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MiR-423-5p Inhibition Exerts Protective Effects on Angiotensin II-Induced Cardiomyocyte Hypertrophy

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Angiotensin II (Ang II) is a kind of bioactive peptide, which can contribute to cardiac hypertrophy. MicroRNAs (miRNAs) play critical role in various heart diseases. The cardioprotective effect of miR-423-5p inhibition has been confirmed by previous studies. But its role in cardiac hypertrophy induced by Ang II is unknown. This study focused on the potential of miR-423-5p in cardiomyocyte hypertrophy under the treatment of Ang II. Our results revealed that miR-423-5p expression was upregulated in Ang II-treated human cardiomyocytes (HCMs). Importantly, miR-423-5p knockdown suppressed Ang II-induced cardiomyocyte hypertrophy and oxidative stress in HCMs. Bioinformatics analysis and luciferase reporter assay confirmed that the suppressor of Ty 6 homolog (SUPT6H) was a target gene of miR-423-5p. Interestingly, SUPT6H knockdown aggravated cardiomyocyte hypertrophy and oxidative stress in Ang II-stimulated HCMs, which were then reversed by silenced miR-423-5p. In conclusion, miR-423-5p knockdown exerts its protective effects on Ang II-induced cardiomyocyte hypertrophy in HCMs via modulating SUPT6H expression.

Keywords: angiotensin II; cardiomyocyte; heart failure; hypertrophy; miR-423-5p
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Introduction

Cardiac hypertrophy is mainly characterized by thickening myocardium and decreased the heart chamber (Nakamura and Sadoshima 2018). Many pathological processes like hypertension and myocardial infarction contribute to the development of it, and prolonged cardiac hypertrophy can easily progress to arrhythmias, heart failure and sudden cardiac death (Thompson 2009; Dey et al. 2018). Although numerous molecules have been identified to be related to the modulation of cardiac hypertrophy, more molecules need to be urgently discovered and studied to improve the treatment. Angiotensin II (Ang II) has been demonstrated to be the strongest inducer of cardiac hypertrophy (Sun et al. 2013; Lyu et al. 2015). Thus, Ang II treated-human cardiomyocytes (HCMs) was confirmed as a research model for exploring the development of cardiac hypertrophy.

MicroRNAs (miRNAs) are endogenous non-coding RNAs with 20-23 nucleotides and regulate the transcription of target gene mRNAs (Bartel 2004). Many miRNAs have been shown to play important roles in the occurrence and development of various cardiovascular diseases, including cardiac hypertrophy (Orenes-Piñero et al. 2013). For example, miR-625-5p suppresses cardiac hypertrophy via signal transducer and activator of transcription 3 (STAT3) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) axis (Cai and Chen 2019). MiR-29a mitigates isoproterenol hydrochloride-induced cardiac hypertrophy response (Zhang et al. 2019). MiR-338-5p improves the pathogenesis of pathological cardiac hypertrophy (Li et al. 2019). In recent years, miR-423-5p has been revealed to alleviate hypoxia/reoxygenation induced cardiomyocyte apoptosis and was abnormally expressed in acute myocardial infarc-

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tion (Nabiałek et al. 2013; Zhu and Lu 2019). Interestingly, numerous studies have mentioned that miR-423-5p is closely related to the progression of heart failure (Tijsen et al. 2010; Shah et al. 2018; Peterlin et al. 2020). However, its concrete role and mechanism in Ang II-induced cardiomyocyte hypertrophy has not been illustrated yet.

The purpose of this study was to investigate the effect miR-423-5p on Ang II-induced cardiomyocyte hypertrophy and confirm the possible mechanisms underlying it in HCMs.

Materials and Methods

Cell culture and treatment

HCMs were obtained from Shanghai Huzhen Biotechnology Co., Ltd. (Shanghai, China) and cultured in human cardiomyocytes primary cell culture media (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with fetal bovine serum (FBS; 5%) and penicillin/streptomycin (1%) in a humid incubator at 37°C with 5% CO_2. Ang II-stimulated cardiomyocyte was established by adding angiotensin II (Ang II; 1 mmol/L; Sigma-Aldrich, Shanghai, China) for 48 h.

Cell transfection

MiR-423-5p inhibitor, NC (negative control) inhibitor, sh-SUPT6H and corresponding sh-NC were produced by GenePharma (Shanghai, China). HCMs were transfected with above plasmids for 48 h for the experiments using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RNA was isolated from fresh tissues and cultured cardiomyocytes using RNA Purification kit (Invitrogen). Total RNA (1 μg) was reverse transcribed into cDNA with the reverse transcriptase amplification kit (Fermentas, New York, NY, USA). Next, real-time PCR was performed using PowerUp™ SYBR™ Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) on the ABI 7900HT sequence detector (Applied Biosystems). GAPDH and U6 served as the reference genes. mRNA and miRNA expression was calculated based on the $2^{-\Delta\Delta Ct}$ method. Sequences of primers are provided in Table 1.

