miR-216a exacerbates TGF-β-induced myofibroblast transdifferentiation via PTEN/AKT signaling

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Abstract. Myofibroblast transdifferentiation is an important feature of cardiac fibrosis. Previous studies have indicated that *microRNA-216a* (*miR-216a*) is upregulated in response to transforming growth factor-β (TGF-β) in kidney cells and can activate Smad3; however, its role in myofibroblast transdifferentiation remains unclear. The present study aimed to investigate the role of *miR-216a* in TGF-β-induced myofibroblast transdifferentiation, and to determine the underlying mechanisms. Adult mouse cardiac fibroblasts were treated with TGF-β to induce myofibroblast transdifferentiation. An antagomir and agomir of *miR-216a* were used to inhibit or overexpress *miR-216a* in cardiac fibroblasts, respectively. Myofibroblast transdifferentiation was evaluated based on the levels of fibrotic markers and α-smooth muscle actin expression. The *miR-216a* antagomir attenuated, whereas the *miR-216a* agomir promoted TGF-β-induced myofibroblast transdifferentiation. Mechanistically, *miR-216a* accelerated myofibroblast transdifferentiation via the AKT/glycogen synthase kinase 3β signaling pathway, independent of the canonical Smad3 pathway. In addition, it was observed that *miR-216a* activated AKT via the downregulation of PTEN. In conclusion, *miR-216a* was involved in the regulation of TGF-β-induced myofibroblast transdifferentiation, suggesting that targeting *miR-216a* may aid in developing effective interventions for the treatment of cardiac fibrosis.

Introduction

Cardiac fibrosis is a common pathologic component of various cardiovascular disorders, defined as the excessive deposition of extracellular matrix (ECM) and the disturbance of myocardial stiffness, which subsequently results in systolic and/or diastolic dysfunction of the heart (1). Increased accumulation of myocardial ECM also impairs the electrical conduction system and contributes to arrhythmogenesis (2,3). Activation and transdifferentiation of cardiac fibroblasts to myofibroblasts is a crucial event in cardiac fibrosis, and is responsible for the excessive synthesis of ECM (1). Therefore, improved understanding of the pathogenesis of myofibroblast transdifferentiation and the identification of novel therapeutic targets may be of great therapeutic interest for the treatment of cardiac fibrosis.

Transforming growth factor-β (TGF-β) is the most widely known fibrogenic growth factor associated with cardiac fibrosis, and promotes the transdifferentiation of cardiac fibroblasts to myofibroblasts (4,5). In response to cardiovascular insults, bioactive TGF-β is induced and released from latent stores, subsequently binding to TGF-β receptors, resulting in the activation of the canonical Smad-dependent signaling pathway and the induction of a profibrotic gene program (6). In addition, TGF-β can stimulate myofibroblast transdifferentiation and promote ECM synthesis via non-canonical pathways, including mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) (7,8). These non-canonical pathways coordinate with the Smad-dependent canonical pathway to induce cardiac fibrosis (9). Furthermore, negative regulators of AKT or p38 have been reported to inhibit myofibroblast transdifferentiation and protect against cardiac fibrosis (8,10). Thus, targeting TGF-β signaling, via canonical or non-canonical pathways, may aid in developing efficacious interventions against fibrosis.

MicroRNAs (miRNAs) are a class of small noncoding RNA molecules that function as negative regulators of gene expression by binding to the 3'-untranslated region (UTR) of target mRNAs (11,12). Emerging evidence suggests that miRNAs regulate the expression of key genes involved in fibrotic diseases, particularly cardiac fibrosis (13,14). Previous studies have demonstrated that *miR-133a* is downregulated in transverse aortic constriction or isoproterenol-induced fibrotic hearts, and that miR-133a overexpression can reduce collagen deposition and improve cardiac dysfunction (15). Pan *et al* (16) observed that forced expression of *miR-101a/b* suppressed the proliferation and collagen production in rat neonatal cardiac fibroblasts. Additionally, results from Nagpal *et al* (17)

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demonstrated that *miR-125b* was important for the induction of cardiac fibrosis, and that the inhibition of *miR-125b* may represent a novel therapeutic approach for the treatment of cardiac fibrosis. These studies indicated a central role for miRNAs in cardiac fibrosis.

miRNA-216a lies in the second intron of a noncoding RNA (RP23-298H6.1-001) located on the mouse chromosome 11 (18). The majority of previous studies into *miR-216*a have focused on tumors, identifying *miR-216a* as a potential biomarker for certain types of cancer (19,20). Xia *et al* (20) reported that *miR-216a* contributed to hepatocarcinogenesis and tumor recurrence in hepatocellular carcinoma. Recent studies, however, have suggested that the functions of *miR-216a* extend beyond the regulation of tumors, and that it serves important roles in other pathophysiological processes. For example, Yang *et al* (21,22) reported that *miR-216a* promotes endothelial senescence and inflammation, and M1 macrophage polarization via Smad3. Additionally, it was observed that miR-216a levels were increased in mouse renal mesangial cells following stimulation with TGF-β (23). The present study hypothesized that *miR-216a* may be involved in the pathogenesis of myofibroblast transdifferentiation and cardiac fibrosis.

