

Original Article

SNF5 alleviates Ang II-induced myocardial hypertrophy by inactivation of MAPK/mTOR pathway

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Abstract: Inhibition of Angiotensin II (Ang II)-induced hypertrophy has been known as a novel aspect of treating hypertrophic cardiomyopathy. This study was aimed to explore the role of SNF5 in Ang II-induced myocardial hypertrophy. Rat cardiomyocyte H9c2 was used and transfected with SNF5 expressing plasmid or small interfering RNA (siRNA) against SNF5. After the transfection, cells were treated with Ang II to induce myocardial hypertrophy. Subsequently, cell viability and apoptosis were respectively measured by 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) and flow cytometry. Further, the expressions of ANP, β -MHC, p-38 and mTOR proteins were detected by Western blotting. Overexpression of SNF5 significantly improved cell viability, suppressed apoptosis and down-regulated the levels of ANP and β -MHC ($P < 0.05$ or $P < 0.01$). In contrast with SNF overexpression, SNF5 suppression showed the inverse effects. Besides, the phosphorylation of p38 and mTOR was suppressed by SNF5 overexpression, while was promoted by SNF5 suppression ($P < 0.05$ or $P < 0.01$). SNF5 alleviated Ang II-induced cardiomyocyte hypertrophy. The functional effects of SNF5 on cardiomyocyte might be implemented by blocking MAPK/mTOR pathway.

Keywords: Hypertrophic cardiomyopathy, SNF5, apoptosis, ANP, β -MHC, MAPK/mTOR pathway

Introduction

Hypertrophic cardiomyopathy, a common genetic cardiac disease, affects one out of 500 individuals from the general population [1]. Patients with hypertrophic cardiomyopathy always suffer from dyspnea, angina, palpitation, syncope, even sudden cardiac death. Besides, microvascular ischemia is one of the hallmarks of hypertrophic cardiomyopathy and has been associated with important disease-related complications that impact clinical outcome, including adverse left ventricular remodeling and systolic dysfunction [2, 3]. Angiotensin II (Ang II), a pivotal component of renin-angiotensin system, plays a critical role in cardiac diseases [4]. Ang II has been suggested to work as an autocrine/paracrine factor regulating growth of local tissues such as blood vessel, kidney and heart [5]. Moreover, studies have also identified Ang II as a critical mediator in hypertrophic cardiomyopathy, that Ang II can cause hyperplasia in cardiomyocyte [6, 7]. Thus, inhibition of Ang II-induced myocardial

hypertrophy might be an aspect of preventing and treating this disease.

SNF5 is one of the core subunits of the SWI/SNF complex, and it is required for remodeling the activity of SWI/SNF [8]. Moreover, SNF5 is also known as a bona fide tumor suppressor based on genetic evidence that majority of rhabdoid tumors contain bi-allelic inactivating mutations in the *SNF5* (*SMARCB1*) locus [9]. McKenna *et al.* found that, SNF5 could drive cancer formation by targeting Bridging Integrator 1 (BIN1) [10]. Lin *et al.* demonstrated that, knockdown of SNF5 in melanoma cell lines resulted in significant chemoresistance, and loss of SNF5 correlated with poor patient survival [11]. However, to date, there still no investigation has linked SNF5 with cardiac diseases, especially with hypertrophic cardiomyopathy.

Thus, this study was aimed to explore whether SNF5 played a role in Ang II-induced myocardial hypertrophy. In the current study, rat cardio-

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myocyte H9c2 was transfected with SNF5 expressing plasmid or small interfering RNA (siRNA) against SNF5. Afterward, Ang II was added into cells to establish a model of myocardial hypertrophy. Cell viability, apoptosis, and the expression changes of ANP and β -MHC were measured to reveal the effects of SNF5 on Ang II-induced hypertrophy and apoptosis. Further, the expressions of p-38 and mTOR proteins were measured to reveal the underlying mechanism of SNF5 on Ang II-induced myocardial hypertrophy. These studies might provide a basic understanding of SNF5 in hypertrophic cardiomyopathy.

