

Effect of miR-9 on myocardial fibrosis in rats *via* TGF- β 1/Smads signaling pathway

X. JIN¹, L.-L. YU², C.-X. YU¹

¹Department of Cardiology, Yantai Hospital of Traditional Chinese Medicine, Yantai, China

²Department of Breast Surgery, Yantaishan Hospital, Yantai, China

Xin Jin and Lili Yu contributed equally to this work

Abstract. – OBJECTIVE: To observe the effect of micro ribonucleic acid (miR)-9 on the myocardial fibrosis (MF) in rats and explore its mechanism of action.

MATERIALS AND METHODS: A total of 30 rats were selected to prepare the models of MF and randomly divided into Sham group, Model group and MiR-9 group. The rat heart weight (HW)/body weight (BW) and left ventricular weight (LVW)/BW were determined. The content of hydroxyproline (Hyp) in the myocardial tissues of left ventricle was measured using a spectrophotometer. Enzyme-linked immunosorbent assay (ELISA) was performed to determine the levels of type I and type III collagens in myocardial tissues, and the level of miR-9 in myocardial tissues was determined *via* reverse transcription-polymerase chain reaction (RT-PCR). Finally, Western blotting was conducted to measure the levels of transforming growth factor- β 1 (TGF- β 1) and Smads2 proteins.

RESULTS: Compared with Sham group, Model group exhibited increased HW/BW and LVW/BW and markedly raised Hyp content and type I collagen, type III collagen, TGF- β 1 protein and Smads2 protein levels, but significantly declined miR-9 expression levels in rats. In MiR-9 group, the rats had substantially decreased HW/BW, LVW/BW, Hyp content, and type I and type III collagen levels as well as lowered TGF- β 1 protein and Smads2 protein levels.

CONCLUSIONS: MiR-9 can alleviate the MF in rats by inhibiting the TGF- β 1/Smads signaling pathway.

Key Words:

Myocardial fibrosis, MiR-9, TGF- β 1, Smads, Collagen.

Introduction

Myocardial fibrosis (MF), an interstitial disease, is the common manifestation of multiple cardiovas-

cular diseases at end edge, and its main pathological features include the excessive proliferation of myocardial fibroblasts and excessive deposition of collagens, an extracellular matrix component¹⁻³. The heart of organisms is composed of cardiac myocytes and cardiac interstitial cells^{4,5}. Cardiac myocytes, a kind of highly differentiated terminal cells without proliferation ability, make up about 80% of the heart volume. Cardiac interstitial cells, as the main source of extracellular matrix, account for approximately 75% of the total heart cells and comprise myocardial fibroblasts, neurocytes and macrophages^{4,5}. The increased collagen synthesis caused by over-proliferation of cardiac fibroblasts destroys the normal physiological structure of myocardial interstitium, thus inducing heart failure or sudden death⁶. Currently, there is a scarcity of effective clinical drugs for preventing or treating MF, because of its complex pathogenesis, so it is extremely urgent to seek drugs for the targeted therapy of MF. Transforming growth factor- β 1 (TGF- β 1), one of the important cytokines in MF, can promote the proliferation of myocardial fibroblasts, inhibit the degradation of collagenases and accelerate MF process^{7,8}. After binding to cell surface receptors, TGF- β 1 stimulates intracellular signal transduction in which Smads2 acts as a crucial substrate, and the stimulated Smads2 will form transcription complexes to enter cell nucleus, thereby regulating gene expression⁹. Hence, suppressing the activity of TGF- β 1 is one of effective ways to treat MF. Micro ribonucleic acids (miRNAs) have become the study focuses in recent years and play a key role in the development, proliferation, differentiation, apoptosis and aging of organisms, only 2% of them in biological genomes subject to coding transcription, and the remaining to non-coding transcription¹⁰. MiRNAs, a kind of conservative non-coding RNAs with 20-24 nucleotides in length, mainly bind to 3'-UTRs of their target gene messen-

ger RNAs (mRNAs) and can repress the target gene transcription or facilitate the degradation of target gene mRNA¹¹. Extensive literatures have proven that miRNAs can participate in modulating the proliferation and differentiation of fibroblasts, the secretion of cytokines, or collagen metabolism and other pathogenic processes of MF. Therefore, this experiment is intended to study the effect of miR-9 regulation on rat MF and its mechanism of action, so as to provide a new idea and strategy for the targeted therapy of MF.