Western blot analysis

Proteins from the HCMs were extracted with lysis buffer (Sigma-Aldrich). Total proteins of 50 μg were used for electrophoresis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime, Shanghai, China). Subsequently, the isolated proteins on the SDS-PAGE gel were transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking, membranes were probed with primary antibodies at 4°C overnight and the corresponding horseradish peroxidase-conjugated secondary antibodies for 1 h. Finally, the blots were detected by enhanced luminol-based chemiluminescent (ECL). The primary antibodies included anti-Nppa (ab209232, 1:1,000); anti-Myh7 (ab172967, 1:1,000); anti-SOD (ab68155, 1:1,000); anti-CAT (ab209211, 1:2,000); anti-MDA (ab283311, 1:1,000); anti-PA2G4 (ab180602, 1:1,000); anti-SUPT6H (ab32820, 1:500); anti-SOX12 (ab54371, 1:500); and anti-GAPDH (ab8245, 1:500). All antibodies were procured from Abcam (Cambridge, UK).

(3H)-leucine incorporation

After the treatment, HCMs were incubated with (3H)-leucine (1 μCi/ml) for 24 h, washed with phosphate-buffered saline (PBS), added with 10% trichloroacetic acid, and incubated for another 30 min. Subsequently, cells were lysed with NaOH (1 mol/L) overnight at 4°C. (3H)-leucine incorporation was assessed by a β counter.

Measurement of antioxidative activities

The supernatant from the samples was transferred into new tubes for evaluation of superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) activities. SOD activities were measured with the SOD assay kit, CAT activity was determined with a commercial colorimetric kit, and MDA level was determined with the MDA kit. All the commercial kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Measurement of reactive oxygen species (ROS) production

After washing with PBS, cells were incubated with DCFH-DA (10.0 μM) at 37°C for 20 min without the light. Then, the levels of ROS were detected with a Reactive Oxygen Species Assay Kit (Beyotime).

Table 1. Primers used for quantitative RT-PCR (human).

Name	Forward primer sequence (5′ - 3′)	Reverse primer sequence (5′ - 3′)
miR-423-5p	CGAAGTTCCCTTTGTCATCCT	GTGCAGGGTCCGAGGTATTC
Nppa	ACCCTGGGCTTCTTCCTCGTCTT	GCGGCCCCTGCTTCCTCA
Myh7	GCCCTTTGACCTCAAGAAAG	CTTCACAGTCACCGTCTTG
PA2G4	CGCCAATAGAAGGTATGCTG	CTGTGGGATTCTGGATAATGG
SUPT6H	TCCTGAAGATCCTGAAGCAG	AGACTTTGGGACCCATGTG
SOX12	CAGGACTCGGAGAAGATCC	TTGTAGTCCGGGTAATCCG
U6	ATACAGAGAAAGTTAGCACGG	GGAATGCTTCAAAGAGTTGTG
GAPDH	TCAAGATCATCAGCAATGCC	CGATACCAAAGTTGTCATGGA

Luciferase reporter assay

The binding site between miR-423-5p and SUPT6H 3′UTR was predicted from the starBase (https://starbase.sysu.edu.cn/), amplified using PCR, and cloned into luciferase reporter vector pmirGLO (Promega, Madison, WI, USA) to obtain SUPT6H 3' UTR-Wt and SUPT6H 3' UTR-Mut. Luciferase reporter plasmids and miR-423-5p inhibitor or NC inhibitor were cotransfected into HCMs using Lipofectamine 2000 (Invitrogen). After transfecting for 48 h, a Luciferase reporter gene assay kit (Promega) was used to determine the Luciferase activities.

Immunofluorescence

For the immunofluorescence staining, the HCMs on coverslips were fixed with paraformaldehyde (4%) in PBS on ice for 15 min and permeabilized with Triton X-100 (0.2%; Sigma-Aldrich) for 10 min. Monolayers were subsequently washed in blocking solution (Sigma-Aldrich) and incubated with the primary antibody against α-actinin (1:100, ab108198) at 4°C for 48 h and the fluorescent secondary antibody (1:200, Invitrogen) for 1 h. Hoechst was conducted to perform the nuclear staining. A fluorescence microscope was used to observe the cells.

Statistical analysis

All statistical values were shown as mean ± SD. All determinations were performed at least in triplicate. Differences between two groups were analyzed using *t*-test. Differences among multiple groups were carried out by two-way ANOVA followed by Tukey's *post-hoc* test. All the *p*-values less than 0.05 were considered significant. SPSS 21.0 (SPSS Inc., Chicago, IL, USA) was used to conduct the statistical analysis.

Results

MiR-423-5p inhibitor suppresses Ang II-induced cardiomyocyte hypertrophy

MiR-423-5p expression was upregulated in Ang II-treated HCMs and downregulated in the cells transfected with miR-423-5p inhibitor (Fig. 1A). Additionally, immunofluorescence staining revealed that the treatment of Ang II obviously increased the cell surface area. However, the cell surface area of Ang II-treated HCMs was then decreased by miR-423-5p knockdown (Fig. 1B). The changes on mRNA and protein expression of myocardial hypertrophy markers (Nppa and Myh7) were detected by RT-qPCR and western blotting. It was demonstrated that the upregulation of Nppa and Myh7 expression induced by Ang II was suppressed by miR-423-5p knockdown (Fig. 1C, D). Furthermore, miR-423-5p knockdown also markedly inhibited Ang II-induced (3H)-leucine incorporation in HCMs. (Fig. 1E). The above results demonstrated that silencing miR-423-5p restrained cardiomyocyte hypertrophy in Ang II-treated HCMs.

