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Original Article

CKAP4 participates in tryptase-induced phenotypic conversion in atrial fibroblasts through PAR2/p38/JNK pathway

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Abstract: Our previous study found that tryptase activated atrial fibroblasts, increased collagen synthesis in atrial fibroblasts through protease activated receptor-2 (PAR2) receptors. Recent studies showed that cytoskeleton-associated protein 4 (CKAP4) played an important role in ventricular fibroblast activation. The present study aimed to investigate the role of CKAP4 in tryptase-induced atrial fibroblast activation, atrial fibrosis, and molecular regulatory mechanisms. We cultured atrial fibroblasts in vitro, gave cells tryptase stimulation, then overexpressed or silenced PAR2 and CKAP4 genes in the cells. Their effects on atrial fibroblast proliferation, migration, extracellular matrix remodeling (Collagen I and fibronectin) and downstream key molecules (TGF-β1, c-jun and c-fos, JNK, p38) were investigated. The results showed that the expression of CKAP4 was significantly increased by tryptase and further increased by pcDNA3.1-PAR2, but decreased by FALLRY-NH2 and PAR2 siRNA. CKAP4 overexpression significantly increased the cell proliferation, migration and levels of Collagen I and fibronectin, matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinases-1 (TIMP-1) levels in atrial fibroblasts, while CKAP4 siRNA significantly reduced them. CKAP4 overexpression significantly increased the expression of TGF-β1, c-jun and c-fos, and activated the JNK/p38 pathway, which were suppressed by CKAP4 siRNA. In conclusion, CKAP4 is involved in tryptase-induced phenotypic conversion in atrial fibroblasts through PAR2/p38/JNK pathway, which may provide novel targets in the prevention of atrial fibrosis.

Keywords: Cytoskeleton-associated protein 4, tryptase, atrial fibrosis, atrial fibroblasts, protease activated receptor 2

Introduction

Atrial fibrosis is a complex pathophysiological process involving Renin-Angiotensin-System (RAS) systems, transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF), inflammation and oxidative stress [1-6]. In theory, intervention on these key signaling systems of atrial fibrosis can delay the progress of atrial fibrosis and prevent/treat atrial fibrillation. However, recent studies have found that interventions targeting some essential factors, such as TGF-β and CTGF that play an important role in atrial fibrosis, failed to achieve desired outcomes [7, 8]. It is possible that other endogenous (external) factors that cause atrial fibrosis may play a more important role in the formation of atrial fibrosis and the occurrence/maintenance of atrial fibrillation. Recent studies have found that tryptase can activate ventricular fibroblasts [9] and exacerbate ventricular myofibrosis in spontaneously hypertensive rats [10]. Our previous study also found that tryptase activated atrial fibroblasts, increased collagen synthesis in atrial fibroblasts, and leaded to extracellular matrix remodeling through protease activated receptor-2 (PAR2) receptors [11]. However, the mechanism of the action of tryptase/PAR2 needs further exploration.

Cytoskeleton-associated protein 4 (CKAP4) is a type II transmembrane protein, consisting of an N-terminal intracellular domain, a single transmembrane domain, and a C-terminal extracellular domain. CKAP4 is mainly located in the endoplasmic reticulum and is involved in the regulation of various biological activities in the cell. It is a receptor for tissue-type plasminogen

activator (tPA), surface protein A, and anti-proliferative factor (APF). CKAP4 is mainly expressed on the surface of vascular smooth muscle cells, type II alveolar wall cells, bladder epithelial cells [12]. Recent studies showed that CKAP4 was up-regulated in activated ventricular fibroblasts after ischemic injury and played an important role in ventricular fibroblast activation [13-15]. These results suggest that the role of CKAP4 in atrial fibrosis needs to be further studied. Based on our previous findings [11], we proposed a hypothesis that tryptase activates atrial fibroblasts and promotes atrial fibrosis through the PAR2/CKAP4 pathway. To verify this hypothesis, we cultured atrial fibroblasts in vitro, gave cells tryptase stimulation, then over-expressed or silenced PAR2 and CKAP4 genes in the cells. Their effects on atrial fibroblast proliferation, migration, extracellular matrix (ECM) remodeling and downstream key molecules were investigated. The present study demonstrated the role of tryptase, PAR2, and CKAP4 in atrial fibroblast activation, atrial fibrosis, and molecular regulatory mechanisms, and explored new mechanisms of atrial fibrosis and new strategies for the prevention and treatment of atrial fibrillation.

Materials and methods

Atrial fibroblasts preparation

Atrial fibroblasts were harvested from the right atrium of male neonatal Sprague-Dawley rats (2-3 days) and cultured in DMEM containing 10% fetal bovine serum (FBS). The study was approved by the Animal Care and Use Committee of Tongji University School of Medicine (approval reference number: 2019-0644) and the experiments were performed in accordance with the guidelines in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 85-23, revised 1996). Rats were sacrificed by cervical dislocation and put into a beaker containing 75% alcohol for 10 seconds. After the heart was completely removed and the residual blood was squeezed out, it was put into DMEM medium and cut into pieces with scissors. The heart tissue was then suspended with 0.125% trypsin and transfered to a centrifuge tube and kept in 37°C water bath for 10 min. Afterwards, the supernatant and precipitate were sucked out and put into two new centrifuge tubes. DMEM medium containing 20% fetal bovine serum was added to the centrifuge tube with supernatant to terminate digestion, and 0.125% trypsin was added into the centrifuge tube with precipitation. After 10 min of shaking digestion at 37°C, trypsin was added into the sedimentation tube for repeated digestion; 2.5 ml 0.125% trypsin and 2.5 ml 0.125% collagenase II were added into the centrifuge tube containing precipitation, and the supernatant and precipitate were sucked out respectively and put into two new centrifuge tubes; trypsin reduction and type II collagenase were added to the sedimentation tube; The digestion was repeated for 3 times. After digestion, DMEM medium containing 20% fetal bovine serum was added into the centrifuge tube containing supernatant to terminate digestion, and centrifuged at 4°C at 1,2000 × g for 10 min. The precipitates in each centrifuge tube were sucked out and put into a new test tube. DMEM medium containing 20% fetal bovine serum was added. The cells were blown and seeded into the culture bottle. Penicillin/Streptomycin antibiotic was added in the ratio of 1:100 to prevent bacterial growth. The culture bottle was put into the cell incubator with the temperature of 37°C and 5% CO_2 for 90 min. Fibroblasts in the culture bottle had adhered to the wall, while myocardial cells were suspended in the culture medium. To inhibit the growth of cardiomyocytes, the medium was aspirated, and the DMEM medium containing 20% fetal bovine serum and 10 mmol/L BrdU were added. The cultured rat cardiac fibroblasts were passaged for 2-3 generations according to the ratio of 1:3. Under the inverted microscope, cells with polygonal or spindle shaped were observed; the cytoplasm was transparent, the nucleus was large, oval, and often contained 2-3 nuclei. The purity of fibroblasts was about 95%. Fibroblast specific markers (vimentin and DDR2) were used to identify the purity of cardiac fibroblasts.