Materials and Methods

Experiment Animals

A total of 30 specific-pathogen-free (SPF)-grade male Sprague-Dawley (SD) rats weighing 200-230 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) with the certificate No. SCXK (Jing) 2014-0001. This study was approved by the Animal Ethics Committee of Yantai Hospital of Traditional Chinese Medicine Animal Center.

Drugs, Reagents and instruments

Isoprenaline (ISO) was purchased from Sigma, and miR-9 analogs from Sangon Biotech Co., Ltd. (Shanghai, China).

Reagents

The hydroxyproline (Hyp) kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), type I and type III collagen kits from Shanghai YuanMu Biological Technology Co., Ltd. (Shanghai, China), reverse transcription-polymerase chain reaction (RT-PCR) kit from Thermo Fisher Scientific (Waltham, MA, USA), miR-9 primers from Invitrogen, the monoclonal antibodies of TGF- β 1, Smads2, and horseradish peroxidase (HRP) goat anti-rabbit secondary antibodies from Cell Signaling Technology, Inc. (Danvers, MA, USA). 3,3'-diaminobenzidine (DAB) development solution was provided by Beyotime Biotechnology (Shanghai, China) and polyvinylidene difluoride (PVDF) membranes by EMD Millipore (Billerica, MA, USA).

Instruments

Spectrophotometer were obtained by (Shanghai Xinmao Instrument Co., Ltd. Shanghai, China), PCR instrument from (LongGene, Hangzhou, China), gel imaging system (UVP, Waltham, MA, USA), nucleic acid electrophoresis instrument

and wet protein transfer system were purchased by Bio-Rad (Hercules, CA, USA).

Methods

Establishment of Rat Models of MF

After 7 d of adaptive breeding, the rats were randomly divided into three groups: Sham group (n=10), Model group (n=10) and MiR-9 group (n=10). Except those in Sham group, the rats in Model group and MiR-9 group were treated through the subcutaneous injection with 5 mg/kg ISO on their backs. Furthermore, the rats in Sham group and MiR-9 group were injected with PBS and miR-9 analog, respectively.

Determination of Heart Weight (HW)/Body Weight (BW)

After the last intervention, the rats were fasted for 24 h to measure BW. Then, they were anesthetized using 10% chloral hydrate, and their thoracic cavities were opened to take out the hearts. After removing great vessels of heart and epicardial adipose tissues, the heart tissues were washed with normal saline, and the excess normal saline was absorbed by gauze, followed by the measurement of HW and left ventricular weight (LVW) and the calculation of HW/BW and LVW/BW.

Measurement of the Level of Hyp Via Spectrophotometer

Hyp is one of main collagen tissue components, and its content is substantially increased in MF of tissues. A 50 mg sample of left ventricular myocardial tissues was obtained from each group, added with 1 mL hydrolysis solution, mixed evenly and bathed in boiling water for 20 min at PH 6.0-6.8, followed by the addition of 3 mL diluted hydrolysis solution and activated carbon. Then, they were mixed again and centrifuged, and the supernatant was reserved. The resulting supernatant was added with 0.5 mL reagent I, II and III, followed by bathing in 60°C water for 15 min and centrifuging, and the supernatant was taken. Finally, the absorbency was determined at 550 nm, and the content of Hyp ($\mu\text{g/mL}$) was calculated according to the instruction.

