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The targeted regulation of miR-26a on PTEN-PI3K/AKT signaling pathway in myocardial fibrosis after myocardial infarction

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Abstract. – OBJECTIVE: MiR-26a is involved in regulating myocardial remodeling and it is also related to organ fibrosis. Its role in myocardial fibrosis process is still controversy. As a definite target gene of miR-26a, phosphatase and tensin homology does on chromosome ten (PTEN) plays a role in regulating PTEN-PI3K/AKT signaling pathway. This study explored the function of miR-26a in regulating PTEN-PI3K/AKT signaling pathway, MMP-9 expression, and myocardial fibrosis after acute myocardial infarction (AMI).

MATERIALS AND METHODS: AMI mod established on Sprague-Dawley (SD) rats droxyproline, COL1A1, miR-26a, PTEN, p- and MMP-9 expressions in myocardial tissue week, 2 weeks, and 4 weeks aft eling we detected. Human cardiac fibr CF) wer cultured *in vitro* to detect m oa, PT p-AKT, MMP-9, COL1A1, and α-S expres s in the process of myofibrobl t enti P5). HCF in P5 we ansi ith mi oa mimics or inhibito est miR-2 TEN, p-AKT, MMP-9, COL1A1 SMA expr ns.

RESULTS: J ox line, COL miR-26a, p-AKT, and MMP-9 ov pressed, while PTEN downreg d in myoca tissue during the proces myocardial fib after AMI. MiR-26a EN, p-A MMP-9, C L1A1, and α-SMA ex ion o ally enhanced, while PTEN decline g the cess of HCF differentiating i ofibrol t. MiR-26a elevation suped ex sion, and increased p-AKT, M 9, CO d α-SMA levels. MiR-26a reduction signi antly upregulated PTEN level, PI3K/AKT signaling pathway activity, d MMP-9, COL1A1, and α-SMA protein expression.

CONCLUSIONS: MiR-26a upregulation may a role in myocardial fibrosis after AMI by suppressing PTEN, enhancing PI3K/AKT signaling pathway and MMP-9 levels.

Key Words:
miR-26a, PTEN, PI3K/AKT, AMI, Myocardial fibrosis.

Introducti

Myocard infa (MI) refers to myocardial ne is caused coronary artery acute or t hypoxia-is ia, featured as sev and persistent retrosternal pain in clinic, c plicated wi ardiac arrhythmia, shock, or h failure. It ne of the important reasons of ac dea . MI caused by myocardial hypox d is a type of cardiovascular disease which seriously harms human health c uses heart failure. It is characterized as pathogenesis and critical dangerous; thus, not timely treatment may lead to high disability and lethality rates[3-5]. Insufficient blood supply induced MI is easy to cause oxidative stress, leading to myocardial tissue necrosis, inflammatory reaction[6], and pathological myocardial remodeling[7]. Myocardial fibrosis is an important pathological change after MI. Cardiac physiological structures are destroyed, and myocardial interstitial extracellular matrix excessive proliferation and accumulation under the effect of multiple pro-fibrogenic factors after MI, leading to increased heart tissue stiffness reduced myocardial systolic and diastolic function abnormality of reserve, and eventually resulting in abnormal cardiac electrophysiological function, malignant arrhythmia, and sudden death[8,9]. Extracellular matrix protein abnormal proliferation and accumulation is related to the enhancement of cardiac fibroblasts activation, proliferation, differentiation to myofibroblasts, and collagen secretion[10]. MicroRNAs is a kind of endogenous single non-coding RNAs at the length of 22-25 nucleotides. They are involved in all types of biological processes including energy metabolism, cell growth, survival, and differentiation by identifying the 3'-UTR sequence of target gene

