Indian Journal of Biochemistry & Biophysics
Vol. 62, January 2025, pp. 7-16
DOI: 10.56042/ijbb.v62i1.12103

CircHelz inhibition protects against angiotensin II-induced cardiac fibrosis *via* miR-29b modulation

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Received 27 June 2024; *revised 22 July 2024*

Cardiac fibrosis is a critical pathological process underlying numerous cardiovascular diseases and contributes to heart failure and other severe complications. Circular RNAs (circRNAs) have been established as functional regulators of cardiovascular diseases, yet their specific roles in cardiac fibrosis remain unclear. This study was developed to explore the CircHelz/miR-29b regulatory axis in angiotensin II (Ang II)-induced cardiac fibrosis. CircHelz levels in Ang II-induced cardiac fibroblasts (CFs) were detected by qPCR, and siRNA was employed to disrupt its expression. The functional roles of CircHelz were explored through 5-Ethynyl-2′-deoxyuridine (EdU) uptake, Transwell, and immunofluorescence assays. Bioinformatics and dual-luciferase reporter assays were used to identify and verify CircHelz binding partners. Following Ang II stimulation and CircHelz silencing of mouse CFs, the cells were transfected with anmiR-29b-inhibitor, and the levels of fibrosis-related indicators, cytokines, and adhesion factors were assessed by Western blotting and qPCR. The results showed increased CircHelz expression after Ang II treatment. Mechanistically, CircHelz was identified as an endogenous sponge capable of targeting miR-29b and thereby interfering with its expression. CircHelz silencing promoted proliferation, migration and α-SMA expression in CFs by enhancing the activity of miR-29b, and reduced the expression levels of cardiac fibrosis related indicators. These findings support the ability of CircHelz to bind to miR-29b as a competing endogenous RNA, thereby promoting cardiac fibrosis. As such, CircHelz inhibition holds promise as a therapeutic strategy for Ang II-induced cardiac fibrosis.

Keywords: Angiotensin II, Cardiac fibroblasts, Cardiacfibrosis, CircHelz, miR-29b

Cardiacfibrosis is a pathological condition that arises due to the excessive proliferation of fibroblasts and their transformation into myofibroblasts[1,2]. When activated, myofibroblasts express higher levels of α-smooth muscle actin (α-SMA)[3], and drive the deposition of higher levels of structural components of the extracellular matrix (ECM), including type I and III collagen (Col I and Col III), the former of which is the most abundant cardiac ECM protein[4]. When the biogenesis of the ECM is excessive, this can compromise the normal architecture of the cardiac system, contributing to the progressive deterioration of normal cardiac function[5]. In the clinic, cardiac fibrosis has been identified as an important pathological basis for the incidence of many forms of cardiovascular disease[6]. There is thus an essential need for efforts to better clarify the molecular and cellular mechanisms that govern the incidence of cardiac fibrosis, as novel therapeutic targets have the potential to lead to better prognostic outcomes for patients with various cardiovascular diseases.

Circular RNAs (circRNAs) are endogenous transcripts that are present at high levels *in vivo* and that have recently been established as important regulators of gene expression[7]. These circRNAs often harbor binding sites for microRNAs (miRNAs) such that they can bind these miRNAs, inhibiting their functionality and thereby indirectly modulating translational activity[8], thus influencing diverse pathological and physiological processes. Several reports have documented a potential role for this competing endogenous RNA (ceRNA) mechanism as a process through which circRNAs regulate the pathology of cardiac fibrosis[9-11]. It has been reported that CircHelz, is associated with cardiac fibrosis,

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Abbreviations: Ang II, Angiotensin II; ceRNA, Competing endogenous RNA; CFs, Cardiac fibroblasts; circRNAs, Circular RNAs; Col I and Col III, Type I and III collagen; ECM, Extracellular matrix; miRNAs, MicroRNAs; α-SMA, α-smooth muscle actin

suggesting that it is capable of serving as a sponge for miR-133a-3p in ischemic murine heart tissue, thereby driving NLRP3 inflammasome activation and exacerbating myocardial injury[12]. In addition, reduced CircHelz expression can reportedly mitigate cardiac fibrosisby inhibiting the nuclear translocation of YAP1[13]. However, the mechanism by which CircHelz influences cardiac fibrosis poorly understood.

MicroRNAs (miRNAs) are an abundant class of small noncoding RNAs approximately 22 nucleotides long[14,15]. MiRNAs function as post-transcriptional negative regulators of the expression of most genes, thereby enabling them to regulate virtually all cellular processes to varying extents[16,17]. Indeed, miRNAs have been linked to many pathological conditions[18,19], including cardiac fibrosis[20]. For example, miR-29b is firmly established as an inhibitor of cardiac fibrosis that is capable of activating Notch signaling to inhibit ventricular remodeling[21]. Through the Smad3-miR-29b regulatory pathway, miR-29b is also capable of protecting against cardiac fibrosis[22]. The severity of Ang II-induced fibrosis was also alleviated by overexpressing miR-29b[23]. Research focused on miR-29b thus offers great promise as an approach to effectively preventing cardiac fibrosis. The regulatory relationships between miR-29b and potential circRNAs with ceRNA-like functions in the context of cardiac fibrosis, however, remain largely unknown.