Materials and methods

Cell culture and transfection

The immortalized rat cardiomyocyte H9c2 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen, Carlsbad, CA, USA) [12]. Cells were incubated in humidified atmosphere at 37°C with 5% CO₂.

A SNF5 expressing plasmid pc-SNF5 was prepared by sub-cloning the full-length wild-type SNF5 coding sequence into pcDNA3.1. The empty construct pcDNA3.1 was transfected as a control. siRNA against SNF and control siRNA was synthesized by GenePharma (Shanghai, China), and the target sequence for siRNA against SNF was: 5'-GGA GAA CTC ACC AGA GAA GTT AAC TTC TCT-3'. Lipofection 2000 reagent (Invitrogen, USA) was used in the transfection, according to the manufacturer's protocol. Stable SNF5 transfection was generated under G418 (Gibco, Paisley, UK) selection as described [13].

Ang II administration

For Ang II treatment, H9c2 cells were seeded into 6-well plates or 96-well plates. After 24 h of incubation, cells were treated with different concentrations (0-10⁻⁶ M) of Ang II for 24 h. The control group was added phosphate-buffered saline (PBS) in medium.

Measurement of cell surface area

After Ang II treatment, cell surface area was measured according to the method introduced in a previous study [14]. Briefly, cells were photographed under a microscope with a digital camera (Olympus IX-81, Olympus, Japan). The cell surface areas were analyzed by Image-Pro Plus 7.0 (Olympus, Tokyo, Japan). A hundred of cells were randomly selected from 5 different fields.

Cell viability assay

Cell viability was performed by the classic 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were firstly transfected with SNF5 expressing plasmid or siRNA against SNF, and then treated with Ang II. Afterward, 20 μ L MTT (0.5 mg/mL; Sigma Aldrich, St. Louis, MO) was added into cells and incubated for 4 h at 37°C. After the incubation, 150 μ L dimethyl sulfoxide (DMSO; Ponsure, Shanghai, China) was added and the plates were shaken violently for 10 min. The absorbance was measured at 570 nm using a Multiskan EX (Thermo, Finland) [15].

Apoptosis assay

Cell apoptosis was measured by using an Annexin V: FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA), according to the manufacturer's recommendations. After transfection and administration of Ang II, cells in each sample were collected and re-suspended in 200 μ L Annexin-binding buffer containing 10 μ L Annexin V-FITC. At 30 min after the incubation in the dark, 5 μ L PI and 300 μ L phosphate buffer saline (PBS) were added into cells, and the apoptotic cells were distinguished under a flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA, USA) immediately [23].

Real-time quantitative PCR (qPCR)

After transfection and administration of Ang II, cells were collected and total RNA in cells were extracted by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). Reverse transcription was performed by Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). Fast-Start Universal SYBR Green Master (ROX) was used in qPCR and each qPCR was carried out in triplicate for a total of 20 μ L reaction mixtures

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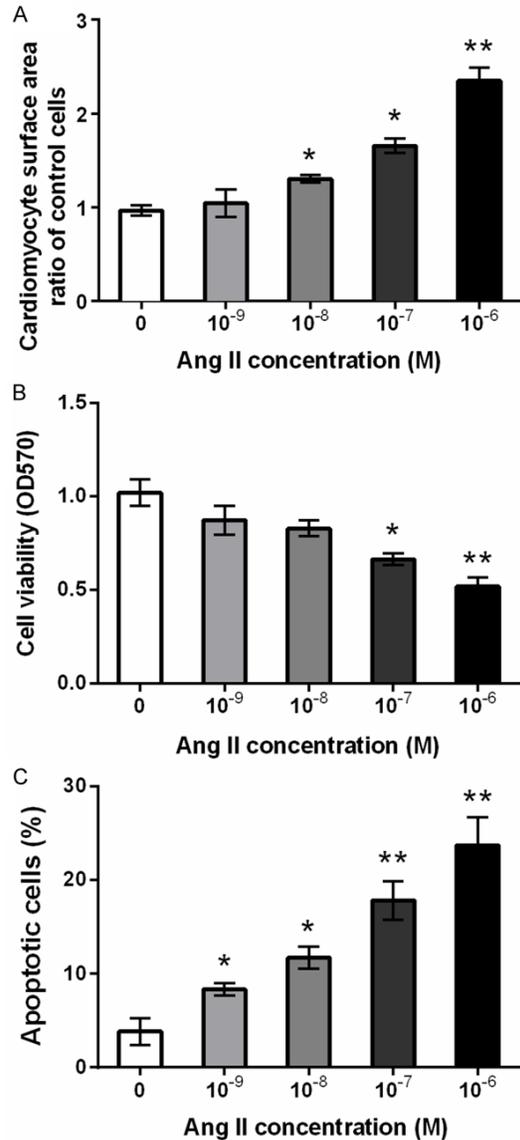