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mRNA to suppress mRNA translation or direct degrade mRNA[11]. Numerous studies[12-14] showed that myocardial fibrosis process was regulated by a series of signaling pathways and miRNAs. It was found that miR-26a was involved in the regulation of myocardial remodeling[15], and also associated with organ fibrosis[16,17]. However, whether it participates in myocardial fibrosis process is still unclear. Matrix metalloproteinases (MMPs) play an important role in the degradation of extracellular matrix and organ fibrosis. As one of important members, MMP-9 activity elevated throughout the course of myocardial fibrosis[18,19]. It was showed that PI3K/AKT signaling pathway played a critical role in regulating MMP-9 expression[20]. Abnormal activation of PI3K/AKT signaling pathway is closely related to the occurrence of myocardial infarction after MI[21,22]. Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) is the most important substrate of phosphatase and tensin homology located on chromosome ten (PTEN). PTEN suppresses PI3K/AKT signaling pathway activation through dephosphorylating PIP3[23]. Downregulation of PTEN and excessive activation of PI3K/AKT signaling pathway are confirmed to part[illegible] in the occurrence of myocardial fibrosis[illegible]s the definite target gene of miR-26a, PTEN expression and function is negatively regulated miR-26a[25]. The role of miR-[illegible]gulati[illegible] PTEN-PI3K/AKT signalin[illegible]athwa[illegible]MMP-9 expression, and myocar[illegible]fibrosis[illegible]lack of investigation.

Mat[illegible]nd Meth[illegible]

Main R[illegible]ents and [illegible]rials

He[illegible] male Sprague[illegible]wley (SD) rats wei[illegible]d 220-[illegible]g and at 6-7 weeks old were pur[illegible]d f[illegible] Heilon[illegible]iang Laboratory Animal Center, [illegible]emy of[illegible]itary Medical Sciences [illegible]ong[illegible] Ch[illegible]. Human cardiac fibroblasts (H[illegible] and [illegible]ecific medium Fibroblast Medi[illegible]2 were [illegible]ught from ScienCell (Carlsbad, [illegible]). Opti-MEM I medium was got from [illegible]ibco (Rockville, MD, USA). RNA extraction kit [illegible]s acquired from Omega (Norcross, GA, USA). [illegible]rse transcription kit and Real-time PCR kit we[illegible]e from TaKaRa (Dalian, Liaoning, China). Oligonucleotide for transfection and PCR primers were designed and synthetized by Gene Pharma (Shanghai, China). PTEN primary antibody was got from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MMP-9 and COL1A1 primary antibodies were from Abcam (Cambridge, MA, USA). P-AKT and α-SMA antibodies were from Cell Signaling Technology (CST, Danvers, MA, USA). Hydroxyproline ELISA kit was from ML-BIO (Shanghai, China). Rats were used [illegible] experiments, and all procedures were [illegible] by the Animal Ethics Committee of [illegible]ongjiang Hospital.

Rat MI Model Establish[illegible]t

The experimental rats[illegible]e randomly [illegible]ly divided into Sham[illegible]oup a[illegible]MI gr[illegible] with 15 in each gr[illegible]p. [illegible]p was [illegible]rther divided into post[illegible]ative[illegible]ek, 2 w[illegible], and 4 weeks subgr[illegible] with 5 in[illegible] s[illegible]oup. SD rats were a[illegible]ed by 10%[illegible]al hydrate (3 mg/g) i[illegible]aperi[illegible]l injection[illegible] and fixed on the pl[illegible] foam boa[illegible]fter no righting reaction[illegible]mbs were c[illegible]cted with, electrocardiogram (ECG) monitor. The neck skin was i[illegible]ed under a[illegible]ic condition and the muscle w[illegible]eparated t[illegible]pose the trachea. An animal bre[illegible]g mac[illegible] was connected for assisted respir[illegible] endotracheal intubation, with the breathing ratio at 1:2, respiratory frequency [illegible]bpm, and tidal volume at 10-12 mL. The [illegible]s opened between 3rd and 4th left ribs to expose the heart. The left anterior descending coronary artery was identified between the pulmonary arterial cone and aorta, and ligated using 6-0 no damage stitches. ECG monitor exhibited ST segment arch lift for 0.1 mV or T wave high, pale myocardium, and abate pulse were applied to confirm the MI model success. Then, the incision was closed and penicillin was adopted to prevent infection. The rats in sham group received the same operation without left anterior descending coronary artery ligation. The rats were anesthetized by 10% chloral hydrate intraperitoneal injection and received cardiac ultrasonography at 1 week, 2 weeks, and 4 weeks after surgery, respectively. Left ventricular end systolic diameter and left ventricular diastolic diameter were recorded at the level of papillary muscle prior to mitral valve through the left ventricular short axis view. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were automatically calculated. The tissue samples at MI region were extracted from the rats at 1 week, 2 weeks, and 4 weeks after modeling, and stored in liquid nitrogen and then in -80°C cryogenic refrigerator.