This study aimed to determine whether CircHelz acts as a molecular sponge to regulate miR-29b,thereby influencing cardiac fibrosis. Our results ultimately demonstrated the ability of CircHelz to induce CF proliferation through reductions in miR-29b expression and activity, in turn driving indirect increases in Col I, Col III, and α-SMA expression. CircHelz silencing promoted the expression of miR-29b, while inhibiting that of fibrosis markers, cytokines, and adhesion molecules. Further targeted research focused on circRNA-mediated miR-29b regulation will thus highlight novel therapeutic targets for the more effective management of cardiac fibrosis.

Materials and Methods

Cell culture and modeling

Murine CFs(CP-M074;Pricella,Wuhan, China) were obtained from Wuhan Pricella Biotechnology Co., Ltd. and cultured in a 5% CO_2 incubator at 37°C in complete culture medium (CM-M074;Pricella) consisting of Dulbecco's Modified Eagle Medium (DMEM) containing 1% fibroblast growth supplement-2, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. Fibrosis modeling was achieved by treating these cells with 1 μM angiotensin II (Ang II; Sigma, MO, USA) for 24 h.

Cell transfection

Murine CFs from passage 3 were used for all transfection experiments. Customized CircHelz-specific siRNAs (si-CircHelz-1, si-CircHelz-2 and si-CircHelz-3), negative control (scrambled siRNA, si-NC), miR-29b inhibitor, NC inhibitor, miR-29b mimic, and NC mimic were purchased from GenePharma Co., Ltd. (Shanghai, China). The sequences were as follows: si-CircHelz-1, 5′-GCCCATAAGTACTATTAAT-3′; si-CircHelz-2, 5′-GAGGCTAUGTCTCATTTAT-3; and si-CircHelz-3, 5′-CAGGCCTAGAAATGCATAT-3′; si-NC, 5′-CTGCCTCATTTGTGCTTAA-3′; miR-29b inhibitor, 5′-CUGGUUUCACAUGGUGGCUUAGA UU-3′; NC inhibitor, 5′-GAUGGCAUUCGAU CAGUUCUA-3′; miR-29b mimic, 5′-UAGCACC AUUUGAAAUCAGUGUU-3′; and NC mimic, 5′-UCACCGGGUGUAAAUCAGCUUG-3′. CFs were transfected with 50 nM concentrations of these appropriate siRNAs, mimics, or inhibitors for 48h with Lipofectamine 3000 (Invitrogen, CA, USA) based on provided directions.

qPCR

The qPCR was conducted according to previous studys[24,25]. Specifically, TRIzol (TaKaRa, Beijing, China) was used to extract cellular RNA, followed by the use of the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa) to synthesize cDNA for circRNAs, while miRNA cDNA synthesis was achieved with a miRNA RT reagent Kit (Sangon Biotech, Shanghai, China) *via* stem-loop RT-PCR. The SYBR Premix Ex Taq (TaKaRa) was used as directed for qPCR analyses, with GAPDH orU6 as internal controls. Analyses were performed with the following primers: CircHelz, forward, 5′-GACAGAGTGGCTGTGTGG AG-3′, reverse, 5′-CTCACTGACTCACGGGGTTT-3′; α-SMA, forward, 5′-TGCTGGACTCTGGAGATG GTGTG-3′, reverse, 5′-CCGCAGTAGTCACGAA GGAATACC-3′; Coll I, forward, 5′-GCTCCTCTTA GGGGCCACT-3′, reverse, 5′-CCACGTCTCACCAT' TGGGG-3′; Coll III, forward, 5′-CTGTAACATGGA AACTGGGGAAA-3′, reverse, 5′-CCATAGCTGA ACTGAAAACCACC-3′; transforming growth factor-β1 (TGF-β1), forward, 5'-GGACCGCAACAACG

CCATCTATGAGA-3', reverse, 5′-CTTGGTTCA GCCACTGCCGTACAACT-3′; interleukin-6 (IL-6), forward, 5′-TCCTTCCTACCCCAATTTCC-3′, reverse, 5′-TCTTGGTCCTTAGCCACTCC-3′; intercellular Adhesion Molecule-1 (ICAM-1), forward, 5′-TTCACA CTGAATGCCAGCTC-3′, reverse, 5′-GTCTGCT GAGACCCCTCTTG-3′; vascular Cell Adhesion Molecule-1 (VCAM-1), forward, 5′-GACATTTAC CCAGTTTACAGGC-3′, reverse, 5′-TGACGGGAGT AAAGGTTACTTC-3′;GAPDH, 5′-CTTCTCCTGCA GCCTCGT-3′, reverse, 5′-GCCCAATACGGCCAA ATCAG-3′; miR-29b, forward, 5'-GCTGGTTTCAT ATGGTGGTTTA-3′, reverse, 5′-CTGGTTTCACATG GTGGCTTAGA-3′; U6, forward, 5′-CCTATTGGAG GCTCACTCACG-3′, reverse, 5′-GCTTGGTGGTACA CCTCTGTC-3′; U6, forward, 5′-CTCGCTTCGGCAG CACA-3′, reverse, 5′-AACGCTTCACGAATTTGCGT-3′. Relative expression levels were compared with the $2^{-\Delta\Delta CT}$ method, and all analyses were conducted in triplicate.