Materials and methods

Reagents. TGF-β (cat. no. ab50036) was purchased from Abcam. AKT inhibitor MK2206 (cat. no. HY-10358) was purchased from MedChemExpress LLC. The antagomir (5'-CACAGU UGCCAGCUGAGAUUA-3') and the agomir (5'-UAAUCU CAGCUGGCAACUGUG-3') of *miR-216a*, their negative controls (antagomir control, cat. no. miR3N0000001-4-5; agomir control, cat. no. miR4N0000001-4-5), small interfering (si)RNA against PTEN (si*Pten*; 5'-TTCCGCCAC TGAACATTGGAA-3') and negative control siRNA (cat. no. siN0000003-1-10) were generated by Guangzhou RiboBio Co., Ltd. Alexa Fluor® 488-goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:200; cat. no. A11008) for immunofluorescence detection was obtained from Pierce (Thermo Fisher Scientific, Inc.). Primary antibodies for total (T)-AKT (1:1,000; cat. no. 4691), phosphorylated (P)-AKT (1:1,000; cat. no. 4060), T-glycogen synthase kinase 3β (GSK3β; 1:1,000; cat. no. 9315), P-GSK3β (1:1,000; cat. no. 9323P), T-Smad3 (1:1,000; cat. no. 9513s), P-Smad3 (1:1,000; cat. no. 8769), PTEN (1:1,000; cat. no. 9559) and GAPDH (1:1,000; cat. no. 2118) were purchased from Cell Signaling Technology, Inc. The anti-TGF-β receptor II (TGFBR2; 1:1,000; cat. no. ab61213) antibody was obtained from Abcam.

Cell culture and treatments. All of the animal experimental protocols were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University (approval no. 20171003) and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (24). A total of 40 male C57BL/6 mice (age, 8-10 weeks; body weight, 23-28 g) were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences. The animals were allowed free access to food/water in a specific pathogen-free, environmentally controlled barrier conditions (temperature, 20-25˚C; humidity, 45-55%; 12-h light/dark cycle) for 1 week prior to commencing the study. Adult mouse cardiac fibroblasts were isolated as previously described (25). In brief, left ventricles were harvested and digested in 0.125% trypsin and collagenase. The culture was then collected and suspended in DMEM/F12 (HyClone; GE Healthcare Life Sciences) medium with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences) at 37˚C for 90 min. The adherent fibroblasts were prepared for the subsequent experiments following synchronization for 12 h. The *miR-216a* antagomir and agomir, and their negative controls were all diluted with DMEM/F12 medium and then were mixed with Lipofectamine RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 20 min at room temperature. Then, when the cells had grown to 70-80% confluency, they were incubated with the mixture at a final concentration of 50 nM at 37˚C for 24 h, followed with TGF-β stimulation for an additional 24 h. To inhibit AKT activity, cardiac fibroblasts were pretreated with MK2206 (1 μM) for 24 h (26). PTEN knockdown was performed using si*Pten*, and the efficiency of the knockdown was assessed via western blotting. Briefly, the si*Pten* and its negative control were diluted with DMEM/F12 medium and then mixed with Lipofectamine RNAiMAX reagent for 20 min at room temperature. Then, the cells (at 40-50% confluency) were incubated with the mixture at a final concentration of 50 nM at 37˚C for 4 h, followed by miR-216a antagomir transfection for 24 h and TGF-β stimulation for an additional 24 h as aforementioned.

Western blotting. Western blotting was performed as previously described (27,28). Briefly, cultured cardiac fibroblasts were lysed in RIPA lysis buffer (50 mM Tris-HCl, 0.5% NP-40, 250 mM NaCl, 5 mM EDTA and 50 mM NaF) and the protein concentration was evaluated using a Rapid Gold BCA Protein Assay kit from Pierce (cat. no. A53225; Thermo Fisher Scientific, Inc.). Total proteins (50 μg) were loaded, separated via 10% SDS-PAGE and electrically transferred to PVDF membranes (cat. no. IPFL00010; EMD Millipore). Non-specific binding was blocked with 5% non-fat milk at room temperature for 1 h. Then, the proteins were incubated with the indicated antibodies at 4˚C overnight, followed by incubation with secondary antibodies (IRDye® 800CW conjugated goat anti-mouse IgG; 1:1,000; cat. no. 925-32210; LI-COR Biosciences) at room temperature for 1 h in the dark. Proteins were scanned and quantified using an Odyssey Infrared Imaging System (Odyssey version 3.0 Software; LI-COR Biosciences) in a blinded manner, and target proteins were normalized to GAPDH or the corresponding total proteins.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from fibroblasts using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and transcribed to cDNA using a Maxima First Strand cDNA Synthesis kit (Roche) according to the manufacturer's protocols. Levels of *miR-216a* were detected using a BulgeLoop™ miRNA RT-qPCR System (Guangzhou RiboBio Co., Ltd.). The thermocycling conditions were as follows: 95˚C for 10, then 40 cycles of 95˚C for 2 sec, 60˚C for 20 sec and 70˚C for 10 sec. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method as previously described (29). Total mRNA levels were normalized to GAPDH, and *miR-216a*