Figure 1. Effects of Ang II on H9c2 cells. H9c2 cells were treated with 0, 10⁻⁹, 10⁻⁸, 10⁻⁷ or 10⁻⁶ M of Ang II, and then cell size, viability and apoptosis were respectively analyzed by (A) Image-Pro Plus software, (B) MTT and (C) flow cytometry. Ang II, Angiotensin II; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide; **P* < 0.05; ***P* < 0.01.

on ABI PRISM 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Data were analyzed according to the classic 2^{-ΔΔCt} method [16], and were normalized to GAPDH expression in each sample. All primers were designed and synthesized from GenePharma (Shanghai, China). The sequences of the primers were as follows, SNF5 forward: 5'-CCT CTC TCA ACG CTG TCC AAC TG-3', reverse: 5' ATC TTG GCG AGG ATG TGC TTG TCT

T-3'; GAPDH forward: 5'-GCA CCG TCA AGG CTG AGA AC-3', reverse: 5'-TGG TGA AGA CGC CAG TGG A-3'.

Western blot

Cellular protein was extracted using Radio Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime, Shanghai, China) Quantitation of protein was conducted by using BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Equal amounts of proteins were resolved over sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane. The membranes were blocked within 5% skim milk for 1 h at room temperature, and then incubated with primary antibodies at 4°C overnight. SNF5 (ab-58209), ANP (ab14348) and β-MHC (ab1708-67) were purchased from Abcam (Cambridge, MA); p-p38 (sc-7973), t-p38 (sc-535), p-mTOR (sc-293132), t-mTOR (sc-293089) and GAPDH (sc-365062) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Bands were visualized by the enhanced chemiluminescence (ECL) reagent (GE Healthcare, Little Chalfont, UK) and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, CA, USA) [17].

Statistical analysis

All data were expressed as mean ± standard derivations (SD) from three independent assays. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). The *P*-values were calculated using a student's *t* test. A *P*-value of < 0.05 was considered as statistical difference.

Results

Effects of Ang II on cardiomyocyte H9c2

Different concentrations of Ang II were added into H9c2 cells, and then cell size, viability and apoptosis were respectively measured by Image-Pro Plus software, MTT and flow cytometry. We found that, Ang II significantly increased cell surface area, inhibited cell viability and induced apoptosis (*P* < 0.05 or *P* < 0.01; **Figure 1A-C**), in a dose-dependent manner. These

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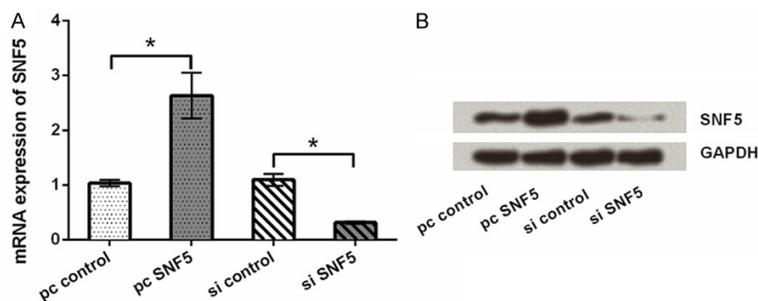


Figure 2. Effects of transfection on SNF5 expression. H9c2 cells were transfected with pc SNF5, si SNF5 or their corresponding controls, and then transfection efficiency was verified by (A) qPCR and (B) Western blot. qPCR, quantitative PCR; * $P < 0.05$.

data indicated that, Ang II could induce H9c2 cells hypertrophy and apoptosis. In addition, 10^{-6} concentration of Ang II was used for establishing the model of myocardial hypertrophy for the following experiments.

