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Original Article

Sp1-induced LncRNA CTBP1-AS2 is a novel regulator in cardiomyocyte hypertrophy by interacting with FUS to stabilize TLR4☆

Xiaojia Luo [a,b], Sen He [a], Yongmei Hu [b], Jianxiong Liu [b], Xiaoping Chen [a,*]

[a] Department of Cardiovascular Medicine, West China Hospital, Sichuan University, Chengdu, Sichuan, 610041, China
[b] Department of Cardiovascular Medicine, Chengdu Second People's Hospital, Chengdu, Sichuan, 610017, China

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ABSTRACT

Cardiomyocyte hypertrophy is a heart response adapting to increasing cardiac load. Prolonged cardiomyocyte hypertrophy indicates a higher risk of heart failure or even cardiac death. Long noncoding RNAs have been largely reported to modulate human diseases. CTPB1-AS2 is a newly discovered lncRNA reported as an oncogene in papillary thyroid cancer, but its function in cardiomyocyte hypertrophy has never been probed. Toll-like receptor 4 (TLR4) is recognized to play important roles in cardiomyocyte hypertrophy. The present study aimed to investigate the role of CTBP1-AS2 in cardiomyocyte hypertrophy. First, we discovered the low expression of CTBP1-AS2 in normal heart tissues in GETx database. Then, we established cardiomyocyte hypertrophy models on mice and cardiomyocytes through transverse aortic constriction surgery and Ang II treatment. We revealed the up-regulation of CTBP1-AS2 and TLR4 in cardiomyocyte hypertrophy models. Also, we confirmed the positive correlation between CTBP1-AS2 and TLR4 expressions in cardiomyocyte hypertrophy tissues. Loss-of-function assays confirmed that inhibiting CTBP1-AS2 attenuated the Ang II-induced cardiomyocyte hypertrophy. Mechanism research showed that CTBP1-AS2 stabilized TLR4 mRNA by recruiting FUS. Rescue assays certified that CTBP1-AS2 regulated cardiomyocyte hypertrophy through TLR4. Finally, we found Sp1 as an upstream activator for CTBP1-AS2 expression. In conclusion, our study uncovered CTBP1-AS2 as a novel regulator of cardiomyocyte hypertrophy through regulating TLR4, providing a new potential treatment target for cardiomyocyte hypertrophy.

1. Introduction

Cardiac hypertrophy is known to be an adaptive response to accumulated heart load, featured with enlarged size of cardiomyocytes and increased synthesis of contractile proteins [1,2]. Cardiac hypertrophy could result from persistent hypertrophic stresses, including hypertension, valvular heart disease, myocarditis, and ischemia [3,4]. Sustained hypertrophy could eventually lead to cell death and irreversible remodeling of cardiac structure, so as to cause heart failure and even sudden death [1,4–6].

Long noncoding RNAs, with more than 200 bases, are a group of RNA transcripts lacking in coding potentials [7–9]. The regulation of lncRNAs on gene expression at transcriptional, posttranscriptional, as well as epigenetic level has been increasingly documented [10]. At posttranscriptional level, lncRNAs interact with RNA binding proteins to regulate the mRNA stability of target genes. Fused in sarcoma (FUS) acts as an essential regulator of mRNA stability in cytoplasm [11,12]. LncRNAs have been discovered to recruit FUS to stabilize target mRNAs in human diseases [13,14]. However, the role of FUS as an mRNA stabilizer in cardiac hypertrophy has never been explored. Additionally, the activation of lncRNAs through transcription factors has been demonstrated in mounting reports [15–18]. Past decades witnessed the rising role of lncRNAs in a considerable number of cancers and diseases [19–22], including cardiac hypertrophy [23–26]. LncRNA CTBP1-AS2 was initially identified to be closely related to the reoccurrence in papillary thyroid cancer [27]. But no study has revealed the function of CTBP1-AS2 in cardiac hypertrophy.

Toll-like receptor 4 (TLR4), a member of toll-like receptors (TLRs), is known to be a pivotal regulator of sterile inflammation [28]. TLRs are acknowledged to be major regulators of sterile inflammation, a commonly observed phenomenon in cardiac hypertrophy contributing to disease progression [29]. Accumulating studies have revealed the role of TLR4 in cardiac hypertrophy [30–32]. But its association with CTBP1-AS2 and FUS has not been explored.

The present study tried to investigate the role of CTBP1-AS2 in cardiomyocyte hypertrophy and its regulation on TLR4.

☆ Conflicts: none.

* Corresponding author at: Department of Cardiovascular Medicine, West China Hospital, Sichuan University, No. 37, Guoxue Lane, Wuhou District, Chengdu, Sichuan, 610041, China.
E-mail address: chengk14481@163.com (X. Chen).

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1054-8807/

2. Materials and methods

2.1. Animal Experiments

The healthy male C57BL/6 mice weighting from 20 g to 25 g were provided by Vital River Laboratory Animal Company (Beijing, China). All mice were kept under the standard conditions with an atmosphere of around 21°C and 55%–60% humidity. All mice had free access to food and water. To establish the model of cardiac stress overload, transverse aortic constriction (TAC) surgery was performed on the mice for 4 weeks referring to the former descriptions [33]. The animal assays had gained the permission of the Institutional Animal Care and Use Committee of West China Hospital.

2.2. Cell culture

The neonatal rat ventricular myocytes (NRVMs) were extracted from 2-day-old Sprague–Dawley male rats (Laboratory Animal Center of Soochow University, Suchow, China). The preparation of NRVMs was conducted as former description [34]. Total NRVMs underwent 24-h culturing in Dulbecco's modified Eagle media (HyClone, Logan, UT, USA) containing 1% of insulin-transferrin-selenium (BD Biosciences, CA, USA), 100 μg/ml streptomycin, and 100 U/ml penicillin before transfection or the drug treatment of Angiotensin II (Ang II) (1 μM; Sigma-Aldrich, St. Louis, MO, USA) or PBS.

2.3. Cell transfection

Specific siRNAs targeting CTBP1-AS2 (siCTBP1-AS2#1/2), FUS (siFUS#1/2), or Sp1 (siSp1) were used to knock down CTBP1-AS2 and Sp1. pcDNA3.1 with the full sequence of TLR4 or FUS was used to overexpress TLR4 or FUS. si-NC and pcDNA3.1 were negative controls. All plasmids were obtained from GenePharma Co., Ltd. (Shanghai, China). The transfection of these plasmids into NRVMs was performed using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) obeying the manufacturer's guide.

