modulation of Pak1/JNK/c-Jun expression. These findings might aid in understanding the application of E2 and Cdc42 in the treatment of myocardial fibrosis, and therapies that target Cdc42 might alleviate the progression of myocardial fibrosis. However, the present study has several limitations. Primary cardiac fibroblasts from female and male animal models were not used for validation in the present study, and it is uncertain whether the effect of E2 on Cdc42 is sex-dependent. Considering physiological and pathological differences between females and males, it is necessary to evaluate primary cardiac fibroblasts from female and male animal models separately in the future. In addition, the results of the present study were not validated in animal models of myocardial fibrosis, and thus, the specific therapeutic effect of Cdc42 on HF and myocardial fibrosis was not assessed at the whole-organ level in the heart. Future evaluation in animal models and patients is warranted.

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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

JX and FW analyzed the data and wrote the draft of the original article. JX, FW and YL performed cell culture and functional verification, and YL and YH collected clinical samples and performed ELISAs. PL and YZ performed quantitative PCR. KS and GX designed the experiments and revised the paper. JX and KS confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved and supervised by the Ethics Committees of Affiliated Suzhou Hospital of Nanjing Medical University, Gusu School (Suzhou, China; approval no. K-2021-GSKY20210202). Oral informed consent was obtained from all subjects involved in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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