Cell Culture and Grouping

The cell culture dish was coated by poly-L-lysine at 2 μg/cm^2 and incubated at 37°C overnight. HCF cells were seeded into the dish at 5000 cells/cm^2 and maintained in Fibroblast Medium-2. The cells were digested by enzyme when the fusion reached 70-80% and named P1. The cells were then passaged or used for experiments. HCF cells in P5 were divided into four groups, including NC mimics, miR-26a mimics, NC inhibitor, and miR-26a inhibitor. Lipofectamine 2000 and oligonucleotide were diluted in Opti-MEM I and transfected to the cells for 6 h at 37°C and 5% CO_2. The cells were further cultured for 48 h for the following experiments.

qRT-PCR

Total RNA was extracted using the kit from OMEGA. The cells were added with 350 μl TRK buffer and 350 μl 70% ethanol. After blending, the solution was moved to the filtration column and centrifuged at 10,000 rpm for 1 min. After washed by 500 μl Wash buffer I for 1 time and 500 μl Wash buffer II for 2 times, the membrane was air dried at r[illegible] temperature and added with 40 μl RNas[illegible] water for 2 min. The solved RNA was m[illegible]d to the EP tube after centrifuged at 10,000 [illegible] for 1 min. The reverse transcription syst[illegible] in 20 μl contained 2 μg total [illegible]μl dNT[illegible] (10 mmol/L), 4 μl RT bu[illegible] (5 × [illegible] μl RT primer (1 μmol/L), 2 μl[illegible]erse tra[illegible]riptase, 0.5 μl RNase inhibit[illegible]a[illegible]N[illegible]. The reverse trans[illegible]tion [illegible]on was performed at 16°C [illegible]0 min, 42[illegible] for 15 min, and 85°C for [illegible]. The obtaine[illegible]NA was stored at -20[illegible] refri[illegible]tor. PCR amplification was [illegible]formed us[illegible] DNA as template under [illegible]effect of TaqDN[illegible]olymerase. The pri[illegible]s sequ[illegible]s used were as follows. miR-26a[illegible]5′-T[illegible]GATCCGTCAGAAATTCTCTC-CCGA[illegible] miR[illegible]P$_R$: 5′-GGTCTAGAT-[illegible]AAC[illegible]TGC[illegible]TTGGTGC-3′; U6P$_F$: 5′-[illegible]CGCT[illegible]CAGCACA-3′, U6P$_R$: 5′-AAC-G[illegible]CACGA[illegible]TTTGCGT-3′; PTENP$_F$: 5′-CT-[illegible]AGCTAAAGGT-3′, PTENP$_R$: 5′-TCACC[illegible]CACACAGGTAACGG-3′; MMP9P$_F$: TGTACCGCTATGGTTACACTCG-3′, MMP-[illegible]5′-GGCAGGGACAGTTGCTTCT-3′; COL1A1P$_F$: 5′-GTGCGATGACGTGATCTGTGA-3′, COL1A1P$_R$: 5′-CGGTGGTTTCTTGGTCGGT-3′; α-SMAP$_F$: 5′-AAAAGACAGCTACGTGGGT-GA-3′, α-SMAP$_R$: 5′-GCCATGTTCTATCGGG-TACTTCT-3′; β-actinP$_F$: 5′-GCACTCTTCCAG-CCTTCC-3′, β-actinP$_R$: 5′-AGAAAGGGTGTA-ACGCAACTAAG-3′. PCR reaction system in 10 μl volume was composed of 4.5 μl 2 × SYBR Green Mixture, 0.5 μl primer (5 μmol/L), 1 μl cDNA, and 3.5 μl ddH_2O. The reaction was started at 95°C for 5 min, followed by 40 cycles [illegible] for 15 s and 60°C for 1 min.