2.4. Measurement of the cell size

The washed NRVMs were fixed for 30 min by 4% paraformaldehyde at room temperature. Then, all cells were permeabilized for 30 min using 0.5% Triton X-100 dissolved in PBS, and blocked for an hour using 2% BSA. Staining of the cells was conducted overnight by the use of anti-α-actinin (1:200, A7811; Sigma) under 4°C. Subsequently, the washed cells were incubated using Alexa Fluor 594-conjugated secondary antibody (1:200, A21205; Life Technologies) as well as 4′,6′-diamino-2-phenylindole (1 μg/ml, D9542; Sigma). EVOS FL auto cell imaging system (Life Technologies) was used to capture the images. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to measure the cell surface area.

2.5. Determinations of protein/DNA ratio

Protein density was examined based on BCA method (P0012; Beyotime, Shanghai, China) following the manufacturer's instruction. Concentration of DNA was measured using fluorescence assay (DNAQF; Sigma, St. Louis, MO, USA) in the light of manufacturer's guide. The protein/DNA ratio was evaluated to measure potential protein synthesis.

2.6. Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

First, TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the RNA samples from NRVMs. 5× All-In-One RT MasterMix (abmGood, Vancouver, Canada) was utilized to reversely transcribe the RNA into cDNA. RT-qPCR was performed on the ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). EvaGreen RT-qPCR Mastermix Kit (abmGood, Vancouver, Canada) was used to examine the level of CTBP1-AS2, ANP, BNP, as well as β-MHC, with GAPDH as normalized control. Data analysis was performed based on the $2^{-\Delta\Delta CT}$ method. The primer sequences were listed as follows:

CTBP1-AS2 Forward: 5′-GAGCCCTGATTTACCGTCCG-3′
Reverse: 5′-AAGGGGAGACGTAGGCTTCT-3′.
TLR4 Forward: 5′-TCTGGGGAGGCACATCTTCT-3′
Reverse: 5′-AGGTCCAAGTTGCCGTTTCT-3′.
ANP Forward: 5′-CTCCCAGGCCATATTGGAG-3′
Reverse: 5′-TCCAGGTGGTCTAGCAGGTT-3′.
BNP Forward: 5′-GAGTCCTTCGGTCTCAAGGC-3′
Reverse: 5′-CAACTTCAGTGCGTTACAGCC-3′.
β-MHC Forward: 5′-CCTCGCAATATCAAGGGAAA-3′
Reverse: 5′-TACAGGTGCATCAGCTCCAG-3′.
FUS Forward: 5′-CTCCGGGAAACTGTGGCGTG-3′
Reverse: 5′-ACAAAGTGGTCGTTGAGGGCA-3′.
Sp1 Forward: 5′-TCCAAGGCTGTGGGAAAGTG-3′
Reverse: 5′-CATAGGCTCTTGCTCACCTGT-3′.
GAPDH Forward: 5′-AGTGCCAGCCTCGTCTCATA-3′
Reverse: 5′-GGTAACCAGGCGTCCGATAC-3′.

2.7. Western blot analysis

Cultured NRVMs were lysed in standard sample buffer. The protein density was examined by the BCA Protein Assay Kit (Beyotime, Shanghai, China). Later, total samples underwent electrophoresis with 10% SDS-PAGE and were then transferred onto the nitrocellulose filter membrane. Primary antibodies applied were: anti-TLR4 (Abcam, Invitrogen, OR, USA); anti-ANP, anti-BNP and anti-β-MHC (Santa Cruz Biotechnology, Inc., Dallas, TX, USA); and anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA). Analyses of protein bands were carried out on the Odyssey version (v.)1.2 software.

2.8. RNA immunoprecipitation (RIP)

RIP assay was conducted using Magna RIP RNA-Binding Protein Immunoprecipitation reagent kit (Merck Millipore, Billerica, MA, USA). Total cells were lysed in 100 μl of lysis buffer for 30 min with proteinase inhibitors and the ribonuclease inhibitors on ice. The cell lysates were incubated at 4°C overnight with antibodies against FUS (ab70381, Abcam) or IgG (ab205719, Abcam) with the addition of protein A/G-beads. After washing the beads, the immunoprecipitated RNAs were subjected to isolation and purification, and then evaluated by RT-qPCR.

2.9. Luciferase reporter assays

The full sequence of CTBP1-AS2 promoter was generated into pGL3 luciferase reporter vector. For luciferase reporter assay, the NRVMs were cultured in the 24-well culture plates and then were transfected with siSp1 or siNC with the use of Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Firefly luciferase activities were assessed using a Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA), with Renilla luciferase activities as normalized control.

2.10. Chromatin immunoprecipitation (ChIP)

To perform ChIP assay, an EZ-ChIP Kit (Millipore, USA) was utilized. In short, the fixation of NRVMs was conducted using formaldehyde, and then cells were incubated to form the DNA-protein cross-links for 10 min. Sonication of cell lysates was carried out for the generation of chromatin fragments ranging from 200 to 300 bp, and the cell lysates were then immunoprecipitated with antibodies against Sp1 (Cell Signaling Technology, USA). The precipitated chromatin DNA was purified and subjected to the analysis of enrichment using quantitative PCR analysis.

2.11. Bioinformatics research

The expression of CTBP1-AS2 in normal heart tissues was obtained from the GETx (V7) database (https://gtexportal.org/home/gene/CTBP1-AS2). The potential interactions of FUS with CTBP1-AS2 and TLR4, and the binding motif of FUS were obtained by StarBase 3.0 (http://starbase.sysu.edu.cn/index.php). To be specific, we used RBP-target search (RBP-lncRNA), entered "CTBP1-AS2" in target gene, and found at page 6 that FUS was a potential interacting protein with CTBP1-AS2. Also in RBP-target search (RBP-mRNA), we entered "TLR4" in target gene and found at page 1 that FUS was a potential interacting protein with TLR4. The binding sites on CTBP1-AS2 for FUS were identified by comparing the binding motif of FUS with the 3′UTR sequences of TLR4 and CTBP1-AS2. The sequences of the 3′UTR of TLR4 and CTBP1-AS2 were obtained from NCBI (https://www.ncbi.nlm.nih.gov/gene/). The binding motif of Sp1 was obtained from JASPAR tool (http://jaspar.genereg.net/). The binding sites on CTBP1-AS2 promoter for Sp1 were predicted by JASPAR based on the promoter sequences of CTBP1-

