



Original Article

Naringenin promotes angiogenesis of ischemic myocardium after myocardial infarction through miR-223-3p/IGF1R axis

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ABSTRACT

Introduction: Naringenin exerts a protective effect on myocardial ischemia and reperfusion. It has been reported that miR-223-3p is a potential target for the treatment of myocardial infarction (MI). In view of the unreported correlation between Naringenin and miR-223-3p, this study was designed to confirm that the ameliorative effects of Naringenin on MI is directly related to the regulation of miR-223-3p.

Methods: Through electrocardiogram detection, Masson pathological staining and immunohistochemistry of angiogenesis-related factors, alleviative effects of Naringenin on heart function, myocardial injury and angiogenesis in MI mice were observed individually. Hypoxic HUVECs were selected in the *in vitro* experimental model. The cell viability, angiogenesis and migration ability were analyzed to fathom out the pro-angiogenesis potential of Naringenin. The effect of Naringenin on miR-223-3p, as well as the downstream molecular mechanism was verified through bioinformatics analysis and rescue experiments.

Results: Naringenin improved heart functions of MI mice, reduced degree of myocardial fibrosis, stimulated expressions of angiogenic factors and down-regulated level of miR-223-3p in myocardial tissue. In *in vitro* experiments, Naringenin increased the viability of hypoxic HUVECs, as well as the abilities of tube formation and migration, and further inhibited the expression of miR-223-3p. In the rescue trial, miR-223-3p mimic reversed the therapeutic effect of Naringenin. Type 1 insulin-like growth factor receptor (IGF1R), as a downstream target gene of miR-223-3p, partially offset the cellular regulatory effects of miR-223-3p after overexpression of IGF1R.

Conclusions: Naringenin improves the angiogenesis of hypoxic HUVECs by regulating the miR-223-3p/IGF1R axis, and has the potential to promote myocardial angiogenesis in MI mice.

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1. Introduction

Myocardial infarction (MI) refers to the sudden interruption of coronary artery or branch blood flow on the basis of coronary artery disease, causing severe and long-lasting acute ischemia of the corresponding myocardium, which eventually causes myocardial ischemia, hypoxia and even necrosis [15]. Therefore, recanalization

of blood vessels, establishment of effective collateral circulation, and improvement of blood circulation in the ischemic area are the first measures to save ischemic myocardium, improve the prognosis of MI and reduce mortality [15,29]. After half a century of exploration and efforts by researchers, the clinical treatment of cardiovascular diseases has made great progress. However, the incidence of cardiovascular disease remains high, and it is still the first cause of pathological death in the aging population [1].

Thrombolytic and interventional therapies based on the principle of vascular recanalization have been widely applied in clinical practice, in which great progress has been made [23]. However, severe ischemia-reperfusion injury, failure of myocardial cell regeneration, hypofunction of the heart, and even the immediate induction of heart failure are still vital factors for the high recurrence rate and mortality of MI, restricting the use of existing methods in the treatment of MI [18]. Based on the data of existing

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research, we found that the current therapy using bone marrow mesenchymal stem cell reduced the area of MI and improved heart function [13]. However, the proliferation of new cardiomyocytes in the infarct area is only one aspect of the repair of MI, and another important aspect is the regeneration of blood vessels in the MI area [16]. New blood vessels are the basis for the nutrition and oxygen supply of tissue structures such as newly formed cardiomyocytes, which promote the recovery of hibernating cardiomyocytes by increasing tissue perfusion, and ultimately exert a protective effect on heart [2,3]. Therefore, exploring other therapeutic approaches dedicated to the vascular recanalization and promotion of myocardial angiogenesis is of great benefit to the treatment of MI.

Naringenin is a monomer belonging to dihydroflavonoids [17]. Pharmacological studies at home and abroad have shown that Naringenin has anti-inflammatory, anti-oxidant, anti-fibrosis, anti-tumor, anti-arrhythmic, anti-cough, prevention of atherosclerosis, immune regulation of fat metabolism, and estrogen-like pharmacological activities [17,20,21]. Naringenin, with a rich source, is widely found in grapefruit, tomatoes, grapes and citrus fruits of the Rutaceae family. It can be developed for use in medicine, food and other fields. Analytical materials on the clinical therapeutic potential of Naringenin point out that [7,19,25] Naringenin has a significant protective effect on cardiovascular system, and is a potential regulator of atherosclerosis. However, no researchers have clarified the efficacy of Naringenin in the treatment of MI based on the angiogenesis mechanism.

MicroRNA (miRNA) is a type of endogenous non-coding RNA, the role of which is to inhibit the translation of target mRNA, or to degrade target mRNA, and negatively regulate the expression of target genes [26]. Studies have found that miRNA is the core of the gene regulatory network, with a wide range of regulatory functions in human life activities, playing an essential role in a variety of physiological and pathological processes such as angiogenesis and cardiovascular diseases. Therefore, exploring miRNAs closely related to angiogenesis is likely to discover new mechanisms for regulating angiogenesis, which may be applied in the treatment of MI. Studies have pointed out that miR-223 is significantly highly expressed in MI, and there are reports that inhibiting miR-223-3p is a potential method for the treatment of MI [12]. Therefore, based on the purpose of new drug development, we analyzed the therapeutic effect of Naringenin on ischemic myocardial angiogenesis after MI, and verified whether this effect is related to the regulation of miR-223-3p.

2. Experimental methods & experimental materials

2.1. MI mouse model construction and treatment

The SPF grade C57 mice (male, 18–21 g) provided by Vital River Laboratories were raised in a comfortable environment with suitable temperature and they could eat and drink freely. The animal experiment has been approved by animal experiment ethics committee of Cangzhou Central Hospital (license number: 20191112-047), the whole process of which strictly followed the committee's requirements. Forty mice were randomly divided into four groups (10 mice for each group): Sham group (the mice were only opened with thorax and received no other treatment); Model group (mice received MI treatment); Model + Nar-L group (mice received MI treatment and then received 25 mg/kg/d Naringenin); Model + Nar-H group (mice received MI treatment and then received 75 mg/kg/d Naringenin).

The MI mouse model was constructed with reference to Li's experimental process [11], and the left anterior descending coronary artery (LAD) of the mice was ligated. The main steps are recorded as follows. First, the mice were anesthetized by

intraperitoneal injection of 25 mg/kg anesthetic (P3761, Sigma, USA) and temporarily lost muscle response. Then, the left front chest of the mice was opened by surgical scissors. Afterwards, the internal organs of the thoracic cavity were bluntly separated and the LAD was exposed. Subsequently, the blood vessels were ligated using 8-0 surgical thread. After that, the opened chest cavity was closed layer by layer. Finally, the mice were placed on a heating pad until awake. The mice in the Sham group only had their chest open without vascular ligation.

