MiR-106b and MiR-15b Modulate Apoptosis and Angiogenesis in Myocardial Infarction

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Key Words

Myocardial infarction • MicroRNA • Bioinformatics • Regulatory network • Apoptosis • Angiogenesis

Abstract

Background: MicroRNAs (miRNAs) are identified as crucial gene regulators in response to myocardial infarction (MI). However, the overall relationships between miRNAs and the gene targets which contribute to the cellular phenotypes in MI are not fully elucidated. To make a better understanding towards functional roles of miRNAs in MI, useful information was mined through bioinformatic techniques. **Method:** MI-related miRNAs were retrieved from publications, and PicTar, TargetScanS, and miRanda programs were used to predict their gene targets. Gene ontology (GO) and pathway analyses of gene targets were applied to uncover functional roles of miRNAs. The miRNA-gene networks were illustrated by Pajek tool. Finally, validation experiments were performed towards two important miRNAs in the networks. **Result:** Up to 119 MI-related miRNAs were retrieved from publications. GO and pathway analyses for their predicted gene targets demonstrated that these dysregulated

miRNAs were enriched in cardiovascular-related phenotypes. Through illustrating miRNA-gene networks, overall relationships between miRNAs and gene targets were detected especially in processes of apoptosis and angiogenesis. Moreover, experimental data supported bioinformatic predictions that miR-106b served as an anti-apoptotic modulator through inhibition of p21 expression and miR-15b displayed anti-angiogenesis activity. **Conclusion:** The miRNAs played essential roles in pathological processes of MI. Further, miR-106b and miR-15b maybe mediated as robust regulators in apoptosis or angiogenesis following MI, respectively.

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Introduction

MicroRNAs (miRNAs) comprise a novel class of endogenous, small non-coding RNAs that negatively regulate gene expression. At the post-transcriptional level, they act as negative regulators by binding to the 3'-Untranslated region (UTR) of targeted mRNA through base pairing, which results in the cleavage of targeted mRNA or translation inhibition [1]. Increasing evidences suggest that miRNAs play crucial roles in nearly all

important biological processes [2-3].

Myocardial infarction (MI), also known as heart attack, is the most frequent cause of heart failure. In particular, acute MI represents an enormous clinical challenge as loss of myocardium. After the acute phase, a chronic change of ventricular remodeling occurs. This remodeling is maladaptive and associated with persistent cardiomyocyte apoptosis, angiogenesis, interstitial fibrosis, cardiac hypertrophy, wall thinning, and ventricular chamber enlargement, which contribute to the development of depressed cardiac function, clinical heart failure, and increased mortality [4-6].

Mounting evidences show that miRNAs mediate as essential modulators in pathological processes of MI, such as apoptosis, angiogenesis, interstitial fibrosis, and cardiac hypertrophy. These studies involve microarray analysis using animal model of MI or heart pathological samples from MI patients [7-9], and some particular investigations which detect specific miRNA functions associated with this disease [10-12]. These results are meaningful to make recognition of miRNAs involved in MI. However, it is a pity that these evidences are scattered, which are not informative enough to take a more comprehensive view of the correlations between miRNAs and MI.

The present study thus integrated these findings using bioinformatic techniques to give a better understanding of functional roles of miRNAs in MI. Furthermore, validation experiments were conducted towards some important bioinformatics-based predictions, especially in the pathological processes of apoptosis and angiogenesis in MI.

Materials and Methods

Retrieval of miRNAs involved in MI

The miRNAs involved in MI were retrieved manually by searching PubMed for the documents which contained miRNAs related to MI (Myocardial infarction [Mesh] AND microRNAs [Mesh]) or any other publications. Moreover, HMDD (Human MicroRNA Disease Database) was also consulted to acquire the relevant miRNAs [3]. The obtained miRNAs were further treated by miRBase, and the miRNA was removed if it was not included in this database [13].

Prediction of putative gene targets

Pictar, TargetScanS, and miRanda programs were used to search gene targets for the obtained miRNAs [14]. Furthermore, defined gene targets were subjected to search across Unigene database to make sure whether they may have the potential to express in heart tissue under physiological or pathological conditions.

GO and functional enrichment analyses

The gene targets were classified into functional groups with DAVID (Database for Annotation, Visualization, and Integrated Discovery) according to biological process (BP) classification of Gene Ontology (GO) [15]. As input, the GenBank ID of each gene target was used. As output, significant processes (GOTERM) with $P < 0.05$ were generated. In addition, related biological processes were clustered with the functional annotation clustering algorithms of DAVID. For each clustered process, this resulted in an Enrichment Score, the $-\log$ value of the geometric mean of the member's P values. The gene targets not incorporated into any significant process were checked one by one in order to avoid omitting the one which was actually involved in a specific pathological category, such as apoptosis or angiogenesis. All the details can be freely found at <http://david.abcc.ncifcrf.gov/>.

