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Inhibition of circHIPK3 prevents angiotensin II-induced cardiac fibrosis by sponging miR-29b-3p

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ABSTRACT

Background: Circular RNAs (circRNAs) are emerging as powerful regulators of cardiac development and disease. Nevertheless, detailed studies describing circRNA-mediated regulation of cardiac fibroblasts (CFs) biology and their role in cardiac fibrosis remain limited.
Methods: PCR and Sanger sequencing were performed to identify the expression of circHIPK3 in CFs. Edu corporation assays, Transwell migration assays, and immunofluorescence staining assays were conducted to detect the function of circHIPK3 in CFs in vitro. Bioinformatics analysis, dual luciferase activity assays, RNA immunoprecipitation, and fluorescent in situ hybridization experiments were conducted to investigate the mechanism of circHIPK3-mediated cardiac fibrosis. Echocardiographic analysis, Sirius Red staining and immunofluorescence staining were performed to investigate the function of circHIPK3 in angiotensin II (Ang II) induced cardiac fibrosis in vivo.
Results: circHIPK3 expression markedly increased in CFs and heart tissues after the treatment of Ang II. circHIPK3 silencing attenuates CFs proliferation, migration and the upregulation of a-SMA expression levels induced by Ang II in vitro. circHIPK3 acted as a miR-29b-3p sponge and overexpression of circHIPK3 effectively reverses miR-29b-3p-induced inhibition of CFs proliferation and migration and alters the expression levels of miR-29b-3p targeting genes (a-SMA, COL1A1, COL3A1) in vitro. Combination of circHIPK3 silencing and miR-29b-3p overexpression had a stronger effect on cardiac fibrosis suppression in vivo than did circHIPK3 silencing or miR-29b-3p overexpression alone.
Conclusions: Our data suggest that circHIPK3 serves as a miR-29b-3p sponge to regulate CF proliferation, migration and development of cardiac fibrosis, revealing a potential new target for the prevention of Ang II-induced cardiac fibrosis.

1. Introduction

Cardiac fibrosis is a common pathological process across cardiovascular disease [1–3]. A critical event in cardiac fibrosis is the activation of cardiac fibroblasts (CFs), which proliferate, and differentiate to myofibroblasts [3–6]. Activated myofibroblasts express α-smooth muscle actin (α-SMA) and promote the formation of extracellular matrix (ECM) proteins, such as collagen types I and III [5,7]. In a healthy heart, the ECM undergoes a balanced turnover through synthesis and degradation of collagen proteins. However, excessive myofibroblasts are activated and an overabundance of ECM components is produced under pathological conditions of the heart, which ultimately lead to cardiac remodeling [4,8,9]. Identifying the cellular and molecular mechanisms that regulate cardiac fibrosis is critical for the development of novel therapeutic approaches for cardiac remodeling.

Circular RNAs (circRNAs) are an emerging class of noncoding RNAs that are generated by the back-splicing of a single pre-mRNA [10,11]. Next-generation sequencing has revealed that circRNAs are abundant and widespread in human and animal tissues, including within the cardiovascular system [12,13]. circRNAs have significantly longer half-lives and are more resistant to RNase R digestion than linear RNA due to their stable covalently closed loop structure. Moreover, circRNAs may sponge with RNA-binding proteins, such as the argonaute 2 protein(AGO2) [11]. Emerging evidence illustrates that circRNAs mediate gene expression and cell function by binding and inhibiting miRNAs [14,15]. circRNAs play an pivotal role in a vast range of developmental and physiological processes, including fibrosis, cell proliferation, migration and differentiation. Recently, circHIPK3 has been found to be abundant and widespread across several human and animals tissues [16]. circHIPK3 regulates cell growth and migration by sponging multiple miRNAs both in normal cell and cancer cells, including hepatocellular carcinoma [17], colorectal cancer [18], retinal vascular [19], and lens

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epithelial cells [20]. However, little is known about the function of circHIPK3 in the heart.

MicroRNAs (miRNAs) are small RNA molecules that contain 18–25 nucleotides [21]. miRNAs control gene expression levels either by inhibiting gene translation or by facilitating the degradation of mRNA [21,22]. Many experimental and clinical studies have revealed that miRNAs participates in the development of various cardiac disorders, which subsequently leads to cardiac remodeling [9,23]. For example, miR133 [24], miR-29 [25], miR-338 [26] and miR-30 [27] serve as suppressive factor in cardiac fibrosis. However, miR-21 [28], miR-122 [29] and miR-499 [30] promote cardiac fibrosis. However, whether circHIPK3 can act as a "miRNA sponge" or not in cardiac fibrosis is remains unknown.

In this study, we show that circHIPK3 is abundant and markedly upregulated in CFs and heart tissues treated with Ang II. circHIPK3 silencing attenuated CF proliferation, migration and the upregulation of a-SMA expression levels induced by Ang II in vitro. At the molecular level, the circHIPK3 acts as a sponge toward miR-29b-3p. Furthermore, overexpression of circHIPK3 effectively reverses miR-29b-3p-induced inhibition of CF function and influences the expression levels of miR-29b-3p targeting genes (a-SMA, COL1A1, COL3A1) in vitro. Importantly, the combination of circHIPK3 silencing and miR-29b-3p overexpression had a stronger effect on cardiac fibrosis suppression in vivo than did circHIPK3 silencing or miR-7 overexpression alone. Our findings indicate that circHIPK3 serves as a miR-29b-3p sponge to regulate CF proliferation, and migration and the development of cardiac fibrosis.

2. Methods

Methods are available in the online-only Data supplement.