Naringenin (A0147) is an experimental reagent purchased from Chengdu Must Bio-technology co., ltd. Naringenin was dissolved by DMSO reagent and used for animal injection or cell intervention. Animals received Naringenin treatment by intraperitoneal injection every day for 4 weeks (w). 2 h (h) after the last injection, the researchers used a small animal electrocardiograph (XR-RM-6280, www.softmaze.com, China) to test the heart function of the mice, and calculated two evaluation indexes, Ejection Fraction (EF) and Fraction Shortening (FS).

2.2. Masson staining

After the ECG test, all mice were quickly put to death. The heart tissues were quickly removed and cleaned. Part of the heart was fixed in 4% paraformaldehyde, while the rest was cryopreserved. The fixed tissues were embedded in paraffin after 24 h and made into sections for staining. Before staining, the paraffin fixing the myocardial tissue in the section was firstly washed off. And then the tissues received a series of stains (potassium dichromate, iron hematoxylin, 1% hydrochloric acid ethanol, ponceau, aniline blue, 1% glacial acetic acid). Finally, the tissue on the section was dehydrated and fixed with a cover glass. Finally, image acquisition was completed in the Leica DMI8 high-speed imager (magnification: × 200).

2.3. Immunohistochemistry

The same deparaffinized myocardial tissue sections were immersed in 3% H₂O₂ solution to remove endogenous peroxidase in the myocardium. After completing antigen retrieval and sealing, the tissue was covered by anti-CD34 (1:3000, ab81289) and anti-CD31 (1:2000, ab182981) antibodies from Abcam at 37 °C for 2 h respectively. After washing, the tissue incubated with diluted horseradish peroxidase (HRP)-conjugated Goat Anti-Rabbit antibody (1:1000, ab6721) at 37 °C for 1 h. DAB Horseradish Peroxidase Color Development Kit (P0202, Solarbio, China) facilitated CD34 or CD31 complexes in tissues to present brown positive expression. The image was also captured by Leica DMI8 high-speed imager (magnification: × 200). The positive cell rate (%) was calculated by counting the proportion of positive cells in the total number of cells.

2.4. The processing of human umbilical endothelial cells (HUVECs)

HUVECs (ATCC® PCS-100-013™, USA) were first inoculated in Vascular Cell Basal Medium (ATCC® PCS-100-030™) supplemented with 10% fetal bovine serum (ATCC® 30-2020™). Then the cells were cultivated in an incubator (Heracell 150i, ThermoFisher, USA) at 37 °C with 5% CO₂, and hypoxically treated with 94% N₂, 5% CO₂ and 1% O₂ for 2 h.

Naringenin treatment or transfection needed to be performed 24 h before the cells were subjected to hypoxic modeling. Specifically, 10 μM Naringenin solution fully reacted with HUVECs under normoxic or hypoxic condition for 24 h. MiR-223-3p mimic (miR 10000892-1-5) and mimic control (miR1N0000001-1-5), and the transfection reagent RiboFECT CP Transfection Kit (C10511-05) were provided by RIBOBIO. The overexpression plasmid of Type 1

Table 1
Primers for qRT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
miR-223-3p	CCCAGTCGTATCCAGTGCAA	GTCGTATCCAGTGCGTGCCG
IGF1R	AGGATATTGGGCTTTACAACCTG	GAGGTAACAGAGGTCAGCATTTT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
GAPDH	TGTGGCATCAATGGATTGG	ACACCATGTATTCCGGGTCAAT

insulin-like growth factor receptor (IGF1R) was constructed by amplifying IGF1R and inserting it into the pcDNA3.1 (+) vector (VT1001, YouBio, China). The transfection efficiency after 48 h was verified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

2.5. qRT-PCR

The RNA in myocardial tissue and HUVECs was successfully extracted and reversely transcribed into cDNA with the participation of Total RNA Extraction Kit (R1200, Solarbio, China) and RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo-Scientific, USA). The following steps on qRT-PCR referred to the existing reports [22]. Concretely, cDNA, SYBR Green stain, primers (Sangon, China) and DEPC water were added to Real-Time PCR Instruments (ABI, USA), followed by a series of reactions and tests. The final gene mRNA level was obtained by calculating $2^{-\Delta\Delta CT}$ [22]. U6 was appeared as an internal reference for miR-223-3p, while GAPDH was used as an internal reference for other genes. The primer sequence was shown in Table 1.

2.6. Cell counting Kit-8 (CCK-8)

1×10^5 HUVECs (after drug treatment or transfection) were inoculated in 96-well plates covered with medium. After 24 h of normal culture, 10 μ L of CCK-8 detection solution (HY-K0301, MCE, USA) was added to each well containing HUVECs and reacted for 4 h. Next, the 96-well plates were placed in the Molecular Devices SpectraMax iD5 microplate reader to test the absorbance (OD value) at 450 nm.

2.7. Angiogenesis experiment

Angiogenesis experiment was conducted to determine the viability and basic function of HUVECs. Matrigel (354230, BD) was put into a 4 °C refrigerator to dissolve one day before experiment. The bottom of the Ibidi angiogenesis slide (81501) was covered with 10 μ L Matrigel, and then placed in a Petri dish. After Matrigel was coagulated, 1×10^4 HUVECs were seeded on angiogenesis slides. Finally, the changes of angiogenesis in HUVECs after 24 h were observed through a microscope (magnification $\times 100$).

2.8. Cell scratch test

Before the formal experiment, we used a marker pen to draw parallel lines (0.8 cm apart) across the holes on the back of the sterilized Petri dish (6-well plate). Then 5×10^5 /group HUVECs were inoculated in a Petri dish. After 24 h, we drew two parallel lines perpendicular to the parallel lines and along the center, and washed the HUVECs in the middle of the parallel lines. After normal culture for 24 h again, we observed and counted the number of migrated cells through a microscope with a field of view of 100 times magnification.

2.9. Bioinformatics analysis & verification experiment

Based on the functional mechanism of miRNA-mRNA, the downstream target genes of miR-223-3p were harvested by four different databases (miRDB, <http://www.mirdb.org/>; TargetScan, <http://www.targetscan.org/>; Starbase, <http://starbase.sysu.edu.cn/>; miRTarBase, <http://mirtarbase.cuhk.edu.cn/>). The common target genes were obtained by Venn diagram analysis of the results of four databases.

The verification experiment on whether miR-223-3p and IGF1R could bind in HUVECs was completed by dual luciferase reporter assay. For co-transfection, the plasmids composed of IGF1R wild-type (WT; 5'-CCUGCCCAAACCCUUAACUGACA-3') and IGF1R mutant (MUT; 5'-CCUGCCCAAACCCUUAACCAAUA-3') which was structured by the reporter gene vector pmirGLO (E1330, Promega, USA) and miR-223-3p mimic/mimic control were used. Next, the transfected HUVECs were reacted with the reagents in the dual luciferase reporter kit (E1910, Promega, USA) in accordance with the operating instructions. The terminated HUVECs were added to the Promega GloMax 20/20 Luminometer to test the luciferase activity.