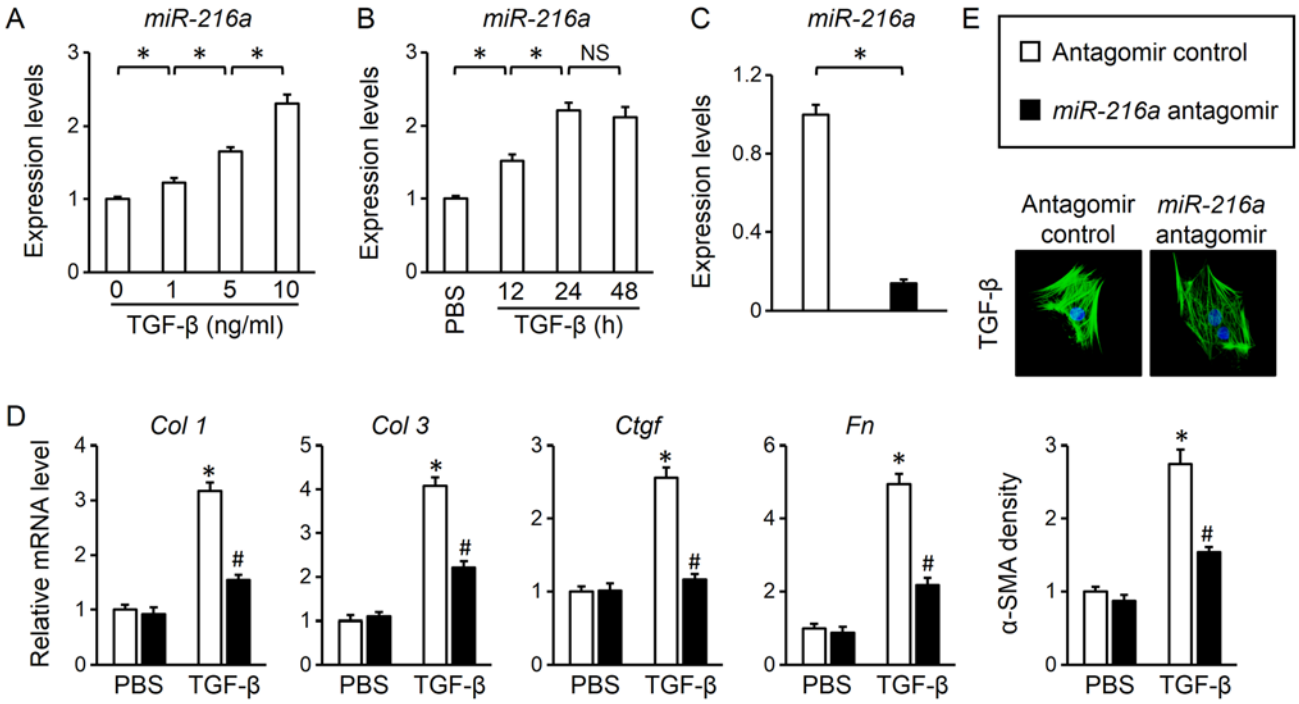

Figure 1. *miR-216a* inhibition attenuates TGF-β-induced myofibroblast transdifferentiation. (A and B) Relative expression levels of *miR-216a* in TGF-β-treated mouse cardiac fibroblasts (n=6). (C) *miR-216a* levels following transfection with *miR-216a* antagomir (n=6). (D) Relative mRNA levels of fibrotic markers following *miR-216a* antagomir transfection in the presence or absence of TGF-β (n=6). (E) Representative immunofluorescence images of α-SMA staining and quantification (n=5). Magnification, x400. Data are presented as the mean ± standard error of the mean. *P<0.05 vs. antagomir control + PBS; #P<0.05 vs. antagomir control + TGF-β. *miR-216a*, microRNA-216a; TGF-β, transforming growth factor-β; SMA, smooth muscle actin; NS, not significant; *Col*, collagen; *Ctgf*, connective tissue growth factor; *Fn*, fibronectin.