3. Results

3.1. Identification and validation of circRNAs

Recent studies showed that circHIPK3 is particularly abundant in the hearts of mice, rats and humans [12]. CircBase retrieval revealed that circHIPK3 (mmu_circ_0001052) is derived from the HIPK3 gene Exon2 (Supplementary Fig. 2A). The full sequence of circHIPK3 is listed in the Supplementary material (Supplementary Fig. 1A–B). Gel electrophoresis verified PCR product of circHIPK3 and sanger sequencing experiments further confirmed the head-to-tail splicing of this product (Fig. 1A). We then investigated the location of circHIPK3 expression in tissues and cells. The circhipk3 expression levels in various tissues (heart, liver, spleen, lung, kidney, brain) were analyzed using qRT-PCR with divergent primers. We noted that circHIPK3 was abundant in most tissues (heart, spleen, lung, kidney, brain) but not in the liver (Supplementary Fig. 2B). Fluorescence in situ hybridization (FISH) assays revealed that circHIPK3 is mainly localized in the cytoplasm (Fig. 1D). qRT-PCR analysis of nuclear and cytoplasmic RNAs further showed that circHIPK3 was mainly expressed in the cytoplasm of CFs (Supplementary Fig. 2F). Next, we investigated the stability of circHIPK3 in CFs. Total RNA was extracted at various time points (0, 6, 12, 18, and 24 h) after treatment with Actinomycin D. qRT-PCR analysis revealed that HIPK3 mRNA was easily degraded with a half-life of 6 h, while circHIPK3 was highly stable with a half-life exceeding 24 h. (Supplementary Fig. 2E). Similarly, it was confirmed that circHIPK3 was resistant to digestion with RNase R, while HIPK3 mRNA was easily degraded (Supplementary Fig. 2C–D).

The expression of circHIPK3 in CFs showed an significant increase at 24 h and remained elevated until 48 h after the treatment of both TGF-β and Ang II (Fig. 1B). FISH further confirmed that circHIPK3 expression

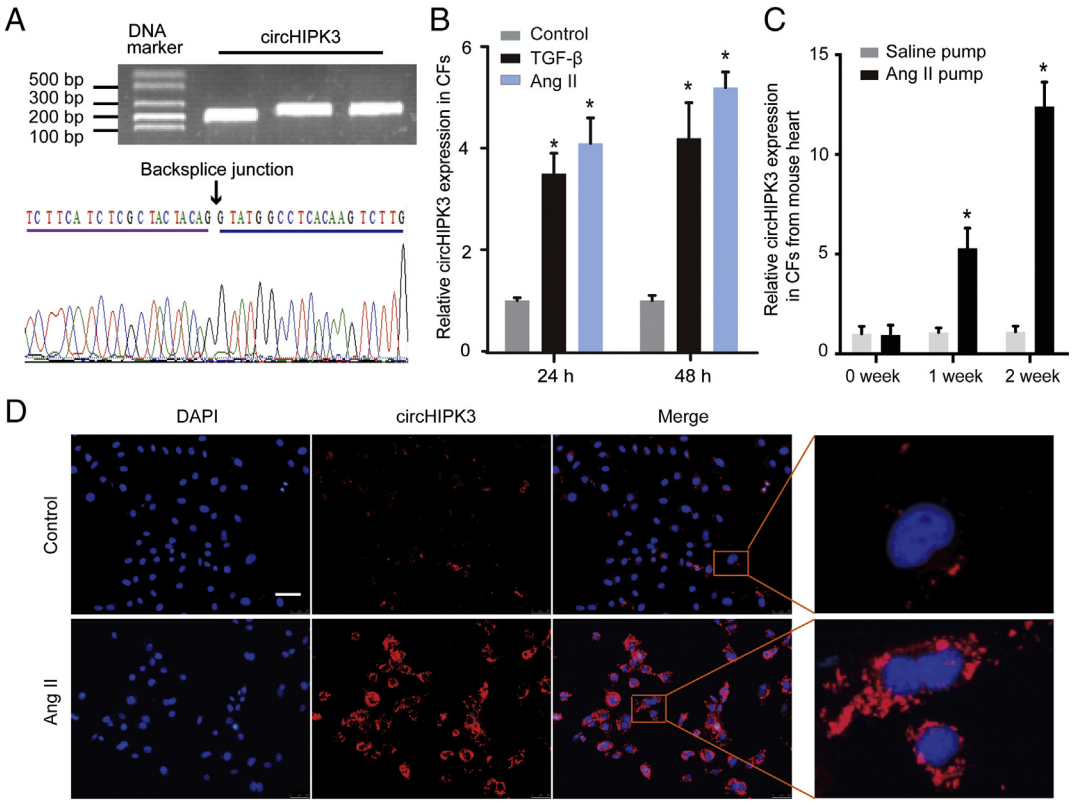


Fig. 1. Ang II upregulated the expression of circHIPK3 in CFs and mouse heart tissues. (A) The expression of circHIPK3 was validated by PCR followed by agarose gel electrophoresis and Sanger sequencing. Arrows represent divergent primers binding to the genomic region of circHIPK3. (B) qRT–PCR assays were performed to detect the abundance of circHIPK3 in CFs after Ang II (10 mmol/mL) and TGF-β (5 ng/mL) treatment at 24 h and 48 h. (C) qRT–PCR assays were conducted to detect abundance of circHIPK3 in CFs from mouse heart tissues after Ang II pump and or saline pump treatment at 0, 1 and 2 weeks. (D) FISH was conducted to indicate the location and abundance of circHIPK3 in CFs after Ang II (10 mmol/mL) or PBS (Control) treatment at 48 h. Scale bar = 50 μm. Data are expressed as the means ± SDs from three independent experiments.

markedly increased in CFs after the treatment of Ang II at 48 h (Fig. 1D). Furthermore, qRT-PCR showed that the expression of circHIPK3 significantly upregulated in CFs from mouse heart tissues in Ang II pump group compared with Saline pump group (Fig. 1C).