2.10. Western blot

The protein in HUVECs was extracted by Solarbio's ordinary RIPA lysate. After completing the detection of concentration by BCA Protein Assay Kit (PC0020, Solarbio), the protein was transferred to a 0.45 μ m PVDF membrane (05317, Millipore, USA) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and

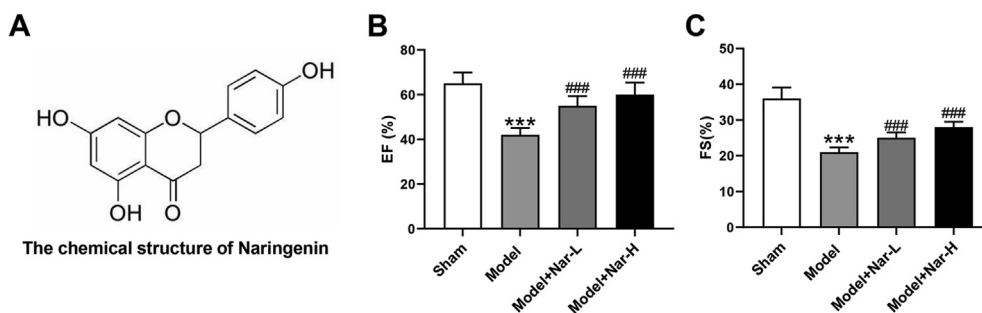


Fig. 1. Naringenin improved heart function in mice with MI (A) The chemical structure of Naringenin (B–C) The cardiac function of mice was evaluated by detecting the values of EF and FS (n = 10 mice/group). All experiments were repeated three times to average. ***p < 0.001 vs Sham; ###p < 0.001 vs Model. **Abbreviations:** MI, myocardial infarction; Nar, Naringenin; Nar-L, low-dose Naringenin; Nar-H, high-dose Naringenin; EF, Ejection Fractions; FS, Fraction Shortening.

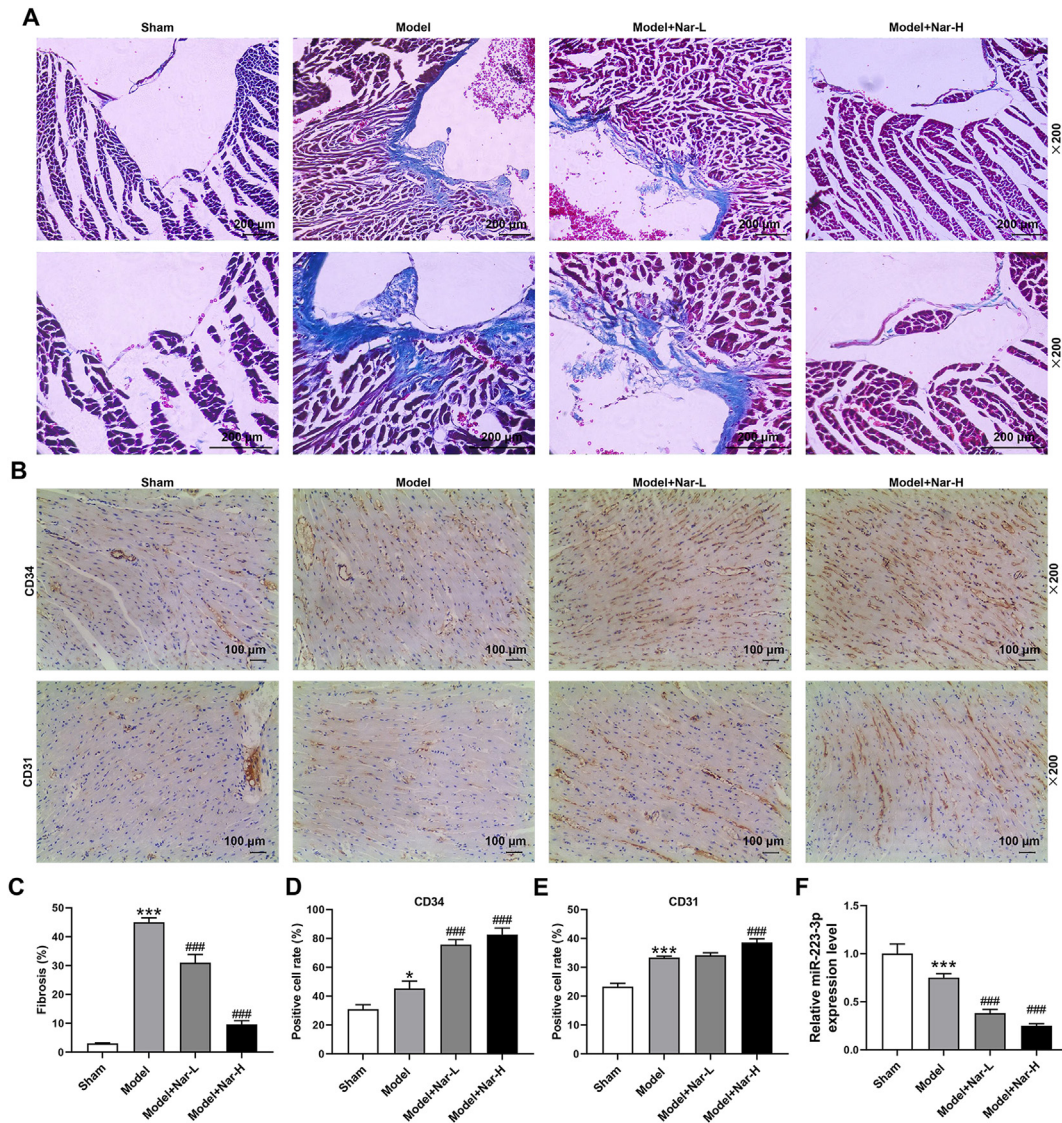


Fig. 2. Naringenin reduced the degree of myocardial fibrosis in model mice, stimulated the expression of angiogenic factors and down-regulated miR-223-3p expression (A and C) The degree of myocardial fibrosis in mice was observed by Masson staining and the fibrosis percentage was calculated (B, D and E) The expressions of CD34 and CD31 in mouse myocardial tissue were measured by immunohistochemistry and the relative positive cell rates were calculated (F) The expression of miR-223-3p in mouse myocardial tissue was detected by qRT-PCR. U6 was an internal reference. All experiments were repeated three times to average. **p* < 0.05, ****p* < 0.001 vs Sham; ###*p* < 0.001 vs Model. **Abbreviations:** MI, myocardial infarction; Nar, Naringenin; Nar-L, low-dose Naringenin; Nar-H, high-dose Naringenin.