Atrial fibroblasts treatments

In experiment 1, fibroblasts were divided into seven groups: Control; Tryptase; Tryptase+FSLLRY-NH2; Tryptase+pcDNA3.1; Tryptase+PAR2+/+; Tryptase+shNC; Tryptase+PAR2-/-. Atrial fibroblasts in Control group received no treatment; fibroblasts in Tryptase group were incubated with 10 mg/ml tryptase (Promega, Beijing, China); fibroblasts in Tryptase+FSLLRY-

NH2 group were incubated with 10 mg/ml tryptase and FSLLRY-NH2 (100 μM); fibroblasts in Tryptase+pcDNA3.1 group were incubated with 10 mg/ml tryptase and received empty pcDNA3.1 transfection; fibroblasts in Tryptase+PAR2+/+ group were incubated with 10 mg/ml tryptase and received pcDNA3.1-PAR2 transfection; fibroblasts in Tryptase+shNC group were incubated with 10 mg/ml tryptase and received non-target siRNA transfection; fibroblasts in Tryptase+PAR2-/- group were incubated with 10 mg/ml tryptase and received PAR2 siRNA transfection.

In experiment 2, fibroblasts were divided into six groups: Control; Tryptase; Tryptase+LvCon; Tryptase+LvCAKP4; Tryptase+NT-siRNA; Tryptase+CKAP4-siRNA. Atrial fibroblasts in Control, Tryptase, Tryptase+LvCon and Tryptase+NT-siRNA groups received treatment as previously described. Fibroblasts in Tryptase+LvCAKP4 group were incubated with 10 mg/ml tryptase and received pcDNA3.1-CAKP4 transfection; fibroblasts in Tryptase+CKAP4-siRNA group were incubated with 10 mg/ml tryptase and received CKAP4 siRNA transfection.

Plasmid construction and small-interference RNA (siRNA) Transfection

For plasmid construction, empty Control and pcDNA3.1-PAR2 vectors were transfected into fibroblasts using Lipofectamine 2000 (Thermo, United States) according to the manufacturer's instructions. CKAP4 expression plasmid was purchased from Addgene (#80977; Addgene) and subcloned into pcDNA3.1(+) with 3xFLAG tag at the C terminus. Truncated CKAP4 constructs were generated by DNA synthesis from Integrated DNA Technologies, Inc (Coralville, Iowa, USA), as described by Lyu et al. [13].

Similarly to Shahjee et al. [16], for siRNA transfection, PAR2 Stealth RNAi siRNA, CKAP4 Stealth RNAi siRNA and Stealth RNAi Negative Control High GC were purchased from Invitrogen Inc (Carlsbad, CA, USA). Sequence of PAR2 siRNA was as follows. 5'-GCUCUGCAAGGUGCUCAUUGGCUUU-3'; Sequence of CKAP4 siRNA was as follows. 5'-AACUUUUGAGUCCAUCUUGAGAA-3'. Atrial fibroblasts were transferred to a sterile cuvette with PAR2 siRNA, CKAP4 siRNA or scrambled non-target siRNA and electroporated using a Bio-Rad Gene Pulser Xcell, then incubated in growth medium overnight in a 37°C/5% CO_2 atmosphere.

Cell proliferation and migration measurement

For cell proliferation measurement, atrial fibroblasts were seeded in 6-well plates. 5-bromo-2'-deoxyuridine (BrdU) incorporation cell proliferation assay (Cell Biolabs, Inc., San Diego, CA, USA) were performed in accordance with the manufacturer's instructions when cells were incubated with tryptase for 0 h, 12 h, 24 h and 48 h. Briefly, we firstly prepared a cell suspension containing 1.0×10^6 cells/ml in medium, then added 100 μL per well to a 96-well cell culture plate and incubated them overnight at 37°C and 5% CO_2 in a humidified incubator. Next, we added 10 μL of 10X BrdU Solution to wells and incubate at 37°C and 5% CO_2 in a humidified incubator for 4 hours, then slowly aspirate wells by pipette and add 100 μL PBS for three times. 100 μL Fix/Denature Solution were then added and incubated for 30 minutes at 37°C. 100 μL Antibody Diluent were added and incubated for 1 hour at room temperature. 100 μL of diluted Anti-BrdU Antibody was added to each tested well and incubated at room temperature for 1 hour on an orbital shaker. 100 μL of the diluted Secondary Antibody HRP Conjugate was added to each well and incubated at room temperature for 1 hour on an orbital shaker. 100 μL of Substrate Solution was added to each well and incubated at room temperature on an orbital shaker for 20 minutes. The enzyme reaction was terminated by adding 100 μL of Stop Solution into each well. Finally, the absorbance of each well was read on a spectrophotometer using 450 nm as the primary wave length.

For migration measurement, atrial fibroblasts were seeded in a 6-well plate. About 5×10^5 cells were added into each well. The next day, a p200 pipet tip was used to create a cell-free zone (20 mm in length; 700 μm in width). Cells were washed with PBS three times to remove the scratched cells, and serum-free medium was added. Next, they were cultured in 5% CO_2 incubator at 37°C. Samples were taken at 0, 6, 12 and 24 hours. The cells were then incubated in 1% serum culture medium. 24 h later, the Leica DFC 420c camera (Leica Microsystems, Wetzlar, Germany) was used to record the migration length (μm) of cells.

Western blotting and MMPs and TIMP-1 measurement by enzyme-linked immunosorbent assays (ELISA)

Cells were treated with RIPA Lysis Buffer (Beyotime, Shanghai, China), and the lysate were centrifuged at 12,000 r/min for 10 min. The protein in the supernatant was used for Western blotting with the method we previously reported 7. The density of blots was quantified by Quantity One software (Bio-Rad, Hercules, CA, USA).

As described in our previous study 11, MMPs (-1, -3), TIMP-1 and Collagen I levels in the cell culture medium were measured by ELISA kits (R&D Systems, Minneapolis, USA) according to manuals. First of all, cells were centrifuged for 20 minutes at 12000 × g and the supernatant was collected. 40 μl sample diluent was added into the sample well on the enzyme-coated plate, and then 10 μl sample was added. After sealing the plate with the sealing film, they were incubated at 37°C for 30 minutes. 50 μl of enzyme-labeled reagent was added into each well. 50 μl of chromogenic agent A and 50 μl of chromogenic agent B were added into each well. They were shaken gently and mixed well, then kept away from light at 37°C for 15 minutes. 50 μl of termination solution was added to each well to terminate the reaction (the blue color turned to yellow immediately). The absorbance of each well was measured by an ELISA plate reader at 490 nm (Biotek, Vermont, USA) at 450 nm. Levels of MMPs, TIMP-1 and Collagen I in the samples were calculated by comparison with the respective standard curves.

