γ-catenin alleviates cardiac fibrosis through inhibiting phosphorylation of GSK-3β

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Abstract
Cardiac fibrosis is a common pathological change of many cardiovascular diseases. β-catenin has been shown to promote fibrosis. However, the precise role of its homolog γ-catenin in the process of fibrosis remains largely unclear. In this study, we found that the expression of γ-catenin was significantly decreased in angiotensin II (Ang II)-induced cardiac fibrosis model, contrary to most reports of β-catenin. Overexpression of γ-catenin in cardiac fibroblasts (CFs) significantly inhibited the expression of α-smooth muscle actin (α-SMA), whereas knocking down the expression of γ-catenin with siRNA promoted the occurrence of cardiac fibrosis. Mechanistically, γ-catenin could bind to GSK-3β to inhibit the phosphorylation of GSK-3β, therefore preventing cardiac fibrosis. Our study shows that γ-catenin is an important protective factor in cardiac fibrosis, which provides a new potential target for the treatment of cardiac fibrosis.

Keywords: γ-catenin, cardiac fibrosis, GSK-3β

Introduction
The cells of the heart are composed of cardiomyocytes and non-cardiomyocytes, including cardiac fibroblasts (CFs), endothelial cells and smooth muscle cells, of which over 90% are CFs[1]. CFs, a fundamental cell type in the heart, play a role in regulating the homeostasis of the extracellular matrix (ECM). However, once stimulated, CFs transform to myofibroblasts[2]. Myofibroblasts, mesenchymal cells within the connective tissue and ultimately responsible for scar tissue development, secrete the highly contractile proteins such as α-smooth muscle actin (α-SMA) and finally result in cardiac fibrosis[3]. Cardiac fibrosis is a common pathological process that occurs in many different heart attacks. It may increase myocardial stiffness and impede ventricular diastolic and systolic function, ultimately leading to heart failure. Superabundant accumulation of collagen fibers in the heart is one of the main proofs of cardiac fibrosis[4]. Despite the treatment with antihypertensive drugs, matrix metalloproteinase inhibitors, microRNA intervention, and stem cell transplantation, could alleviate cardiac fibrosis, the treatment has not yielded satisfactory effects in the clinic[4-9].

β-catenin is a key downstream for Wnt signaling...
pathway, and the Wnt/β-catenin signaling pathway is an essential positive regulated signaling network for cardiovascular diseases[6–9]. β-catenin increases expression of target genes associated with cell adhesion, and involves in the regulation of angiogenesis, intimal thickening, and atherosclerosis[10–13]. Studies have also shown that β-catenin plays a key regulatory role in myocardial injury in rats with coronary heart disease[14]. In addition, β-catenin plays a crucial role in heart failure caused by cardiac pressure afterload induced cardiac hypertrophy[15]. It can also crosstalk with the transforming growth factor-β (TGF-β) signaling pathway to exacerbate cardiac fibrosis and aggravate chronic heart failure[16–17]. These reports mentioned above indicate that β-catenin is a key risk factor in cardiovascular disease, promoting the occurrence of cardiac fibrosis. Interestingly, however, our experiment found that the expression of γ-catenin, one of the catenin protein family and a homologue of β-catenin, was significantly reduced at the cellular level of cardiac fibrosis. This phenomenon has attracted our attention.

One of the catenin family, γ-catenin (also called plakoglobin), plays an essential role in cell junctions[18–19]. At present, there are few reports on γ-catenin in cardiovascular diseases. Although β-catenin and γ-catenin have a compensatory effect, it also has reported that γ-catenin overexpression suppresses β-catenin activity and they play contrary roles in liver fibrosis caused by liver tumors[20]. In the pathological model of cardiac fibrosis, the two expressions are exactly the opposite. Therefore, we hypothesize that γ-catenin may play a protective part in cardiac fibrosis, which is different from β-catenin.

Here we report that the expression of γ-catenin is significantly decreased in angiotensin II (Ang II)-stimulated fibrosis models from different sources of fibroblasts. The level of cardiac fibrosis could be suppressed after γ-catenin overexpression. We found that γ-catenin regulates the phosphorylation of GSK-3β by regulating its interaction with GSK-3β, and thus alleviates the progression of cardiac fibrosis.