electroporation. The PVDF membrane carrying the protein was then subjected to following series of treatments: blocked by 5% BSA blocking solution (SW3015, Solarbio); incubated with protein antibody at 4 °C for 24 h; incubated with HRP-conjugated secondary antibody at room temperature for 1 h; underwent color development and corresponding analysis. Specifically, the PVDF membrane was fully covered with a developing solution (WBULS0500, Millipore, USA) for 1 min (min), and then photographed, followed by the analysis of protein content with BIO-RAD Gel Doc™ XR + instrument (USA). GAPDH acted as a housekeeping gene. The following protein antibodies were used in this experiment: anti-IGF1R (Abcam ab182408; 1/1000; 156 kDa), anti-phosphorylated-PI3K (p-PI3K; Abcam ab32089; 1/1000; 126 kDa), anti-PI3K (Cell Signaling Technology #4292; 1/1000; 85 kDa), anti-p-Akt (Abcam ab38449; 1/1000; 56 kDa), anti-Akt (Abcam ab8805; 1/500; 55 kDa), anti-GAPDH (Abcam ab8245; 1/5000; 36 kDa), Goat Anti-Rabbit IgG (ab6721; 1/5000), and Goat Anti-Mouse IgG (ab205719; 1/5000) antibodies.

2.11. Data analysis

The data collected in the above experiments were analyzed by GraphPad Prism professional data analysis software (version 8.0). Specific analysis methods included independent sample analysis (comparison of differences between two groups), one-way analysis of variance (ANOVA) (comparison of differences between multiple groups) and Bonferroni post-hoc analysis (pairwise comparison). *P* < 0.05 was perceived statistically significant.

3. Result

3.1. Naringenin alleviated the degree of myocardial damage in MI mice, stimulated the expressions of angiogenic factors and down-regulated miR-223-3p expression

The chemical structure of Naringenin was presented in Fig. 1A. In animal experiments, some MI mice were treated with

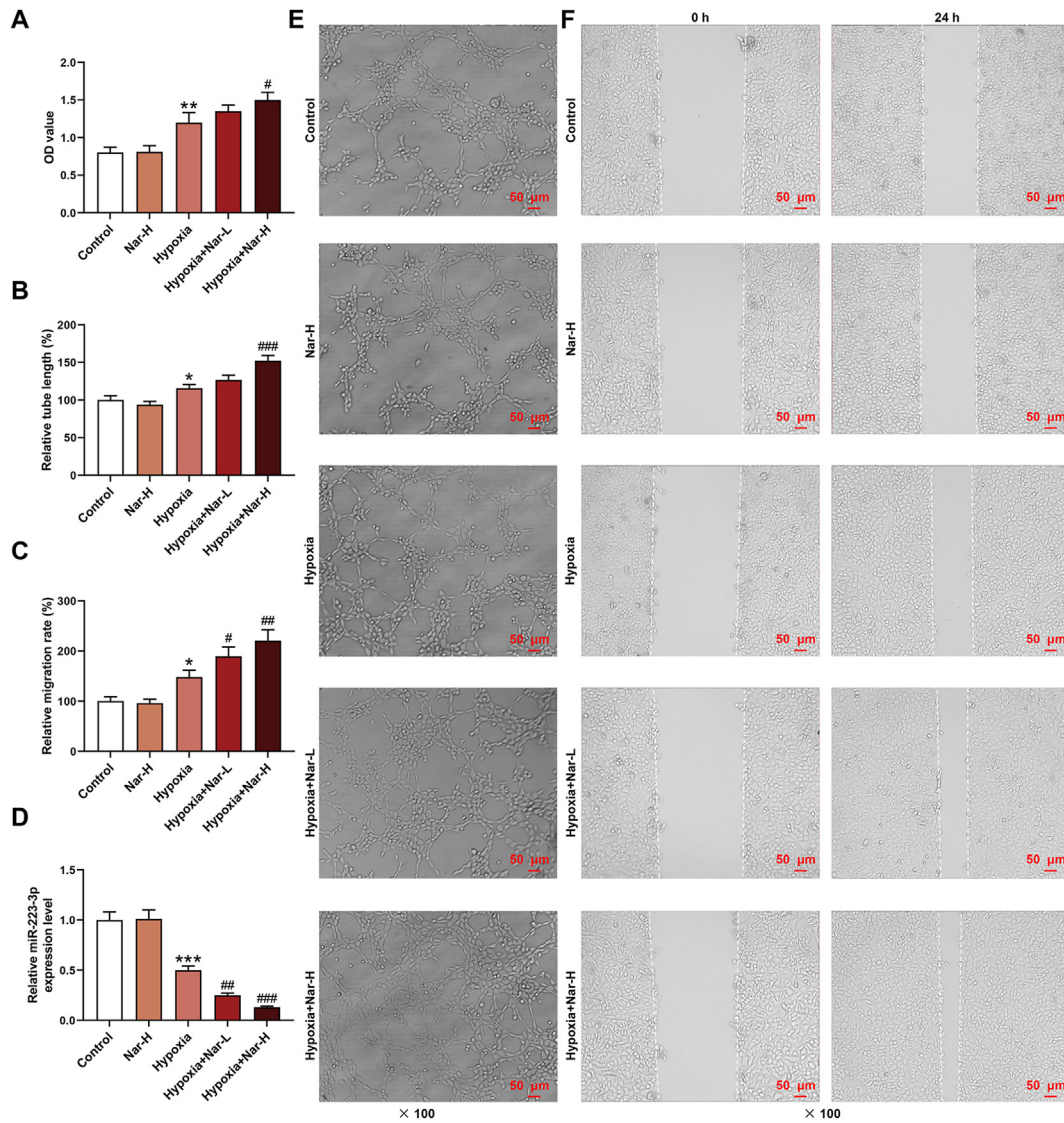


Fig. 3. Naringenin promoted the viability, tube formation and migration of hypoxic HUVECs, and further inhibited the expression of miR-223-3p (A) The effect of Naringenin on the viability of HUVECs in normoxic or hypoxic condition was tested by CCK-8 experiment (B and E) The effect of Naringenin on tube formation ability of HUVECs in normoxic or hypoxic condition was determined by tube formation experiments. The image was enlarged 100 times (C and F) The effect of Naringenin on the migration ability of HUVECs in normoxic or hypoxic condition was tested by scratch test. The image was enlarged 100 times (D) The regulation of Naringenin on miR-223-3p expression in HUVECs in normoxic or hypoxic condition was quantified by qRT-PCR. U6 was the internal reference. All experiments were repeated three times to average. ** $p < 0.01$, *** $p < 0.001$ vs Control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs Hypoxia. **Abbreviations:** Nar, Naringenin; Nar-L, low-dose Naringenin; Nar-H, high-dose Naringenin.