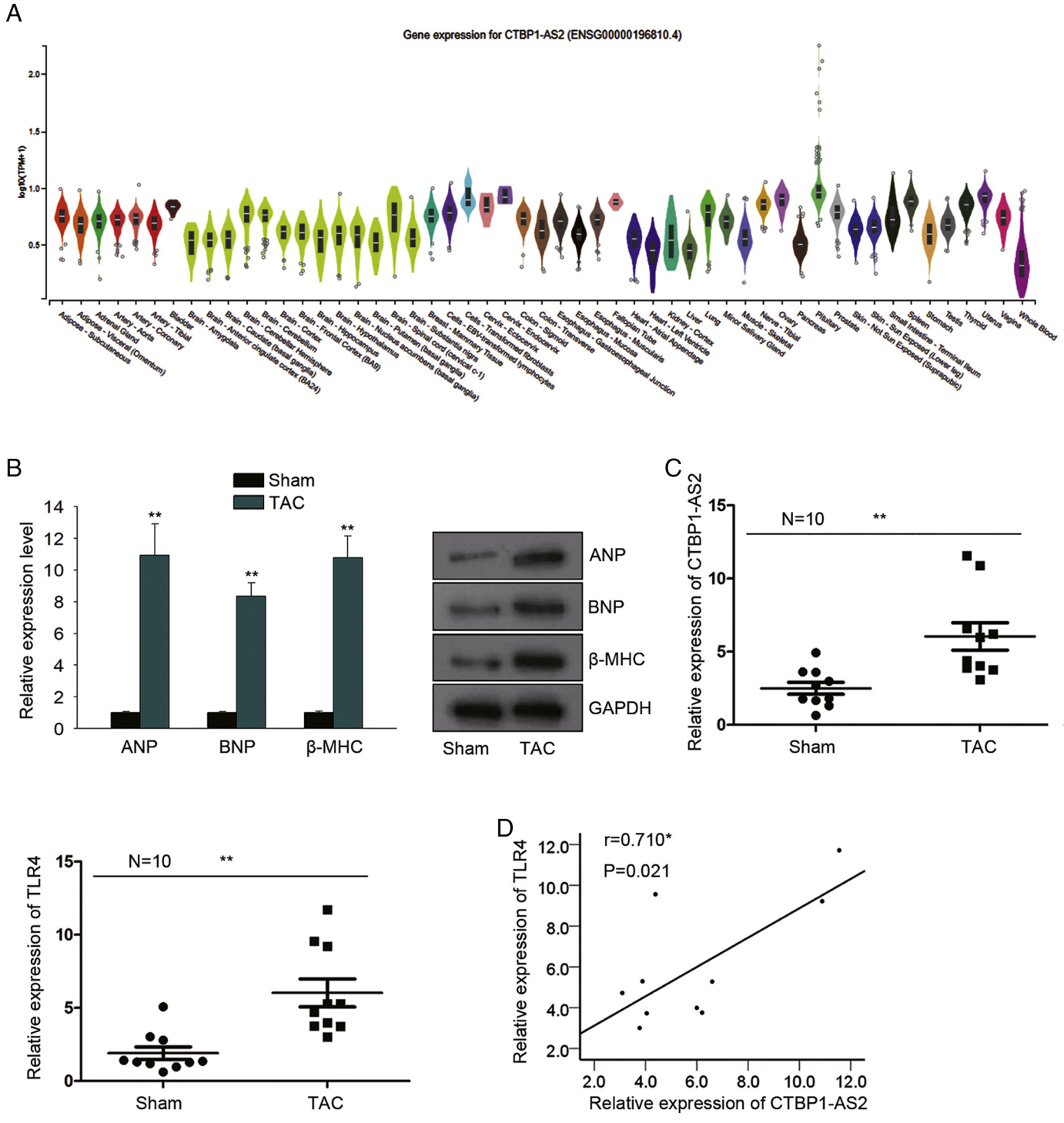

Fig. 1. CTBP1-AS2 was up-regulated in cardiomyocyte hypertrophy mice tissues and positively correlated with TLR4. (A) The low expression of CTBP1-AS2 in normal heart tissues was obtained from GETx database. (B) RT-qPCR and Western blot analyses were used to detect the expressions of ANP, BNP, and β-MHC in the heart tissues from mice which received TAC operation and sham operation. (C) RT-qPCR analysis was used to detect the expressions of CTBP1-AS2 and TLR4 in the heart tissues from mice which received TAC operation and sham operation. (D) Spearman's correlation analysis showed the positive correlation between CTBP1-AS2 and TLR4 in cardiac hypertrophy tissues (r=0.710, P=.021). $^{**}P$<.01.

AS2 and the binding motif of Sp1. The promoter sequences were obtained from Ensembl (http://asia.ensembl.org/index.html).

2.12. Statistical analysis

The presentation of data was on the basis of mean ± standard error. The expression correlation was examined by Spearman's correlation analysis. Double-sided Student's *t* tests were carried out for analyzing two-group comparisons or variance. Tukey's test was carried out for multigroup assays. Statistical significance was determined by $P<.05$. All the experiments were repeated for at least three times. Data analysis was conducted on the IBM SPSS software (version 24; IBM Corp., Armonk, NY, USA).

3. Results

3.1. CTBP1-AS2 was up-regulated in cardiomyocyte hypertrophy mice tissues and positively correlated with TLR4

First, we tried to figure out the association of CTBP1-AS2 in cardiomyocyte hypertrophy. Through browsing the GETx database (https://gtexportal.org/home/gene/CTBP1-AS2), we found that CTBP1-AS2 was only expressed at a median of 1.84 TPM in normal heart tissues (left