3.2. circHIPK3 silencing suppresses CFs proliferation, migration and cardiac fibrosis induced by Ang II in vitro

Because Ang II significantly affected circHIPK3 expression in CFs, we aimed to clarify whether circHIPK3 was involved in this process. Cell proliferation and migration and a-SMA expression levels were evaluated following a 48 h treatment with Ang II (1.0 μmol/L) in cells transfected with si-HIPK3 and si-circHIPK3 to further determine the functional effects of circHIPK3 silencing. After 48 h, qRT-PCR analysis showed that si-circHIPK3 transfection significantly downregulated circHIPK3 expression, while HIPK3 mRNA expression was not affected (Fig. 2A).

The EdU assay showed that circHIPK3 silencing significantly suppresses the proliferation of CFs and effectively reverses the upregulation of proliferation induced by Ang II, but there was no difference in

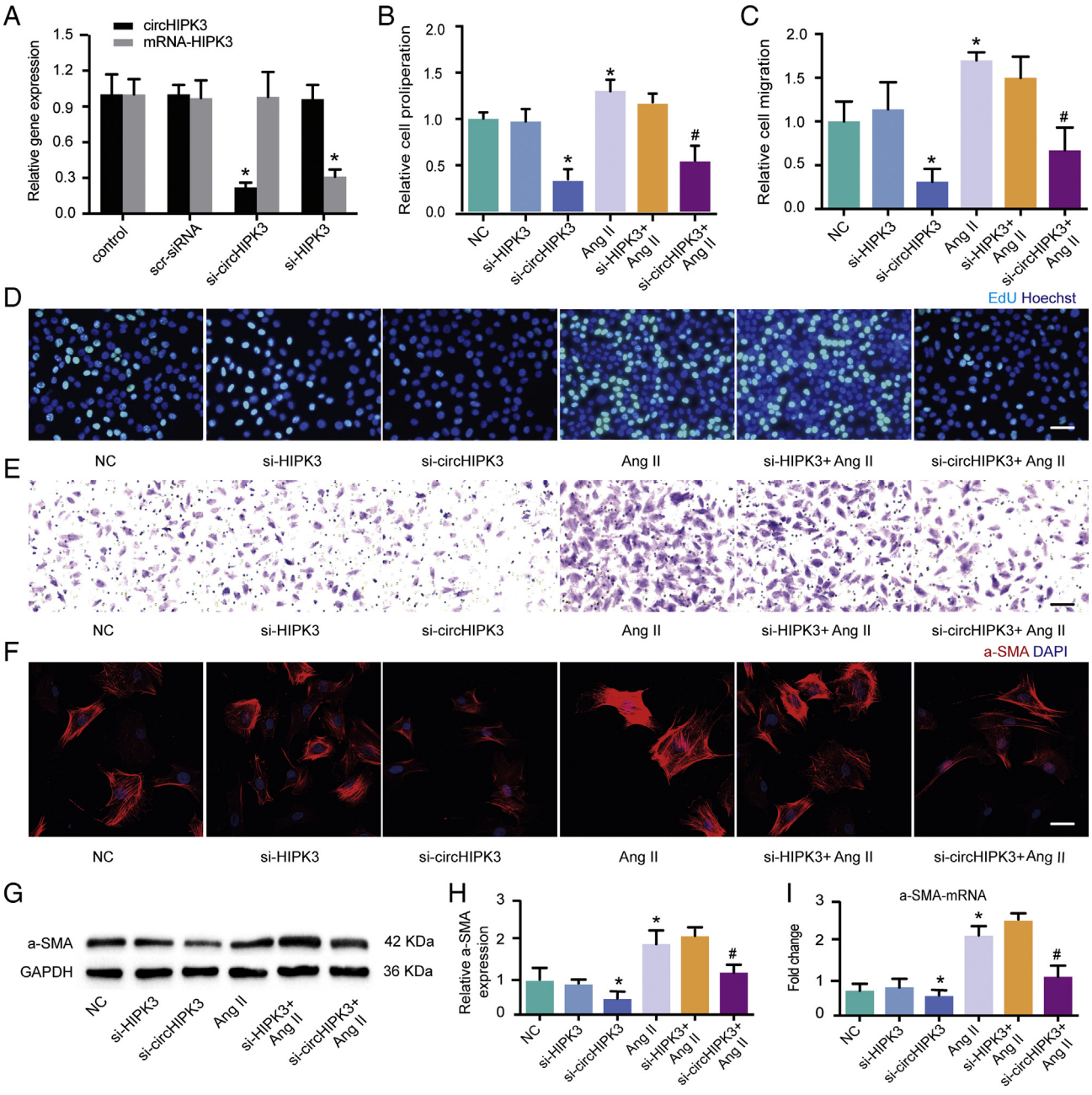


Fig. 2. circHIPK3 silencing suppresses CFs proliferation, migration and cardiac fibrosis induced by Ang II in vitro. CFs were transfected with scrambled (Scr) siRNA, siRNA targeting the backsplice sequence of circHIPK3 (si-circHIPK3), or siRNA targeting the sequence only existing in HIPK3 linear transcript (si-HIPK3) with or without Ang II (10 mmol/mL) treatment for 48 h. (A) CFs were transfected with scrambled (Scr) siRNA, si-circHIPK3, or si-HIPK3 for 48 h. qRT-PCR assays were performed to detect circHIPK3 and mRNA-HIPK3 expression. (B) Quantification analysis of cell proliferation using EdU assay data. (C) Quantification analysis of cell migration using transwell assay data. (D) Cell proliferation was detected using the EdU incorporation assay. Scale bar = 100 μm. (E) Cell migration was detected using the transwell assay. Scale bar = 100 μm. (F) Representative immunofluorescence images of a-SMA. Red signals represent a-SMA protein and blue signals represent nuclei. Scale bar = 25 μm. (G) a-SMA expression levels were detected by western blot analysis. GAPDH was used as an internal loading control. (H) Quantitative analysis of a-SMA protein expression. (I) qRT-PCR assays were performed to detect a-SMA mRNA expression levels. Data are expressed as the means ± SDs from three independent experiments.*$p < 0.01$ vs. control; #$p < 0.01$ vs. Ang II.

cells transfected with si-HIPK3 (Fig. 2B, D). Transwell migration assays also revealed that circHIPK3 silencing significantly reduces the migration of CFs and effectively reverses the upregulation of migration induced by Ang II (Fig. 2C, E). The expression of a-SMA was significantly downregulated in CFs transfected with si-circHIPK3 but upregulated in cells after Ang II treatment (Fig. 2F). Furthermore, western blot and qRT-PCR analysis showed that circHIPK3 silencing effectively reversed the upregulation of a-SMA expression levels induced by Ang II (Fig. 2G–I).