Statistical analyses

Data were expressed as mean ± SD. Statistical analysis was performed using analysis of variance (ANOVA) followed by post-hoc test with the software SPSS 17.0 (SPSS, USA). $P<0.05$ was considered statistically significant.

Results

Changes in the cell proliferation, migration and levels of Collagen I and fibronectin in atrial fibroblasts by PAR2 overexpression or siRNA

Figure 1 sketches the changes in the cell proliferation (A), migration (B) and levels of Collagen I and fibronectin (C) in atrial fibroblasts by PAR2 overexpression or siRNA. PAR2 inhibitor FALLRY-NH2 and PAR2 siRNA significantly abolished the effect of tryptase on cell proliferation, migration length and the expression of Collagen I and fibronectin in atrial fibroblasts ($P<0.05$ compared to Tryptase group).

Overexpression of PAR2 caused by pcDNA3.1, on the other hand, further promoted cell proliferation, migration and the expression of Collagen I and fibronectin ($P<0.05$ compared to Tryptase group). Treatment with empty pcDNA3.1 or non-target siRNA did not significantly change these parameters ($P>0.05$ compared to Tryptase group).

Changes in MMPs and TIMP-1 levels in atrial fibroblasts by PAR2 overexpression or siRNA

The expression of MMPs (MMP-1, MMP-3) and TIMP-1 in atrial fibroblasts and their levels in the culture medium were measured after cells were treated with tryptase and PAR2 overexpression or siRNA. As illustrated in **Figure 2**, the expression of MMP-1 and TIMP-1 and their levels in the culture medium were significantly changed by tryptase. PAR2 inhibitor FALLRY-NH2 and PAR2 siRNA significantly abolished the effect of tryptase on these two parameters ($P<0.05$ compared to Tryptase group), while overexpression of PAR2 further promoted the expression of MMP-1, but decreased the expression of TIMP-1. The expression of MMP-3 and their levels in the culture medium were not significantly changed by these treatments. Treatment with empty pcDNA3.1 or non-target siRNA did not significantly change the expression of MMPs (MMP-1, MMP-3) or TIMP-1 in atrial fibroblasts or their levels in the culture medium ($P>0.05$ compared to Tryptase group).

Changes in expression of CKAP4 and JNK/p38 pathway in atrial fibroblasts by tryptase, PAR2 overexpression and siRNA

To investigate the effect of tryptase and PAR2 on the expression of CKAP4 and JNK/p38 pathway in atrial fibroblasts, we measured the expression levels of CKAP4, the phosphorylation level of JNK and p38 in atrial fibroblasts after they were treated with tryptase, PAR2 overexpression or siRNA. As depicted in **Figure 3A** and **3B**, the expression of CKAP4 was significantly increased by tryptase and further in-

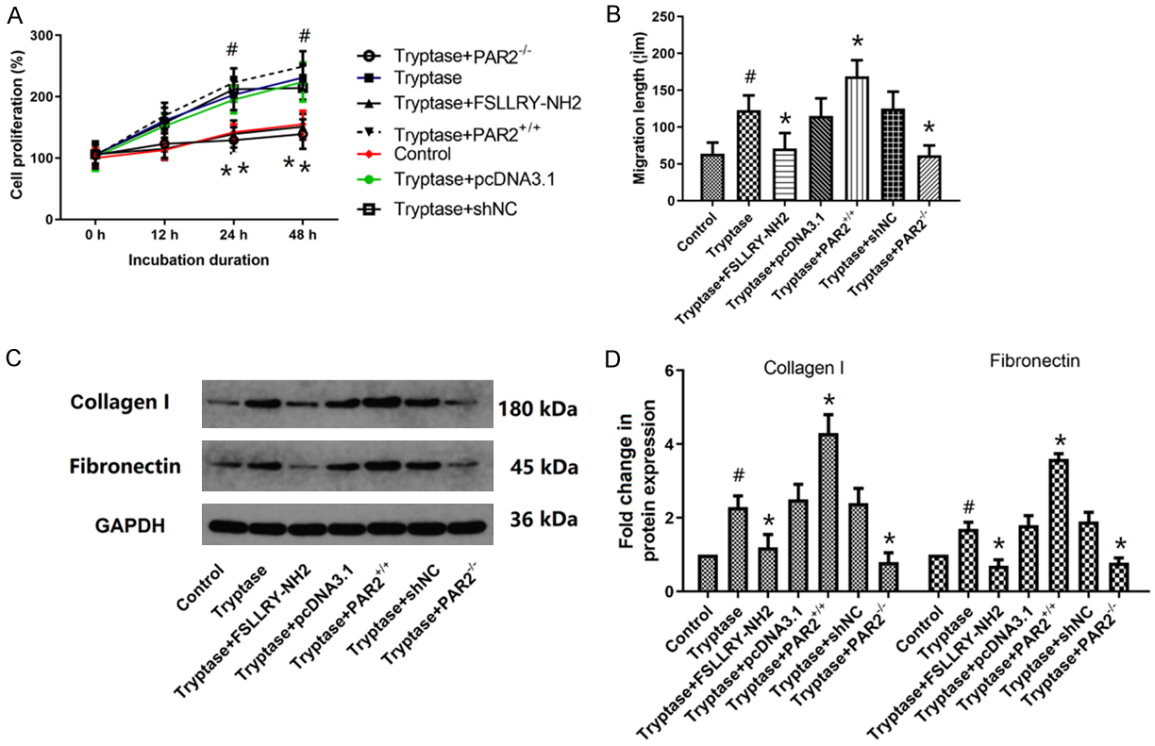

Figure 1. Effects of PAR2 overexpression or siRNA on the cell proliferation, migration and levels of Collagen I and fibronectin in atrial fibroblasts. The figure sketches the changes in the cell proliferation (A), migration (B) and levels of Collagen I and fibronectin (C and D) in atrial fibroblasts by PAR2 overexpression or siRNA. PAR2 inhibitor FALLRY-NH2 and PAR2 siRNA significantly abolished the effect of tryptase on cell proliferation, migration length and the expression of Collagen I and fibronectin. Overexpression of PAR2 promoted cell proliferation, migration and the expression of Collagen I and fibronectin. Values are expressed as Mean ± SD. #P<0.05 compared with Control; *P<0.05 compared with Tryptase group. N=12.