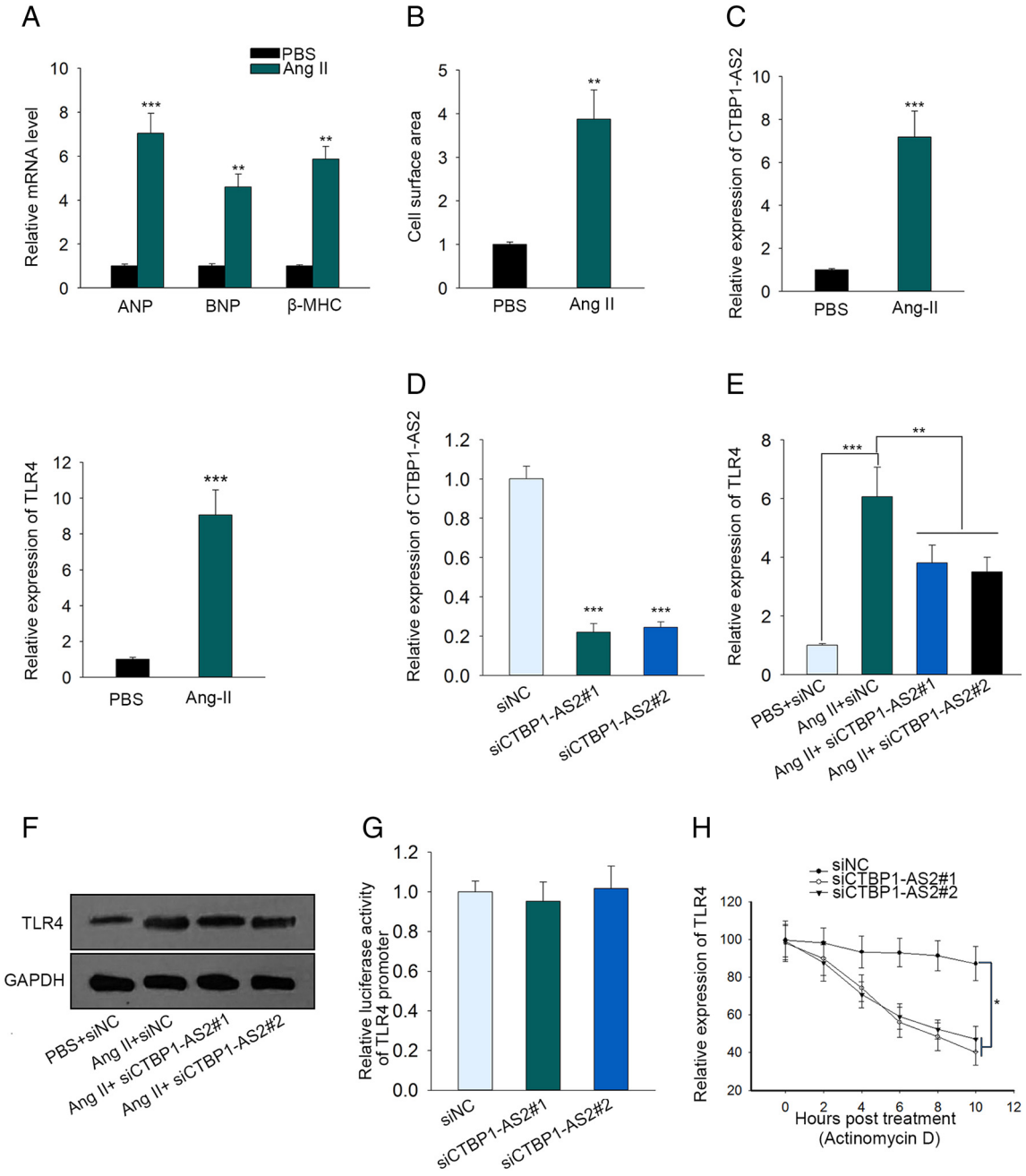

Fig. 2. CTBP1-AS2 was up-regulated in Ang II-induced cardiomyocyte hypertrophy cells and stabilized TLR4 mRNA. (A) The cardiomyocyte hypertrophy models were created by Ang II treatment in NRVMs. The expression levels of ANP, BNP, and β-MHC were detected in Ang II treatment group and PBS treatment group by RT-qPCR. (B) The cell surface of Ang II treated NRVMs was validated to be augmented. (C) RT-qPCR was used to examine levels of CTBP1-AS2 and TLR4 mRNA in NRVMs. (D) Transfection efficiency of siCTBP1-AS2#1/2 into NRVMs was evaluated by RT-qPCR, with siNC as negative control. (E–F) Transfected cells were treated with Ang II or PBS as demanded. RT-qPCR and Western blot were conducted to assess the effect of CTBP1-AS2 on mRNA and protein levels of TLR4. (G) Luciferase reporter assay showed that silencing CTBP1-AS2 had no effect on the luciferase activity of TLR4 promoter. (H) RT-qPCR after actinomycin D treatment showed that silencing CTBP1-AS2 impaired mRNA stability of TLR4. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$.

ventricle) (Fig. 1A). Then, we established cardiomyocyte hypertrophy models in mice through TAC surgery, with sham-operated mice as control. To confirm the construction of animal model, we detected the levels of hypertrophic markers (ANP, BNP, and β-MHC) in the mice tissues. Expectedly, results of RT-qPCR and Western blot analyses showed that the hypertrophic markers were all elevated in the tissues from mice receiving TAC surgery compared with tissues from sham-operated mice (Fig. 1B), indicating the successful establishment of cardiac hypertrophy model in mice. Thereafter, we detected the expression of CTBP1-AS2 and found that CTBP1-AS2 was highly expressed in the cardiomyocyte hypertrophy tissues induced by TAC surgery compared with the sham group (Fig. 1C). These results indicated that CTBP1-AS2 might participate in cardiomyocyte hypertrophy. Since TLR4 has been widely reported as a pivotal regulator of cardiac hypertrophy [30–32], we speculated that CTBP1-AS2 might be related to TLR4. Through RT-qPCR analysis, we also confirmed the up-regulation of TLR4 in cardiomyocyte hypertrophy mice tissues (Fig. 1C). Furthermore, we validated through Spearman's correlation analysis that CTBP1-AS2 was positively correlated with TLR4 expression in cardiomyocyte hypertrophy tissues induced by TAC surgery (Fig. 1D). Together, these results suggested that CTBP1-AS2 was up-regulated in cardiomyocyte hypertrophy tissues and positively related to TLR4.

3.2. CTBP1-AS2 was up-regulated in Ang II-induced cardiomyocyte hypertrophy cells and stabilized TLR4 mRNA

Next, we interrogated the role of CTBP1-AS2 in cardiac hypertrophy in vitro. The cardiomyocyte hypertrophy model was established in NRVMs with the treatment of Ang II. It was found that ANP, BNP, and β-MHC levels were elevated, and the cell surface area was expanded in NRVMs after Ang II treatment (Fig. 2A–B), confirming the establishment of cardiomyocyte hypertrophy model. Through RT-qPCR analysis, it was observed that both CTBP1-AS2 and TLR4 were significantly up-regulated by Ang-II treatment in NRVMs (Fig. 2C).