3.3. circHIPK3 acts as a miR-29b-3p sponge in CFs

To determine whether circHIPK3 can serves as a binding platform for AGO2 and miRNA, we conducted RNA immunoprecipitation assays

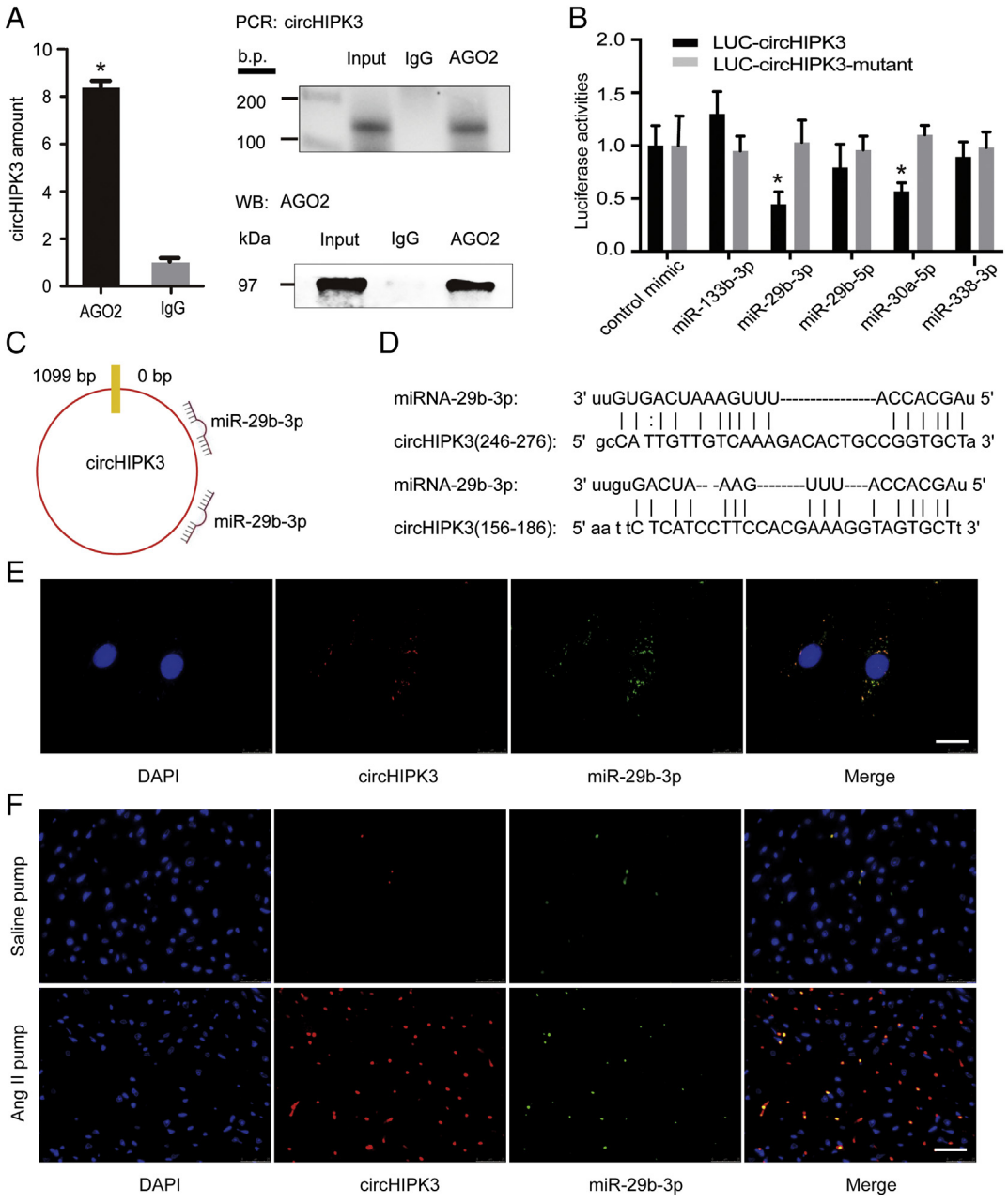


Fig. 3. circHIPK3 acts as a miR-29b-3p sponge in CFs. (A) Ago2 RNA immunoprecipitation (RIP) assays were performed to immunoprecipitate circHIPK3 in CFs using Ago2 or IgG antibodies, and the circHIPK3 levels in the immunoprecipitate were detected by qRT-PCR assays. PCR detection of immunoprecipitated circHIPK3 and western blot detection of immunoprecipitated AGO2 protein are shown. *$p < 0.01$ vs. IgG. (B) Luciferase reporter assays were conducted to detect the luciferase activity of LUC-circHIPK3 or LUC-circHIPK3-mutant in HEK-293 T cells transfected with six miRNA mimics to identify miRNAs that were able to bind to the circHIPK3 sequence. *$p < 0.01$ vs. LUC-circHIPK3-mutant. (C) circHIPK3 contains two sites that are complementary to miR-29b-3p, as determined by the bioinformatics analysis. (D) Schematic representation of the target sites in circHIPK3 for miR-29b-3p. (E) FISH was performed to colocalize circHIK3 (red) and miR-29b-3p (green) in CFs using probes. The circHIPK3 probe was labeled with cy-3, and the miR-29b-3p probe was labeled with FAM. Scale bar = 25 μm. (F) FISH was performed to colocalize circHIK3 (red) and miR-29b-3p (green) in mouse heart tissues after Ang II pump and or saline pump treatment for 2 weeks. Data are expressed as the means ± SDs from three independent experiments.