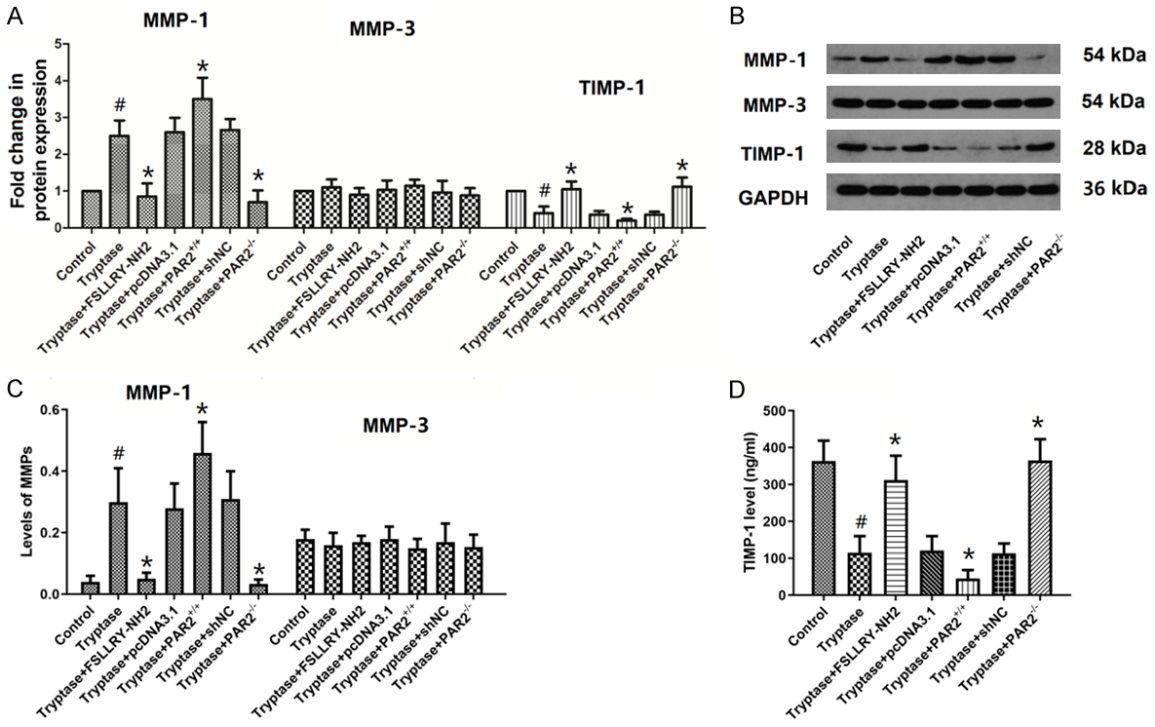

Figure 2. Effects of PAR2 overexpression or siRNA on the levels of MMPs and TIMP-1 levels in atrial fibroblasts. A. It shows the relative protein levels of MMP-1, MMP-3, and TIMP-1. B. It shows the representative blots. C. It shows the levels of MMP-1 and MMP-3 in the culture medium. D. It shows the levels of TIMP-1 in the culture medium. The expression of MMP-1 and TIMP-1 and their levels in the culture medium were significantly changed by tryptase. FALLRY-NH2 and PAR2 siRNA significantly abolished the effect of tryptase, while overexpression of PAR2 promoted the expression of MMP-1, but decreased the expression of TIMP-1. Values are expressed as Mean ± SD. #P<0.05 compared with Control; *P<0.05 compared with Tryptase group. N=12.