Effects of transfection on SNF5 expression

H9c2 cells were transfected with pc SNF5, si SNF5 or their corresponding controls, and then the transfection efficiency was verified by qPCR and Western blot analyses. As results showed in **Figure 2A** and **2B**, both the mRNA and protein levels of SNF5 were significantly up-regulated by pc SNF5 ($P < 0.05$), while was down-regulated by si SNF5 ($P < 0.05$). Thus, the expression of SNF5 was successfully overexpressed or suppressed in H9c2 cells.

SNF5 overexpression alleviated Ang II-induced myocardial hypertrophy

In order to explore the role of SNF5 in Ang II-induced myocardial hypertrophy, H9c2 cells were firstly transfected with pc SNF5, si SNF5 or their corresponding controls, and then treated with 10^{-6} M of Ang II for 24 h. We found that (**Figure 3A-C**), overexpression of SNF5 significantly enhanced cell viability ($P < 0.05$) and suppressed apoptosis ($P < 0.01$). However, knock-down of SNF5 significantly inhibited cell viability and induced apoptosis (both $P < 0.05$). Besides, results in **Figure 3D** and **3E** showed that, both the levels of ANP and β -MHC were down-regulated by SNF5 overexpression, while were up-regulated by SNF5 suppression (both $P < 0.05$). These data revealed that, SNF5 might alleviate Ang II-induced hypertrophy and apoptosis.

SNF5 overexpression alleviated Ang II-induced myocardial hypertrophy by inactivation of MAPK/mTOR pathway

To further investigate the underlying mechanisms in which SNF5 affected Ang II-induced myocardial hypertrophy, H9c2 cells were transfected with pc SNF5 or si SNF5, and treated with 10^{-6} M of Ang II. Afterward, the expression levels of p38 and mTOR in cells were measured by Western blotting.

Figure 4A and **4B** showed that, Ang II together with/without SNF5 knock-down significantly up-regulated the increased the phosphorylation of p38 and mTOR (all $P < 0.05$). In contrast, Ang II together with SNF5 overexpression significantly decreased phosphorylation of these two factors ($P < 0.05$ or $P < 0.01$). Basing on these findings, we inferred that MAPK/mTOR pathway might be implicated in the impacts of SNF5 on Ang II-induced myocardial hypertrophy.

Discussion

In the present study, the expression of SNF5 in H9c2 cells was dysregulated and then cells were treated with Ang II, to explore the effects of SNF5 on Ang II-induced myocardial hypertrophy. We found that, overexpression of SNF5 could notably enhance cell viability, suppress apoptosis and down-regulate the expression of ANP and β -MHC proteins. In contrast, SNF5 suppression showed the inverse effects towards cell viability, apoptosis as well as ANP and β -MHC expressions. Further, the expression of MAPK/mTOR pathway proteins, *i.e.*, p38 and mTOR, were regulated by the Ang II and dysregulated SNF5.