Then, we sought to explore whether CTBP1-AS2 regulated TLR4 expression in cardiomyocyte hypertrophy by knocking down CTBP1-AS2. Subsequently, two siRNAs against CTBP1-AS2 (siCTBP1-AS2#1/2) were transfected into NRVMs, causing an obvious knockdown of CTBP1-AS2 (Fig. 2D). It was discovered that TLR4 mRNA and protein levels were reduced by CTBP1-AS2 silencing (Fig. 2E-F). Furthermore, we found that silencing CTBP1-AS2 had no effect on the luciferase activity of TLR4 promoter in the NRVMs with Ang II treatment (Fig. 2G), indicating that CTBP1-AS2 regulated TLR4 at posttranscriptional level. Hence, we tried to detect whether CTBP1-AS2 could regulate mRNA stability of TLR4. After treating NRVMs with Actinomycin D to block mRNA generation, the expression of TLR4 mRNA was evaluated by RT-qPCR over time. Results showed that silencing CTBP1-AS2 shortened the half-life of TLR4 mRNA (Fig. 2H). These results indicated that CTBP1-AS2 was up-regulated in Ang II-induced cardiomyocyte hypertrophy cells and stabilized TLR4 mRNA.

3.3. Suppression of CTBP1-AS2 retarded the Ang II-induced cardiomyocyte hypertrophy

Then we inquired the effect of CTBP1-AS2 on cardiomyocyte hypertrophy. First, we validated that the mRNA and protein levels of three hypertrophic markers, including ANP, BNP, and β-MHC, were induced under Ang II treatment, and the inductive effect was impaired by silencing CTBP1-AS2 in NRVMs (Fig. 3A–B). In addition, CTBP1-AS2 knockdown abrogated the promoting effect of Ang II treatment on cell

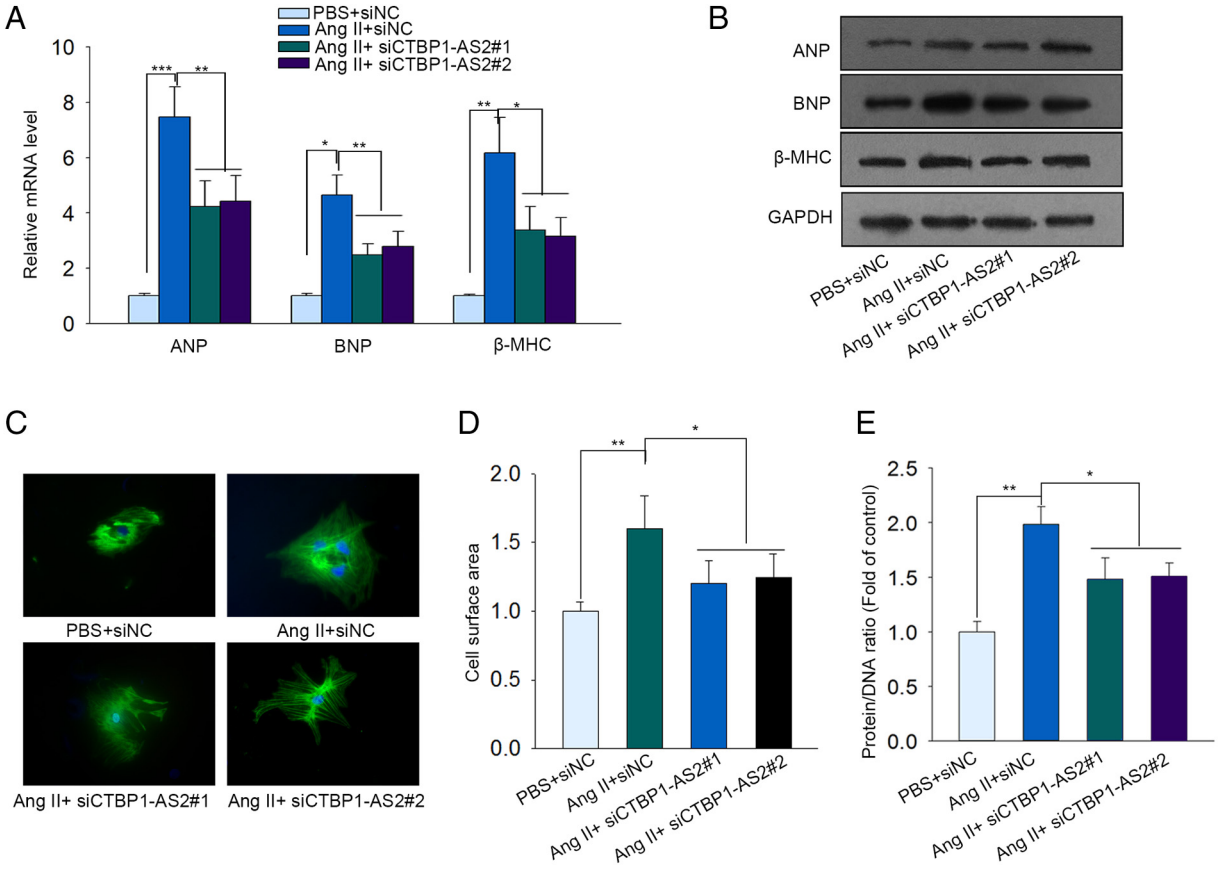

Fig. 3. Suppression of CTBP1-AS2 retarded the Ang II-induced cardiomyocyte hypertrophy. (A–B) RT-qPCR and Western blot were carried out to probe the effect of CTBP1-AS2 on the mRNA and protein levels of cardiomyocyte hypertrophy-related proteins (ANP, BNP, and β-MHC). (C–D) Cell surface area was measured with immunofluorescence under the knockdown of CTBP1-AS2 in Ang II treated NRVMs cells. (E) Protein/DNA ratio was evaluated with BCA method and fluorescence assay with the silencing of CTBP1-AS2 in Ang II treated NRVMs cells. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$.