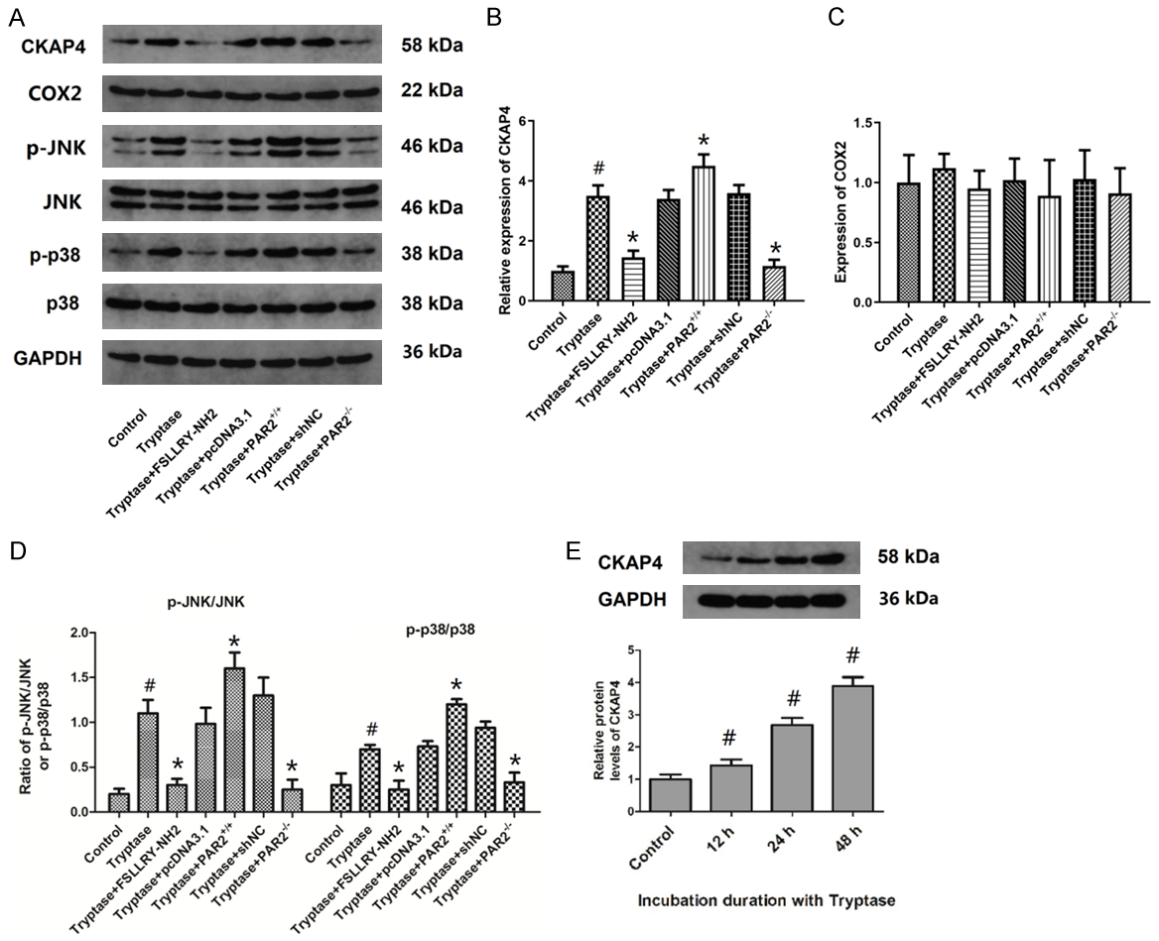

Figure 3. Changes in expression of CKAP4 and JNK/p38 pathway in atrial fibroblasts. A. It shows the representative blots of CKAP4, COX2, p-JNK, JNK, p-p38, p38 and GAPDH. B. It shows the relative protein levels of CKAP4. The expression of CKAP4 was significantly increased by tryptase and further increased by PAR2 overexpression, but decreased by PAR2 inhibitor FALLRY-NH2 and PAR2 siRNA. C. It shows the relative protein levels of COX2, which was not changed among groups. D. It shows the ratios of p-JNK/JNK or p-p38/p38. They were significantly increased by tryptase and further increased by PAR2 overexpression, but decreased by FALLRY-NH2 and PAR2 siRNA. E. It shows the representative blots and of relative protein levels of CKAP4 in atrial fibroblasts after they were treated with tryptase for in 12 h, 24 h, 48 h. The expression of CKAP4 increased in a duration-dependent manner. Values are expressed as Mean ± SD. #P<0.05 compared with Control; *P<0.05 compared with Tryptase group. N=12.

creased by PAR2 overexpression (P<0.05 compared to Control), but decreased by PAR2 inhibitor FALLRY-NH2 and PAR2 siRNA. As depicted in **Figure 3C**, the expression of COX2 did not change by these treatments. As shown in **Figure 3D**, the ratios of p-JNK/JNK and p-p38/p38 were significantly increased by tryptase (P<0.05 compared to Control) and further increased by PAR2 overexpression (P<0.05 compared to Tryptase), but decreased by PAR2 inhibitor FALLRY-NH2 and PAR2 siRNA (P<0.05 compared to Tryptase). Treatment with empty pcDNA3.1 or non-target siRNA did not significantly change the expression of CKAP4 and JNK/p38 pathway in atrial fibroblasts (P>0.05 compared to Tryptase group). As illustrated in

Figure 3E, after we treated atrial fibroblasts with tryptase for different durations, the expression of CKAP4 increased in a duration-dependent manner.

Changes in the cell proliferation, migration and levels of Collagen I and fibronectin in atrial fibroblasts by CKAP4 overexpression or siRNA

Figure 4 shows the changes in the cell proliferation (A), migration (B) and levels of Collagen I and fibronectin (E) in atrial fibroblasts by CKAP4 overexpression or siRNA. The release level of Collagen I into the cell culture medium was shown in **Figure 4F**. The representative blots were demonstrated in **Figure 4C**. **Figure 4D** sketches the expression of CKAP4 by CKAP4 overexpression or siRNA. CKAP4 siRNA significantly abolished the effect of tryptase on cell proliferation, migration length and the expression of Collagen I and fibronectin in atrial fibroblasts ($P<0.05$ compared to CKAP4 group). Overexpression of CKAP4 caused by pcDNA3.1, on the other hand, further promoted cell proliferation, migration and the expression of Collagen I and fibronectin ($P<0.05$ compared to Tryptase group). The expression of CKAP4 was significantly increased by CKAP4 overexpression, but significantly decreased by CKAP4 siRNA. Treatment with empty pcDNA3.1 or non-target siRNA did not significantly change these parameters ($P>0.05$ compared to Tryptase group).

Changes in MMP-1 and TIMP-1 levels in atrial fibroblasts by CKAP4 overexpression or siRNA

The expression of MMP-1 and TIMP-1 in atrial fibroblasts and their levels in the culture medium were measured after cells were treated with tryptase and CKAP4 overexpression or siRNA. As illustrated in **Figure 5**, overexpression of CKAP4 further promoted the expression of MMP-1, but decreased the expression of TIMP-1 ($P<0.05$ compared to Tryptase group), while CKAP4 siRNA significantly abolished the effect of tryptase on the expression of MMP-1 and TIMP-1 and their levels in the culture medium ($P<0.05$ compared to Tryptase group).

Changes in TGF-β1, c-jun, c-fos and JNK/p38 pathway in atrial fibroblasts by CKAP4 overexpression and siRNA

As illustrated in **Figure 6**, the expression of TGF-β1, c-jun and c-fos was significantly increased by tryptase ($P<0.05$ compared to Control), further increased by CKAP4 overexpression ($P<0.05$ compared to Tryptase group), but decreased by CKAP4 siRNA ($P<0.05$ compared to Tryptase group). The ratios of p-JNK/JNK and p-p38/p38 were significantly increased by CKAP4 overexpression ($P<0.05$ compared to Tryptase), but decreased by CKAP4 siRNA ($P<0.05$ compared to Tryptase). Treatment with empty pcDNA3.1 or non-target siRNA did not significantly change the expression of TGF-β1, c-jun and c-fos and JNK/p38 pathway in atrial fibroblasts ($P>0.05$ compared to Tryptase group).

Discussion

Atrial fibrosis refers to the pathological process of atrial muscle cell interstitial abnormal collagen deposition or collagen composition changes due to imbalance of collagen production and degradation, which leads to abnormal atrial electrical conduction and mechanical function [6]. Its main features are an increase in the number of atrial fibroblasts and the remodeling of the extracellular matrix (ECM) of the myocardium. Regardless of the cause, once the atrial fibrosis starts, it will be a continuous and progressive pathological process [2-6]. Boldt et al. [17] found that compared with those with sinus rhythm, patients with atrial fibrillation had increased levels of collagen I, collagen III, and fibronectin in the left atrium; A study on the animal model of atrial fibrillation in dogs established with left ventricular pacing also confirmed that atrial fibrillation was accompanied with increased atrial fibrosis [18]. Atrial fibrosis increases atrial stiffness, the spacing between myocardial cells, and the anisotropy of atrial conduction, which is the histological basis for the structural and electrical reconstruction of atrial fibrillation [1-6]. Clinical studies have found that the severity of atrial fibrosis is closely related to the success of catheter ablation in patients with atrial fibrillation [19, 20]. Therefore, how to effectively intervene and delay the progress of atrial fibrosis is very important for the prevention and treatment of atrial fibrillation, and understanding of the mechanism of atrial fibrosis is even more critical [21]. For the first time, the present study revealed that tryptase may promote the phenotypic conversion of atrial fibroblasts by PAR2/CKAP4 and p38/JNK pathways, which expands our understanding of the mechanism of atrial fibrosis.