levels were normalized to U6. The primer sequences were as follows: Mouse collagen 1 (*Col 1*), forward, 5'-AGGCTTCAG TGGTTTGGATG-3' and reverse, 5'-CACCAACAGCAC CATCGTTA-3'; mouse collagen 3 (*Col 3*), forward, 5'-CCC AACCCAGAGATCCCATT-3' and reverse, 5'-GAAGCACAG GAGCAGGTGTAGA-3'; mouse connective tissue growth factor (*Ctgf*), forward, 5'-TGTGTGATGAGCCCAAGGAC-3' and reverse, 5'-AGTTGGCTCGCATCATAGTTG-3'; mouse fibronectin (*Fn*), forward, 5'-CCGGTGGCTGTCAGTCAGA-3' and reverse, 5'-CCGTTCCCACTGCTGATTTATC-3'; mouse *miR-216a*, forward, 5'-CATGATCAGCTGGGCCAAGACACA GTTGCCAGCTG-3' and reverse, 5'-TAATCTCAGCTGGCA A-3'; mouse GAPDH, forward, 5'-CGTGCCGCCTGGAGA AACC-3' and reverse, 5'-TGGAAGAGTGGGAGTTGCTGT TG-3', and U6, forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Immunofluorescence staining. Immunofluorescence staining was performed to detect the expression of the myofibroblast transdifferentiation biomarker α-smooth muscle actin (α-SMA) in mouse cardiac fibroblasts, as previously described (30). In brief, cardiac coverslips were fixed with 4% paraformaldehyde for 15 min at room temperature and then permeabilized with 0.2% Triton X-100, followed by incubation with 10% goat serum (GeneTex, Inc.) for 1 h at room temperature. The cells were then incubated with anti-α-SMA (1:100; cat. no. ab5694; Abcam) at 4˚C overnight, followed by incubation with the secondary antibody for 1 h at room temperature. The nuclei were stained with DAPI at room temperature for 30 sec. Images were captured using an Olympus DX51 fluorescence microscope (magnification, x400; Olympus Corporation) and quantified using Image-Pro Plus 6.0 (Media Cybernetics, Inc.). A total of 10-15 fields of view were observed per coverslip.

Bioinformatic prediction. The online database TargetScanMouse (Release 7.1, http://www.targetscan.org/mmu_71/) was employed for target prediction and analysis of *miR-216a* (31).

Statistical analysis. Results were presented as the mean ± standard error of the mean. Unpaired Student's t-tests (two-tailed) were used to compare differences between two groups. One-way ANOVA followed by a Tukey's post hoc test was performed to determine differences across multiple groups. All data were analyzed using SPSS 22.0 software (IBM Corporation) P<0.05 was considered to indicate a statistically significant difference.

Results

miR-216a inhibition attenuates TGF-β-induced myofibroblast transdifferentiation. Alterations in the expression of *miR-216a* were evaluated in TGF-β-treated adult mouse cardiac fibroblasts. As presented in Fig. 1A, TGF-β incubation increased *miR-216a* levels in a dose-dependent manner. In addition, *miR-216a* levels were increased in a time-dependent manner, albeit with no significant difference between 24 and 48 h (Fig. 1B). Therefore, a 24-h treatment period with 10 ng/ml of TGF-β was selected for further experiments. An antagomir was used to inhibit *miR-216a* expression in cultured adult mouse cardiac fibroblasts; the efficiency of transfection was demonstrated via RT-qPCR analysis (Fig. 1C). As presented in Fig. 1D, *miR-216*