Determination of the Levels of Type I and Type III Collagens in Heart Tissues of Rats Via Enzyme-Linked Immunosorbent Assay (ELISA)

The double-antibody sandwich ELISA was employed to measure the levels of type I and type

III collagens in the heart tissues of rats. The obtained myocardial tissues were pounded to pieces in normal saline and centrifuged, and the supernatant was taken. Next, the standard sample was diluted for preparation according to the instruction. The diluted sample solution was added into wells and then 50 μ L biotin were added to mark the antibodies, followed by incubation for 1 h. After being cleaned, the resulting products were added with horseradish peroxidase (HRP) to mark the antibodies, followed by inoculation for 30 min and drying with absorbent paper. Finally, the substrate A and B solutions were added, and the absorbency of each well was measured at 450 nm.

Detection of the Level of miR-9 in the Heart Tissues of Rats Via RT-PCR

A 50 mg sample of myocardial tissues were collected from rats and added with 0.5 mL TRIzol reagent for lysis and 0.1 mL chloroform, followed by mixing evenly and centrifuging into the two layers of water and phenol. The transparent upper layer was taken and added with the same volume of ISO. They were put upside down to mix evenly and centrifuged to obtain the deposit, namely total RNA. The concentration of RNA was measured using an ultraviolet spectrophotometer. Complementary deoxyribonucleic acid (cDNA) was synthesized using RevertAid™ First Strand cDNA Synthesis Kit, and the synthesized cDNA was stored at -80°C for standby use. Then, the cDNA was utilized for PCR in accordance with the instruction in PCR Master Mix Kit. The primer sequence and bp value are shown in Table I. The optical density of each band was analyzed using ImageJ software.

Determination of the Levels of TGF- β 1 and Smads2 Proteins via Western blotting

Rat myocardial tissues (50 mg) were taken from each group and added with 1 mL pre-cooled radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) to fully lyse the tissues, followed by centrifuging. The supernatant was obtained. Different concentrations of bovine serum albumin (BSA) standard solutions were prepared to determine the content of proteins. The protein sample (30 mg/well) was loaded for electrophoresis with 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, followed by transferring onto PVDF membrane rinsed by methanol in ice at 300 mA. After that, the polyvinylidene difluoride (PVDF)

Table I. List of primers.

Gene name	5'---3'
miR-9	UCUUUGGUUAUCUAGCUGUAUGA
β -actin	UUCUCCGAACGUGUCACGUTT ACGUGACACUGGCGGAGAATT

membrane was sealed with 5% skim milk powder and added with the primary antibody overnight. On the next day, the secondary antibody was added for incubation for 1 h, followed by development with DAB development solution. Finally, the optical density of bands was analyzed using ImageJ software.

Statistical Analysis

Data were statistically analyzed using Graphpad Prism 6.0 (La Jolla, CA, USA) and expressed as (\bar{x} ±s). *t*-test was performed for analysis. p <0.05 suggested that the difference was statistically significant.

Results

MiR-9 Down-Regulated HW/BW of MF Rats

Compared with those in Sham group, the rats in Model group after establishment of model were prone to irritation and dysphoria, and had obviously raised HW/BW and LVW/BW ($*p$ <0.05, $**p$ <0.01). After the intervention *via* administering miR-9 analogs, the rats in MiR-9 group exhibited significantly decreased HW/BW and LVW/BW compared with those in Model group ($\#p$ <0.05) (Table II).

MiR-9 Lowered the Content of Hyp in Rat Myocardial Tissues

The Hyp content was markedly higher in the rat myocardium of Model group than in that of Sham group ($**p$ <0.01). After the intervention with miR-9, the content of Hyp was significantly increased by 0.026 μ g/g, and there was a

Table II. Analysis of HW/BW and LVW/BW.