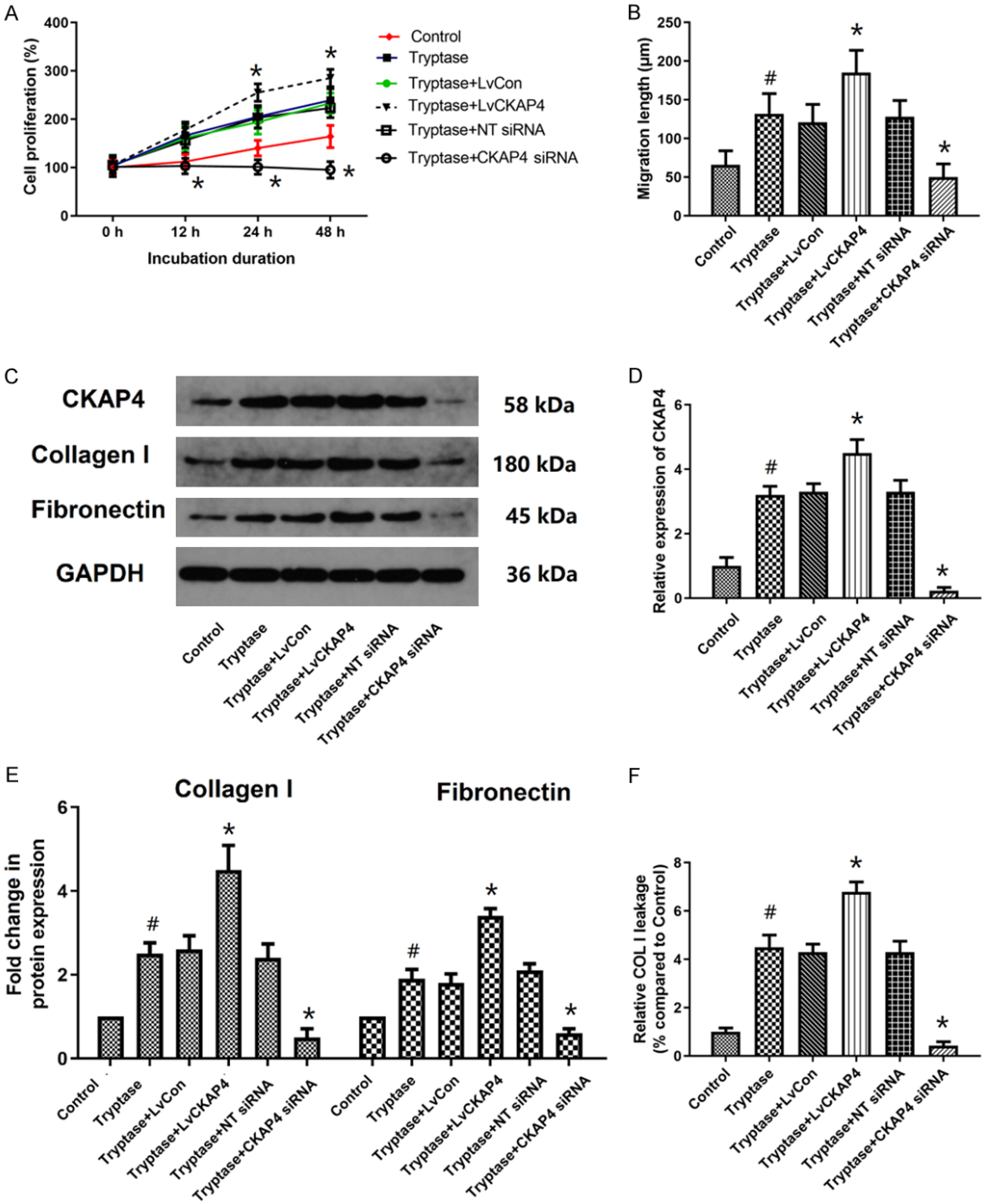

Figure 4. Effects of CKAP4 overexpression or siRNA on cell proliferation, migration and levels of Collagen I and fibronectin in atrial fibroblasts. The picture sketches the changes in the cell proliferation (A), migration (B) and levels of CKAP4, Collagen I and fibronectin (C-E) relative Collagen I leakage (F) in atrial fibroblasts by CKAP4 overexpression or siRNA. CKAP4 siRNA significantly abolished the effect of tryptase on cell proliferation, migration length, the expression of Collagen I and fibronectin and the Collagen I leakage in atrial fibroblasts. Overexpression of CKAP4 further promoted cell proliferation, migration and the expression of Collagen I and fibronectin. The expression of CKAP4 was significantly increased by CKAP4 overexpression, but significantly decreased by CKAP4 siRNA. Values are expressed as Mean ± SD. #P<0.05 compared with Control; *P<0.05 compared with Tryptase group. N=12.

In our previous study [11], we have found that tryptase significantly increased the cell proliferation, the protein levels of Collagen I and fibronectin, migration ability and MMP (-1, -2)