Analysis of biological pathways

Biological pathways were analyzed statistically according to KEGG and Biocarta databases in DAVID platform. Further, GenMAPP was used to view the gene targets involved in specific biological pathways, such as apoptosis, angiogenesis, fibrosis, and hypertrophy. Details can be freely found at <http://www.genmapp.org/>.

Illustration of networks between miRNAs and gene targets

According to classification results of GO and biological pathways, specific gene targets associated with apoptosis and angiogenesis were collected to develop the miRNA-gene networks. The miRNA-gene networks were visualized by publicly available tool Pajek (<http://vlado.fmf.uni-lj.si/pub/networks/pajek/>) [16].

Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs, ScienCell Research Laboratories, USA) were cultured in complete endothelial cell medium (ECM) as described previously [17]. For experiments, cells of passage No. 4–6 were grown until confluence. H9c2 cells were obtained from ATCC and cultured according to manufacture's instructions. For experimental hypoxia, cells were placed in an airtight humidified chamber with 37°C, 5% CO₂, and 95% N₂. The corresponding normoxia control cells were cultured in a humidified incubator with 37°C, 5% CO₂, and 21% O₂.

Transfection of miRNA mimic, miRNA inhibitor and adenovirus

The miR-106b mimic (cat. no. MSY0000825), miR-15b mimic (cat. no. MSY0000784), miR mimic scramble (cat. no. 1027280), miR-106b inhibitor (cat. no. MIN0000825), and miR inhibitor scramble (cat. no. 1027271) used in our study were designed by and purchased from Qiagen. Cells were transiently transfected with a final concentration of 25 nM or 50 nM for miR mimic and 100 nM for miR inhibitor using Lipofectamine 2000 (Invitrogen). Recombinant adenoviruses expressing p21 or control viruses were constructed with the Ad-Easy system and cultures were routinely infected at a multiplicity of infection

of five with an infection efficiency up to 80%.

q-RT-PCR

Total RNA was purified from cells with Trizol reagent (Invitrogen). The primers used were as follows: VEGF: 5'-TTG CCT TGC TGC TCT ACC TC-3' (forward), 5'-TGC ATG GTG ATG TTG GAC TC-3' (reverse); Ang2: 5'-GAC GGC TGT GAT GAT AGAAAT AGG-3' (forward), 5'-GAC TGT AGT TGG ATG ATG TGC TTG-3' (reverse); GAPDH: 5'-GAC CCC TTC ATT GAC CTC-3' (forward), 5'-GCT AAG CAG TTG GTG GTG-3' (reverse). The primers for miRNAs (cat. no. MS00000301 for miR-106b; cat. no. MS00013097 for miR-15b) used in our study were designed by and purchased from Qiagen. The quantification was performed with SYBR Green (Qiagen). Each sample was analyzed in triplicate using Bio-Rad iQ5 Multicolor Real-Time PCR Detection System. The comparative threshold cycle (CT) method was used to determine gene relative expression. U6 was chosen as an internal control gene for miRNA.

Immunoblot analysis

Immunoblot was performed according to previous protocols [17]. In brief, total proteins were extracted from cells using cell lysis buffer. Protein (40–60 µg) from each sample was separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with primary antibodies and then with horseradish peroxidase (HRP)-conjugated secondary antibodies. The enhanced chemiluminescence signal was detected using a densitometry program (Gel-pro 4.5 Analyzer, Media Cybernetics). To quantify the protein signal, we subtracted background and normalized the value to β-actin.

Construction of 3'-UTR reporter and luciferase assay

3'-UTR of p21 fragments were PCR-amplified from rat genomic DNA and cloned into a vector at the XbaI site 3' of the luciferase gene (pGL3; Promega, Madison, WI). The primers used for PCR amplification were as follows: 5'-GGG TCT AGA TTT AAG TCC TTC TCA ACC CAA G-3' (sense); 5'-GGG AGA TCT GGC TAA AGA TAA ACA GTC CAG C-3' (antisense). H9c2 cells were seeded in 96-well plates and transfected with 50 nM miR mimic or 100 nM miR inhibitor, 100 ng of luciferase vector (pGL3 constructs), and 25 ng of Renilla vector (pRL-TK) using Lipofectamine 2000 (Invitrogen). Twenty-four hours later, cells were lysed and ratios between firefly luciferase and Renilla luciferase activity were measured with a dual-luciferase assay (Promega).