Naringenin. The results displayed that MI treatment did obvious myocardial damage to mice: EF and FS values were significantly decreased ($p < 0.001$, Fig. 1B–C), and obvious fibrotic lesions appeared around the infarcted myocardial tissue ($p < 0.001$, Fig. 2A and C). Naringenin treatment alleviated the above-mentioned damage, high-dose Naringenin in particular. EF and FS, representing the heart function of mice, were prominently elevated ($p < 0.001$, Fig. 1B–C), and degree of fibrosis around the infarcted myocardial tissue was also improved ($p < 0.001$, Fig. 2A and C). In response to our concern about angiogenesis in the infarct area, we observed the positive expression of angiogenic factors CD34 and CD31 through immunohistochemical staining. Thereinto, the positive expressions of CD34 and CD31 in the myocardial tissue of model mice were increased, followed by the growth in relative positive cell rate ($p < 0.05$, Fig. 2B, D and 2E). Moreover, high-dose Naringenin further increased the positive rates of CD34 and CD31 ($p < 0.001$, Fig. 2B, D and 2E). These results suggest that Naringenin

may alleviate MI by stimulating vascular regeneration. Additionally, we also analyzed the effect of Naringenin on miR-223-3p in the myocardium of MI mice. Compared with sham-operated mice, the expression of miR-223-3p in the myocardium of MI mice was down-regulated, and Naringenin further inhibited the mRNA level of miR-223-3p in the myocardium of model mice ($p < 0.001$, Fig. 2F).

3.2. Naringenin promoted the viability, tube formation and migration of hypoxic HUVECs, and further inhibited the expression of miR-223-3p

In *in vitro* studies, short-term hypoxic treatment promoted the viability of HUVECs ($p < 0.01$, Fig. 3A), and enhanced the abilities of tube formation and migration in HUVECs ($p < 0.01$, Fig. 3B–C and 3E–F), but inhibited the expression of miR-223-3p in HUVECs ($p < 0.001$, Fig. 3D). However, pretreatment with high-dose

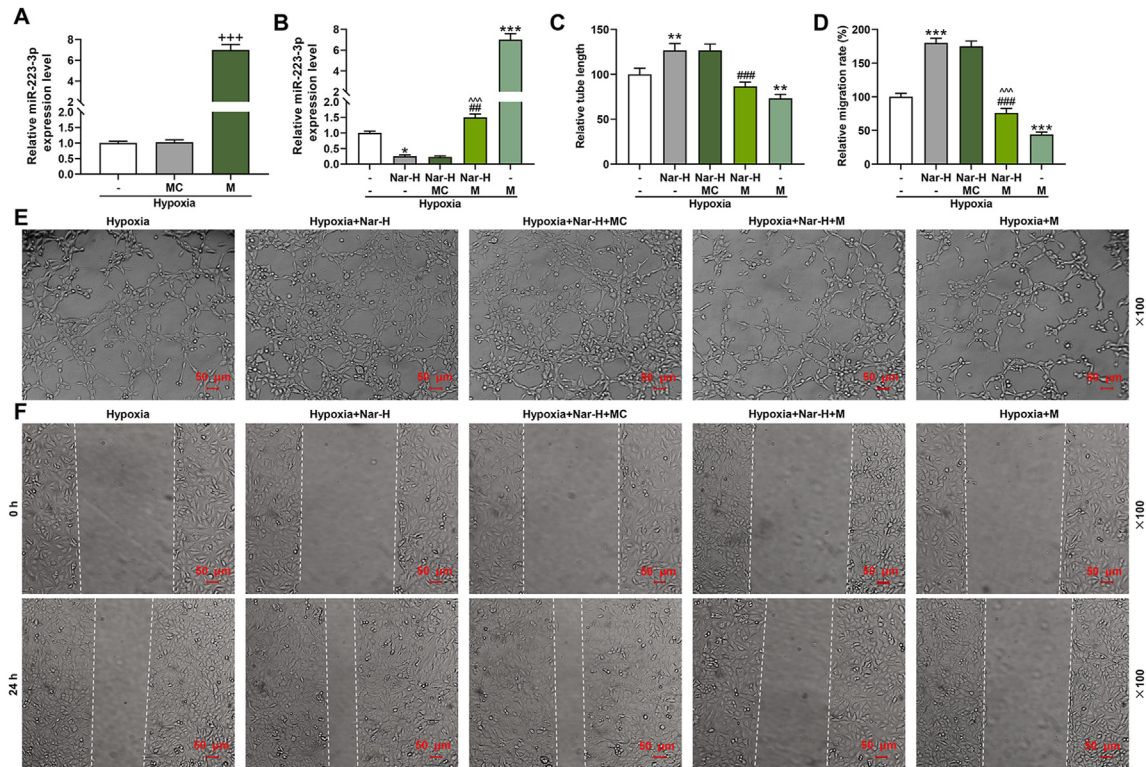


Fig. 4. MiR-223-3p reversed the regulation of Naringenin in hypoxic HUVECs (A) The transfection efficiency of miR-223-3p mimic was tested by qRT-PCR. U6 was the internal reference (B) The effects of miR-223-3p mimic and Naringenin on the expression of miR-223-3p were quantified by qRT-PCR. U6 was the internal reference (C and E) The effect of Naringenin on the tube-forming ability of hypoxic HUVECs was measured by tube formation experiments. The image was enlarged 100 times (D and F) The effect of Naringenin on the migration ability of hypoxic HUVECs was detected by scratch test. The image was enlarged 100 times. All experiments were repeated three times to average. +++*p* < 0.001 vs MC; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs Control; ##*p* < 0.01, ###*p* < 0.001 vs Nar-H + MC; ~*p* < 0.001 vs M. **Abbreviations:** MC, mimic control; M, miR-223-3p mimic; Nar, Naringenin; Nar-H, high-dose Naringenin.

Naringenin further aggravated the above-mentioned cellular response under hypoxic condition, although high-dose Naringenin did not significantly affect these cell phenotypes under normoxic condition. Compared with those in the Hypoxia group, the viability, tube formation and migration abilities of HUVECs in the Hypoxia + Nar-L group were further enhanced (*p* < 0.05, Fig. 3A–C and 3E–F), whereas the expression of miR-223-3p was continuously suppressed (*p* < 0.001, Fig. 3D). Low-dose Naringenin exerted a weak regulatory effect, and only promoted the migration of HUVECs yet inhibited miR-223-3p expression (*p* < 0.01, Fig. 3C–D and 3F).