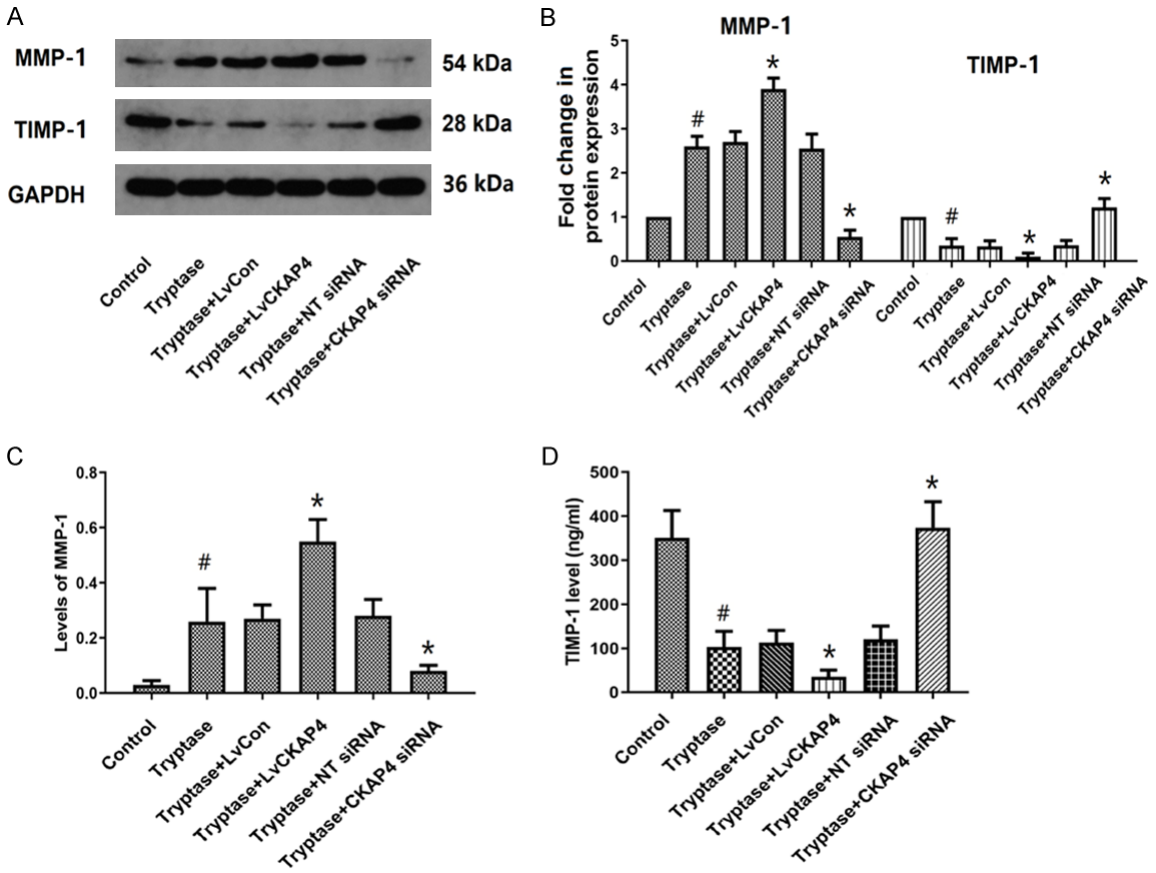

Figure 5. Effects of CKAP4 overexpression or siRNA on the levels of MMP-1 and TIMP-1 levels in atrial fibroblasts. A. It shows the representative blots of MMP-1 and TIMP-1. B. Shows their relative protein levels. C and D. They show the levels of MMP-1 and TIMP-1 in the culture medium. Overexpression of CKAP4 promoted the expression of MMP-1, but decreased the expression of TIMP-1, while CKAP4 siRNA significantly abolished the effect of tryptase on the expression of MMP-1 and TIMP-1 and their levels in the culture medium. Values are expressed as Mean ± SD. #P<0.05 compared with Control; *P<0.05 compared with Tryptase group. N=12.

levels of atrial fibroblasts. The TIMP (-1, -2) levels of atrial fibroblasts were significantly decreased by tryptase. PAR2 antagonist FSLLRY-NH2 significantly abolished these profibrotic effects of tryptase, suggesting that tryptase may promote the phenotypic conversion of atrial fibroblasts by activating PAR2. However, the underlying mechanism of the action of tryptase is not clear yet. To further confirm the involvement of PAR2 in the action of tryptase, we treated atrial fibroblasts with PAR2 inhibitor FALLRY-NH2, pcDNA3.1-PAR2 vector and PAR2 siRNA, then measured the cell proliferation, migration and levels of Collagen I and fibronectin, MMPs and TIMP-1 levels in the atrial fibroblasts. The results showed that PAR2 inhibitor and PAR2 siRNA significantly suppressed the cell proliferation and phenotypic conversion of atrial fibroblasts. On the other hand, overexpression of PAR2 by pcDNA3.1-PAR2 vector significantly enhanced the cell proliferation and phenotypic conversion of atrial fibroblasts. These results confirmed that tryptase induced the cell proliferation and phenotypic conversion of atrial fibroblasts through PAR2.

Next, to further clarify the mechanism of PAR2's effect on phenotypic conversion of atrial fibroblasts, we explored its effect on the expression of CKAP4, COX2, and the p38/JNK pathway. The expression of COX2 did not change by these treatments. COX-2, the major isoform of COXs, plays an important role in mediating inflammatory response in cardiac fibrosis. It can be induced by stimulation with various growth factors and cytokines in fibroblasts [22]. The result that its expression was not changed by tryptase or PAR2 overexpression or

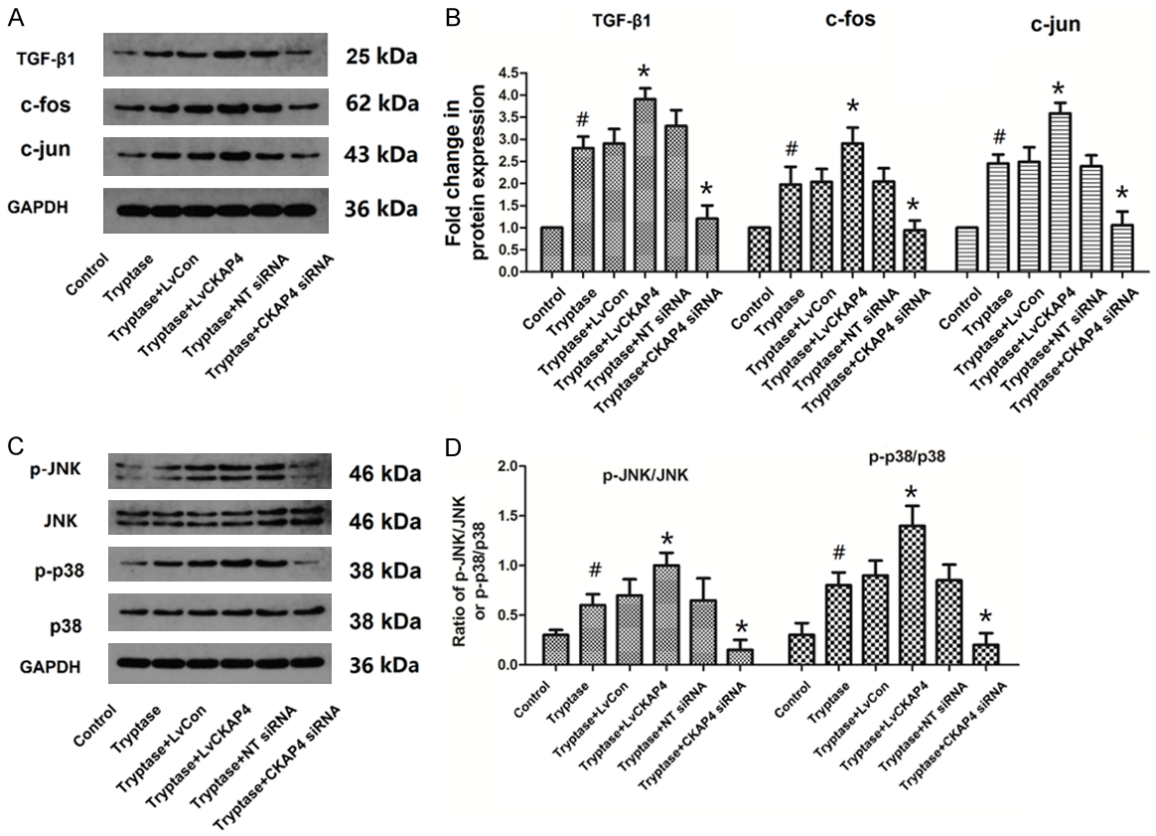

Figure 6. Effects of CKAP4 overexpression or siRNA on TGF-β1, c-jun, c-fos and JNK/p38 pathway in atrial fibroblasts in atrial fibroblasts. A. It shows the representative blots of TGF-β1, c-jun and c-fos. B. It shows their relative protein levels. Their expression was significantly increased by tryptase, further increased by CKAP4 overexpression, but decreased by CKAP4 siRNA. C. It shows the representative blots of p-JNK, JNK, p-p38 and p38. D. It shows the ratios of p-JNK/JNK or p-p38/p38. The ratios of p-JNK/JNK and p-p38/p38 were significantly increased by CKAP4 overexpression, but decreased by CKAP4 siRNA. Values are expressed as Mean ± SD. #P<0.05 compared with Control; *P<0.05 compared with Tryptase group. N=12.