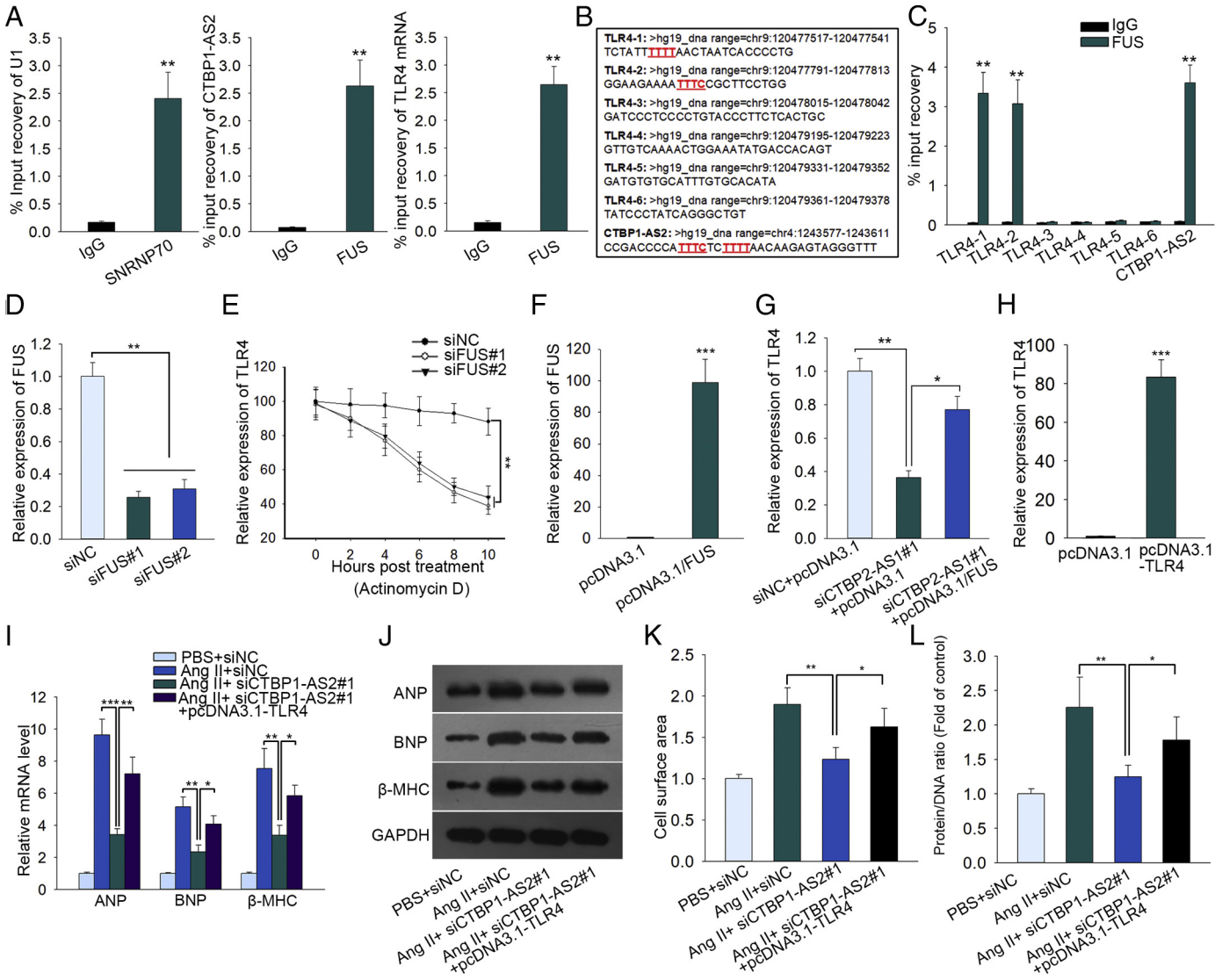

Fig. 4. CTBP1-AS2 promoted cardiomyocyte hypertrophy through recruiting FUS to stabilize TLR4. (A) RIP assay confirmed that CTBP1-AS2 and TLR4 could both interact with FUS. The interaction of U1 and SNRNP7 was positive control. (B) The predicted binding sites on TLR4 mRNA and CTBP1-AS2 for FUS. (C) RIP assay confirmed the specific binding sites on TLR4 mRNA and CTBP1-AS2 for FUS. (D) The knockdown efficiency of FUS was confirmed by RT-qPCR. (E) RT-qPCR after actinomycin D treatment showed that silencing FUS impaired mRNA stability of TLR4. (F) The overexpression efficiency of FUS was confirmed by RT-qPCR. (G) RT-qPCR results showed that overexpressing FUS rescued the inhibitive effect of silencing CTBP1-AS2 on TLR4 expression. (H) Transfection of pcDNA3.1-TLR4 into NRVMs cells was examined by RT-qPCR. (I-J) mRNA and protein levels of ANP, BNP, and β-MHC were evaluated by RT-qPCR and Western blot in Ang II-treated NRVMs transfected with siNC, siCTBP1-AS2, or siCTBP1-AS2 plus pcDNA3.1-TLR4, with PBS-treated NRVMs transfected with siNC as control. (K) Cell surface area was determined in Ang II-treated NRVMs transfected with siNC, siCTBP1-AS2, or siCTBP1-AS2 plus pcDNA3.1-TLR4, with PBS-treated NRVMs cells transfected with siNC as control. (L) Protein/DNA ratio was assessed in Ang II-treated NRVMs cells transfected with siNC, siCTBP1-AS2, or siCTBP1-AS2 plus pcDNA3.1-TLR4, with PBS-treated NRVMs transfected with siNC as control. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$.

surface area (Fig. 3C–D). Concordantly, inhibiting CTBP1-AS2 expression reversed the effect of Ang II on increasing protein/DNA ratio in NRVMs (Fig. 3E). Collectively, results suggested that knockdown of CTBP1-AS2 attenuated the Ang II-induced cardiac hypertrophy.

3.4. CTBP1-AS2 stabilized TLR4 by recruiting FUS to promote cardiomyocyte hypertrophy

Furthermore, we explored the mechanism whereby CTBP1-AS2 stabilized TLR4. By browsing Starbase (http://starbase.sysu.edu.cn/index.php), we discovered that FUS was a shared RNA binding protein (RBP) for CTBP1-AS2 and TLR4. FUS is known to function as an mRNA stabilizer [11,12]. Therefore, we speculated that CTBP1-AS2 could regulate TLR4 mRNA stability through FUS. RIP assay revealed that CTBP1-AS2 and TLR4 mRNA were abundant in the precipitates of FUS antibody (Fig. 4A). Furthermore, combining the Starbase data and mRNA motif of FUS, six putative binding sites on TLR4 mRNA and one on CTBP1-AS2 for FUS were identified (Fig. 4B). We designed the primers according to these binding sites and conducted RIP assay. It was confirmed that TLR4-1 and TLR4-2 was the responsible binding sites for FUS and that CTBP1-AS2 interacted with FUS at the predicted sites (Fig. 4C). Then, we silenced FUS to evaluate its influence on TLR4 mRNA stability (Fig. 4D). Results showed that the half-life of TLR4 mRNA was also shortened by knocking down FUS (Fig. 4E). Finally, the overexpression of FUS was confirmed by RT-qPCR (Fig. 4F), and we validated that overexpressing FUS impaired the inhibitive effect of CTBP1-AS2 on TLR4 expression (Fig. 4G).