3.3. MiR-223-3p reversed the regulation of Naringenin on hypoxic HUVECs

In the previous experiment, we observed the inhibitory effect of Naringenin on the expression of miR-223-3p. Therefore, we designed a rescue experiment using miR-223-3p mimic to verify the regulation of Naringenin on miR-223-3p. The experiments on transfection efficiency showed that the transfection of miR-223-3p mimic significantly increased the expression of miR-223-3p in HUVECs (*p* < 0.001, Fig. 4A). The following verification results exhibited that miR-223-3p mimic produced an opposite cellular effect to Naringenin, not only up-regulating its own mRNA level, but also significantly reducing the abilities of tube formation and migration in hypoxic HUVECs (*p* < 0.01, Fig. 4B–F). More importantly, miR-223-3p mimic partially offset the regulation of Naringenin on the above-mentioned cell functions (*p* < 0.01, Fig. 4B–F). These results confirmed our conjecture that Naringenin exerted a therapeutic effect by inhibiting miR-223-3p expression.

3.4. IGF1R, the downstream target gene of miR-223-3p, was a potential mechanism for Naringenin to exert the protective effect on HUVECs.

We predicted the target genes of miR-223-3p through multiple target prediction websites. Through the method of intersection, 22 common target genes were finally obtained (Fig 5A). By consulting the literature, IGF1R aroused our interest. IGF1R played an important role in MI-induced angiogenesis [8], and Naringenin stimulated HUVECs angiogenesis by inhibiting miR-223-3p expression, but whether the role of IGF1R was related to Naringenin still required further research. Fig. 5B showed the base sequence to which miR-223-3p and IGF1R could bind. The verification results in Fig. 5C suggested that the luciferase activity of IGF1R-MUT did not change, while that of the IGF1R-WT was prominently decreased after the transfection of miR-223-3p mimic, thus proving the binding relationship between IGF1R and miR-223-3p (*p* < 0.001). Next, we tested the expressions of IGF1R in myocardial tissue and HUVECs. IGF1R expression was significantly up-regulated in the myocardium of model mice, and Naringenin treatment further strengthened this regulatory effect (*p* < 0.001, Fig 5D). In hypoxic HUVECs, the pretreatment of Naringenin further increased the level of IGF1R. On the contrary, miR-223-3p mimic inhibited the expression of IGF1R and prevented the effect of Naringenin (*p* < 0.001, Fig 5E–G).

3.5. Overexpression of IGF1R reversed the regulation of miR-223-3p in hypoxic HUVECs.

After constructing the IGF1R overexpression plasmid, we first tested the regulatory efficiency of the plasmid. Both qRT-PCR and

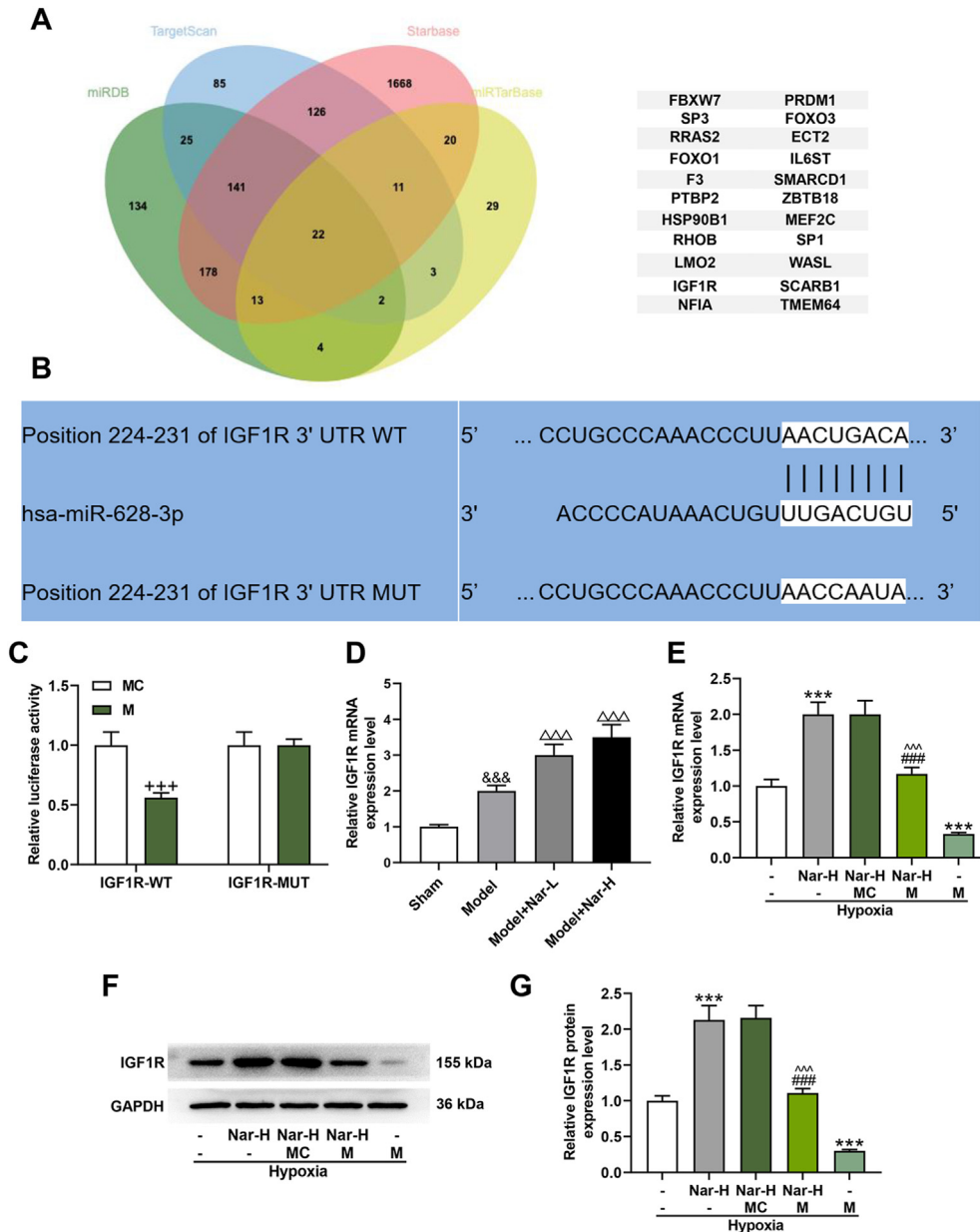


Fig. 5. IGF1R, a downstream target gene of miR-223-3p, was a potential mechanism for Naringenin to exert the protective effect on HUVECs (A) The target genes of miR-223-3p were screened through multiple target gene prediction websites (B–C) The binding relationship between miR-223-3p and IGF1R was predicted and verified (D) The expression of IGF1R in mouse myocardium was verified by qRT-PCR. GAPDH was an internal control (E–G) The expression of IGF1R in hypoxic HUVECs was detected by qRT-PCR and Western blot. GAPDH was an internal control. All experiments were repeated three times to average. $+++p < 0.001$ vs MC; $***p < 0.001$ vs Control; $###p < 0.001$ vs Nar-H + MC; ${}^{\sim}p < 0.001$ vs M; $\&\&\&p < 0.001$ vs Sham; $\Delta\Delta\Delta p < 0.001$ vs Model. **Abbreviations:** MC, mimic control; M, miR-223-3p mimic; Nar, Naringenin; Nar-L, low-dose Naringenin; Nar-H, high-dose Naringenin; IGF1R, Type 1 insulin-like growth factor receptor.