Western Blot

The cell lysis in SDS was [illegible]boiled [illegible]5 min and quantified using BCA[illegible]. A tot[illegible]60 μg sample was separat[illegible]y sodium d[illegible] sulphate-polyacrylami[illegible]gel [illegible]trophor[illegible] (SDS-PAGE) and t[illegible]ns[illegible]d [illegible]olyvin[illegible]idene difluoride (PVDF[illegible]embr[illegible]After bl[illegible]ed by 5% skim milk[illegible]oom temp[illegible]re[illegible]1 h, the membrane[illegible]bated in p[illegible]y antibody at 4°C ove[illegible]ght a[illegible]shed by p[illegible]osphate-buffered sal[illegible]Tween (P[illegible] for three times. Next, the [illegible]ane was in[illegible]ed in horseradish p[illegible]idase (HRP) labeled secondary antibody a[illegible]om temper[illegible]e for 60 min and washed by P[illegible] for three[illegible]es. At last, the membrane wa[illegible]ted by[illegible]L reagent and developed. The data w[illegible]ed using Quantity One software (Bio-Rad).

[illegible]dial Hydroxyproline Content Measurement

A total of 100 mg myocardial tissue was digested and hydrolyzed to obtain the supernatant. Hydroxyproline content was determined by ELISA. In brief, 50 μl standard substrate or diluted sample were added to the plate and incubated at 37°C for 30 min. After washed by washing buffer for 5 times, 50 μl enzyme-labeled reagent were added to each well at 37°C for 30 min. The plate was washed by washing buffer for 5 times and treated by 50 μl color developing agent A and 50 μl color developing agent B at 37°C for 15 min. Next, the plate was added with 50 μl stop buffer and read at 450 nm.

Statistical Analysis

All data analysis was performed on SPSS 18.0 software (SPSSS Inc., Chicago, IL, USA). The measurement data was presented as mean ± standard deviation, while the enumeration data was depicted as percentage. Data between groups were compared by x^2-test or one-way ANOVA when necessary. LSD was performed for post-hoc test. $p < 0.05$ was considered as statistical significance.

Results

The degree of Myocardial Fibrosis Gradually Increased After MI

LVEF and LVFS values in each time point showed no statistical difference in sham group ($p > 0.05$). LVEF and LVFS levels in AMI group were significantly lower than that in sham group in each time point ($p < 0.05$). Their levels gradually declined following time extension, suggesting MI modeling success (Figure 1A and B). Hydroxyproline content and COL1A1 expression in myocardium at 1 week, 2 weeks, and 4 weeks after MI modeling were significantly higher than that in sham group with time dependence (Figure 1C and D). It suggested that myocardial function reduced together with MI aggravated after MI modeling.

MiR-26a Expression Elevated During the Process of Myocardial Fibrosis

qRT-PCR detection revealed that miR-26a level in AMI group at 1 week, 2 weeks, and 4 weeks after modeling was higher than that in sham group with time dependence (Figure 2A). PTEN mRNA and protein expression in infarcted myocardium in AMI group were markedly lower than in sham group following time extension (Figure 2B and C), indicating that [illegible] reduction may be related to miR-26a [illegible] sion. AKT phosphorylation gradual[illegible]nhanced in the process of myocardial fibros[illegible]ading to downstream MMP-9 protein [illegible]vel e[illegible]ed. It demonstrated that miR-26a [illegible]ation ma[illegible]y a role in downregulating P[illegible], enhancing AKT signaling pathw[illegible]tivity [illegible] increas[illegible] MMP-9 expression.

MiR-26a En[illegible]ed in th[illegible] of HCF Cell [illegible]iation

Cardiac f[illegible]roblas[illegible]n spontane[illegible]usly differentiate to [illegible]fibroblasts[illegible]er *in vitro* cultivation. Th[illegible]ion of α-SM[illegible]e specific marker of m[illegible]broblasts, was upregulated upon passage n[illegible]er (Figure [illegible] and B). COL1A1 level also