Group	HW/BW (mg/g)	LVW/BW (mg/g)
Sham group	2.45±0.18	1.86±0.27
Model group	3.98 ± 0.34*	2.63±0.18**
MiR-9 group	2.93±0.30#	2.24±0.12#

Table III. Analysis of Hyp.

Group	Hyp (μg/g)
Sham group	0.084±0.006
Model group	0.134±0.005**
MiR-9 group	0.108±0.012##

statistical difference from that in Model group (***p*<0.01, Table III). It suggests that miR-9 can inhibit MF in rats by decreasing the production of collagen.

MiR-9 Suppressed the Expressions of Type I and III Collagens in Rat Myocardial Tissues

Model group showed substantially higher type I and III collagen content in rat myocardial tissues than Sham group (**p*<0.05, ***p*<0.01), and the intervention with miR-9 notably repressed the expressions of type I and III collagens (#*p*<0.05, ##*p*<0.01). The comparison showed a statistical difference between MiR-9 group and Model group (Table IV). The results further reveal that the intervention can inhibit the expressions of collagens.

The Expression of miR-9 Declined in the Rat Model of MF

It was found in this study that miR-9 was closely related to MF in rats. According to the RT-PCR results, Model group had a markedly decreased miR-9 expression compared with Sham group (***p*<0.01, Figure 1A), but after the intervention with miR-9, the expression of miR-9 was substantially increased (##*p*<0.01), and MF in rats was significantly suppressed (Figure 1B).

Table IV. Analysis of type I collagen and type III collagen.

Group	Type I collagen (ng/L)	Type III collagen (ng/L)
Sham group	3.23±0.38	2.14±0.34
Model group	7.25±1.12**	4.12 ± 0.47*
MiR-9 group	4.45±0.46#	2.96±0.58##

MiR-9 Regulated the TGF-β1/Smads Signaling Pathway to Inhibit the Expressions of TGF-β1/Smads2 Proteins

To further explore the mechanism by which miR-9 inhibited MF in rats, Western blotting was performed to determine the levels of TGF-β1 and Smads2 proteins in the TGF-β1/Smads signaling pathway generally recognized as the canonical one in MF (Figure 2A). Compared with Sham group, Model group exhibited significantly increased TGF-β1 and Smads2 expression levels in rat myocardial tissues (***p*<0.01, **p*<0.05). The levels of TGF-β1 and Smads2 expressions in the rat myocardial tissues of miR-9 group were significantly decreased, compared with those in Model group (#*p*<0.01, Figure 2B).

Discussion

MF is the pathological result of multiple heart diseases at terminal stage, and it is mainly caused by the aberrant proliferation of myocardial fibroblasts. The activated myocardial fibroblasts produce larger numbers of collagens that are deposited in myocardial tissue gap, leading to ventricular remodeling, increasing cardiac load and inducing arrhythmia and heart failure in severe cases^{14, 15}. The TGF-β1/Smads2 sig-

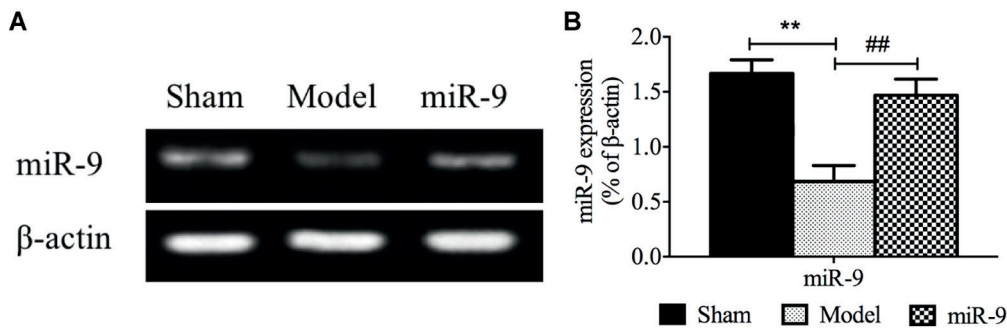


Figure 1. MiR-9 was downregulated in myocardial fibrosis model rats. **A**, The bands of PCR. **B**, Analysis of PCR. (***p*<0.01, ##*p*<0.01).