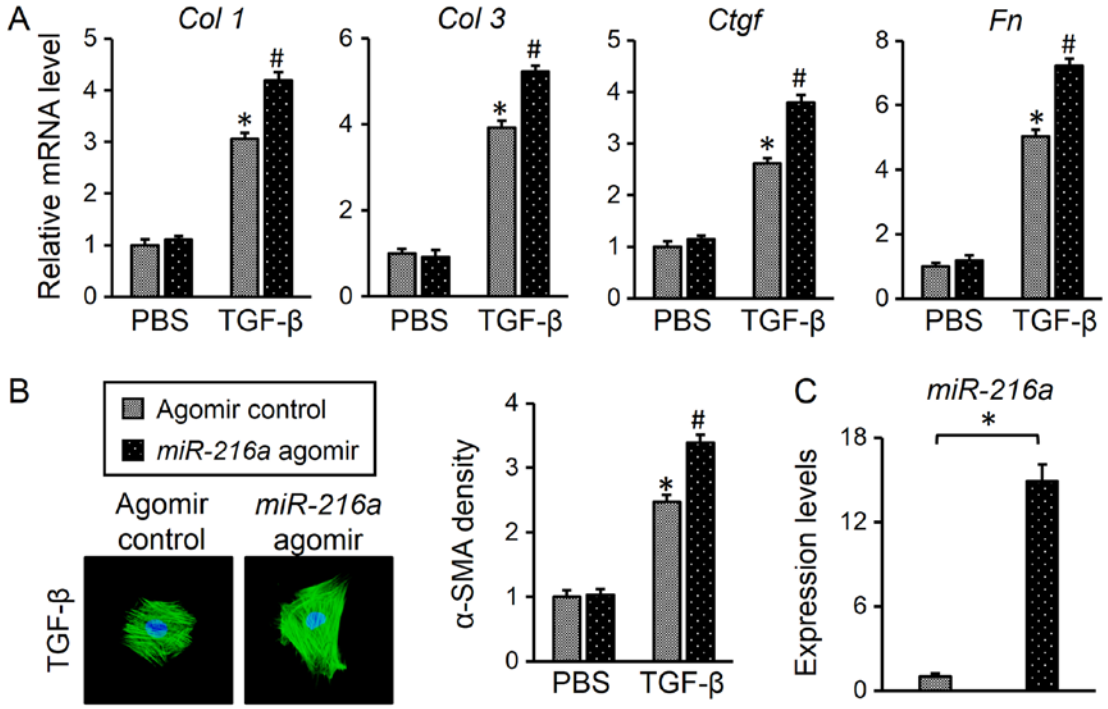

Figure 2. *miR-216a* activation promotes TGF-β-induced myofibroblast transdifferentiation. (A) Relative mRNA levels of fibrotic markers following *miR-216a* agomir transfection in the presence or absence of TGF-β (n=6). (B) Representative immunofluorescence images of α-SMA staining and quantification (n=5). Magnification, x400. (C) *miR-216a* levels following transfection with *miR-216a* agomir (n=6). Data are presented as the mean ± standard error of the mean. *P<0.05 vs. agomir control + PBS; #P<0.05 vs. agomir control + TGF-β. *miR-216a*, microRNA-216a; TGF-β, transforming growth factor-β; SMA, smooth muscle actin; *Col*, collagen; *Ctgf*, connective tissue growth factor; *Fn*, fibronectin.

inhibition suppressed collagen synthesis in response to TGF-β stimulation, as determined by the significantly decreased mRNA levels of fibrotic markers, *Col 1*, *Col 3*, *Ctgf* and *Fn* compared with the control. Increased α-SMA expression in response to TGF-β is a hallmark of myofibroblast transdifferentiation (27). Of note, it was observed that *miR-216a* inhibition also significantly reduced the TGF-β-induced expression of α-SMA and myofibroblast transdifferentiation (Fig. 1E). Thus, the results indicated that *miR-216a* was upregulated following TGF-β treatment, and that *miR-216a* inhibition attenuated TGF-β-induced myofibroblast transdifferentiation.

miR-216a activation exacerbates TGF-β-induced myofibroblast transdifferentiation. Next, the present study investigated whether *miR-216a* overexpression promoted myofibroblast transdifferentiation in response to TGF-β. Cardiac fibroblasts were transfected with a *miR-216a* agomir (Fig. 2C), and it was observed that *miR-216a* upregulation enhanced the TGF-β-induced increase in the expression of fibrotic markers compared with the control (Fig. 2A). In addition, it was determined that the *miR-216a* agomir further promoted α-SMA expression in response to TGF-β compared with the control (Fig. 2B). Collectively, these findings suggested that *miR-216a* is required for the induction of myofibroblast transdifferentiation.

AKT/GSK3β is involved in the regulation of myofibroblast transdifferentiation by miR-216a. A previous study reported that TGF-β/Smad is the most common signaling pathway responsible for myofibroblast transdifferentiation, and that Smad3 is a critical and necessary mediator in this process (6). In the present study, however, the results demonstrated that *miR-216a* inhibition did not significantly affect the phosphorylation of Smad3 (Fig. 3A and B). TGFBR2 is the primary receptor of TGF-β and mediates its profibrotic effect. Furthermore, bioinformatics analysis using TargetScan indicated that the *TGFBR2* gene may be a target for *miR-216a* (data not shown). Therefore, the expression levels of TGFBR2 were analyzed via western blotting; however, the protein levels of TGFBR2 were not altered by the up- or downregulation of *miR-216a*, with or without TGF-β treatment (Fig. 3A, C, E and F).

In addition to the canonical Smad-dependent signaling, TGF-β also activates Smad-independent pathways, including the AKT pathway (8). Previous studies have reported that AKT and its downstream target GSK3β also contribute to the regulation of myofibroblast transdifferentiation (8,32). Therefore, the phosphorylation status of AKT and GSK3β was examined in the present study, and the results demonstrated that the *miR-216a* antagomir significantly inhibited TGF-β-induced AKT/GSK3β activation compared with the control (Fig. 3D); by contrast, the *miR-216a* agomir further promoted AKT/GSK3β phosphorylation following treatment with TGF-β (Fig. 3G). Additionally, inhibition of AKT with MK2206 significantly attenuated the *miR-216a* agomir-mediated increase in myofibroblast transdifferentiation in response to TGF-β stimulation, as determined by the decreased mRNA levels of *Col 1* and *Col 3* (Fig. 3H). Collectively, these results indicated that *miR-216a* promoted TGF-β-induced myofibroblast transdifferentiation via activating AKT/GSK3β.

miR-216a activates AKT via inhibition of PTEN. Finally, the possible mechanisms underlying the *miR-216a*-mediated