Currently, an increasing number of investigations have linked SNF5 with cell survival. Studies of mouse models for SNF5 inactivation indicated that SNF5 has a dual role depending on the cell type: in some cell lineages SNF5 disruption is lethal by driving apoptosis, while in others it impairs differentiation or promotes cell growth [18]. *In vitro*, it has also highlighted that SNF5 could regulate the balance between cell proliferation and differentiation [19]. In the present study, we found that SNF5 was impli-

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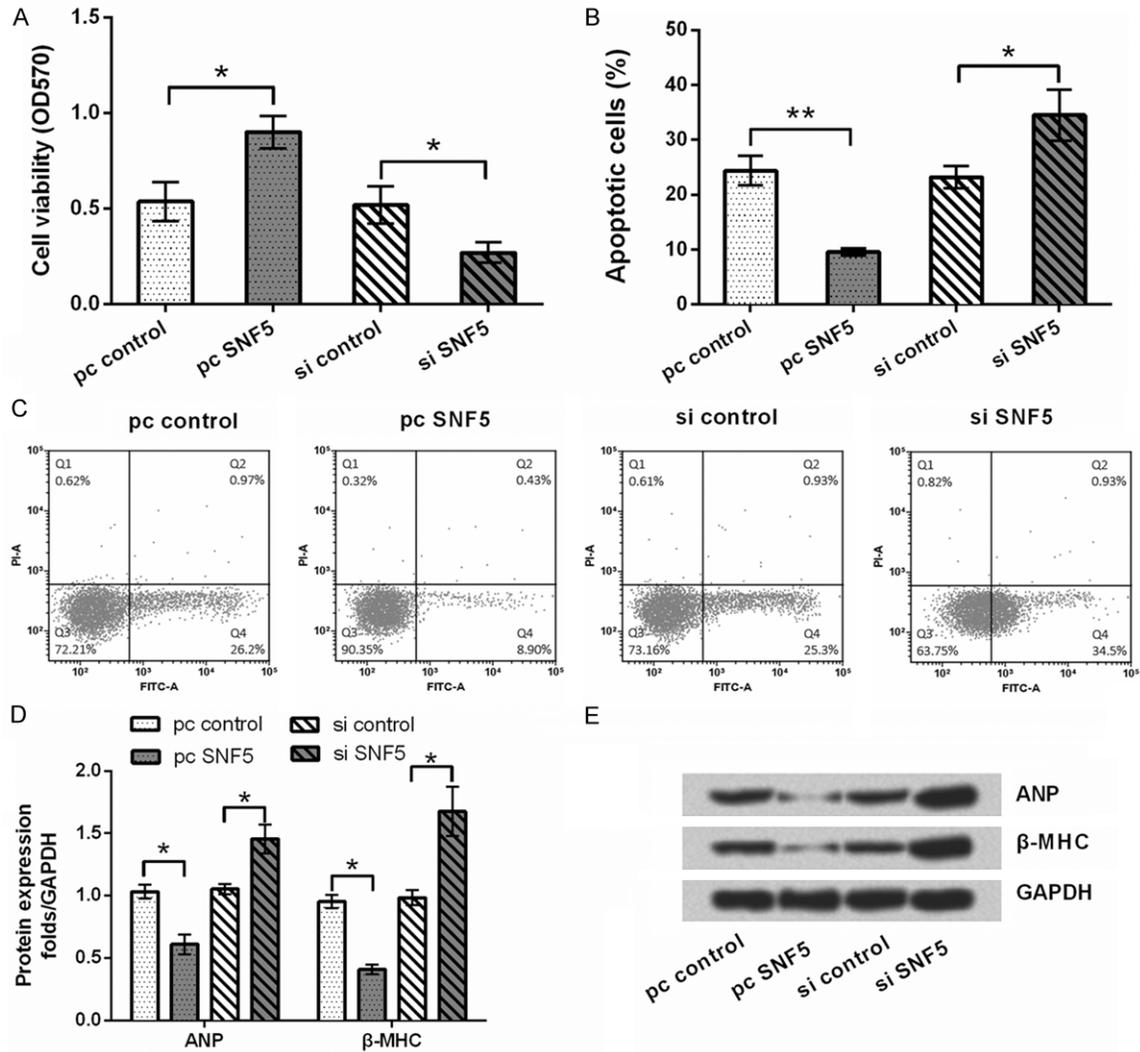


Figure 3. SNF5 overexpression alleviated Ang II-induced myocardial hypertrophy. H9c2 cells were transfected with pc SNF5, si SNF5 or their corresponding controls, and then cells were treated with 10^{-6} M of Ang II for 24 h. Subsequently cell viability and apoptosis were respectively measured by (A) MTT and (B and C) flow cytometry. The expression of ANP and β -MHC proteins was measured by (D and E) Western blot. Ang II, Angiotensin II; MTT, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide; * $P < 0.05$; ** $P < 0.01$.