EdU uptake

A Click-iTEdU Alexa Fluor-594 Imaging Kit (Invitrogen) was used to assess murine CFs proliferation. Briefly, following the transfection of siRNAs or inhibitors, the CFs were transferred into 6-well plates and incubated for 48 h, followed by treatment with 1 μM Ang II for 24 h. Then, 100 μL of EdU was added to each well for an additional 2 h incubation at 37°C, followed by fixation using 4% paraformaldehyde, rinsing with BSA containing 3% glycine, and incubation at room temperature (25°C) in 0.5% TritonX-100 and 1x click-it reaction solution while protected from light. Cells were then counterstained with Hoechst 33342 in the dark for 20 min, rinsed thrice with PBS, and imaged *via* fluorescence microscopy.

Cell migration

Following treatment with Ang II for 24 h as above, 1×10^5 cells were added to the upper compartment of an insert with 8 μm pores (Millipore, MA, USA) in serum-free medium. The lower chamber was filled with media containing 10% FBS. Following 24 h incubation, methanol fixation and 0.1% crystal violet staining of cells that had migrated across the membrane was performed.

Immunofluorescence

CFs were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. After washing three times with phosphate-buffered saline (PBS),the cells were permeabilized with 0.2% Triton X-100 (PBST) for 20 min at room temperature, blocked for 1 h (PBST) using 10% goat serum in PBST, and incubated overnight in 10% goat serum containing 1:100 anti-α-SMA (1:100, Sigma, USA) at 4°C. A Cy3-labeled secondary antibody was then used for the immunostaining of these cells for 1 h, followed by DAPI nuclear counterstaining for 5 min and imaging with an Olympus fluorescence microscope.

Luciferase reporter assays

The full-length 3'-untranslated region (3'-UTR) sequence of CircHelzharboring wild-type (WT) or mutated (Mut) miR-29b binding sites was inserted into the pMIRGLO vector (Promega, WI, USA), after which all plasmids were co-transfected into CFs along with miR-29b mimic or NC mimic constructs using Lipofectamine 3000 (Invitrogen). Following a 48-h incubation, a Dual Glo Luciferase Assay System (Promega) was employed to quantify luciferase activity. These analyses were conducted in triplicate.

Western blotting analysis

Western blotting was conducted as per previous studies[26,27]. Briefly, RIPA lysis buffer supplemented with 1 mM PMSF (Sigma) was used to extract total CF protein, followed by the separation of these proteins (40 μg/sample) *via* SDS-PAGE. Proteins were electrophoretically separated, transferred onto PVDF membranes, and these blots were blocked at room temperature for 1 h using TBST containing 5% skim milk. Blots were next probed overnight with antibodies specific for α-SMA (1:1000, Sigma), Col I (1:1000, Cell Signaling Technology, MA, USA), Col III (1:1000, Cell Signaling Technology), or GAPDH (1:1000, Cell Signaling Technology) at 4°C, followed by three washes with TBST and incubation for 1 h at room temperature using horseradish peroxidase (HRP)-labeled anti-rabbit IgG (IgG; 1:5000, Cell Signaling Technology). An ECL chemiluminescence kit (Thermo Fisher, MA, USA) was then used for protein band detection, with densitometric quantification being performed with the Imagelab Software (Bio-Rad), using GAPDH as a loading control.

Statistical Analysis

Results are means ± SD and were compared with GraphPad Prism 5. Analyses were conducted in triplicate, and results were compared using Student's

t-tests or one-way ANOVA with Bonferroni's post-test. $P< 0.05$ served as the cut-off to define statistical significance.

Results

CircHelz knockdown significantly suppresses the activity of CFs

At a range of time points following Ang II treatment (0, 6, 12, 24, 48 h), qPCR analyses of CFs were conducted, revealing significantly higher levels of CircHelz at 24 which remained elevated at 48 h post-stimulation (Fig. 1A). Accordingly, CircHelz silencing was performed following a 24 h Ang II treatment interval, with the successful knockdown of this circRNA having been confirmed *via* qPCR (Fig. 1B). The si-CircHelz-3 that exhibited the best knockdown efficiency was used to all subsequent assays. The silencing of CircHelz resulted in a significant drop in CF proliferation as determined through an EdU uptake assay, effectively reversing the enhanced proliferation stimulated by Ang II (Fig. 1C & D). Transwell migration assays additionally demonstrated the ability of CircHelz knockdown to significantly suppress the increase in CF migratory activity detected in response to Ang II (Fig. 1E & F). Taken together, these results indicate that CircHelz expression was increased in Ang II-induced CFs, and its reduction could significantly inhibit the migration and proliferation of CFs.