Endothelial cell tube formation assay

Sterile 24-well plates were coated with 200 µl Matrigel and incubated at 37°C for 1 h to form gels. After polymerization of the gels, 1×10^5 HUVECs were seeded into each well and incubated with 1.0 ml DMEM containing 1% FBS, and then exposed to normoxia or hypoxia. After 16 h, each well was washed in PBS and fixed with 4% paraformaldehyde. Four different fields were chosen randomly in each well and photographs were taken. The length of the tubes was quantified using Image-Pro Plus software (Media Cybernetics, L.P., Silver Spring, MD, USA).

Cell migration assay

Migration assays were performed using Costar Transwell plates with 6.5 mm-diameter polycarbonate filters (8 µm pore-size). The lower surface of the filter was coated with 10 µg of gelatin. Fresh ECM containing 5% FBS were placed in the lower wells. HUVECs with a final concentration of 1×10^6 cells/ml were seeded onto the upper surface of the filter. Then, the chamber was incubated at normoxia or hypoxia. After 24 h, nonmigrating cells were removed from the upper surface of the filter and migration was observed using an inverted microscope. Images were captured with digital camera and cells were quantified by counting the cells that migrated to the lower side. Four randomly chosen fields were counted per well.

VEGF and Ang2 ELISA

VEGF and Ang2 protein levels were detected in conditioned media using VEGF and Ang2 ELISA assays according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Each sample was tested in duplicate. Concentrations were reported in pg/ml.

Apoptosis ELISA

Cells were seeded in 96-well plates for 24 h, and then were transfected with miR-106b inhibitor or miR scramble. At the indicated time point, quantification of cell apoptosis was determined by an ELISA that measured cytoplasmic histone-DNA fragments produced during apoptosis (Roche). The enrichment of histone-DNA fragments was expressed as fold change in absorbance compared with control (normoxic miR inhibitor scramble group).

Statistical analysis

All results were presented as means ± S.E.M. Differences between groups were evaluated for statistical significance using Student's t or ANOVA tests. In each case, significance was defined as $P < 0.05$.

Results

The miRNAs involved in MI

One hundred and nineteen miRNAs correlated to MI were retrieved (Table 1.), all of which were from documented experimental results. In several studies, miRNA microarray analysis has been performed using animal models of MI [7-8, 18-19] or heart pathological samples from patients [9]. Some particular investigations detect specific miRNA function associated with MI using cell lines or animal models, for example, the function of miRNAs in apoptosis [10-11, 20-21], fibrosis [19, 22], or angiogenesis [23-25].

General feature of GO and pathway analyses

To assess the functional roles of MI-related miRNAs, their gene targets were predicted (data not shown), and

overrepresented BP classes were determined with DAVID according to GO classification. We should be aware that the same gene may belong to different GO processes. The annotated clusters were listed according to their Enrichment scores, and the first thirty were presented (Table 2.). We found that by far the most prominently enriched BP process in the gene targets was regulation of cellular processes with the Enrichment score of 22.5. Significantly, many processes which associated closely with MI were also predominated (Enrichment score>2.0), such as angiogenesis (cluster 16) and regulation of apoptosis (cluster 26). Moreover, biological pathways were analyzed statistically with KEGG and Biocarta databases through DAVID platform. It seemed that these gene targets were involved in many pathways correlated with cardiovascular pathology, such as apoptosis, angiogenesis, interstitial fibrosis, and cardiac hypertrophy. Thus, the results of GO and pathway analyses demonstrated that the retrieved miRNAs were enriched in cardiovascular-related functions.

The miRNA-gene network associated with apoptosis

Recent studies indicate that apoptosis plays a role in the process of tissue damage after MI, which is of pathological and therapeutic significance [26]. In the present study, gene targets related to apoptosis were identified, and the network between miRNAs and apoptotic gene targets was constructed (Fig. 1). From the network, we noticed that more apoptotic gene targets were regulated by miR-106b, miR-17, miR-30a, miR-30b, and miR-30c. The miR-106b targeted 14 genes, such as FASTK, BCL11B, and CDKN1A. Secondary to miR-106b, 13 gene targets were regulated by miR-30a, miR-30b, and miR-30c, including ATG5 and PAWR. Some other miRNAs also manifested their potential modulatory activities in this network, and they were mir-15 family members, including miR-195, miR-15b, and miR-16 (7 apoptotic gene targets), and mir-181 family members, including miR-181a, and miR-181c (7 apoptotic gene targets). Recent studies provided evidence that Sox4 is involved in regulation of apoptosis [27]. In the network, SOX4 gene was targeted by 10 miRNAs, more than other gene targets. Bcl-2, an important apoptosis inhibitor, is expressed in cardiomyocytes surrounding infarcted areas soon after the onset of infarction [26]. We found that miR-195, miR-15b, and miR-16 targeted BCL2 gene. Death receptor signaling is also highly activated in MI [26]. Three gene targets associated with death receptor signaling pathway were obtained, including TNFAIP3,