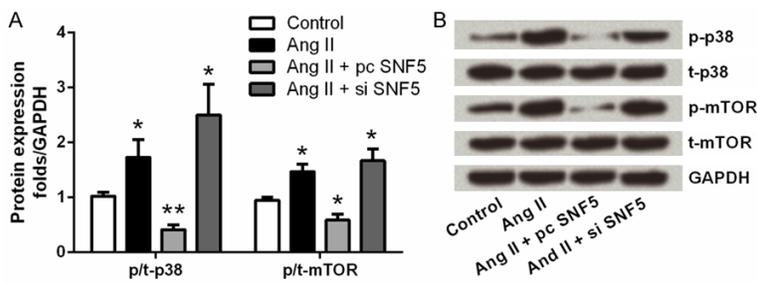


Figure 4. SNF5 overexpression alleviated Ang II-induced myocardial hypertrophy by inactivation of MAPK/mTOR pathway. H9c2 cells were infected with pc SNF5 or si SNF5, and treated with 10^{-6} M of Ang II for 24 h. The expression of p-38 and mTOR proteins was determined by Western blot (A and B). Ang II, Angiotensin II; * $P < 0.05$; ** $P < 0.01$.

cated in H9c2 cells viability and apoptosis. These findings documented that SNF5 might be a critical mediator for cell survival depending upon the cell background. Besides, our data provided the first evidence that SNF5 could protect cardiomyocyte from Ang II-induced apoptosis.

Pathological myocardial hypertrophy is characterized by the increase in cardiomyocyte

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size associated with the re-expression of the so-called foetal gene programme [20]. This programme includes increased ANP and β -MHC gene expression, and these two factors have been identified as two sensitive markers for hypertrophy [21]. Huang *et al.* produced a cardiac hypertrophy model in rat by using Ang II and found that, Ang II treatment up-regulated levels of ANP and β -MHC [22]. In the current study, we found that SNF5 remarkably down-regulated the protein expression of ANP and β -MHC, indicating that SNF5 could alleviate hypertrophy induced by Ang II.

MAPK and mTOR signaling pathways play pivotal roles in mediation of multiple biological processes, including cell proliferation, cell cycle and apoptosis [23-27]. To date, the exact signal transduction mechanism of hypertrophic cardiomyopathy is still unclear. However, studies have found that MAPK and mTOR pathways were involved in the pathogenesis of hypertrophic cardiomyopathy. *In vitro* studies found that, blocking the activation of S6K1 and 4E-BP1 (two downstream proteins of mTOR) exhibited a severe reduction in cell size [28, 29]. In addition, p-38 and its upstream regulatory kinases MKK3 and MKK6 have been identified as effectors of the hypertrophic response have largely been obtained in cultured neonatal rat cardiomyocytes [30]. In the current study, we found that SNF5 could remarkably inhibit the activation of p-38 and mTOR, suggesting that SNF5 alleviated Ang II-induced myocardial hypertrophy via blocking MAPK/mTOR pathway. Similar with our finding, Zhou *et al.* demonstrated that, Exendin-4 attenuated cardiac hypertrophy via MAPK/mTOR signaling pathway activation [31].

Taken together, the present study showed that SNF5 could alleviate Ang II-induced myocardial hypertrophy. The functional effects of SNF5 on cardiomyocyte might be implemented by blocking MAPK/mTOR pathway. Our findings provided the first evidence that SNF5 might be a potential therapeutic target for control of hypertrophic cardiomyopathy. However, more work still needed to strive to confirm the role of SNF5 in this disease.

Disclosure of conflict of interest

None.

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