Silencing of miR-423-5p suppresses Ang II-induced oxidative stress in HCMs

Evaluation of SOD, CAT, MDA, and ROS revealed the changes in oxidative stress. The levels of SOD and CAT were decreased after the Ang II treatment and then significantly increased in the Ang II-treated HCMs transfected with miR-423-5p inhibitor (Fig. 2A, B). Inversely, the level of MDA was upregulated by Ang II and was downregulated by miR-423-5p inhibitor (Fig. 2C). Similarly, the same trend was observed in the levels of SOD, CAT, and MDA proteins in western blotting (Fig. 2D). More directly, the level of ROS was promoted by Ang II and the promotion was subsequently repressed in Ang II + miR-423-5p inhibitor group (Fig. 2E). Collectively, miR-423-5p knockdown suppressed Ang-II-induced oxidative stress in HCMs.

MiR-423-5p targets SUPT6H and leads to its degradation

Three target genes (PA2G4, SUPT6H, and SOX12) of miR-423-5p were predicted by the starBase on the overlapping conditions of microT, miRmap, RNA22, PicTar and TargetScan. RT-qPCR and western blotting showed that the expression and protein levels of PA2G4 and SOX12 showed no significant alteration in the Ang II-treated HCMs. While SUPT6H expression obviously downregulated under the treatment of Ang II in HCMs, thus we chose SUPT6H for the subsequent experiments (Fig. 3A, B). RT-qPCR also revealed that SUPT6H expression and protein levels were higher in the cells silencing miR-423-5p (Fig. 3C). The binding site between miR-423-5p and SUPT6H 3' UTR was predicted by starBase website and cloned into luciferase reporter vector pmirGLO to obtain SUPT6H 3' UTR-Wt and SUPT6H 3' UTR-Mut, as shown in schematic diagram (Fig. 3D). According to the luciferase reporter assay, the luciferase activity of SUPT6H 3' UTR-Wt was significantly increased in the cells silencing miR-423-5p, while the luciferase activity of SUPT6H 3' UTR-Mut remained unchanged after the transfection of miR-423-5p inhibitor or NC inhibitor (Fig. 3E). Collectively, miR-423-5p targeted SUPT6H and negatively regulated SUPT6H expression.

Silenced miR-423-5p represses Ang II-induced cardiomyocyte hypertrophy by SUPT6H

In Ang II-stimulated HCMs, SUPT6H expression was downregulated by SUPT6H knockdown, while miR-423-5p significantly upregulated the SUPT6H expression (Fig. 4A). Particularly, SUPT6H knockdown increased the cell surface area of Ang II-treated HCMs, which was then abolished by miR-423-5p knockdown, as shown by the immunofluorescence staining (Fig. 4B). The mRNA expression and protein levels of Nppa and Myh7 were elevated by SUPT6H knockdown, which was partially reversed by the miR-423-5p inhibitor in Ang II-treated HCMs (Fig. 4C, D). Further, miR-423-5p knockdown also abolished the increase of (3H)-leucine incorporation induced by sh-SUPT6H (Fig. 4E). Thus, miR-423-5p knockdown repressed cardiomyocyte hypertrophy through regulating SUPT6H expression in