Materials and methods

Isolation of neonatal rat CFs

Neonatal rat CFs were isolated from 3-day-old neonatal Sprague-Dawley rats, which were obtained from the Experimental Animal Center of Nanjing Medical University. After wiped with 75% alcohol and anesthetized, these rats' hearts were isolated and minced into small pieces. The mixture was sequentially digested using 0.25% Trypsin-EDTA in 37 ℃ for 5 minutes, and then discarded the supernatant. Then 8–10 mL of trypsin was added, shook and digested at 37 ℃ for 5 minutes. The supernatant of cell suspension was transferred into the medium containing 20% FBS. This process was repeated for 8–10 times until these heart tissues were completely digested. The collected total cell suspensions were centrifuged at 4 ℃ for 5 minutes. Following removing supernatant, Dulbecco's modified eagle medium (DMEM) with 10% FBS medium was added to resuspend pellets. Cells were planted and incubated (5% CO₂) for 3 hours. The culture medium was removed and the adherent cells were fibroblasts. These neonatal CFs were treated by Ang II (100 nmol/L, Sigma-Aldrich, St Louis, USA) and/or phosphorylated-GSK-3β inhibitor, P529 (20 μmol/L, Selleck, USA), for 24 hours for further experiments.

Isolation of mouse embryonic fibroblasts (MEFs)

MEFs were extracted in accordance with a previously reported method[21] and cultured in pre-prepared complete DMEM which contained 10% FBS (Gibco, Carlsbad, USA) and antibiotics (1:100, penicillin-streptomycin, Gibco) as before. HT1080 cells were purchased from ATCC (American Type Culture Collection, Rockville, USA), and cultured in DMEM with 10% FBS.

Isolation of mouse cardiac fibroblasts (MCFs)

Firstly, mouse hearts were isolated from 4-week-old C57BL/6 mice without vessels and atria. Then, myocardial tissues were minced and digested by collagenase II (LS004177, Worthington, USA) for 15 minutes. After removing the visible fragments of tissues, isolated cells were centrifuged at 800 g for 2 minutes, and the cell pellets were washed with PBS for 3 times. Then the pellet was collected and re-suspended by Fibroblast Medium-2(FM-2) with 5% FBS (Scienccell, 2331). Cells were planted in 10 cm tissue-culture dishes (Applied Biological Materials, Vancouver, Canada). Twenty-four hours later, non-adherent cells and micro-fragments of tissue were removed. Cells were digested with Trypsin-EDTA, passaged in a 1:2 or 1:4 ratio. In case that the phenotype of fibroblasts was influenced by passage and cell density, morphology of cells was determined visually under a light microscope by fibroblast marker. Only early 5 passages of the cells were used for further experiments.

Western blotting analysis

Total cell were lysed in RIPA lysis buffer (Beyotime Biotechnology, China) with appropriate volumes including a protease inhibitor cocktail (Thermo Fisher Scientific, USA), and then homogenized and gathered by centrifugation at 12 000 g
for 10 minutes. Equal amounts of cell lysates were loaded and separated on 15% or 10% SDS poly acrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. Immunoblot was performed with anti-γ-catenin (ab184919, 1:1,000, Abcam, USA), anti-GAPDH (AP0063, 1:1,000, Bioworld, China), anti-α-SMA (ab32575, 1:1,000, Abcam), anti-p-GSK-3β (Ser9) (5558, 1:1,000, Cell Signaling Technology, USA), anti-GSK-3β (9315, 1:1,000, Cell Signaling Technology), anti-SMAD3 (9523, 1:1,000, Cell Signaling Technology), and anti-p-SMAD3 (9520, 1:1,000, Cell Signaling Technology) antibodies.