CircHelz knockdown inhibites fibrosis in CFs

To clarify the effect of CircHelz on CF fibrosis, the expression of the fibrosis-related markers α-SMA, Col I, and Col III was determined by qPCR. The results showed that CircHelz silencing significantly reduced the mRNA expression levels of α-SMA, Col I, and Col III that had been raised by Ang II treatment (Fig. 2A-C). Furthermore, immunofluorescence demonstrated that the silencing of CircHelz was also sufficient to reverse Ang II-

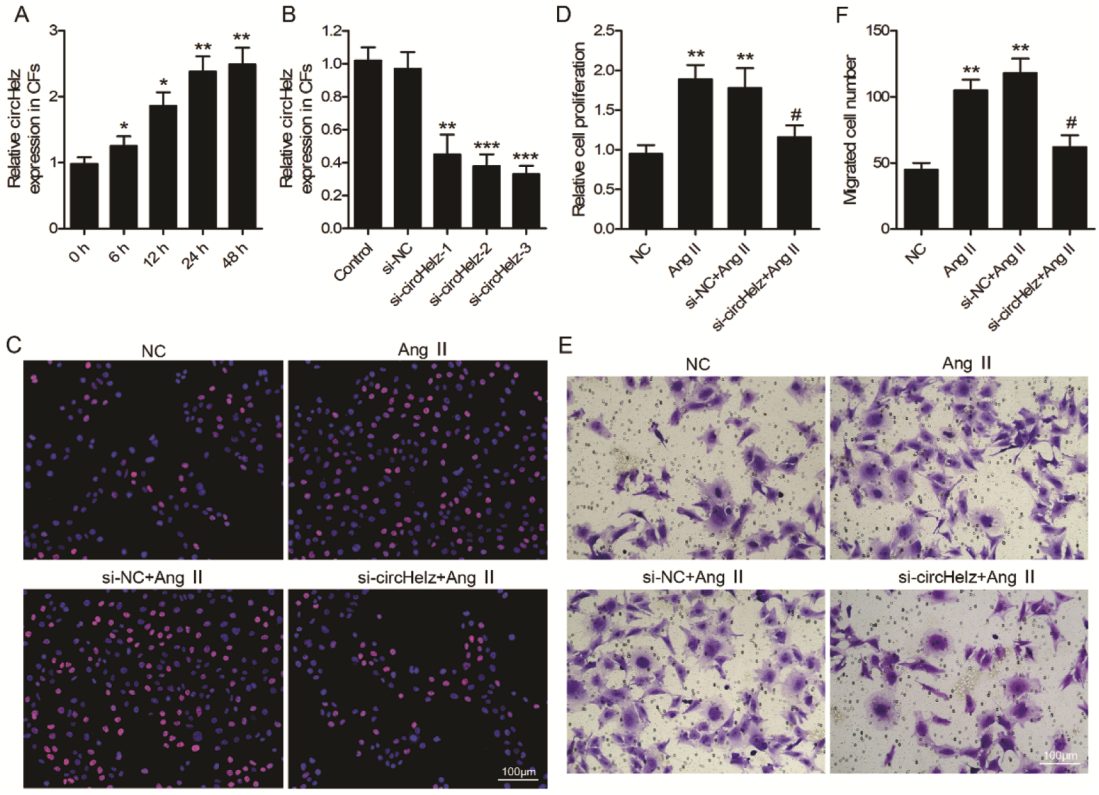

Fig. 1 — CircHelz knockdown suppresses the Ang II-induced proliferative and migratory activity of murine CFs. (A) CFs were stimulated with 1 μM Ang II for varyingtimes, and the expression of CircHelz was quantified by qPCR. n=3, *$P< 0.05$, **$P< 0.01$ *vs.* 0 μM Ang II; (B) CFs were transfected with CircHelz siRNAs (si-CircHelz-1, si-CircHelz-2 and si-CircHelz-3) and the negative control (scrambled siRNA, si-NC) for 48 h. qPCR assays were was used to measure CircHelz expression. n=3, **$P< 0.01$, ***$P< 0.001$ *vs.* si-NC; (C) Cell proliferation was assessed using EdU uptake assays. Scale bar: 100 μm; (D) Quantification of cell proliferation using EdU assay data; (E) Cell migration was assessed by Transwell assays. Scale bar: 100 μm; and (F) Quantification of cell migration using Transwell assay data. n=3, **$P< 0.01$ *vs.* NC; #$P< 0.05$ *vs.* Ang II