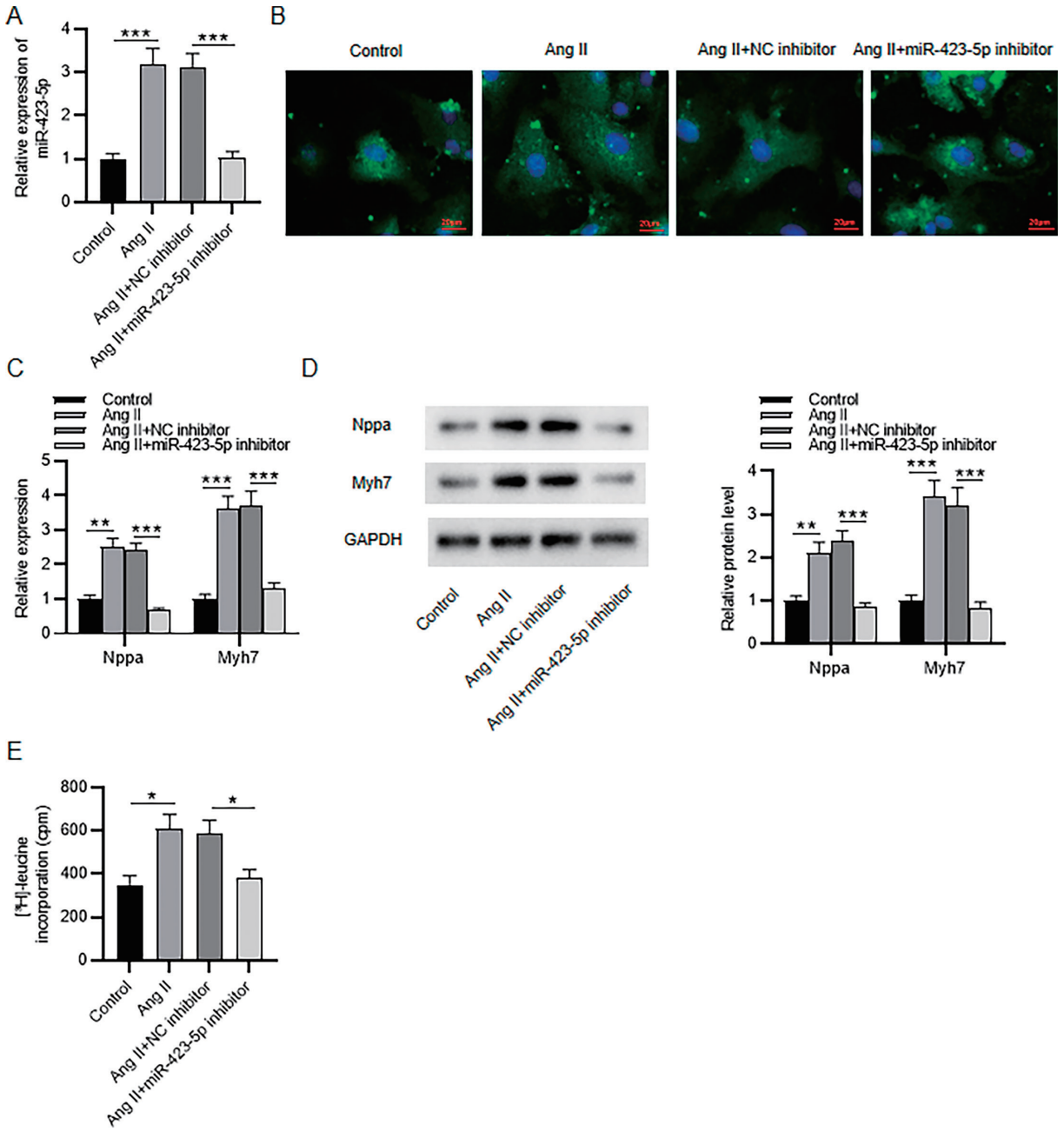

Fig. 1. MiR-423-5p inhibitor suppresses Ang II-induced cardiomyocyte hypertrophy.
(A) MiR-423-5p expression in Control, Ang II, Ang II + NC (negative control) inhibitor and Ang II + miR-423-5p inhibitor groups was measured by RT-qPCR. (B) The morphological changes of cardiomyocytes were detected in Control, Ang II, Ang II + NC inhibitor and Ang II + miR-423-5p inhibitor groups by immunofluorescence staining. (C, D) The mRNA expression (C) and protein levels (D) of Nppa and Myh7 were detected through RT-qPCR and western blotting, respectively. (E) Cardiomyocyte hypertrophy in Control, Ang II, Ang II + NC inhibitor and Ang II + miR-423-5p inhibitor groups was evaluated by (3H)-leucine incorporation assay. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Ang II-stimulated HCMs.

MiR-423-5p inhibitor inhibits oxidative stress in Ang II-stimulated HCMs by SUPT6H

In this part, we investigated the effect of SUPT6H and miR-423-5p knockdown on Ang II-induced oxidative stress in HCMs. As expected, the levels of SOD and CAT were reduced by SUPT6H knockdown, and the reduction was then counteracted by miR-423-5p knockdown (Fig. 5A, B). Conversely, the level of MDA was enhanced in sh-SUPT6H group and then lowered in sh-SUPT6H + miR-423-5p inhibitor group in Ang II-stimulated HCMs (Fig. 5C). Consistently, decreased protein levels of SOD and CAD and increased protein level of MDA were observed in SUPT6H group, which was then partially reversed by miR-423-5p knockdown in Ang II-stimulated HCMs (Fig. 5D).