RNA purification and real-time PCR

Cells were washed by PBS, and Trizol reagent (Takara, Japan) was added. The lysates were gently transferred into sterile enzyme-free EP tubes. A 1/5 volume of chloroform was added and mixed upside down, stood for 10 minutes on ice, and then centrifuged at 10,000 g at 4 °C for 15 minutes. RNAs were collected from the upper solution and transferred into a new sterile enzyme-free EP tube. An equal volume of isopropanol was added and mixed upside down, following by standing at 4 °C for 10 minutes, and then centrifuged at 10,000 g at 4 °C for 15 minutes. Pre-cooled 75% ethanol (prepared with DEPC water) was added after centrifuging at 10,000 g for 15 minutes and washed twice with pre-cooled PBS. An appropriate volume of lysis buffer was added. Cells were placed in a 1.5 mL of the centrifuge tube, spun for 30 minutes and centrifuged for 10 minutes, then the supernatant was collected, detecting the protein concentration. The total protein was diluted to approximately 1 μg/μL with PBS. A volume of specific antibody was added to 500 μg protein, and the antigen-antibody mixture was slowly shaken overnight at 4 °C. The mixture was added with 20 μL of Protein G agarose beads to capture the antigen-antibody complex, and the new mixture was slowly mixed at 4 °C for 4 hours. After washed several times, the agarose bead-antigen antibody complex was suspended with 20 μL loading buffer and mixed gently. The sample was boiled for 5 minutes and analyzed by SDS-PAGE.

siRNA transfection

Expression of γ-catenin was silenced using siRNA oligonucleotide against γ-catenin (GenePharma, China). Neonatal rat CFs were transfected with siRNA oligonucleotide following the instruction of the Lipofectamine 3000 reagent (Invitrogen, USA). When CFs were confluent to approximately 75%, the culture solution was discarded. The cells were washed twice using pre-warmed PBS to remove the remaining serum in the medium, and then opti-MEM was added to each well. Lipofectamine 3000 (shaking well before use) was pipetted and opti-MEM was added, with a gentle mixture and incubation at room temperature for 5 minutes. The siRNA was pipetted into opti-MEM and Lipofectamine 3000 solution. siRNA-transfection reagent was added and mixed to each well with a gentle shaking. The CFs were cultured with the transfection mixture in CO₂ incubator at 37 °C for 4–6 hours. Then it was replaced with a complete culture solution (DMEM containing 10% FBS), with an incubation for 24 hours, and then the drug was added.

Plasmid transfection

The plasmid pcDNA-HA-γ-catenin was purchased from Addgene (Cambridge, USA). CFs were
transfected with the plasmid pcDNA-HA-γ-catenin using the Lipofectamine 3000 reagent (Invitrogen, USA). When CFs were confluent to approximately 75%, the culture solution was discarded. The cells were washed twice using pre-warmed PBS to remove the remaining serum in the medium, and then opti-MEM was added to each well. Lipofectamine 3000 (shaking well before use) was pipetted and opti-MEM was added, with a gentle mixture and incubation at room temperature for 5 minutes. The plasmid was pipetted into opti-MEM and Lipofectamine 3000 solution was added. Plasmid-transfection reagent was added and mixed to each well with a gentle shake. The CFs were placed in CO₂ incubator at 37 °C for 4 – 6 hours. Then it was replaced with a complete culture solution (DMEM containing 10% FBS), with an incubation for 24 hours, and then the drug was added.

Statistical analysis

All the data were expressed as mean±standard deviation (mean±SD). Student's t test was used for comparison between the two groups. One-way ANOVA analysis of Newman-Keuls Multiple Comparison Test was used for comparison between groups. P<0.05 was considered as statistically significant.

Results

Significantly decreased expression of γ-catenin in Ang II-induced cardiac fibrosis

It is reported that β-catenin is significantly increased in the cardiac fibrosis, and Wnt/β-catenin signaling activation is associated with fibrogenesis. β-catenin/GSK-3β signaling pathway is activated in the cardiac remolding. β-catenin, GSK-3β, and TGF-β1-SMAD-3 signaling pathways are essential regulators of cardiac fibrosis in heart diseases[22–24]. These results suggested that β-catenin is a positive regulator in cardiac fibrosis. However, compared with the homolog of β-catenin, γ-catenin was significantly decreased in the cardiac fibrosis. We detected the level of γ-catenin in different fibroblasts. The results showed that γ-catenin was downregulated in Ang II-induced cardiac fibrosis (Fig. 1A–D). Meanwhile, the fibrosis marker α-SMA was significantly increased in the Ang II treatment, as indicated by Western blotting.