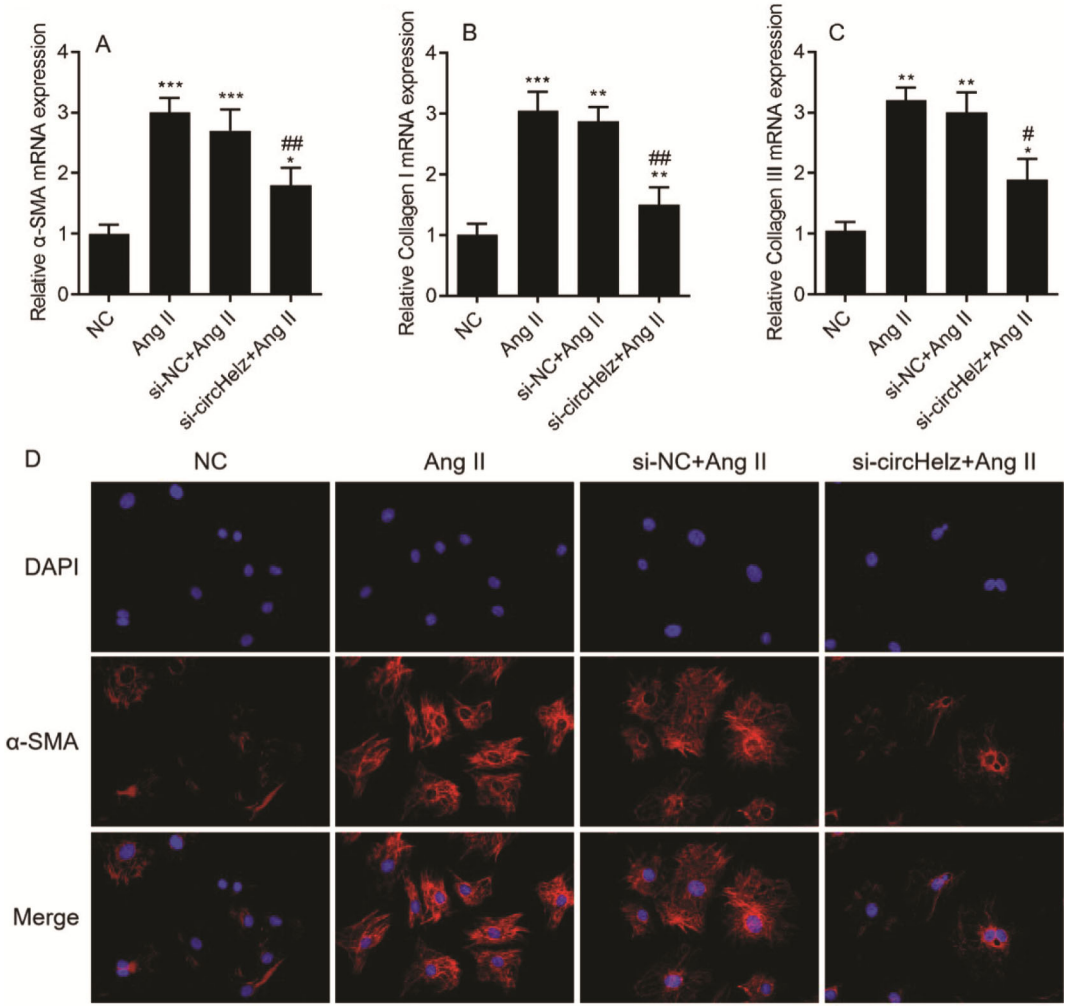

Fig. 2 — CircHelz knockdown inhibits Ang II-induced fibrotic activity in CFs. mRNA expression of (A) α-SMA; (B) collagen I; (C) collagen III. n=3,**P*< 0.05, ***P*< 0.01, ****P*< 0.001 *vs.* NC; #*P*< 0.05, ##*P*< 0.01 *vs.* Ang II; and (D)CFs transfected with si-CircHelz or si-NC were treated with 1 μm Ang II for 24 h. Representative immunofluorescence images of α-SMA. Red signals represent α-SMA protein and blue signals represent nuclei. Scale bar: 25 μm

driven increases in α-SMA expression (Fig. 2D). Together, these findings demonstrate the ability of CircHelz to significantly reduce the expression of fibrosis markers in CFs.

CircHelz directly targets miR-29b

We then explored whether CircHelz could function as a sponge for miRNA in CFs. Firstly, using bioinformatics methods to analyze potential interaction sites between CircHelz and miRNAs, it was found thatmiR-29b contained the greatest number of potential CircHelz binding sites (Fig. 3A). The qPCR results showed that miR-29b mimics and inhibitors were successfully transfected into CFs (Fig. 3B & C). Subsequent luciferase reporter assays validated the ability of miR-29b mimics to suppress the activity of luciferase reporter constructs harboring WT but not Mut versions of the CircHelz 3'-UTR, consistent with the ability of these two factors to directly interact (Fig. 3D). Moreover, qPCR analyses demonstrated that Ang II induced a down-regulation of miR-29b expression in CFs, whereas, CircHelz silencing spurred miR-29b up-regulation (Fig. 3E). As such, CircHelz may be capable of functioning as a ceRNA for miR-29b, thus influencing the pathogenesis of cardiac fibrosis.

CircHelz influences the phenotype of Ang II-induced CFs by regulating miR-29b

To further explore whether sponging between CircHelz and miR-29b regulated proliferation, migration, and fibrosis in CFs, rescue experiments

were performed in Ang II-induced CFs. EdU uptake (Fig. 4A & B) and Transwell migration (Fig. 4C & D) assays showed that silencing of CircHelz significantly inhibited Ang II-induced proliferation and migration in CFs, which could be reversed by the miR-29b inhibitor. Western blotting demonstrated that relative to si-NC+Ang II treatment, CircHelz knockdown was associated with significantly lower levels of Col I, Col III, and α-SMA expression. To gain additional insight into how miR-29b suppresses CF fibrotic phenotypes in this context, CFs in which CircHelz had been knocked down were treated with miR-29b inhibitors. Relative to CircHelz knockdown alone, the simultaneous knockdown of CircHelz and miR-29b could partially restore the protein levels of Col I, Col III, and α-SMA (Fig. 4E-H). In addition, qPCR showed that the miR-29b inhibitor increased the expression of cytokines TGF-β1 and IL-6, as well as that of the adhesion factors VCAM-1 and ICAM-1, which were inhibited by CircHelz silencing (Fig. 5A-D). These findings confirmed CircHelz as a ceRNA for miR-29bin CFs, influencing biological functions, and this regulatory relationship could assist in the elucidation of the pathogenesis of cardiac fibrosis.