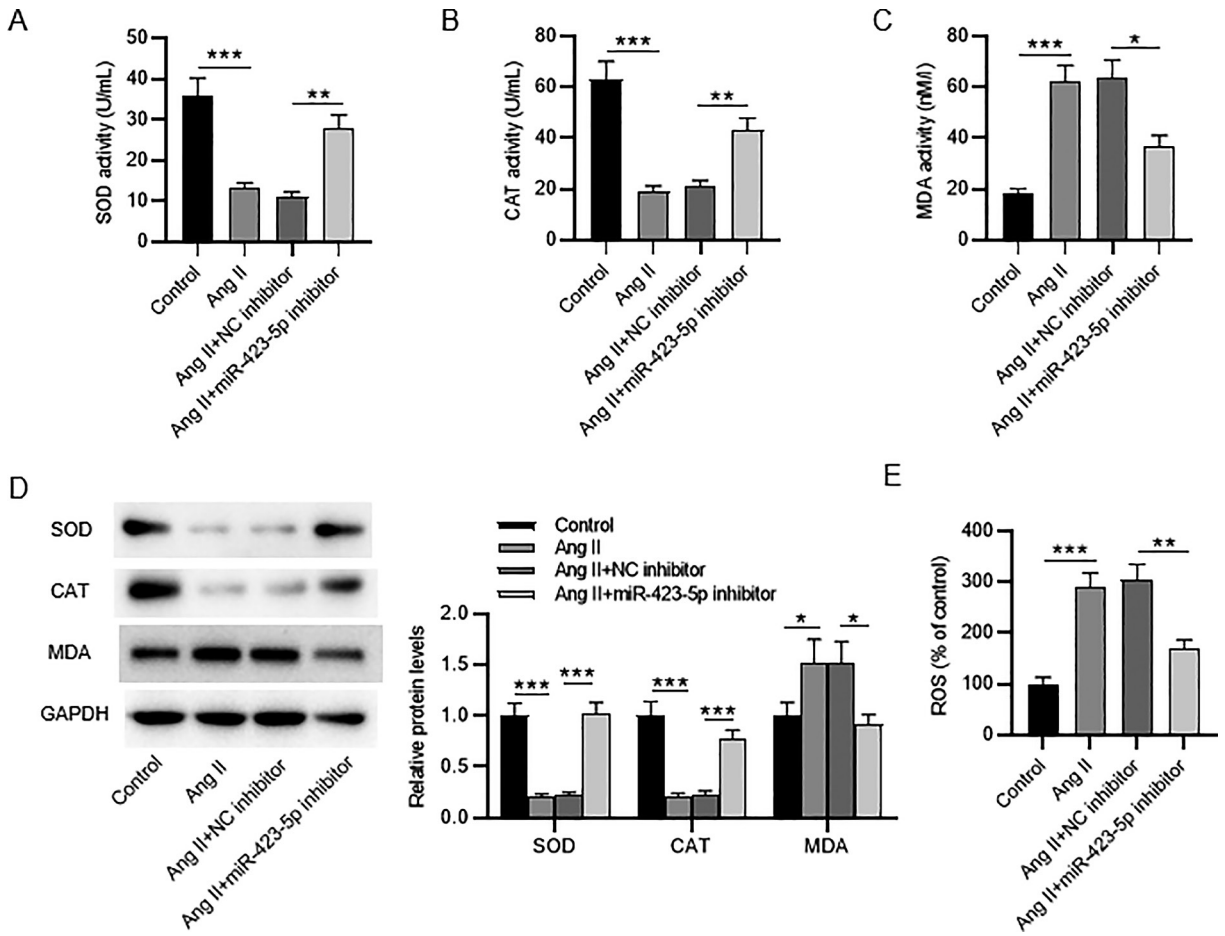

Fig. 2. Silencing of miR-423-5p suppresses Ang II-induced oxidative stress in human cardiomyocytes (HCMs). (A-C) The activities of superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) in Control, Ang II, Ang II + NC inhibitor and Ang II + miR-423-5p inhibitor groups were measured by the corresponding detection kits. (D) The protein levels of oxidative stress-related enzymes in Control, Ang II, Ang II + NC inhibitor and Ang II + miR-423-5p inhibitor groups were detected by western blotting. (E) The production of ROS was detected in HCMs with Control, Ang II, Ang II + NC inhibitor and Ang II + miR-423-5p inhibitor groups. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Additionally, SUPT6H knockdown induced accumulated ROS production in Ang II-treated HCMs, while the transfection of miR-423-5p inhibitor obviously reduced the accumulated ROS production (Fig. 5E). Thus, these results all indicated that miR-423-5p inhibitor attenuated cardiomyocytes injury by inhibiting Ang II-induced oxidative stress by upregulating SUPT6H expression.

Discussion

Sustained cardiac hypertrophy is one of the main causes of heart failure and has become a serious threat to human health. Anti-hypertrophic treatment is an effective strategy for controlling the development of heart failure (Rosca et al. 2013; Nakamura and Sadoshima 2018). Studies have confirmed that the activation of Ang II can lead to cardiomyocyte hypertrophy (Johar et al. 2006). Thus, revealing novel targets that can control Ang II-provoked cardiac hypertrophy is of great significance. In this study, we identified miR-423-5p as a key regulator in cardiac hypertrophy and confirmed SUPT6H as its downstream target.

Recently, substantial recognition has been given to plasma miRNAs as possible functional biomarkers that may serve as key post-transcriptional regulators of gene expression networks in various clinical scenarios, including heart failure. Previous studies have shown that many miRNAs were downregulated in cardiac hypertrophy, like miR-101b (Lee et al. 2017), miR-1 (Seok et al. 2020), and miR-26a-5p (Tang et al. 2020), which mitigate the hypertrophic responses. Inversely, the upregulated miRNAs including miR-22 (Huang et al. 2013), miR-297 (Bao et al. 2017), and miR-217 promoted cardiac hypertrophy and dysfunction (Nie et al. 2018). In a previous study, miR-423-5p has been identified as potential biomarker in the diagnosis and prognosis of heart failure (Goren et al. 2012). In addition, miR-423-5p expression was significantly increased in response to hypertension-induced heart failure (Dickinson et al. 2013). However, researchers then reported that miR-423-5p had no significant effect on systemic ventricular dysfunction after atrial repair for transposition of the great arteries (Tutarel et al. 2013), and left ventricular remodeling after myocardial infarction (Bauters et al. 2013).