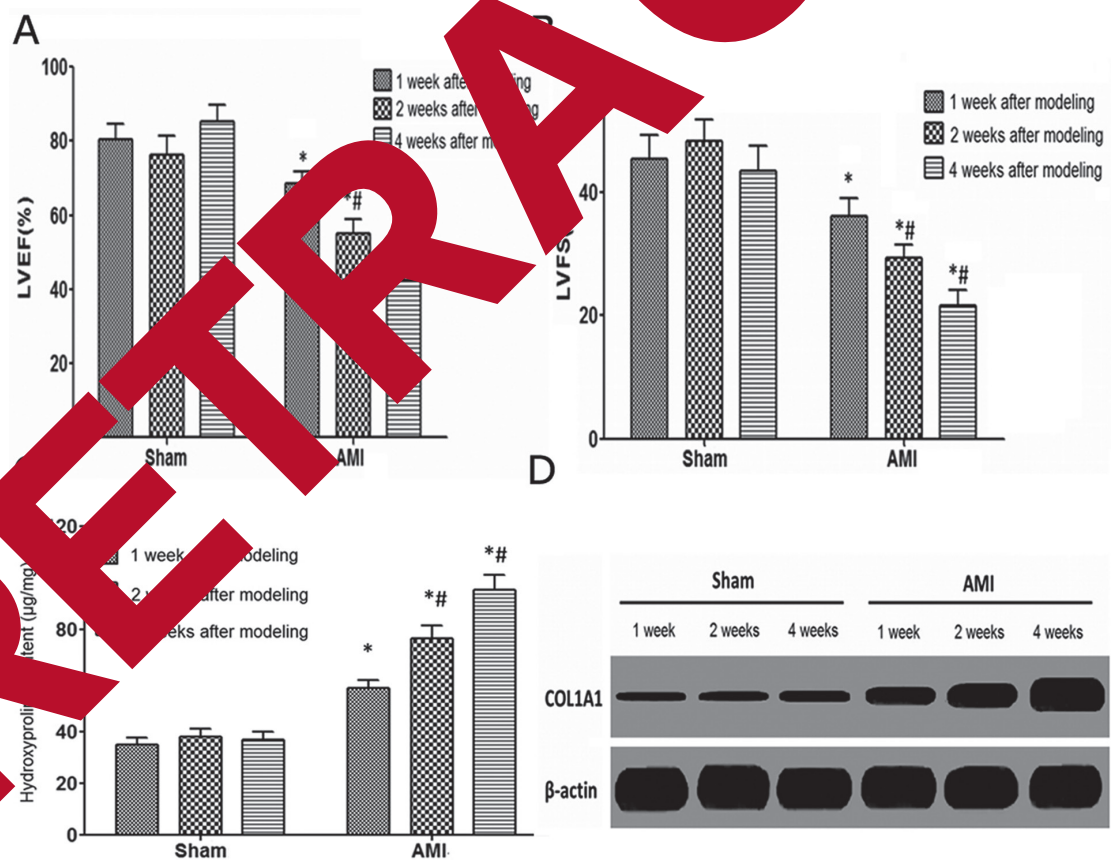

Figure 1. Myocardial fibrosis aggravated after MI. ***A,*** LVEF detected by echocardiography. ***B,*** LVFS detected by echocardiography. ***C,*** Myocardial hydroxyproline content measured by ELISA. ***D,*** COL2A1 protein expression tested by Western blot. $^{*}p < 0.05$ vs. sham group. $^{\#}p < 0.05$ *vs.* 1 week after modeling.

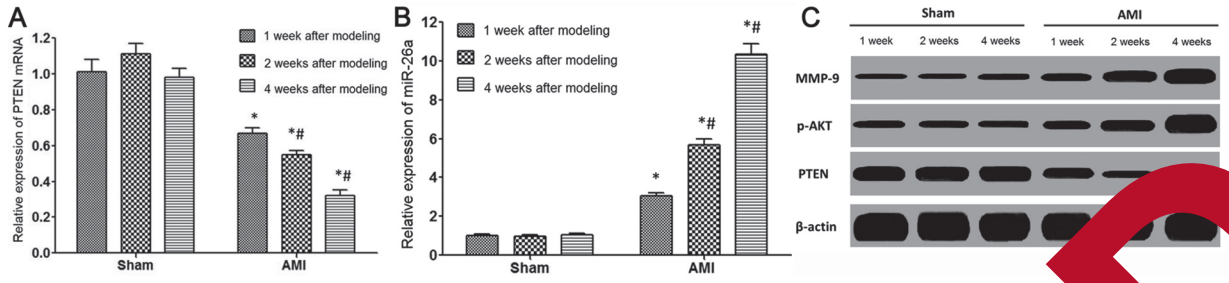

Figure 2. MiR-26a expression elevated during the process of myocardial fibrosis. ***A,*** miR-26a expression detected by qRT-PCR. ***B,*** PTEN mRNA expression detected by qRT-PCR. ***C,*** Protein expression tested by Western blot. $^{*}p < 0.05$ *vs.* Sham group. $^{\#}p < 0.05$ *vs.* 1 week after modeling.