in CFs. Total RNA was pulled down by magnetic beads conjugated with anti-AGO2 or anti-IgG. qRT-PCR analysis showed circHIPK3 was specifically abundant in the immunoprecipitate pulled down by anti-AGO2 but not anti-IgG (Fig. 3A). To identify the miRNAs that interact with circHIPK3 in CFs, we selected miR-133b-3p, miR-29b-3p, miR-29b-5p, miR-30a-5p, and miR-338-3p according to the bioinformatics prediction analysis. Next, each miRNA mimic was cotransfected with the pmirGLO-circHIPK3 or pmirGLO-circHIPK3-mutant into HEK-293 T cells and a dual luciferase reporter assay was carried out after 48 h. Compared to the control mimic group, miR-29b-3p significantly reduced the luciferase reporter activities of pmirGLO-circHIPK3 but did not affect the luciferase activities of the mutant vectors (Fig. 3B).

Bioinformatics analysis was carried out to determine the binding sites between circHIPK3 and miR-29b-3p based on the regRNA2. We found that circHIPK3 has two binding sites for miR-29b-3p (Fig. 3C–D). Next, we performed FISH to determine whether circHIPK3 and miR-29b-3p colocation in CFs. The results revealed that circHIPK3 and miR-29b-3p colocalize in the cytoplasm of CFs (Fig. 3E). The colocalization between circHIPK3 and miR-29b-3p in vivo was increased with the treatment of Ang II compared with saline (Fig. 3F). These results demonstrated that circHIPK3 may acts as a miR-29b-3p sponge in CFs.

3.4. Overexpression of circHIPK3 effectively reverses miR-29b-3p-induced inhibition of CFs cell function in vitro

To determine whether circHIPK3 contributes to cardiac fibrosis by acting as a sponge for miRNA-29b-3p, we overexpressed circHIPK3 and transfected CFs with miR-29b-3p mimics. RT-PCR results indicated that the expression levels of circHIPK3 were significantly increased in CFs cells transfected with circHIPK3 plasmid while mRNA-HIPK3 showed no increase (Supplementary Fig. 3D). RT-PCR results showed that miR-29b-3p were significantly increased in CFs cells transfected with miR-29b-3p mimic compared with control mimic ($p < 0.01$) (Supplementary Fig. 3E). Data from EdU incorporation assays showed that overexpression of circHIPK3 significantly promotes CFs proliferation ($p < 0.01$), while overexpression of miR-29b-3p mimics inhibits cell proliferation ($p < 0.01$) (Supplementary Fig. 4A, D). Overexpression of the miR-29b-3p mimics, in combination with circHIPK3, reversed the inhibition of cell proliferation caused by miR-29b-3p mimics alone (Supplementary Fig. 4A, D). Transwell migration assays revealed that overexpression of circHIPK3 significantly promotes CFs migration ($p < 0.01$), while overexpression of miR-29b-3p mimics inhibits cell migration ($p < 0.01$) (Supplementary Fig. 4B, E). CFs cotransfected with circHIPK3 plasmids and miR-29b-3p mimics reversed the inhibition of cell migration compared to cells transfected with the miR-29b-3p mimics alone (Supplementary Fig. 4B, E).

In addition, immunofluorescence staining indicated that overexpression of circHIPK3 led to the upregulation of a-SMA expression, while overexpression of miR-29b-3p mimics downregulated the expression of a-SMA (Supplementary Fig. 4C). Overexpressed miR-29b-3p mimics together with circHIPK3 reversed the downregulated expression of a-SMA compared to overexpression of the miR-29b-3p mimics alone. Moreover, the expression of COL1A1, COL3A1 and a-SMA was markedly increased in CFs cotransfected with circHIPK3 vectors and the miR-29b-3p mimics compared with the cells transfected with the miR-29b-3p mimics alone (Supplementary Fig. 4F–G). Similarly, qRT-PCR revealed that overexpressed miR-29b-3p, together with circHIPK3, reversed the downregulation of expression of COL1A1, COL3A1 and a-SMA compared with overexpression of the miR-29b-3p mimics alone (Supplementary Fig. 4H).