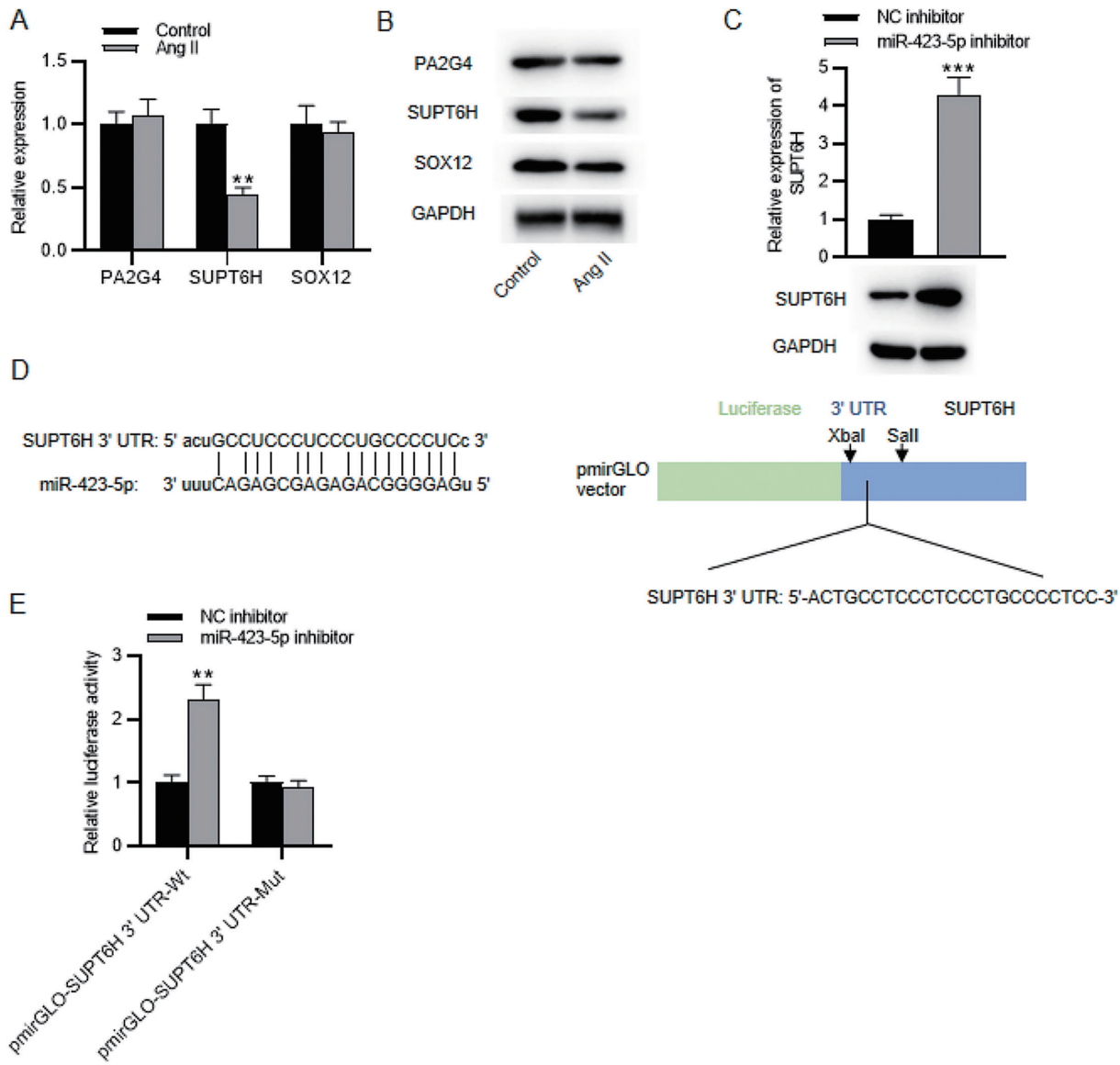

Fig. 3. MiR-423-5p targets SUPT6H and leads to its degradation.
(A, B) The mRNA expression (A) and protein levels (B) of possible targets of miR-423-5p (PA2G4, SUPT6H and SOX12) were evaluated by RT-qPCR and western blotting, respectively. (C) SUPT6H mRNA expression and protein level were evaluated after transfecting cells with NC inhibitor and miR-423-5p inhibitor. (D) The starBase predicted the binding site between SUPT6H 3' UTR and miR-423-5p. (E) A luciferase reporter gene assay revealed the changes of luciferase activity in SUPT6H 3' UTR-Wt and SUPT6H 3' UTR-Mut after transfecting cells with NC inhibitor and miR-423-5p inhibitor. $^{**}p < 0.01$, $^{***}p < 0.001$.

Controversially, subsequent studies further defined miR-423-5p as promising biomarker for various cardiovascular diseases (Rizzacasa et al. 2019; Zhu and Lu 2019), including heart failure (Oliveira-Carvalho et al. 2012, 2013; Shah et al. 2018; D'Alessandra et al. 2020; Peterlin et al. 2020). But its role and mechanisms have not been discussed in Ang II-induced cardiac hypertrophy yet. In this study, miR-423-5p was highly expressed in Ang II-treated HCMs. In addition, miR-423-5p knockdown reduced the cell surface area, inhibited the expression of myocardial hypertrophy markers (Nppa and Myh7), and decreased (3H)-leucine incorporation in Ang II-treated HCMs, indicating the suppressive role of miR-423-5p knockdown in cardiac hypertrophy.