Then, we investigated whether TLR4 was involved in the regulation of CTBP1-AS2 on cardiomyocyte hypertrophy. Rescue assays were performed with the overexpression of TLR4 in NRVMs (Fig. 4H). In Ang II-treated NRVMs, overexpressing TLR4 countervailed the inhibitive effect of siCTBP1-AS2#1 on mRNA and protein levels of ANP, BNP, and β-MHC (Fig. 4I–J). Additionally, the inhibitive effect of CTBP1-AS2 silencing on cell surface area and protein/DNA ratio was impaired by the co-transfection of pcDNA3.1-TLR4 in NRVMs with Ang II treatment

A

Matrix ID	Name	Score	Relative score	Sequence ID	Start	End	Strand	Predicted sequence
MA0079.3	SP1	17.3958	0.999999996047	5'	1570	1580	+	GCCCCGCCCCC

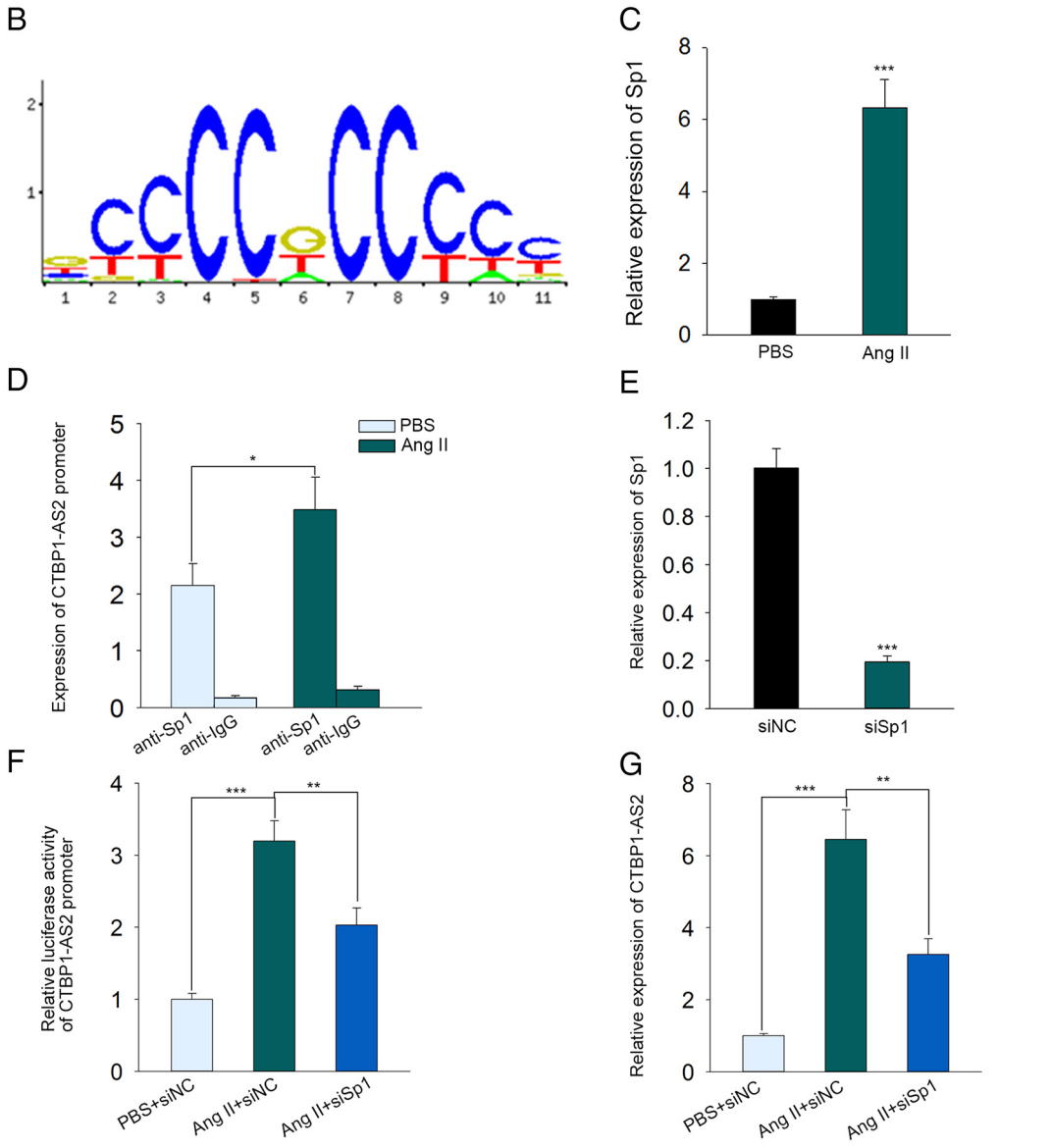

Fig. 5. Sp1 induced the expression of CTBP1-AS2 in cardiomyocyte hypertrophy. (A–B) The binding motif of Sp1 and binding sites on the CTBP1-AS2 promoter for Sp1 were obtained from JASPAR tool. (C) The significant up-regulation of Sp1 in Ang II-induced hypertrophy cardiomyocytes was confirmed by RT-qPCR. (D) ChIP assay was performed to investigate the interaction between Sp1 and CTBP1-AS2 promoter. (E) The knockdown efficiency of Sp1 was examined by RT-qPCR. (F) Luciferase reporter assay was used to evaluate the effect of Sp1 on CTBP1-AS2 promoter. (G) RT-qPCR was carried out to probe the effect of Sp1 on CTBP1-AS2 expression. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$.

(Fig. 4K–L). Jointly, data above indicated that CTBP1-AS2 stabilized TLR4 through recruiting FUS and regulated cardiomyocyte hypertrophy through TLR4.