Fig. 1 γ-catenin expression in Ang II-induced cardiac fibrosis. A–D: CFs, MEFs, MCFs and human fibrosarcoma cells HT1080 were incubated with Ang II for 24 hours, and Western blotting was performed to analyze γ-catenin and α-SMA expression (n=5 per group). E: Immunofluorescence analysis of α-SMA expression in CFs. α-SMA was stained with green, nuclei were counterstained with blue. Scale bars: 50 μm. Data are presented as mean±SD, *P<0.05. CFs: cardiac fibroblasts; MEFs: mouse embryonic fibroblasts; MCFs: mouse cardiac fibroblasts.
and immunofluorescence (Fig. 1A–E). These results indicated that γ-catenin and β-catenin may play different roles in cardiac fibrosis.

**Overexpression of γ-catenin inhibited Ang II-induced cardiac fibrosis**

To study whether γ-catenin and β-catenin play different roles in cardiac fibrosis, we first overexpressed γ-catenin in CFs (Fig. 2A) and showed that γ-catenin significantly inhibited the elevated protein and mRNA expression of α-SMA induced by Ang II (Fig. 2B and C). Overexpressed γ-catenin also reduced the transformation of fibroblasts into myofibroblasts through co-labelling with vimentin and α-SMA (Fig. 2D). These results demonstrated that overexpression of γ-catenin significantly inhibits fibrosis in CFs and confirmed our hypothesis that γ-catenin may play an important protective role in cardiac fibrosis.

**Fig. 2 Overexpressed γ-catenin inhibited Ang II-induced cardiac fibrosis.** A: Representative Western blotting showed successful overexpression of γ-catenin at 24 hours after γ-catenin transfection compared with pcDNA transfection of CFs (n=5 per group). B: pcDNA and γ-catenin were transfected to CFs following Ang II administration for 24 hours, and representative Western blotting analysis of α-SMA expression (n=5 per group). C: mRNA levels of α-SMA were detected in the same treated as in B (n=5 per group). D: pcDNA and γ-catenin were transfected to CFs following Ang II administration for 24 hours. Immunofluorescence analysis of co-localization of vimentin and α-SMA. α-SMA was shown in green, Vimentin in red, and nuclei in blue. Scale bars: 20 μm. Data are presented as mean±SD, *P<0.05.
Knockdown of γ-catenin exacerbated Ang II-induced cardiac fibrosis

To further prove the protective role of γ-catenin in cardiac fibrosis, we knocked down γ-catenin using γ-catenin-targeting siRNA (si-γ-catenin). It showed that siRNA knockdown was successful (Fig. 3A). Results indicated that the increased protein and mRNA expression of α-SMA by Ang II administration was aggravated by γ-catenin down-regulation (Fig. 3B and C). Inhibition of γ-catenin significantly promoted cardiac fibrosis. Taken together, these results demonstrated the positive role of γ-catenin in anti-fibrosis effect.

γ-catenin inhibited GSK-3β phosphorylation by interacting with GSK-3β

It has been reported that β-catenin can interact with GSK-3β. The binding of β-catenin and GSK-3β was enhanced by Ang II treatment. Considering the structural similarity between β-catenin and γ-catenin, we questioned whether γ-catenin interacted with GSK-3β, and how they would respond to Ang II treatment. We predicted the possibility of combining γ-catenin and GSK-3β in bioinformatics software. STRING database results showed that γ-catenin could interact with GSK-3β. To verify the results of the bioinformatics database, co-immunoprecipitation assay was performed. The results indicated that γ-catenin could interact with GSK-3β, and interaction of γ-catenin and GSK-3β was reduced by Ang II treatment as expected (Fig. 4A). Previous studies have shown that GSK-3β is phosphorylated, as inhibited form. To find out whether γ-catenin inhibited GSK-3β phosphorylation by interacting with GSK-3β, we further detected the phosphorylated GSK-3β expression. It demonstrated that the augmented expression of phospho-GSK-3β by Ang II treatment was reduced by γ-catenin overexpression (Fig. 4B). Besides, γ-catenin overexpression also inhibited the phosphorylation of SMAD-3 in Ang II-induced cardiac fibrosis (Fig. 4B). These results indicated that γ-catenin prevented CFs from fibrosis by inhibiting GSK-3β phosphorylation and further inhibiting SMAD-3 phosphorylation.