Both experimental and clinical observations have indicated that oxidative stress is accumulated in the hypertrophic myocardium during heart failure (Keith et al. 1998; Rababa'h et al. 2018). The imbalance between antioxidant defense and production of ROS was the main cause of oxidative stress, which can induce cardiac dysfunction in hypertrophied heart through a complex set of mechanisms and aggravate the progression of heart failure (Seddon et al. 2007; Adebiyi et al. 2016). Here, we found that miR-423-5p knockdown promoted the activities of the antioxidant enzymes like SOD and CAT, and suppressed Ang II-induced MDA accumulation and ROS production in

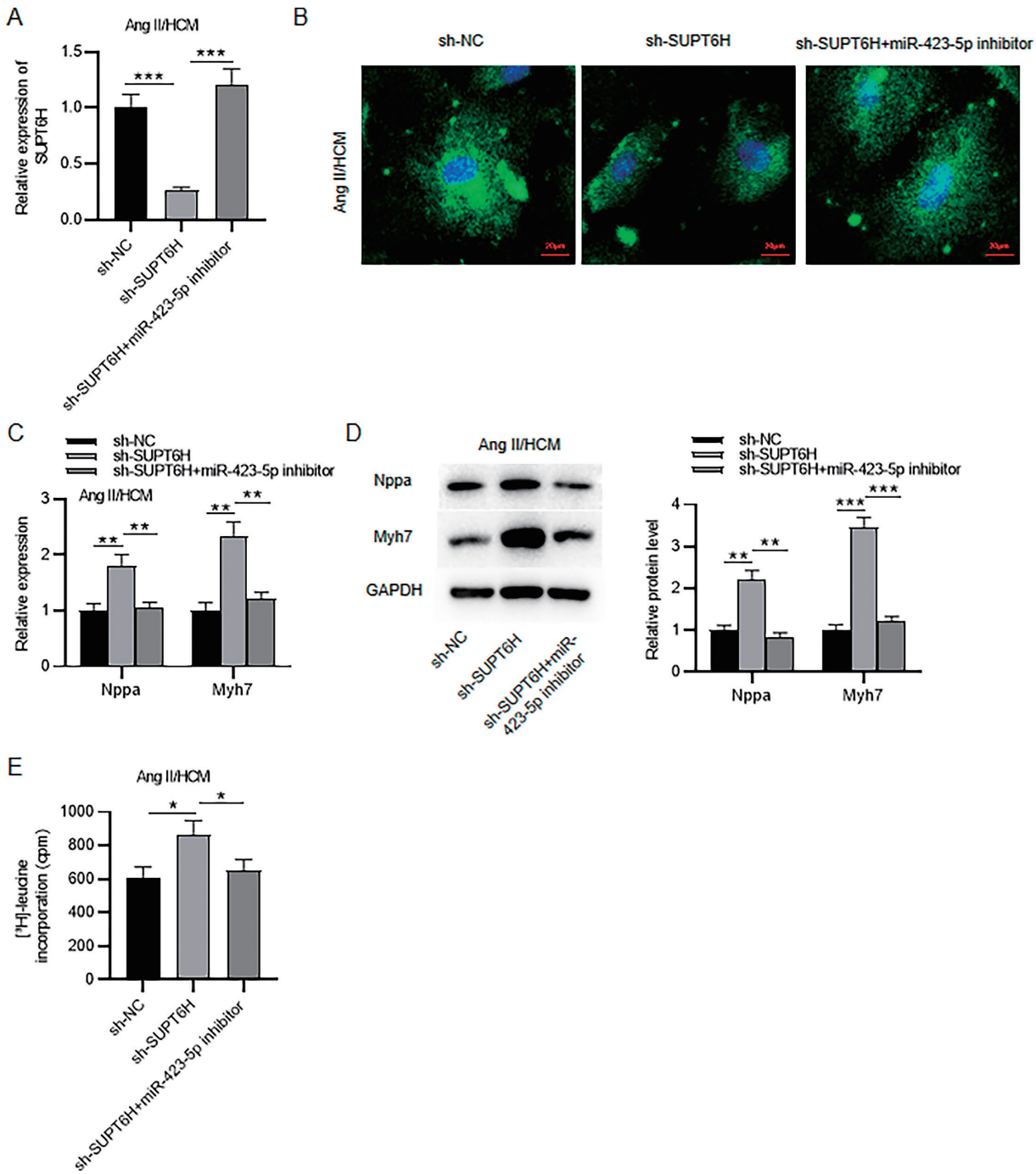

Fig. 4. Silenced miR-423-5p represses Ang II-induced cardiomyocyte hypertrophy by SUPT6H.
(A) MiR-423-5p expression in in sh-NC, sh-SUPT6H, and sh-SUPT6H + miR-423-5p inhibitor groups was measured by RT-qPCR. (B) Immunofluorescence staining of Ang II-treated cardiomyocytes in sh-NC, sh-SUPT6H, and sh-SUPT6H + miR-423-5p inhibitor groups. (C, D) The mRNA expression (C) and protein levels (D) of Nppa and Myh7 were detected through RT-qPCR and western blotting. (E) The (3H)-leucine incorporation of Ang II-treated cardiomyocytes in sh-NC, sh-SUPT6H, and sh-SUPT6H + miR-423-5p inhibitor groups. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

HCMs, indicating a strong inhibitory effect of miR-423-5p knockdown on oxidative stress during cardiac hypertrophy.