elevated according to passage number, revealing that the ability of synthetizing collagen gradually enhanced in the process of cardiac fibroblasts differentiate to myofibroblasts (Figure 3A and B). Further experiments showed that PTEN reduced, while p-AKT and MMP-9 upregulated following miR-26a increase in the differentiation process (Figure 3A and B).

MiR-26a Regulated PI3K/AKT Activity and MMP-9 Expression in HCF Cells

HCF cells in P5 were applied for m mimic or miR-26a inhibitor transfection. I demonstrated that miR-26a upregulation nificantly suppressed PTEN expression, and creased p-AKT, MMP-9, COL α-SM levels (Figure 4A and B) R-26 duction

upregulated PTE evel, ened P /AKT signaling path ctivity, a MMP-9, COL1A1, a protein e ion (Figure 4A and B).

Discussion

ocardial fi sis, resulting from collagen exc e depo n, characterized by elevation of co centration and volume various collagen imbalance and disorder, and myocardial titial structure abnormality, is the common gical feature of various cardiovascular diseases, such as atherosclerosis, hypertension, viral myocarditis, cardiomyopathy, arrhythmia, MI. Myocardial fibrosis and AMI caused ventric-

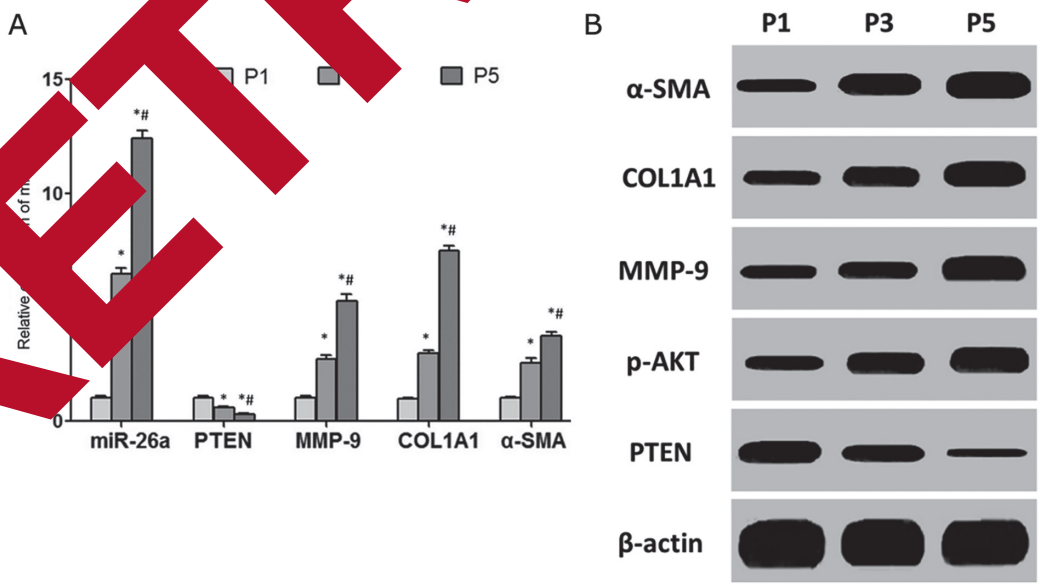

Figure 3. MiR-26a enhanced in the process of HCF cell differentiation. ***A,*** mRNA expression detected by qRT-PCR. ***B,*** Protein expression detected by Western blot. $^{*}p < 0.05$ *vs.* P1. $^{\#}p < 0.05$ *vs.* P3.