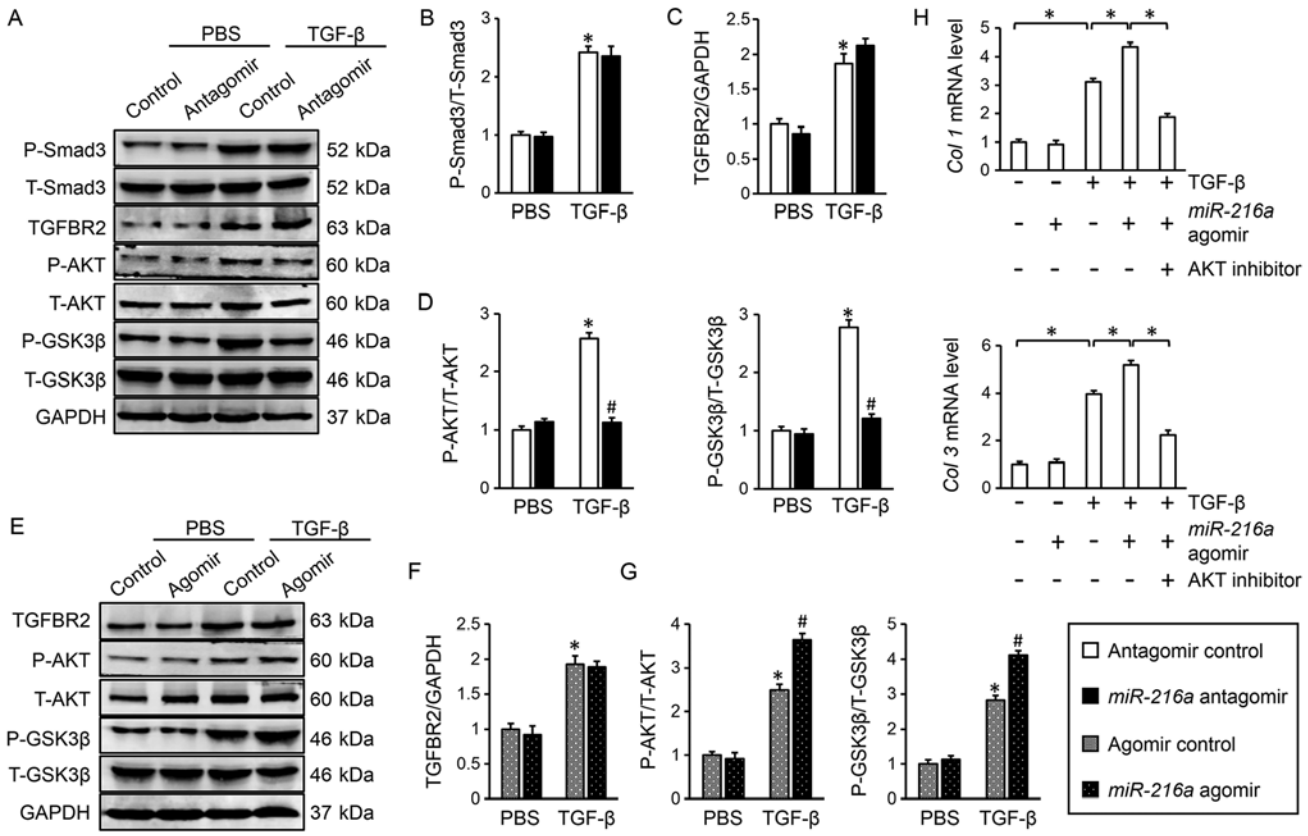

Figure 3. AKT/GSK3β is involved in the regulation of myofibroblast transdifferentiation by *miR-216a*. (A-D) Representative western blot images and quantification following transfection with *miR-216a* antagomir, in the presence or absence of TGF-β (n=6). *P<0.05 vs. antagomir control + PBS; #P<0.05 vs. antagomir control + TGF-β. (E-G) Representative western blot images and quantification following transfection with *miR-216a* agomir, in the presence or absence of TGF-β (n=6). *P<0.05 vs. agomir control + PBS; #P<0.05 vs. agomir control + TGF-β. (H) Relative mRNA levels of *Col 1* and *Col 3* in the presence of AKT inhibitor MK2206 (n=6). *P<0.05, with comparisons indicated by brackets. Data are presented as the mean ± standard error of the mean. GSK3β, glycogen synthase kinase 3β; *miR-216a*, microRNA-216a; TGF-β, transforming growth factor-β; *Col*, collagen; P-, phosphorylated; T-, total; TGFB2R, TGF-β receptor II.

activation of AKT were investigated. miRNAs exert biological regulation on various pathophysiological procedures via complementary binding to cognate mRNA transcripts, and subsequent degradation of the targeted transcripts (12). Among the *miR-216a* target genes predicted using TargetScan was *Pten*, a negative regulator of AKT signaling (Fig. 4A) (33). It was observed that the *miR-216a* agomir further decreased PTEN expression in response to TGF-β in cardiac fibroblasts (Fig. 4B), while *miR-216a* antagomir significantly attenuated TGF-β-induced PTEN reduction compared with the control (Fig. 4C). To determine the role of PTEN in AKT activation by *miR-216a*, the expression of PTEN was knocked down in cardiac fibroblasts using si*Pten* (Fig. 4D). RT-qPCR analysis revealed that PTEN knockdown abolished the *miR-216a* antagomir-mediated effects on TGF-β-induced myofibroblast transdifferentiation, as determined by the mRNA levels of *Col 1* and *Col 3* (Fig. 4E). Thus, the present data suggested that *miR-216a* may activate AKT by inhibiting PTEN.