Subsequently, bioinformatic approaches identified three candidate miR-423-5p targets in cardiomyocytes *in vitro*, and only SUPT6H expression obviously downregulated under the treatment of Ang II in HCMs. Our research then confirmed the binding between miR-423-5p and 3′UTR of SUPT6H. SUPT6H is a histone chaperone, which has been reported to involve in epigenetic regulation, transcriptional elongation, chromatin modifications and cardiac function (Bedi et al. 2015; Duan et al. 2017; Yan et al. 2019). However, its specific role in cardiovascular diseases and miRNA/mRNA networks are unknown. Notably, we found that SUPT6H knockdown promoted cardiomyocyte

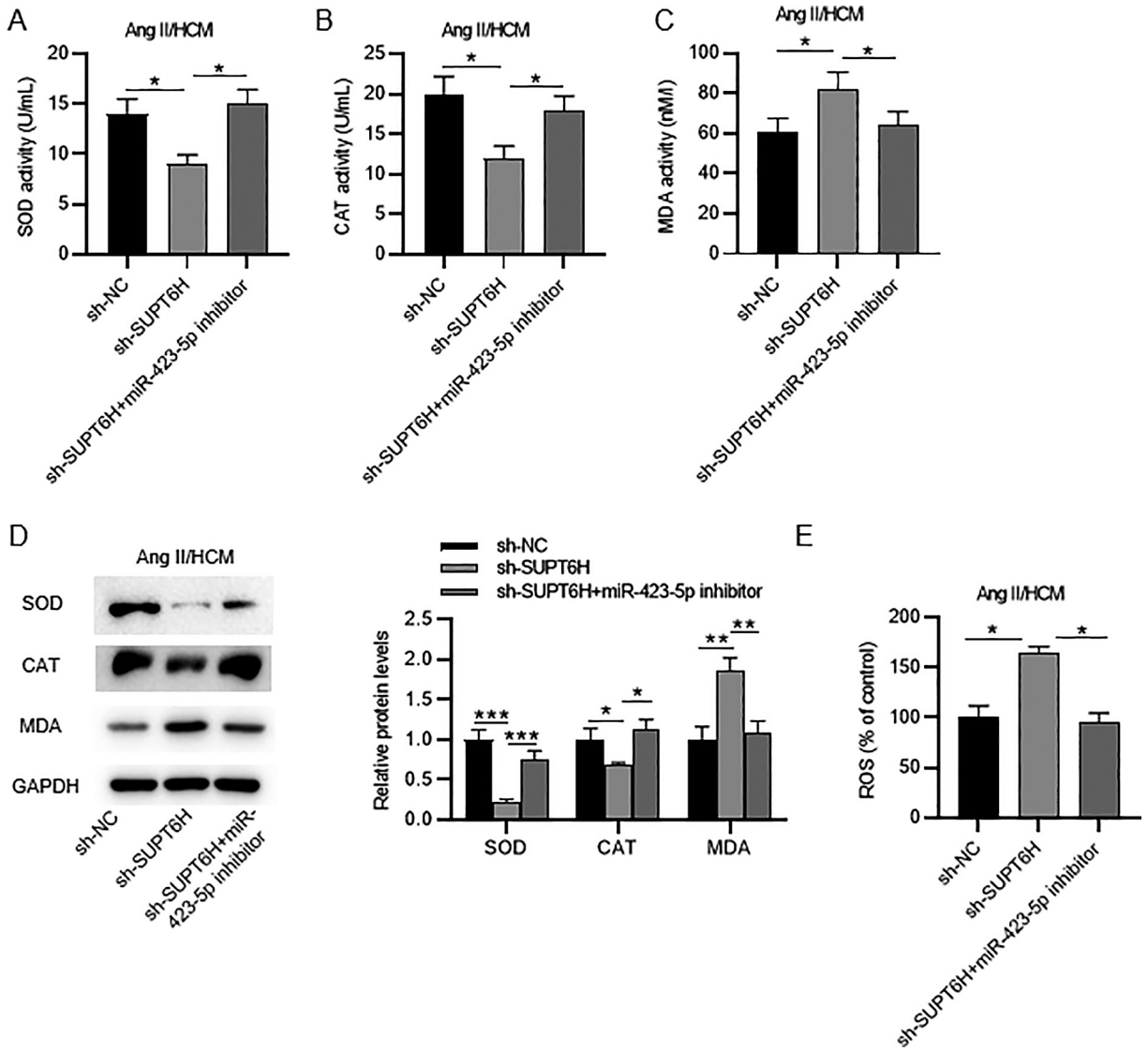

Fig. 5. MiR-423-5p inhibitor inhibits oxidative stress in Ang II-stimulated HCMs by SUPT6H.
(A-C) The activities of superoxide dismutase (A; SOD), catalase (B; CAT), and malondialdehyde (C; MDA) in sh-NC, sh-SUPT6H, and sh-SUPT6H + miR-423-5p inhibitor groups. (D) The protein levels of oxidative stress-related enzymes in sh-NC, sh-SUPT6H, and sh-SUPT6H + miR-423-5p inhibitor groups. (E) The production of ROS in sh-NC, sh-SUPT6H, and sh-SUPT6H + miR-423-5p inhibitor groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

hypertrophy and oxidative stress in Ang II-treated HCMs. Interestingly, miR-423-5p inhibitor then abolished the promotive effect of SUPT6H knockdown on cardiomyocyte hypertrophy and oxidative stress, suggesting that miR-423-5p knockdown alleviated Ang II-induced cardiac hypertrophy by upregulating SUPT6H expression. As for the limitations of our study, we only discussed the effect of miR-423-5p/SUPT6H axis *in vitro*, but did not verify the changes *in vivo*, and we will carry out the study in the future.

In summary, our research revealed the protective effects of miR-423-5p/SUPT6H axis on cardiomyocyte hypertrophy caused by Ang II, thus providing a novel research strategy for myocardial hypertrophy.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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