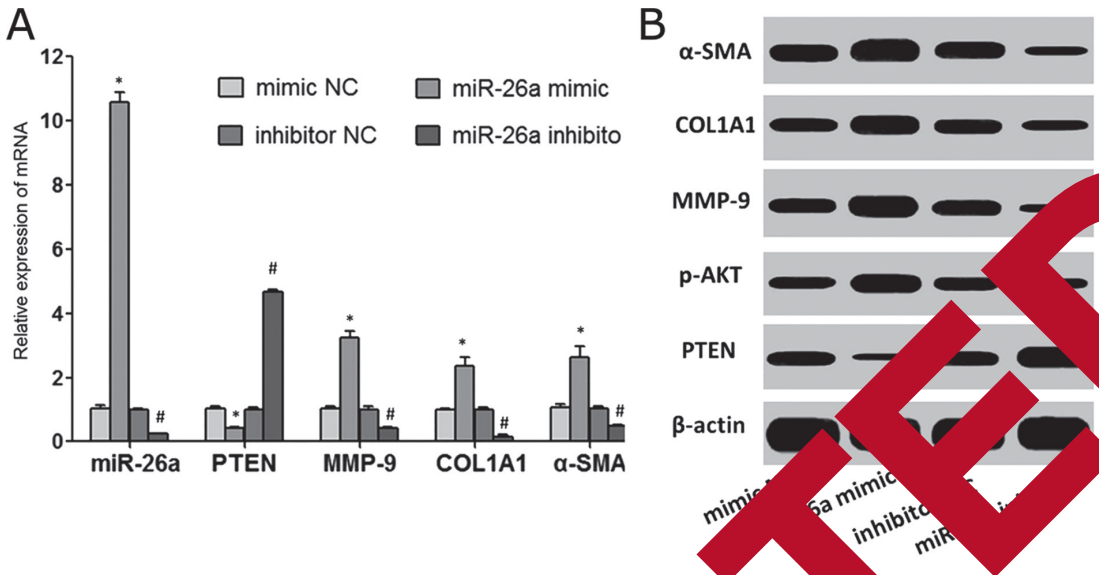

Figure 4. MiR-26a regulated PI3K/AKT activity and MMP-9 expression in HCF cells. A, mRNA expression detected by qRT-PCR. ***B,*** Protein expression detected by Western blot. *$p < 0.05$ *vs* NC. #$p < 0.05$ *vs* inhibitor NC.

ular remodeling are important reasons of heart failure, malignant arrhythmia, and cardiac death. There are numerous types of collagen in my[illegible] dial interstitium, while type I collagen ac[illegible] for more than 80%. It plays a key role in mai[illegible]-ing the intensity of ventricular wall because [illegible] thick fiber, stiffness, and anti-traction. Typ[illegible] collagen content or proportion [illegible] increas[illegible] will reduce the compliance o[illegible] vent[illegible]lar wall, thus, it is reported to part[illegible]te in th[illegible]ocess of myocardial fibrosis[26]. Thi[illegible]dy[illegible] model to observe th[illegible]yocard[illegible]rosis process after MI. It was[illegible]ved that t[illegible]pression of type I collagen[illegible] myocardi[illegible]om AMI group was sig[illegible]nifican[illegible]igher than the sham group wi[illegible]me depend[illegible] which was similar to the[illegible]ts of Hookana e[illegible] Hydroxyproline is t[illegible]ain co[illegible]ment of collagen, while almost all h[illegible]xy[illegible]ne in the animal tissue is derived from c[illegible]n. As a[illegible]ult, hydroxyproline con-[illegible]in my[illegible]rdial[illegible]sues can represent collagen le[illegible]o reflec[illegible]egree of myocardial fibrosis[27]. O[illegible]esults d[illegible]monstrated that hydroxyproline [illegible]igher in the AMI group, confirming myocardial fibrosis after MI. MMPs are a kind of Ca^{2+} dependent extracellular matrix protein hy-[illegible]sis enzyme family that can degrade almost all components of extracellular matrix. MMPs exist in normal myocardial tissue in inactive state and may be quickly activated under the stimulus of ischemia-hypoxia or inflammation[28]. The levels of MMPs with enzyme activity significantly in[illegible]se in the m[illegible]cardial tissue and circulating blo[illegible]uring m[illegible]ardial fibrosis, while MMP-9 is the[illegible]ype[29]. Extracellular collagen is degraded by elevated MMP-9, leading to abnormal collagen reticular formation promoting the [illegible]ce of myocardial fibers[30]. This study observed that MMP-9 level in myocardium from AMI group increased after infarction, suggesting that MMP-9 elevation participated in the process of myocardial fibrosis. It was found that miR-26a was involved in the regulation of myocardial remodeling[15] and associated with organ fibrosis[16,17]. However, whether it participates in myocardial fibrosis is controversy. This study showed that miR-26a upregulated in the myocardium after infarction. It also gradually increased following the degree of myocardial fibrosis, suggesting that miR-26a may promote myocardial fibrosis. As a member of PTP gene family, PTEN locates on 10q23.3 with a transcription product at 515 kb[31]. PTEN can dephosphorylate PIP3 to antagonize the phosphorylation effect of PI3K on PIP2, thus preventing AKT and downstream signaling pathway activation[23]. Several studies demonstrated that PI3K/AKT signaling pathway activation played an important role in upregulating MMP-9[32-34] and promoting myocardial fibrosis[35]. As a negative regulatory factor of PI3K/AKT signaling pathway, PTEN downregulation plays a promoting role in myocardial fibrosis[36]. The expression and function of PTEN is negatively regulated by miR-26a[25]. This study investigated the role of