Discussion

Myofibroblast transdifferentiation enhances collagen synthesis and is responsible for the occurrence of cardiac fibrosis; however, there is no available strategy to effectively suppress this pathological process (1,27). In the present study, it was observed that *miR-216a* expression was upregulated in TGF-β-treated cardiac fibroblasts, which in turn activated the AKT/GSK3β signaling pathway and induced myofibroblast transdifferentiation. Furthermore, it was revealed that AKT inhibition abolished the *miR-216a* agomir-mediated acceleration of myofibroblast transdifferentiation, and that *miR-216a* activated AKT via the inhibition of PTEN. The results indicated that *miR-216a* is involved in myofibroblast transdifferentiation, and that targeting of *miR-216a* may aid the development of efficacious interventions to treat cardiac fibrosis.

In response to mechanical or neurohumoral stimulation, cardiac fibroblasts transdifferentiate into myofibroblasts that produce large amounts of ECM and trigger the fibrotic process (1). Additionally, myofibroblasts secrete various factors that accelerate cardiac remodeling via autocrine and paracrine pathways (34). Nagpal *et al* (17) demonstrated that inhibiting the fibroblast-to-myofibroblast transition is required for the treatment of human cardiac fibrosis. miRNAs are now considered to be important regulators of gene expression in various pathophysiological processes (11). Previous studies have reported that miRNAs are specifically involved in the regulation of cardiac fibrosis (15-17). The present data demonstrated that an *miR-216a* agomir enhanced TGF-β-induced myofibroblast transdifferentiation, whereas an *miR-216a* antagomir inhibited this process and decreased ECM synthesis. It was previously reported that *miR-216a* was upregulated in TGF-β-treated mouse glomerular

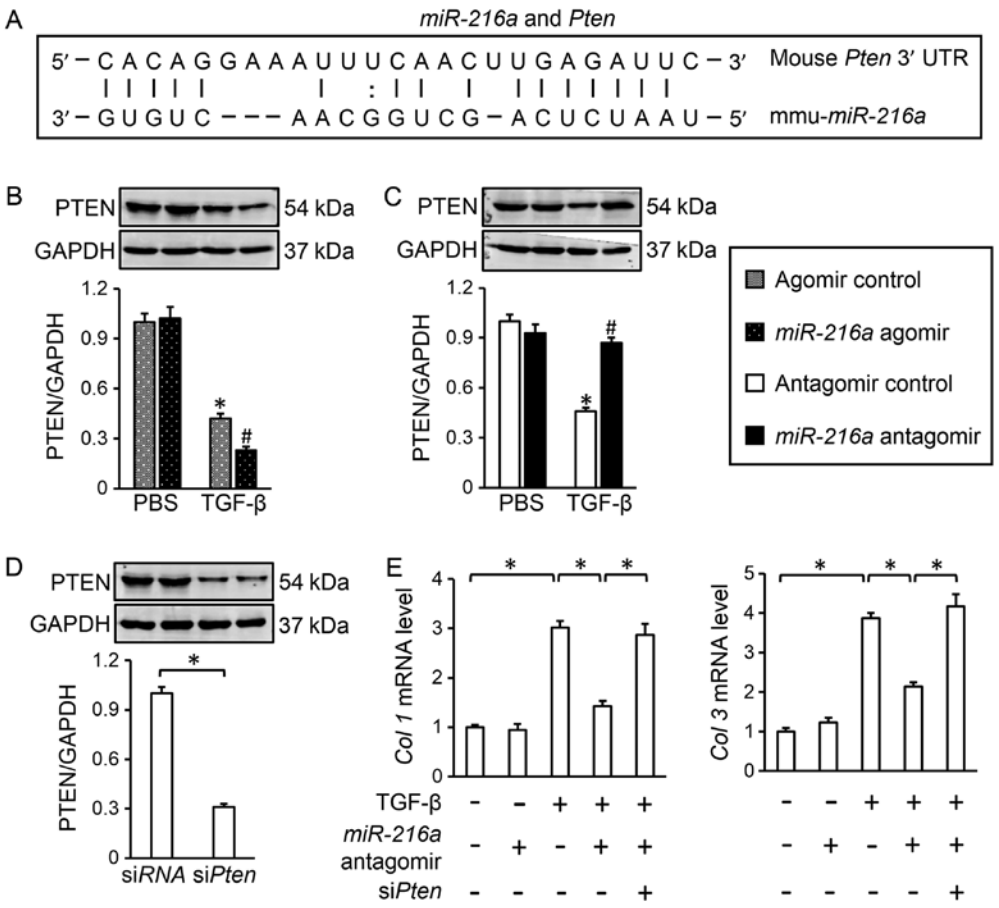

Figure 4. *miR-216a* activates AKT in mouse cardiac fibroblasts via the inhibition of PTEN. (A) Sequence alignment of *miR-216a* with the mouse *Pten* 3'-UTR. (B) Alterations in PTEN protein expression levels following transfection with *miR-216a* agomir or (C) antagomir (n=6). *P<0.05 vs. antagomir/agomir control + PBS; #P<0.05 vs. antagomir/agomir control + TGF-β. (D) Efficiency of si*Pten* as determined via western blotting (n=5). (E) Relative mRNA levels of *Col 1* and *Col 3* in the absence of PTEN (n=6). *P<0.05, with comparisons indicated by brackets. Data are presented as the mean ± standard error of the mean. *miR-216a*, microRNA-216a; UTR, untranslated region; TGF-β, transforming growth factor-β; si, small interfering.