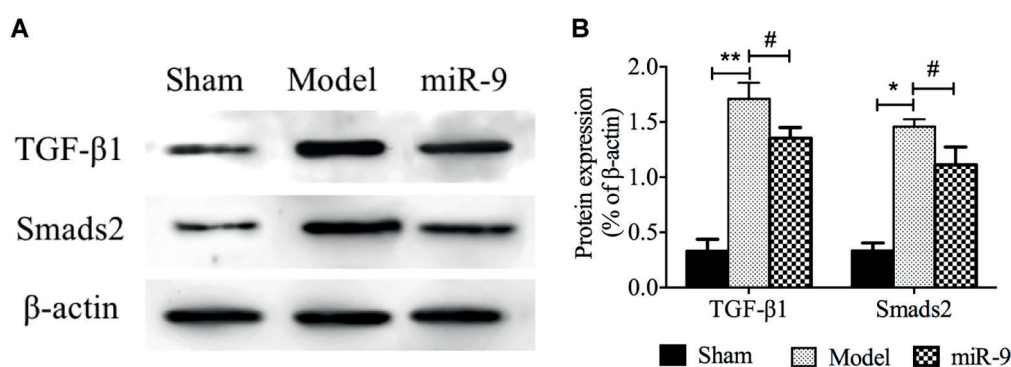


Figure 2. MiR-9 suppresses the levels of TGF- β 1 and Smads2 proteins. **A**, The band of Western blotting. **B**, Analysis of Western blotting (* p <0.05, ** p <0.01, # p <0.05, ## p <0.01).

naling pathway is one of crucial pathways for regulating MF. The over-expression of TGF- β 1, generally acknowledged as a pro-fibrogenic cytokine, promotes the transformation of cardiac fibrocytes into myocardial fibroblasts, thereby accelerating the occurrence and development of MF. Smads, the major downstream molecules of TGF- β 1, have several activating and inhibitory receptors of which Smads2 receptor is the first one to be activated¹⁶. Therefore, an effective treatment way of MF is to suppress the activation of TGF- β 1. In recent years, great progress has been achieved in the regulation of miRNAs in MF, and it has been proven that many miRNAs participate in and regulate MF. Zhang *et al*¹⁷ found that C-Ski, as an inhibitory regulator in the TGF- β 1 signaling pathway, can modulate the proliferation of myocardial fibroblasts. They targeted C-ski using miR-34/miR-93, thereby inhibiting ISO-induced MF in rats and TGF- β 1-induced myocardial fibroblast proliferation. Chen *et al*¹⁸ transfected the miR-33 analog and inhibitor into primarily cultured cardiac fibroblasts, and the results showed that the proliferation of cardiac fibroblasts is repressed after the action of miR-133 inhibitor. Moreover, they verified *via* dual-luciferase reporter gene assay that MMP16 is the target gene of miR-33. It reveals that miR-133 can inhibit MMP16 and activate the p38 MAPK signaling pathway. According to by Ji *et al*¹⁹, knocking down miR-327 can suppress angiotensin II, thus suppressing the differentiation of myocardial fibroblasts. It was found by Verjans *et al*²⁰ that miR-221/miR-222 levels are significantly decreased in the myofibroblasts of rat heart, and negatively correlated with MF degree in MF patients. Additionally, they found that miR-221/

miR-222 family can regulate several receptors in the TGF- β 1 signaling pathway, so they inferred that the miR-221/miR-222 family will become the potential treatment target of MF. In the present study, the rat model of MF was established by subcutaneously injecting of ISO, and the findings showed that HW/BW and LVW/BW in Model group were markedly increased, so were Hyp content and type I and type III collagen levels in myocardial tissues. On the contrary, the HW/BW and LVW/BW in MiR-9 group were significantly lowered, so were Hyp content and type I and type III collagen levels in myocardial tissues. To further probe into the effect of miR-9 on MF in rats, the levels of TGF- β 1 and Smads2 proteins in the TGF- β 1/Smads signaling pathway, generally recognized as the canonical one in MF, were measured, and it was discovered that the levels were suppressed after miR-9 intervention, suggesting that miR-9 may mitigate MF in rats by inhibiting the TGF- β 1/Smads signaling pathway.