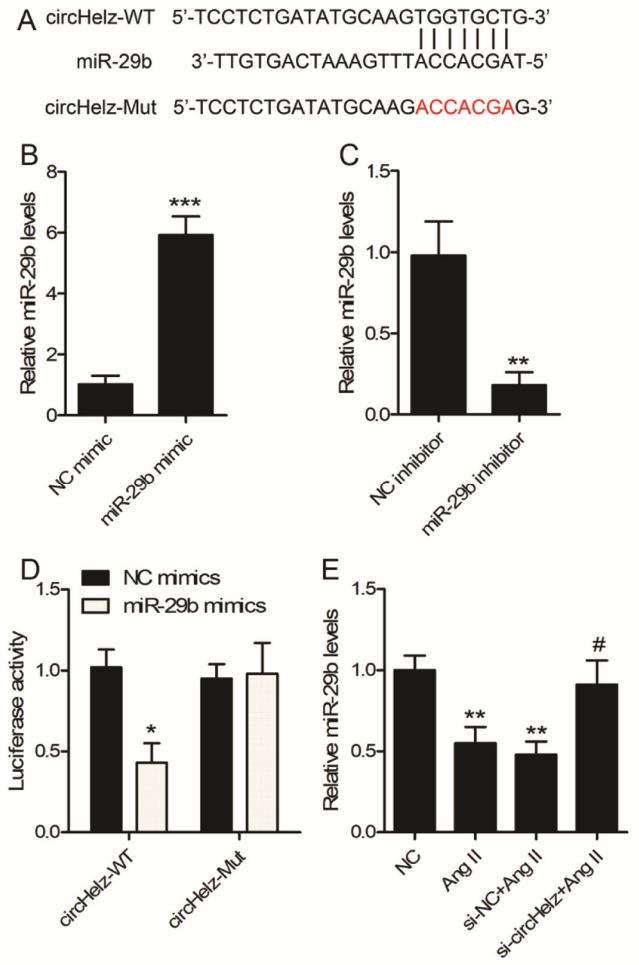

Fig. 3 — CircHelz targets miR-29b. (A) The identified binding site overlap between CircHelz and miR-29b; (B, C) qPCR was used to confirm miR-29b mimic or inhibitor transfection efficiency; (D) Luciferase reporter assays identified miR-29b as a direct target of CircHelz; and (E) qPCR was used to quantify miR-29b expression levels in CFs following si-CircHelz or si-NC transfection and treatment for 24 h in the presence of 1 μm Ang II. n=3, *P< 0.05, **P< 0.01, ***P< 0.001 *vs.* NC controls; #P< 0.05 *vs.* Ang II

Discussion

Here, the regulatory roles that CircHelz plays in the context of cardiac fibrosis were explored at the mechanistic level. CircHelz was up-regulated after Ang II treatment of CFs, and CircHelz silencing was sufficient to suppress CF migration and proliferation while down-regulating fibrosis-related protein expression owing to its status as a ceRNA that sequesters miR-29b. TGF-β1 is a major pro-fibrotic factor[28] that can cause cardiac fibroblasts to transform into myofibroblasts expressingα-SMA[29]. The inflammatory cytokine IL-6 plays a central role in cardiomyocyte hypertrophy and myocardial fibrosis[30], and can promote cardiac fibrosis. Furthermore, VCAM-1 and ICAM-1 promote leukocyte infiltration and inflammation, leading to which myocardial interstitial fibrosis[31]. Knockdown of CircHelz was found to down-regulated the expression of these cytokines and adhesion factors, potentially inhibiting the expression of related inflammatory mediators to alleviate myocardial fibrosis by regulating the expression of miR-29b. As such, CircHelz may offer utility as a novel target for therapeutic interventions designed to treat cardiac fibrosis.

In most known cases, circRNAs function as molecular sponges for miRNAs, thereby modulating their post-transcriptional activity[32]. Recent studies have suggested that circRNAs can play important regulatory roles in cardiovascular diseases, including cardiac fibrosis[33,34]. For example, circNFIB has been shown to act as a ceRNA for miR-433 such that miR-433 mimic-induced fibroblastic proliferation can be mitigated by circNFIB overexpression, thereby protecting against cardiac fibrosis[9]. In diabetic model mice, cardiac fibrosis has also been demonstrated to be regulated by the circRNA_010567/miR-141/TGF-β1 axis[10]. Associations between CircHelz and the pathogenesis of cardiac fibrosis have, to date, primarily been attributed to its ability to interact with certain proteins[13]. Ang II can stimulate cardiac