miR-26a in regulating PTEN-PI3K/AKT signaling pathway, MMP-9 expression, and myocardial fibrosis. The results exhibited that PTEN level reduced in myocardial tissue after infarction, which was similar to Gao et al[36] findings; PTEN expression declined in infarcted myocardial tissue following time extension. Furthermore, AKT phosphorylation also increased in infarcted myocardial tissue, which was in accordance with the upregulation trend of MMP-9. It suggested that miR-26a abnormal elevation may play a role in reducing PTEN, enhancing PI3K/AKT signaling pathway activity, and upregulating MMP-9, which may be a mechanism of its participating in myocardial fibrosis. Cardiac fibroblast is an important component in myocardial tissue that can regulate extracellular matrix synthesis and degradation. MMPs in myocardial tissue are mainly synthetized and secreted by cardiac fibroblasts. Cardiac fibroblasts abnormally activate, increase proliferation and migration, differentiate to secretory myofibroblasts, and enhance the ability of collagen synthesis and secretion, thus playing a critical role in promoting myocardial fibrosis[10]. Upon *in vitro* HCF spontaneous differentiation to myofibroblasts model, this study observed [illegible] 26a expression gradually increased, while PTEN reduced, and PI3K/AKT signaling pathway activity, MMP-9 and COL1A1 expression elevated [illegible] the differentiation process, which is in accordance with the animal model. Zhong et al[37] found that PTEN downregulation was associated with cardiac fibroblasts proliferation [illegible] abilities enhancement. Lorenzen et al[24] reported that reducing PTEN level can promote cardiac fibroblasts survival, proliferation. They suggested that PTEN abnormal expression may be involved in accelerating cardial fibrosis. This study observed that PTEN declined in cardiac fibroblasts in the process of differentiation to myofibroblasts, indicating that PTEN downregulation partake in myocardial fibrosis. [illegible] may [illegible] the common theoretical basis and mechanism as by Zhong et al[37] and Lorenzen et al[24]. In addition, cardiac fibroblasts differentiate muscle fibers in the process of cell differentiation, not only resulting in enhanced collagen synthesis, but also overexpressed MMP-9. The process of cardiac fibroblasts differentiate to myofibroblasts, revealing collagen degradation is strengthened. Vadla et al[38] showed that except collagen synthesis elevation, MMP-2 and MMP-9 with the function of collagen degradation also significantly upregulated in myocardial fibrosis, suggesting the myocardial fibrosis process was the result of collagen synthesis abnormal increase and degradation disorder. To further clarify the influence of miR-26a on myocardial fibrosis, this study transfected miR-26a mimic or miR-26a inhibitor to change miR-26a level in HCF respectively. It was confirmed that miR-26a had the ability to restrain PTEN expression, enhance PI3K/AKT signaling pathway activity, and promote MMP-9 expression.

Conclusions

MiR-26a abnormal elevation inhibits PTEN, strengthened PI3K/AKT signaling pathway activity, and upregulated MMP-9, thus playing a part in facilitating cardiac fibrosis after AMI.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interests.

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