To further prove the regulatory effect of GSK-3β phosphorylation on SMAD-3 in Ang II-induced cardiac fibrosis, Ang II-treated CFs were pre-incubated with inhibitors of GSK-3β phosphorylation (P529). P529 largely attenuated GSK-3β phosphorylation (Fig. 4C). The decrease of phosphorylation of GSK-3β significantly reduced the phosphorylation of SMAD-3, the protein, and mRNA expression of α-SMA (Fig. 4C and D). Together, reducing phosphorylation of GSK-3β (promoting the activity of GSK-3β) inhibits phosphorylation of SMAD-3 and attenuates cardiac fibrosis.

In summary, we found that γ-catenin is an important target for inhibiting fibrosis, which can inhibit the phosphorylation level of GSK-3β and reduce the SMAD-3 pathway to attenuate cardiac fibrosis (Fig. 5). Our research reveals that γ-catenin, as a new protective factor, may serve as putative novel therapeutic targets in treating pathological cardiac fibrosis.

Fig. 3 Depleted expression of γ-catenin exacerbated Ang II-induced cardiac fibrosis. A: Representative Western blotting showed successful depletion of γ-catenin expression 24 hours after si-γ-catenin transfection compared with that with si-scramble transfection (n=5 per group). B: si-scramble and si-γ-catenin were transfected to CFs following Ang II administration for 24 hours, and representative expression levels of α-SMA were analyzed by Western blotting (n=5 per group). C: mRNA levels of α-SMA were detected in the same treated as in B (n=5 per group). Data are presented as means±SD, *P<0.05.
Cardiac fibrosis has been considered as an essential pathological factor that aggravates the cardiovascular diseases, associated with the end stages of various cardiac diseases[25]. The occurrence of cardiac fibrosis is accompanied with excessive accumulation of collagen. Excessive collagen deposition results in the increase of myocardial stiffness, weakens cardiac systolic and diastolic activity, and causes cardiac electrophysiological disorders, ultimately developing into heart failure[1]. Fibroblasts are the major source of extracellular matrix during cardiac fibrosis[1]. Ang II, an oligopeptide leading to vasoconstriction and elevating blood pressure, is increased in fibrotic tissues. In CFs, Ang II increases the level of TGF-β1 via the angiotensin type 1 receptor[26]. In addition, in vivo, TGF-β is essential for the process of Ang II-induced cardiac hypertrophy as well as fibrosis[27]. Cardiac fibrosis demonstrates excess collagen and cardiac structural disorder. Our study found that Ang II increased the secretion of α-SMA in cardiac fibroblast, while overexpression of γ-catenin effectively reduced α-SMA secretion induced by Ang II. Although we have only confirmed the protective effect of γ-catenin at the cellular level, its role is very

**Fig. 4 γ-catenin interacted with GSK-3β and inhibited GSK-3β phosphorylation.** A: CFs were incubated with or without Ang II for 24 hours. Representative co-immunoprecipitation and Western blotting analysis of immunoprecipitated γ-catenin in CFs (n=5 per group). B: pcDNA and γ-catenin were transfected to CFs following Ang II administration for 24 hours. Representative Western blotting analysis of phosphorylated GSK-3β, GSK-3β, phosphorylated SMAD3, SMAD3 and GAPDH expression (n=5 per group). C: Representative Western blotting analysis of phosphorylated GSK-3β, GSK-3β, phosphorylated SMAD3, SMAD3, α-SMA and GAPDH expression in CFs treatment with Ang II and P529 (inhibitor of phosphorylated GSK-3β; 20 μmol/L). D: mRNA levels of α-SMA were detected in the same treated as in C. Data are presented as mean±SD, *P<0.05.