3.5. Sp1 induced the expression of CTBP1-AS2 in cardiomyocyte hypertrophy

Finally, we explored the upstream mechanism underlying the activation of CTBP1-AS2. The induction of lncRNA via transcription factors has been largely reported in various diseases and cancers [15,16]. Sp1 is a typical transcription factor identified to transactivate numerous lncRNAs [17,18]. Therefore, we assumed that CTBP1-AS2 could be activated by Sp1 in cardiomyocyte hypertrophy. We first identified the binding potential between Sp1 and CTBP1-AS2 promoter with the aid of JASPAR tool (http://jaspar.genereg.net/). The binding sites on CTBP1-AS2 promoter and binding motif of Sp1 were presented in Fig. 5A–B. Moreover, we found that Sp1 was significantly up-regulated in Ang II-induced hypertrophy cardiomyocytes (Fig. 5C), indicating that Sp1 might be responsible for the upregulation of CTBP1-AS2 in cardiomyocyte hypertrophy. ChIP assay followed by RT-qPCR analysis confirmed the binding of Sp1 to CTBP1-AS2 promoter and that their interaction could be facilitated by Ang II treatment (Fig. 5D). To further assess the effect of Sp1 on CTBP1-AS2, we knocked down Sp1 in NRVMs (Fig. 5E). It was validated that silencing Sp1 abrogated the inductive of Ang II treatment on the luciferase activity of CTBP1-AS2 promoter (Fig. 5F). Furthermore, the expression level of CTBP1-AS2 stimulated

by Ang II was reversed by of Sp1 knockdown (Fig. 5G). In conclusion, these results implied that Sp1 induced the expression of CTBP1-AS2 in cardiomyocyte hypertrophy.

4. Discussion

Cardiomyocyte hypertrophy occurs when heart adapts to the accumulated cardiac burden, characterized by enlargement of cell size and facilitated protein synthesis [1,2]. It has been noted that various types of prolonged hypertrophic burdens are responsible for cardiac hypertrophy [3,4], and long-term hypotrophy indicates a higher risk of heart failure or even sudden death [1,4–6]. Therefore, further molecular study is of necessity for improving the treatment of cardiomyocyte hypertrophy.

Long noncoding RNAs have long been studied in human cancers and diseases [19–22]. Emerging evidence has proved the significant regulatory roles of lncRNAs in cardiomyocyte hypertrophy [23–26]. CTBP1-AS2 was discovered to serve oncogenic role in papillary thyroid cancer [27]. Nevertheless, its function in cardiomyocyte hypertrophy is yet to be illustrated. The present study found through GETx database that CTBP1-AS2 was at low expression level in normal heart tissues. Then, we established cardiac hypertrophy models in mice through TAC surgery and the in vitro cardiac hypertrophy model through Ang II treatment in NRVMs. We firstly discovered that CTBP1-AS2 was up-regulated in cardiomyocyte hypertrophy mice tissues and cells. Loss-of-function assays suggested that silencing CTBP1-AS2 could attenuate the cardiomyocyte hypertrophy induced by Ang II.

The regulation on lncRNA expression by transcription factors has been increasingly reported during recent years [15,16]. Sp1 is a typical transcription factor largely studied in human diseases [17,18], including in cardiomyocyte hypertrophy [35,36]. Herein, we firstly revealed that the interaction between Sp1 and CTBP1-AS2 promoter was enhanced in Ang II-induced cardiomyocyte hypertrophy, which induced the expression of CTBP1-AS2.

Toll-like receptor 4 (TLR4), an inflammation-related protein [28,29], is unveiled to promote cardiac hypertrophy [30–32]. Accordingly, we confirmed TLR4 up-regulation in cardiomyocyte hypertrophy tissues and cells. Also, we firstly showed the positive relation between CTBP1-AS2 and TLR4 expression in cardiomyocyte hypertrophy tissues. Mechanically, we uncovered that FUS, known to exert mRNA stabilizing function in cytoplasm [11,12], was a shared RNA binding protein for CTBP1-AS2 and TLR4, and proved that CTBP1-AS2 interacted with FUS to stabilize TLR4 mRNA. Previous studies have revealed the stabilization of mRNAs by lncRNAs through interacting with FUS in several human diseases [13,14], but our work was the first to uncover the FUS-dependent mRNA stabilization by CTBP1-AS2 in cardiac hypertrophy.

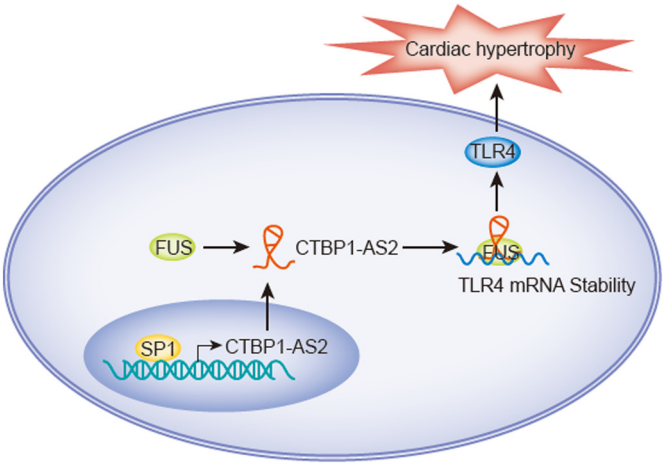

Fig. 6. Scientific schematic. Sp1-induced lncRNA CTBP1-AS2 is a novel regulator in cardiomyocyte hypertrophy by interacting with FUS to stabilize TLR4.

Finally, rescue assays proved that CTBP1-AS2 could regulate cardiomyocyte hypertrophy through TLR4.

In sum, the present study revealed that CTBP1-AS2 was induced by SP1 and was a novel regulator in cardiomyocyte hypertrophy by interacting with FUS to stabilize TLR4 (Fig. 6), casting a new light on the identification of treatment target for cardiomyocyte hypertrophy. However, this study has limitations since the function and mechanism of CTBP1-AS2 were only confirmed in in vitro models, so more in vivo studies are needed in the future for further confirmation of the role of CTBP1-AS2 in cardiac hypertrophy.

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Authors' contributions

Xiaojia Luo was responsible for the article writing, project design, and data collection. Sen He, Yongmei Hu, and Jianxiong Liu contributed to data analysis and result record. Xiaoping Chen prepared the figures.

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