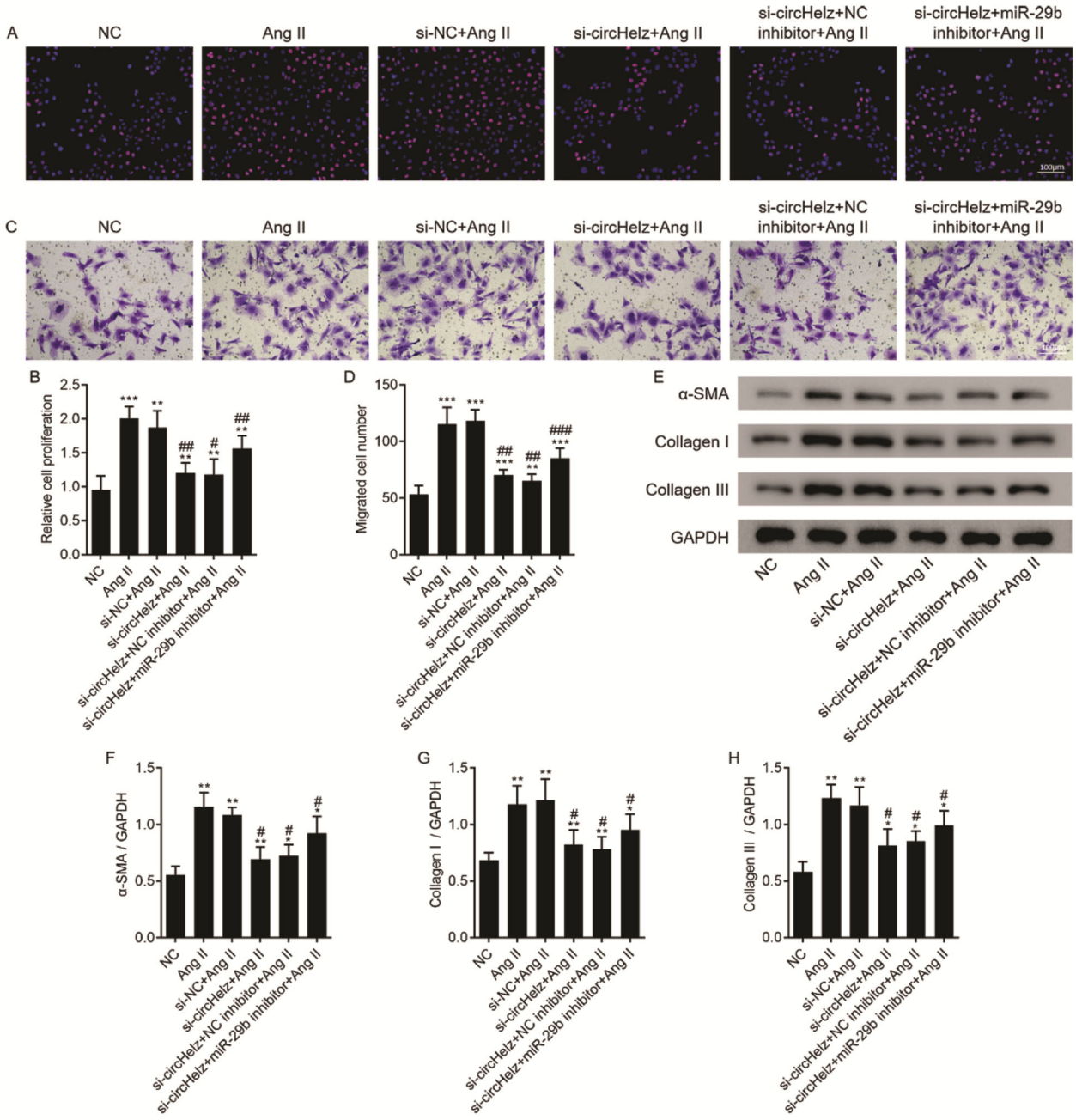

Fig. 4 — CircHelz knockdown inhibits Ang II-induced cardiac fibrosis in CFs *via* the upregulation of miR-29b. (A) Cell proliferation was measured using EdU uptake assays. Scale bar: 100 μm; (B) Quantification of cell proliferation using EdU assay data; (C) Cell migration was assessed using Transwell assays. Scale bar: 100 μm; (D) Quantification of cell migration using Transwell assay data; (E) Western blotting was used to analyze α-SMA, Col I, and Col III levels in these cells, with GAPDH as a loading control. Quantification of the protein levels of (F) α-SMA; (G) Col I; and (H) Col III. n=3, **P*< 0.05, ***P*< 0.01, ****P*< 0.001 *vs.* NC; #*P*< 0.05, ##*P*< 0.01, ###*P*< 0.001 *vs.* Ang II

fibrosis[35]; in the present study, CircHelz expression was found to be up-regulated in Ang II-induced CFs, while knockdown of CircHelz inhibited CF migration and proliferation, as well as the expression of fibrosis-related markers. Similar results have been reported when CircHelz was silenced in a model of myocardial infarction[13]. The inhibition of miR-29b was associated with changes in the fibrotic activity in CFs, albeit not fully revesing these changes to the basal levels detected prior to CircHelz silencing. As such,