western blot results indicated that the level of IGF1R in HUVECs was greatly increased ($p < 0.001$, Fig. 6A–B). Overexpressed IGF1R up-regulated the mRNA and protein levels of IGF1R, increased the abilities of tube formation and migration in hypoxic HUVECs, and activated the phosphorylation of PI3K and Akt ($p < 0.05$, Fig. 6C–D and Fig. 7A–G). As we expected, overexpression of IGF1R reversed the regulation of miR-223-3p mimic in hypoxic HUVECs ($p < 0.01$, Fig. 6C–D and Fig. 7A–G).

4. Discussion

Acute MI is the prominent cause of death in cardiovascular diseases, with high morbidity, disability and mortality, bringing a

great economic burden to the patients [9]. Active and effective revascularization can significantly improve the patient's condition and prognosis. Although great progress has been made in revascularization methods such as surgery, there are still a large number of patients who cannot receive treatment due to complex lesion or intolerance [16]. Therefore, it is particularly important to find more effective MI drugs.

In this experiment, MI mice model and hypoxic HUVECs model were used to evaluate the therapeutic effect of Naringenin. The results were the same as we expected. Naringenin improved the heart function of MI mice, enhanced the angiogenesis ability of HUVECs, and up-regulated the expressions of pro-angiogenic factors. These results confirm that Naringenin exerts a pro-angiogenic

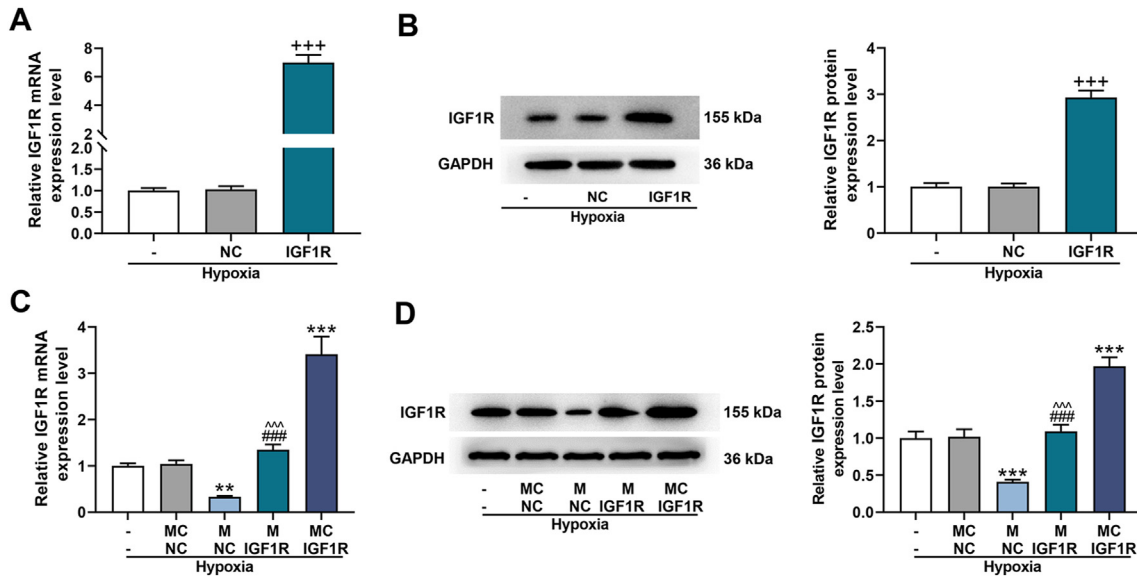


Fig. 6. IGF1R transfection significantly up-regulated the expression of IGF1R in hypoxic HUVECs (A–B) The transfection efficiency of overexpressed IGF1R plasmid was tested by qRT-PCR and Western blot (C–D) The effects of overexpression of IGF1R and miR-223-3p mimic on the level of IGF1R were detected by qRT-PCR and Western blot. GAPDH was an internal control. All experiments were repeated three times to average. ⁺⁺⁺*p* < 0.001 vs MC; ^{***}*p* < 0.001 vs M + NC; ^{###}*p* < 0.001 vs M + NC; [~]*p* < 0.001 vs MC + IGF1R. **Abbreviations:** MC, mimic control; M, miR-223-3p mimic; NC, negative control; IGF1R, overexpressed IGF1R.

effect in the myocardium after MI-induced ischemia. As a kind of monomer component with abundant sources, Naringenin has been widely explored for its application prospects in the field of medicine. Regarding the cardiovascular protective effect of Naringenin, most of the existing studies have clarified its therapeutic effect on myocardial ischemia-reperfusion injury based on energy metabolism and oxidative stress response pathways [28]. Our results expanded the therapeutic role of Naringenin in the cardiovascular field, and innovatively explored from the perspective of angiogenesis. Comparing the studies of other scholars, we found that Naringenin exerted a two-way regulatory effect on angiogenesis in the body, in line with Jawun’s results that Naringenin inhibited angiogenesis in melanoma [4]. Similar to our results, Feng confirmed that Naringenin inhibited the apoptosis of HUVECs induced by high glucose or free fatty acids via PI3K/Akt and JNK/Nrf 2 pathways [7]. The results above suggested that Naringenin exerted a protective effect in HUVECs. In view of the cardiovascular intervention of miRNAs and the conclusions of Zhao et al. [30], we turned the next research target to screen the differentially expressed miRNAs in the cardiovascular system and analyze the regulation of downstream targeted mRNAs.

MiRNAs are highly conserved in evolution, with tissue-specific and sequential expressions. Namely, MiRNAs are only expressed in specific tissues and specific developmental stages, playing pivotal roles in various biological activities of organisms [26]. As a new biological marker for early diagnosis of MI, MiRNA is closely related to the angiogenesis process [27]. More importantly, the research of miRNA omics has opened up a new field for the development of traditional Chinese medicine and monomer pharmacodynamics. As our research subject, miR-223-3p is the core miRNA in the angiogenesis process of ischemic myocardial microvascular endothelial cells (CMECs) [5]. In this study, the expressions of miR-223-3p in model mice and HUVECs were significantly lower than normal. After up-regulation of miR-223-3p expression, the angiogenesis and migration abilities of HUVECs were inhibited; what’s more serious is that miR-223-3p agonists counteracted the protective effect of Naringenin in HUVECs. Our

results suggested that the effect of Naringenin in promoting ischemic myocardial angiogenesis after MI might be related to the regulation of miR-223-3p. Apart from this, we selected IGF1R into the research, for which it played an important role in screening the downstream target gene associated with angiogenesis resulted from MI [8].