mesangial cells, leading to glomerular mesangial cell survival and hypertrophy (18). Additionally, it was observed that *miR-216a* mediated TGF-β-induced collagen expression in kidney cells (23). The heart comprises numerous types of cells; previous studies have identified important roles for various other cell types in the regulation of cardiac fibrosis, in addition to cardiac fibroblasts. For example, activation of M2 macrophages is associated with cardiac fibrosis (35,36). Yang *et al* (22) demonstrated that *miR-216a* promoted M1 macrophage polarization via Smad3 activation. The present study demonstrated that transfection with an *miR-216a* antagomir did not affect Smad3 phosphorylation, but it increased the phosphorylation of AKT/GSK3β. These results suggested that the functional effects of *miR-216a* are cell type-dependent, and that upregulation of *miR-216a* in cardiac fibroblasts may lead to cardiac fibrosis. These data collectively provided rationale for the treatment of myofibroblast transdifferentiation and cardiac fibrosis via the targeting of *miR-216a* in cardiac fibroblasts.

AKT/GSK3β signaling serves an important role in the pathological fibrotic response (8). AKT is phosphorylated and activated in response to fibrotic stimulation, and AKT inhibition alleviates pressure overload-induced cardiac fibrosis (37). Activated AKT phosphorylates and inactivates GSK3β, which is also an important regulator of cardiac fibrosis (32). Lal *et al* (32) previously demonstrated that GSK3β physically interacted with Smad3, inhibiting its transcriptional activity. Specific deletion of GSK3β in cardiac fibroblasts induced a profibrotic myofibroblast phenotype and contributed to the pathogenesis of cardiac fibrosis post-myocardial infarction (32). In the present study, it was revealed that an *miR-216a* agomir further enhanced TGF-β-induced AKT/GSK3β phosphorylation; conversely, an *miR-216a* antagomir inhibited AKT/GSK3β activation. Inhibition of AKT attenuated the *miR-216a* agomir-mediated promotion of myofibroblast transdifferentiation. Of note, an alteration in Smad3 phosphorylation was not observed, suggesting that the AKT/GSK3β pathway also contributes to fibrotic regulation, independent of Smad3. TGFBR2 has been identified as the primary receptor of TGF-β, and it delivers its profibrotic signal from the cell membrane into the cytoplasm (38); however, it was revealed that TGFBR2 protein levels were unchanged following transfection with the agomir or antagomir of *miR-216a*, with or without TGF-β. Collectively, these data indicated that the effects of *miR-216a* on myofibroblast transdifferentiation may be specifically mediated by the downstream, non-canonical AKT/GSK3β signaling pathway independent of TGFBR2. PTEN is the main negative regulator of AKT signaling, and its inhibition results in the accumulation of phosphatidyl (3,4,5)-trisphosphate, mimicking the effect of PI3K activation and inducing the activation of downstream AKT signaling (33). In the present study, it was

observed that *miR-216a* was predicted to bind the 3'-UTR of *Pten*, thus potentially leading to the downregulation of PTEN protein levels and subsequent activation of AKT. Functional experiments confirmed that transfection with the *miR-216a* agomir decreased PTEN levels in combination with TGF-β, whereas the *miR-216a* antagomir alleviated the TGF-β-induced downregulation of PTEN. *Pten* knockdown attenuated the beneficial effects of the *miR-216a* antagomir. Nie *et al* (39) recently reported that PTEN downregulation by *miR-217* enhances the proliferation of fibroblasts and accelerates collagen synthesis. In the present study, it was demonstrated that downregulation of PTEN by *miR-216a* promoted fibrotic progress via activation of the downstream AKT/GSK3β pathway.

In conclusion, it was revealed that *miR-216a* exacerbated TGF-β-induced myofibroblast transdifferentiation via the PTEN-dependent activation of AKT/GSK3β signaling. The present study identified roles and underlying mechanisms of *miR-216a* in myofibroblast transdifferentiation, suggesting that *miR-216a* may be a novel therapeutic target for the treatment of cardiac fibrosis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CQ and BY contributed to the conception and design of the experiments. XL, TY, LW, SL and XZ performed the experiments. CQ, GW, JL and SS analyzed the experimental results and interpreted the data. CQ and BY drafted and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All of the animal experimental protocols were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University (approval no. 20171003).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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