3.5. circHIPK3 silencing attenuates Ang II induced cardiac fibrosis in vivo

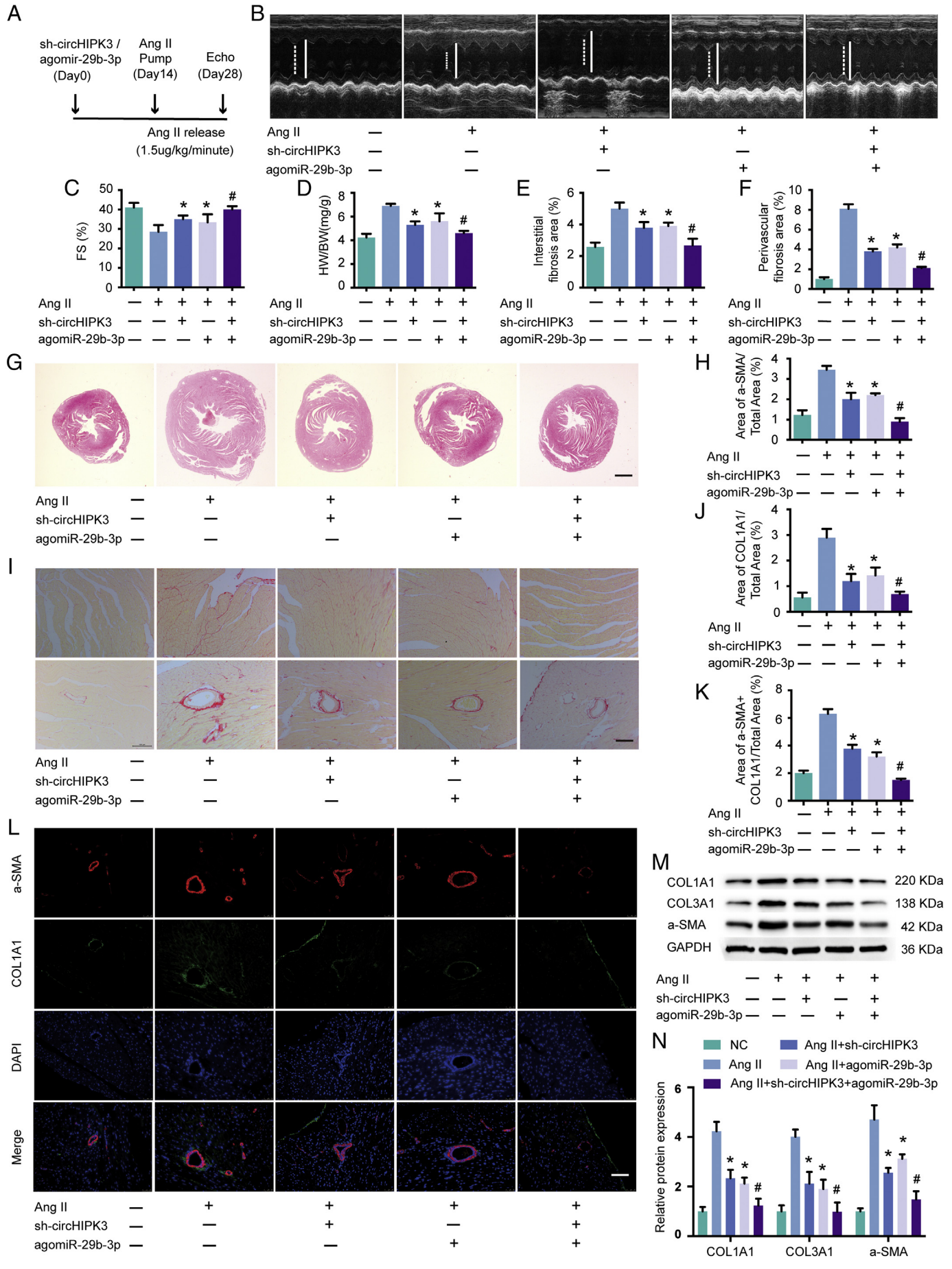
To further assess the role of circHIPK3 during cardiac fibrosis in vivo, we established mouse models by infusing 1.5 μg/kg/min Ang II (Sigma, USA) or saline for two weeks using osmotic mini-pumps. Functional studies of circHIPK3 were performed by administration of intravenous AAV-9 containing sh-circHIPK3 or Scr-shRNA 2 weeks prior to implantation of minipumps (Supplementary Fig. 5A). After two weeks, an echocardiographic analysis showed that circHIPK3 silencing protected from Ang II-induced cardiac hypertrophy and functional impairment, showing better heart function than that of mice injected with Scr-shRNA. sh-circHIPK3 injection significantly decreased circHIPK3 levels in CFs from mouse heart tissues but did not have a linear relationship with mRNA-HIPK3 expression after 4 weeks ($p < 0.05$, Supplementary Fig. 3A). In addition, injection of AAV9 with sh-circHIPK3 decreased the expression of circHIPK3 specifically in CFs but not in cardiomyocytes (CMs) in vivo (Supplementary Fig. 3B). circHIPK3 silencing significantly increased the FS(%) ($p < 0.01$) induced by Ang II (Supplementary Fig. 5C, Supplemental Table 2). Similarly, the Ang II + sh-circHIPK3 group exhibited a significantly reduced ratio of heart weight to body weight (HW/BW) compared with the Ang II alone ($p < 0.01$) and Ang II + Scr-shRNA groups ($p < 0.01$, Supplementary Fig. 5D–E). However, there were no differences in key hypertrophic parameters between the Ang II group and the Ang II + Scr-shRNA group.

Remarkably, myocardial fibrosis was reduced in the Ang II + sh-circHIPK3 group compared to the Ang II alone and Ang II + Scr-shRNA groups, as determined by Sirius Red staining of the left ventricular sections (Supplementary Fig. 5G) and by collagen expression (Supplementary Fig. 5L, M). The Ang II + sh-circHIPK3 group reduced both the interstitial fibrosis area ($p < 0.01$) and the perivascular fibrosis area ($p < 0.01$) compared with the Ang II alone and Ang II + Scr-shRNA groups (Supplementary Fig. 5F, H). Immunofluorescence staining revealed that circHIPK3 silencing reduced the areas of a-SMA/total area, COL1A1/total area and a-SMA + COL1A1/total area induced by Ang II (Supplementary Fig. 5I–L). Similarly, western blot analysis showed that circHIPK3 silencing markedly suppressed the expression of COL1A1, COL3A1 and a-SMA induced by Ang II (Supplementary Fig. 5M–N). However, there were no differences in Sirius Red staining or collagen expression between the Ang II group and the Ang II + Scr-shRNA group. Immunohistochemical analysis of vimentin revealed that the proliferation of CFs in vivo was decreased by injection of AAV9 with sh-circHIPK3 in the Ang II model (Supplementary Fig. 5O–P).