Conclusions

We found that miR-9 inhibits the expressions of collagens by regulating the TGF- β 1/Smads2 signaling pathway, and thereby suppresses MF in rats, providing new evidence that miR-9 serves as the treatment target of MF. There is no doubt that the studies on miRNAs are still faced with difficulties yet to be resolved and numerous challenges, but miRNAs have been applied in tumor patients, which makes the studies in tumor field more promising. Therefore, it is reasonable to believe that miRNAs also possess a broad prospect in the prevention and treatment of MF.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

- 1) BROBERG CS, VALENTE AM, HUANG J, BURCHILL LJ, HOLT J, VAN WOERKOM R, POWELL AJ, PANTELY GA, JEROSCH-HEROLD M. Myocardial fibrosis and its relation to adverse outcome in transposition of the great arteries with a systemic right ventricle. *Int J Cardiol* 2018; 271: 60-65.
- 2) ULAS T. Comment on "Silybum marianum provides cardioprotection and limits adverse remodeling post-myocardial infarction by mitigating oxidative stress and reactive fibrosis". *Int J Cardiol* 2018; 270: 81.
- 3) VILAHUR G, CASANI L, PENNA E, CRESPO J, JUAN-BABOT O, BEN-AICHA S, MENDIETA G, BEJAR MT, BORRELL M, BADIMON L. Silybum marianum provides cardioprotection and limits adverse remodeling post-myocardial infarction by mitigating oxidative stress and reactive fibrosis. *Int J Cardiol* 2018; 270: 28-35.
- 4) LI YL, HAO WJ, CHEN BY, CHEN J, LI GO. Cardiac fibroblast-specific activating transcription factor 3 promotes myocardial repair after myocardial infarction. *Chin Med J (Engl)* 2018; 131: 2302-2309.
- 5) MAO S, WANG L, CHEN P, LAN Y, GUO R, ZHANG M. Nanoparticle-mediated delivery of Tanshinone IIA reduces adverse cardiac remodeling following myocardial infarctions in a mice model: role of NF-kappaB pathway. *Artif Cells Nanomed Biotechnol* 2018: 1-10.
- 6) HUA J, LIU Z, LIU Z, AN D, LAI W, ZHAN Q, ZENG Q, REN H, XU D. Metformin increases cardiac rupture after myocardial infarction via the AMPK-MTOR/PGC-1alpha signaling pathway in rats with acute myocardial infarction. *Med Sci Monit* 2018; 24: 6989-7000.
- 7) CHEN Y, LI T, GAO Q, WANG LY, CUI LQ. MiR-1908 improves cardiac fibrosis after myocardial infarction by targeting TGF-beta1. *Eur Rev Med Pharmacol Sci* 2018; 22: 2061-2069.
- 8) WANG Q, SUI X, CHEN R, MA PY, TENG YL, DING T, SUI DJ, YANG P. Ghrelin ameliorates angiotensin II-induced myocardial fibrosis by upregulating peroxisome proliferator-activated receptor gamma in young male rats. *Biomed Res Int* 2018; 2018: 9897581.
- 9) XIAO Y, YE J, ZHOU Y, HUANG J, LIU X, HUANG B, ZHU L, WU B, ZHANG G, CAI Y. Baicalin inhibits pressure overload-induced cardiac fibrosis through regulating AMPK/TGF-beta/Smads signaling pathway. *Arch Biochem Biophys* 2018; 640: 37-46.