The IGF1R gene is located in chromosome 15 (15q26.3) and contains 21 exons. The product encoded by IGF1R not only has tyrosine kinase activity, but also has anti-apoptotic, mitogenic and transformational properties [24]. IGF1R is a powerful mitogenic factor. The results of experiments conducted by scientists on the cancerous transformation of virus-infected cells revealed that the prerequisite for cells to transform into a cancer phenotype after virus infection was the expression of IGF1R and the gene that coordinate cell movement, adhesion, cell cycle regulation, and metastasis function at the same time [14]. IGF1R has a cancer-promotive effect, acting a key regulatory role in cardiovascular disease [10]: IGF-1 binds to IGF-1R, and plays a multi-effect role in the growth, development and tissue repairing of cardiomyocytes. IGF1R and IGF2 have been identified as protein markers of tip cells (basis of angiogenesis) [6]. These evidences advantageously explain the cellular phenomena we have observed. Overexpression of IGF1R effectively promoted the angiogenesis of HUVECs. At the same time, as a downstream target gene of miR-223-3p, IGF1R reversed the damage of HUVECs caused by miR-223-3p.

Naringenin is widely found in natural plants, with a wide range of medicinal effects, diverse uses, small side effects, and broad application prospects. At present, the physical and chemical properties of Naringenin have been basically clarified. In recent years, domestic and foreign scholars have extensively explored the pharmacodynamics and pharmacological effects of Naringenin. Based on the existing reports, this study creatively demonstrated that Naringenin promoted angiogenesis in ischemic myocardium after MI, and it was clear that this effect was achieved by regulating the miR-223-3p/IGF1R axis. Nevertheless, our research is still dearth of the verification on clinical direction, which is also the goal that we need to overcome in the next step.

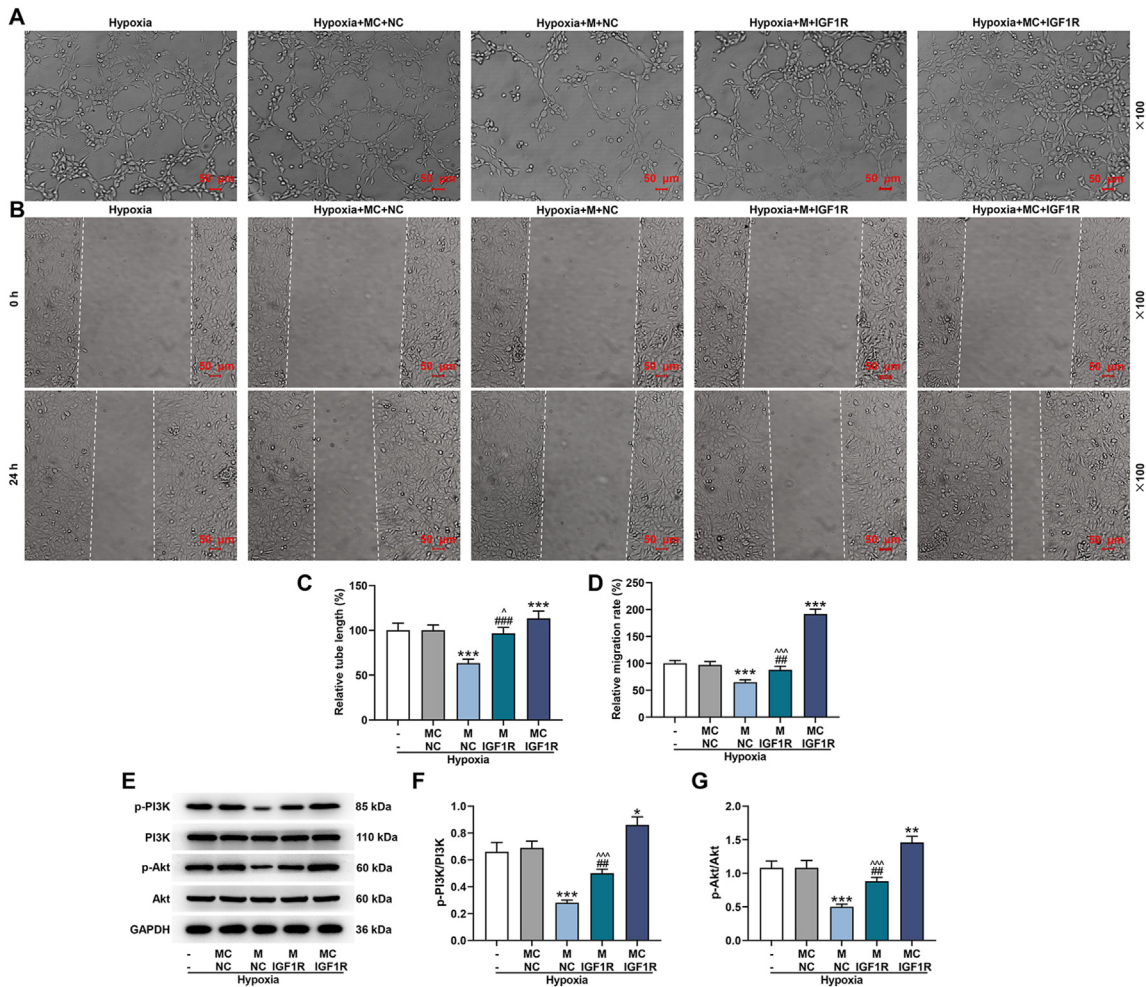


Fig. 7. Overexpression of IGF1R reversed the regulation of miR-223-3p in hypoxic HUVECs (A and C) The effects of overexpressed IGF1R and miR-223-3p mimic on the tube formation ability of hypoxic HUVECs were tested by tube formation experiments. The image was enlarged 100 times (B and D) The effects of overexpression of IGF1R and miR-223-3p mimic on the migration ability of hypoxic HUVECs were evaluated by scratch test. The image was enlarged 100 times (E–G) The regulation of PI3K/Akt pathway in hypoxic HUVECs by overexpression of IGF1R and miR-223-3p mimic was detected by Western blot. GAPDH was an internal control. All experiments were repeated three times to average. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs MC + NC; ### $p < 0.01$, #### $p < 0.001$ vs M + NC; $p < 0.05$, $p < 0.001$ vs MC + IGF1R. **Abbreviations:** MC, mimic control; M, miR-223-3p mimic; NC, negative control; IGF1R, overexpressed IGF1R.

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Authors' contributions

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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