**Discussion**

Cardiac fibrosis has been considered as an essential pathological factor that aggravates the cardiovascular diseases, associated with the end stages of various cardiac diseases[25]. The occurrence of cardiac fibrosis is accompanied with excessive accumulation of collagen. Excessive collagen deposition results in the increase of myocardial stiffness, weakens cardiac systolic and diastolic activity, and causes cardiac electrophysiological disorders, ultimately developing into heart failure[1]. Fibroblasts are the major source of extracellular matrix during cardiac fibrosis[1]. Ang II, an oligopeptide leading to vasoconstriction and elevating blood pressure, is increased in fibrotic tissues. In CFs, Ang II increases the level of TGF-β1 via the angiotensin type 1 receptor[26]. In addition, in vivo, TGF-β is essential for the process of Ang II-induced cardiac hypertrophy as well as fibrosis[27]. Cardiac fibrosis demonstrates excess collagen and cardiac structural disorder. Our study found that Ang II increased the secretion of α-SMA in cardiac fibroblast, while overexpression of γ-catenin effectively reduced α-SMA secretion induced by Ang II. Although we have only confirmed the protective effect of γ-catenin at the cellular level, its role is very
The expression of γ-catenin was significantly decreased under fibrosis stimuli, which promoted the phosphorylation of GSK-3β owing to decrease the interaction of γ-catenin and GSK-3β. GSK-3β negatively regulated the downstream of cardiac fibrosis signaling, so the consequence of GSK-3β inhibition by GSK-3β phosphorylation activated SMAD signaling and enhanced the fibrosis-related gene expression.

Therefore, we will discuss it further at the animal level in the future.

β-catenin and γ-catenin are homologs of armadillo, which are originally recognized to function as cell fate determination and fragment polarity. Similarly to armadillo, β-catenin and γ-catenin play essential roles in cell adhesion and wingless/Wnt signaling pathways[10,28–29]. β-catenin and γ-catenin both exist at the adhesion junction and connect E-cadherin to the actin cytoskeleton. γ-catenin is found in the desmosome as well in which it interacts with desmoglein and desmocollin. The highly conserved central regions of catenin contain 12 armadillo repeats, and these repeats simplify binding to numerous proteins, including E-cadherin, the adenomatous polyposis coli (APC) tumor suppressor, and axin/conductin proteins. It indicates the role of γ-catenin to promote protein interactions[30]. It is reported that, under pathological conditions, β-catenin and γ-catenin has a compensatory effect, knocking out one of them can cause another compensatory increase[31]. However, it also reported that γ-catenin overexpression suppresses β-catenin activity and target genes and they play different roles in the liver tumor Cells and in liver fibrosis[31]. Contrary to the effect of β-catenin, in γ-catenin knockout mice, liver damage and liver fibrosis induced by chemical stimulation are accelerated suggesting γ-catenin and β-catenin may have contrary effects. This report also suggests that γ-catenin may play an important role in fibrosis. We found γ-catenin expression is reduced in the cardiac fibrosis models, contrary to the expression of β-catenin, indicating that γ-catenin may have a protective effect in cardiac fibrosis.

It has been reported that dickkopf-3 inhibits Ang II-induced cardiac hypertrophy and cardiac fibrosis through inhibiting GSK-3β/β-catenin signaling pathway[23]. Additionally, literature also presented that γ-catenin inhibits the role of β-catenin in cell proliferation by activating GSK-3β[32]. Therefore, we speculate that γ-catenin may inhibit the role of β-catenin in cardiac fibrosis by promoting GSK-3β, resulting in preventing the progression of cardiac fibrosis. However, γ-catenin inhibits that the role of β-catenin has not been fully demonstrated in our experiments.

GSK-3β inhibits TGFβ1-SMAD3-induced cardiac fibrosis pathway by inhibiting the phosphorylation of SMAD3. Phosphorylation of GSK3-β (inactivated form) loses its inhibitory effect on SMAD3, and fibrosis is aggravated[31]. All these indicate that GSK-3β plays a certain protective role in the process of cardiac fibrosis. Our experiment demonstrates that γ-catenin can interact with GSK-3β to reduce the fibrosis pathway by inhibiting the phosphorylation of GSK3-β, thereby inhibiting the phosphorylation of SMAD3 and playing a protective role in cardiac fibrosis.

In conclusion, we have clarified the specific role and mechanism of γ-catenin in cardiac fibrosis, and proposed a reasonable conjecture by demonstrating changes in the expression of γ-catenin. We have advanced the theoretical understanding of the interactions of the catenin protein family. At the same time, we also provide a new possible therapeutic target for the treatment of cardiac fibrosis.

Acknowledgments

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