- 10) ZHUANG YS, LIAO YY, LIU BY, FANG ZC, CHEN L, MIN L, CHEN W. MicroRNA-27a mediates the Wnt/beta-catenin pathway to affect the myocardial fibrosis in rats with chronic heart failure. *Cardiovasc Ther* 2018; 20: e12468.
- 11) CHU X, WANG Y, PANG L, HUANG J, SUN X, CHEN X. miR-130 aggravates acute myocardial infarction-induced myocardial injury by targeting PPAR-gamma. *J Cell Biochem* 2018; 119: 7235-7244.
- 12) UMBARAWAN Y, SYAMSUNARNO M, KOITABASHI N, OBINATA H, YAMAGUCHI A, HANAOKA H, HISHIKI T, HAYAKAWA N, SANO M, SUNAGA H, MATSUI H, TSUSHIMA Y, SUEMATSU M, KURABAYASHI M, ISO T. Myocardial fatty acid uptake through CD36 is indispensable for sufficient bioenergetic metabolism to prevent progression of pressure overload-induced heart failure. *Sci Rep* 2018; 8: 12035.
- 13) NAHAR K, KABIR F, ISLAM P, RAHMAN MM, AL MM, FARUK M, SUBHAN N, RAHMAN G, REZA HM, ALAM MA. Cardioprotective effect of Amaranthus tricolor extract in isoprenaline induced myocardial damage in ovariectomized rats. *Biomed Pharmacother* 2018; 103: 1154-1162.
- 14) WANG X, TAN Y, XU B, LU L, ZHAO M, MA J, LIANG H, LIU J, YU S. GPR30 attenuates myocardial fibrosis in diabetic ovariectomized female rats: role of iNOS signaling. *DNA Cell Biol* 2018; 37: 821-830.
- 15) WANG XB, CUI H, DU JB. Sulfur dioxide ameliorates rat myocardial fibrosis by inhibiting endoplasmic reticulum stress. *Histol Histopathol* 2018; 33: 1089-1097.
- 16) JEONG MH, KIM SJ, KANG H, PARK KW, PARK WJ, YANG SY, YANG DK. Cucurbitacin I attenuates cardiomyocyte hypertrophy via inhibition of connective tissue growth factor (CCN2) and TGF-beta/Smads signalings. *PLoS One* 2015; 10: e136236.
- 17) ZHANG C, ZHANG Y, ZHU H, HU J, XIE Z. MiR-34a/miR-93 target c-Ski to modulate the proliferation of rat cardiac fibroblasts and extracellular matrix deposition in vivo and in vitro. *Cell Signal* 2018; 46: 145-153.
- 18) CHEN Z, DING HS, GUO X, SHEN JJ, FAN D, HUANG Y, HUANG CX. MiR-33 promotes myocardial fibrosis by inhibiting MMP16 and stimulating p38 MAPK signaling. *Oncotarget* 2018; 9: 22047-22057.
- 19) JI Y, QIU M, SHEN Y, GAO L, WANG Y, SUN W, LI X, LU Y, KONG X. MicroRNA-327 regulates cardiac hypertrophy and fibrosis induced by pressure overload. *Int J Mol Med* 2018; 41: 1909-1916.
- 20) VERJANS R, PETERS T, BEAUMONT FJ, VAN LEEUWEN R, VAN HERWAARDEN T, VERHESEN W, MUNTS C, BIJNEN M, HENKENS M, DIEZ J, DE WINDT LJ, VAN NIEUWENHOVEN FA, VAN BILSEN M, GOUMANS MJ, HEYMANS S, GONZALEZ A, SCHROEN B. MicroRNA-221/222 family counteracts myocardial fibrosis in pressure overload-induced heart failure. *Hypertension* 2018; 71: 280-288.