3.6. The circHIPK3-miR-29b-3p interaction regulates cardiac fibrosis in vivo

To investigate the interaction between circHIPK3 and miR-29b-3p during cardiac fibrosis in vivo, we established mouse models by infusing 1.5 μg/kg/min Ang II (Sigma, USA) for two weeks using osmotic mini-pumps. Functional studies of circHIPK3 were performed by administration of intravenous of AAV-9 containing sh-circHIPK3 alone, agomiR-29b-3p/antagomir-29b-3p alone, or both 2 weeks prior to mini-pump implantation (Fig. 4A, Supplemental Fig. 5). AgomiR-29b-3p injection significantly increased the expression of miR-29b-3p in CFs from mouse heart at 1 and 2 weeks ($p < 0.05$, Supplementary Fig. 3C). Echocardiographic analysis showed that mice injected with sh-circHIPK3 and agomiR-29b-3p were significantly protected from Ang II-induced cardiac hypertrophy and functional impairment, showing better cardiac function. (Fig. 4B–D, G, Supplemental Table 3). circHIPK3 silencing ($p < 0.01$) or miR-29b-3p overexpression ($p < 0.01$) significantly increased FS (%) and decreased the HW/BW levels induced by Ang II, and the combined group had an even larger effects on FS (%) and HW/BW (Fig. 4C–D, G).

Sirius Red staining analysis showed that the sh-circHIPK3 group, the agomiR-29b-3p group and the combined group all had reduced


A
sh-circHIPK3 / agomir-29b-3p (Day0)
Ang II Pump (Day14)
Echo (Day28)
Ang II release (1.5ug/kg/minute)
B
Ang II
sh-circHIPK3
agomiR-29b-3p
C
FS (%)
D
HW/BW(mg/g)
E
Interstitial fibrosis area (%)
F
Perivascular fibrosis area (%)
G
H
Area of a-SMA/ Total Area (%)
I
J
Area of COL1A1/ Total Area (%)
K
Area of a-SMA+ COL1A1/Total Area (%)
L
a-SMA
COL1A1
DAPI
Merge
M
COL1A1 220 KDa
COL3A1 138 KDa
a-SMA 42 KDa
GAPDH 36 KDa
N
NC
Ang II
Ang II+sh-circHIPK3
Ang II+agomiR-29b-3p
Ang II+sh-circHIPK3+agomiR-29b-3p
Relative protein expression
COL1A1
COL3A1
a-SMA

myocardial fibrosis induced by Ang II (Fig. 4I). The sh-circHIPK3 + agomiR-29b-3p group displayed obvious reductions in both the interstitial fibrosis area and the perivascular fibrosis area compared with the sh-circHIPK3 or agomiR-29b-3p group alone (Fig. 4E–F). But the antagomiR-29b-3p abolished the antifibrotic effect of sh-circHIPK3 in vivo. (Supplementary Fig. 6A–C). Immunofluorescence staining revealed that the sh-circHIPK3 group, the agomiR-29b-3p group and the combined group also had reduced levels of collagen expression level induced by Ang II (Fig. 4L). The sh-circHIPK3 + agomiR-29b-3p group had a significantly reduced area of a-SMA/total area, COL1A1/total area and a-SMA + COL1A1 /total area induced by Ang II compared to the sh-circHIPK3 or agomiR-29b-3p group alone (Fig. 4H, J–K). Likewise, agomiR-29b-3p overexpression or circHIPK3 silencing significantly decreased the expression of miR-29b-3p target proto-oncogenes (COL1A1, COL3A1, a-SMA) at the protein levels (Fig. 4M–N). By contrast, antagomiR-29b-3p rescued the collagen expression level downregulated by circHIPK3 silencing in Ang II model in vivo (Supplementary Fig. 6D–I).

4. Discussions

Our findings reveal that circHIPK3 expression is markedly upregulated in CFs and heart tissues after Ang II treatment. Functionally and mechanistically, circHIPK3 promotes CF proliferation, migration and cardiac fibrosis by sponging miR-29b-3p and upregulating of a-SMA, COL1A1 and COL3A1 expression (Supplementary Fig. 6). Furthermore, circHIPK3 silencing attenuates cardiac fibrosis and improves diastolic function. Our data suggest that circHIPK3 may be a novel therapeutic target for treating cardiac fibrosis.

circRNAs are abundantly expressed and have important regulatory and developmental functions in humans and animals [11]. Several recent studies have suggested that circRNAs may play critical roles in the initiation and progression of cardiovascular diseases [31,32]. A circular RNA, HRCR, has been shown to protect the heart from pathological hypertrophy and heart failure [33], while Cdr1as was found to induce myocardial infarction, and circ-Foxo3 was shown to promote cardiac senescence [14]. circHIPK3 is a known circRNA abundantly expressed in various human and animal tissues. Furthermore, circHIPK3 expression is conserved in human, rat, and mouse cardiac tissue [12]. Several studies have shown that circHIPK3 exerts various biological functions by acting as sponge with multiple RNAs [16]. circHIPK3 increases endothelial proliferation and vascular dysfunction by blocking miR-30a function and mediates retinal vascular dysfunction in diabetes mellitus [19]. Furthermore, circHIPK3 regulates both normal and cancer cell proliferation and migration by sponging miR-124 [16,17]. circHIPK3 also promotes colorectal cancer growth and metastasis by sponging miR-7 [18]. In this study, we demonstrated that circHIPK3 promotes CFs proliferation and migration and upregulates the expression level of a-SMA, COL1A1 and COL3A1 by sponging miR-29b-3p. Bioinformatics analysis showed that miR-133b-3p, miR-29b-3p, miR-29b-5p, miR-30a-5p, and miR-338-3p potentially interact with circHIPK3. To identify the miRNAs that bind to circHIPK3, we performed a dual luciferase reporter assay and validated miR-29b-3p significantly reduced the activity of LUC-circHIPK3 among those miRNAs.