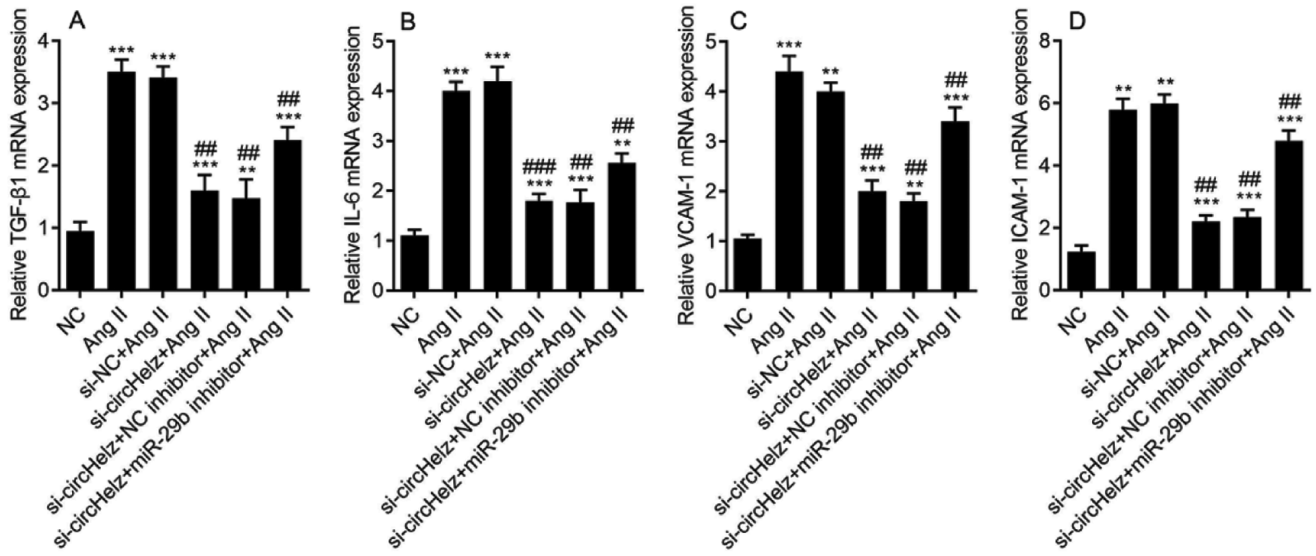

Fig. 5 — CircHelz knockdown inhibits Ang II-induced inflammation in CFs *via* upregulation of miR-29b. mRNA expression of (A) TGF-β1; (B) IL-6; (C) VCAM-1; and (D) ICAM-1. n = 3,***P*< 0.01, ****P*< 0.001 *vs.*NC; ##*P*< 0.01, ###*P*< 0.001 *vs.* Ang II

CircHelz may shape the pathogenesis of cardiac fibrosis by acting as a ceRNA for several miRNAs, as has been demonstrated previously for other circRNAs[36]. Indeed, Bian *et al.*[12] determined that CircHelz was capable of sequestering miR-133a-3p and thereby driving NLRP3 inflammasome activation, contributing to myocardial injury in a murine ischemic heart model system. The present study confirmed that CircHelz acted as its endogenous sponge by inhibiting miR-29b activity, and silencing of CircHelz alleviated Ang II-induced fibrosis in CFs by enhancing the function of miR-29b.

The miR-29 family is associated with both fibrosis and inflammation in human disease processes[37], the family contains three members, miR-29a/b/c[38]. MiR-29b has been reported to have significant therapeutic potential in diseases such as renal fibrosis[39], hepatic fibrosis[40], and pulmonary fibrosis[41]. Recently, miR-29b has been advanced as a promising cardiac fibrosis-related target[42]. Overexpression of miR-29b leads to reduced Col I and α-SMA expression *via* the targeting of SH2B3, thus alleviating cardiac functional impairment[43]. Silencing of miR-29b-3p was sufficient to abrogate the antifibrotic effects of sh-circHIPK3 *in vivo*[44]. Xue *et al.*[45] found that miR-29b-3p could reduce the TGF-β1-induced profibrotic effect by targeting FOS, thereby inhibiting cardiac fibrosis after infarction. In addition, miR-29b ameliorated a trial fibrosis in AF rats by targeting TGFβR1 and inhibiting the activation of the Smad-2/3 pathway[46], suggesting that it may serve as a therapeutic agent for cardiac fibrosis by targeting the TGF-β/Smad3 pathway[23]. These data indicate that miR-29b affects collagen deposition and cardiac fibrosis through multiple regulatory mechanisms. CircHelz may regulate the above signaling pathways through miR-29b, thereby participating in cardiac fibrosis. Given the identification of CircHelz as a sponge-like ceRNA for miR-29b, this circRNA appears to be capable of protecting against Ang II-induced cardiac fibrosis within CFs *via* the up-regulation of miR-29b.

These results should be interpreted in light of some limitations. First, no corresponding *in vivo* analyses of Ang II-induced murine CFs were conducted, so the detection of interactions between CircHelz and miR-29b *in vivo* has yet to be performed. Second, we did not investigate the downstream signaling pathways in depth. While we explored the regulatory role of CircHelz as a molecular sponge for miR-29b, further detailed studies are needed to elucidate the specific downstream signaling pathways involved. Moreover, the mechanism by which CircHelz regulates cardiac fibrosis is not yet fully understood. These circRNAs may act as molecular sponge-like ceRNAs that can sequester several miRNAs[8], but they are also capable of regulating transcription or binding to various RNA-binding proteins[47]. Given these diverse regulatory mechanisms, additional analyses of the mechanistic basis for the functions of CircHelz will be essential.

Conclusion

In conclusion, the study findings demonstrated that CircHelz can act as a ceRNA to bind miR-29b, thereby promoting cardiac fibrosis. CircHelz knockdown was found to inhibit Ang II-induced cardiac fibrosis in CFs through up-regulation of miR-29b.CircHelz inhibition thus holds promise as a therapeutic strategy for the prevention and management of Ang II-induced cardiac fibrosis.

Acknowledgement

This study was supported by the key projects in the field of medical and health in Xiangyang City in 2022 (2022YL50A).

Conflict of interest

All authors declare no conflicts of interest.

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