siRNA suggested that tryptase may regulate phenotypic conversion of atrial fibroblasts through other molecules, not COX2. The p38/JNK pathway plays an important role in fibrosis. Activation of p38 and JNK correlates with macrophage infiltration and fibrosis [23, 24]. Our results showed that the p38/JNK pathway was significantly activated by tryptase and pcDNA3.1-PAR2, but inhibited by FALLRY-NH2 and PAR2 siRNA, indicating the involvement of p38/JNK pathway in the action of tryptase and PAR2.

It's interesting that the expression of CKAP4 was significantly increased by tryptase and further increased by pcDNA3.1-PAR2, but decreased by FALLRY-NH2 and PAR2 siRNA. As far as we know, this is the first study that revealed the relationship between tryptase, PAR2 and CKAP4. It indicated that tryptase can induce the expression of CKAP4 through PAR2. Furthermore, the expression of CKAP4 increased in a duration-dependent manner after cells were treated with tryptase. Most researches on CKAP4 are focused on tumors. It is involved in tumor cell proliferation and differentiation, tumor metastasis and prognosis [12, 25, 26]. In the cardiovascular system, Lyu et al. found that CKAP4 played an important role in regulating the integrity/permeability of vascular endothelial cells and maintaining endothelial homeostasis [13]. It was reported that CKAP4 on the surface of vascular smooth muscle cells is involved in the regulation of tPA on vascular wall damage repair [14]. Gladka et al. revealed that the expression of CKAP4 was up-regulated in activated ventricular fibroblasts and the level of CKAP4 in ventricular tissue increased after ischemic injury [15]; CKAP4 was positively correlated with genes that respond to ventricular

fibroblast activation and the CKAP4 expression is specifically increased in activated fibroblasts [15]. When CKAP4 is inhibited, it leads to an overactivation of myofibroblast-related genes, suggesting the importance of CKAP4 in fibroblast activation. Moreover, there is a positive correlation between CKAP4 and fibroblasts markers in ischemic heart [15]. These results suggest that CKAP4 may be one of the key factors regulating ventricular fibroblast activation.

To confirm the role of CKAP4 in phenotypic conversion of atrial fibroblasts caused by tryptase, we treated atrial fibroblasts with CKAP4 overexpression or siRNA. The results showed that CKAP4 overexpression induced by pcDNA3.1-CKAP4 significantly increased the cell proliferation, migration and levels of Collagen I and fibronectin, MMP-1 and TIMP-1 levels in atrial fibroblasts, while CKAP4 siRNA significantly abolished the effect of tryptase on these parameters. These results provide direct evidence that CKAP4 is involved in the phenotypic conversion of atrial fibroblasts caused by tryptase. As tryptase can significantly activate PAR2, these results also indicated that CKAP4 may serve as a key downstream molecular of PAR2.

TGF-β is a central regulator of ECM deposition in vascular injury and in injury-induced fibrosis in many tissues [27]. TGF-β1 auto-induction is mediated by the transcription factor AP-1, a heterodimer comprised of subunits of the c-Jun, c-Fos and ATF families. A previous study has shown that TGF-β1 induced tissue factor expression in human lung fibroblasts in a PI3K/JNK/Akt-dependent and AP-1-dependent manner [28]. Kruppel-like factor 2 attenuates pulmonary fibrosis and inflammation, possibly through the regulation of AP-1 [29]. The interactions between TGF-β1, AP-1 and JNK/p38 pathway promotes the development of fibrosis. However, the relationship between CKAP4 and TGF-β1, AP-1 and JNK/p38 pathway has not been well investigated yet. Gladka et al. inhibited CKAP4 expression in fibroblasts, and then exposed them to TGF-β. Their results showed that TGF-β induced CKAP4 expression [15]. In the present study, we measured the changes in TGF-β1 and AP-1 subunits c-Jun and c-Fos and JNK/p38 pathway in atrial fibroblasts. The results indicated that tryptase can activate TGF-β1, AP-1 and JNK/p38 pathway through CKAP4. There have been some reports about the relationship between tryptase/PAR2 and TGF-β1, AP-1 and JNK/p38 pathway. There was significant positive correlation between TGF-β1-positive cells and tryptase-positive cells [30]. In a TGF-β1-induced pulmonary fibrosis model, the number of tryptase-positive mast cells was significantly increased in fibrotic lung [31]. In joint capsule fibrosis after traumatic injury, the expression of TGF-β1 and tryptase were both significantly increased [32]. Compared to these reports, the present study provided direct evidence that tryptase induced expression of TGF-β1. The study of Temkin et al. revealed that tryptase activated the MAPK/AP-1 pathway in eosinophils, causing cytokine production and release [33]. The mRNA levels of factors c-fos, c-jun were significantly increased in the tryptase-treated ECV304 cells [34]. In another study, PAR2 agonists activated MAPK/AP-1 pathway, induced proliferation of pancreatic satellite cells, and increased collagen synthesis [35]. Our results are consistent with these studies. This is the first study which revealed that tryptase/PAR2 may activate TGF-β1, AP-1 and JNK/p38 pathway through CKAP4. It expanded our understanding on the regulation of tryptase/PAR2 on TGF-β1, AP-1 and JNK/p38 pathway as well as the phenotypic conversion of atrial fibroblasts.

In conclusion, using plasmid construction and siRNA methods, the present study proved that CKAP4 participates in the action of tryptase on phenotypic conversion of atrial fibroblasts, and the JNK/p38 pathway is involved. CKAP4 may serve as a key molecule target in the prevention of atrial fibrosis and atrial fibrillation. Future in vivo studies can be performed to investigate the effect of CKAP4 inhibition on atrial fibrosis or atrial fibrillation, which may be developed as a novel target in the prevention of atrial fibrosis.

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Disclosure of conflict of interest

None.

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