In this study, circHIPK3 expression was markedly elevated in CFs and heart tissues after Ang II treatment. Silencing of circHIPK3 inhibits CFs proliferation, migration and cardiac fibrosis as demonstrated by loss-of- function assays. In parallel with the reduced cardiac fibrosis and improved diastolic function, we observed a decrease in cardiomyocyte size after circHIPK3 silencing. Our further studies revealed that circHIPK3 exerts regulatory functions through sponging miR-29b-3p to reduce the expression of its target genes (COL1A1, COL3A1, and a-SMA). Previously, the miR-29 family has been observed to prevent excess collagen expression in various organs, particularly through target genes regulation [9]. Overexpression of miR-29 has already been proven to have therapeutic effects in several fibrotic diseases, such as cardiac fibrosis [34], kidney fibrosis [35], liver fibrosis [36], lung fibrosis [37,38]. Several studies have reported that miR-29 acts as an antifibrotic molecule by targeting the expression of multiple extracellular matrix genes including elastin, fibrillin1, COL1A1, COL2A1, COL3A1 and fibronectin [39,40]. Furthermore, another study confirmed the antifibrotic effects of miR-29b in Ang II-induced cardiac fibrosis [41]. In this study, we also demonstrated that miR-29b-3p downregulated the overexpression of COL1A1, COL3A1 and a-SMA induced by Ang II in vitro and vivo. Importantly, circHIPK3 silencing increased the antifibrotic effects of miR-29b-3p, but overexpression of circHIPK3 reversed this activity. In addition, miR-29-3p silencing abolished the effect of sh-circHIPK3 in vivo. These observations suggest that circHIPK3 acts as miR-29b-3p sponge to regulate cardiac fibrosis.

In this study, silencing of circHIPK3 resulted in significant attenuation of angiotensin II-induced cardiac fibrosis and hypertrophy. TGF-β is an important downstream mediator of angiotensin II in inducing myocardial hypertrophy and fibrosis in this in vivo model [42,43]. Bedsides, we found that the expression of circHIPK3 in CFs significantly increased at 24 h after the treatment of TGF-β. The function of TGF-β was affected by circHIPK3 silencing is a possible mechanism resulting in the attenuation of Ang II-induced cardiac fibrosis and hypertrophy. Cardiac dysfunction was induced by Ang II though several mechanisms, such as remodeling, vascular constriction, oxidative stress, inflammation and so on [44,45]. Remodeling adversely impairs ventricular function and increases tissue stiffness [46]. Vascular constriction induced by Ang II contributes to elevated blood pressure and increased cardiac afterload. Consistently, cardiac hypertrophy and remodeling correlate with the deterioration of myocardial function induced by induced Ang II.

A variety of stimulus factors could trigger cardiac fibrosis, including TGF-β1 [47], Ang II [48], endothelin-1 [49], and inflammatory cytokines [50]. Our study revealed that circHIPK3 and miR-29b-3p are involved in Ang II-induced cardiac fibrosis pathways. Furthermore, the expression of circHIPK3 was also markedly increased after TGF-β1 treatment. Whether circHIPK3 and miR-29b-3p are also involved in cardiac fibrosis induced by stimulus factors other than Ang II requires future research. High-throughput circRNA microarray assays were not used to analyze the circRNA expression differences between normal and cardiac fibrosis tissues in this study. Whether other circRNAs participate in

Fig. 4. The circHIPK3-miR-29b-3p interaction regulates cardiac fibrosis in vivo. (A) Study design: adult male C57B/L6 mice were infused with 1.5 μg/kg/min Ang II (Sigma) for two weeks using osmotic mini-pumps (Alzet). Mice were treated with AAV-9 containing sh-circHIPK3, agomiR-29b-3p or sh-circHIPK3 + agomiR-29b-3p 2 weeks prior to mini-pump implantation. Saline-treatment served as the NC group (n = 6 each group). (B) Echocardiographic measurements of mouse hearts were performed two weeks after treatment. (C) Echocardiographic analysis of fractional shortening (FS) as a measure of left ventricular function. (D) Ratio between heart weight and body weight (HW/BW) as a measure of cardiac hypertrophy. (E) Quantitative analysis of interstitial fibrosis area. (F) Quantitative analysis of perivascular fibrosis area. (G) Representative hematoxylin/eosin staining of mouse myocardial tissue. Scale bar = 2 mm. (H) Quantitative analysis of a-SMA/total area. (I) Representative photomicrographs of the left ventricular perivascular and interstitial fibrosis by Sirius Red staining. Scale bar = 100 μm. (J) Quantitative analysis of COL1A1/total area. (K) Quantitative analysis of a-SMA + COL1A1/total area. (L) Representative immunofluorescence images of a-SMA, COL1A1, DAPI and the merged image from mouse myocardial tissue. Scale bar = 100 μm. (M) COL1A1, COL3A1 and a-SMA protein expression levels were detected by western blot analysis. (N) Quantitative analysis of COL1A1, COL3A1 and a-SMA protein expression. Data are expressed as the means ± SDs from six independent experiments. $^{*}p < 0.05$ vs. NC; $^{\#}p < 0.05$ vs. Ang II + sh-circHIPK3 or agomiR-29b-3p.

the Ang II-induced cardiac fibrosis, as well as their roles in regulating miR-29b-3p remains to be further determined.

5. Conclusion

In summary, inhibition of circHIPK3 prevents Ang II-induced cardiac fibrosis and improves diastolic function by sponging miR-29b-3p, thereby providing a novel therapeutic strategy for the prevention of Ang II-induced cardiac fibrosis.

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Conflict of interest

The authors certify that they have no conflict of interest.

Ethical approval

All animal protocols in this study were approved by the Animal Care and Use Committee, Research Institute of Medicine, Shanghai Jiao Tong University, in accordance with the guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85–23, revised 1996).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2019.04.006.

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