Table 1. The miRNAs related to MI disease.				
let-7a	miR-132	miR-194	miR-27b	miR-494
let-7b	miR-133a	miR-195	miR-29a	miR-497
let-7c	miR-133b	miR-199a-3p	miR-29b	miR-499-5p
let-7d	miR-139-5p	miR-199a-5p	miR-29c	miR-532-5p
let-7e	miR-143	miR-199b*	miR-30a	miR-572
let-7f	miR-145	miR-206	miR-30a*	miR-574-3p
let-7g	miR-145*	miR-208	miR-30b	miR-574-5p
miR-140*	miR-146b-5p	miR-21	miR-30c	miR-575
miR-1	miR-149	miR-210	miR-30e*	miR-638
miR-100	miR-149*	miR-214	miR-31	miR-663
miR-101	miR-150	miR-218	miR-320a	miR-663b
miR-103	miR-152	miR-22	miR-328	miR-711
miR-106b	miR-155	miR-22*	miR-335	miR-762
miR-107	miR-15b	miR-221	miR-34a	miR-92a
miR-10a	miR-16	miR-222	miR-361-5p	miR-92b
miR-10b	miR-17	miR-223	miR-365	miR-933
miR-122	miR-181a	miR-23a	miR-378	miR-98
miR-1224-5p	miR-181c	miR-23b	miR-379	miR-99a
miR-1231	miR-181d	miR-23b*	miR-422a	miR-99b
miR-125a-5p	miR-185	miR-24	miR-423-5p	
miR-125b	miR-186	miR-24-2*	miR-424	
miR-126	miR-188-5p	miR-25	miR-451	
miR-127	miR-18a	miR-26a	miR-466	
miR-1291	miR-18b	miR-26b	miR-483	
miR-130a	miR-192	miR-27a	miR-486-5p	

Table 2. First thirty clusters of BP analysis for the gene targets.

Cluster	Category	Enrichment Score
Annotation Cluster 1	Regulation of cellular process	22.46
Annotation Cluster 2	Positive regulation of biological process	12.33
Annotation Cluster 3	Protein metabolic process	11.02
Annotation Cluster 4	Cellular catabolic process	9.83
Annotation Cluster 5	Negative regulation of biological process	9.48
Annotation Cluster 6	Morphogenesis	7.73
Annotation Cluster 7	Chromosome organization	7
Annotation Cluster 8	Regulation of protein metabolic process	5.83
Annotation Cluster 9	Development	5.65
Annotation Cluster 10	Tube development	5.61
Annotation Cluster 11	Embryonic development	5.32
Annotation Cluster 12	Proten ubiquitination	5.23
Annotation Cluster 13	RNA metabolic process	5.16
Annotation Cluster 14	Chromatin modification	4.77
Annotation Cluster 15	Intracellular receptor-mediated signaling pathway	4.63
Annotation Cluster 16	Angiogenesis and vascular development	4.2
Annotation Cluster 17	Localization	4.15
Annotation Cluster 18	Ubiquitin-dependent protein catabolic process	3.91
Annotation Cluster 19	Embryonic morphogenesis	3.89
Annotation Cluster 20	Regulation of development process	3.86
Annotation Cluster 21	Regulation of molecular function	3.81
Annotation Cluster 22	Cell cycle	3.7
Annotation Cluster 23	Gene silencing	3.33
Annotation Cluster 24	Methylation	2.86
Annotation Cluster 25	Intracellular protein transport	2.8
Annotation Cluster 26	Regulation of apoptosis	2.65
Annotation Cluster 27	Tube morphogenesis	2.25
Annotation Cluster 28	Response to chemical stimulus	2.21
Annotation Cluster 29	Regulation of cell growth and cell size	2.19
Annotation Cluster 30	Regulation of fibroblast proliferation	2.16

Fig. 1. The miRNA-gene network associated with apoptosis. To make an overall view of the relationship between miRNAs and apoptosis, the gene targets associated with apoptosis were obtained, and a miRNA-gene network was developed by publicly available tool Pajek.

DAPK1, and FASTK. Additionally, some other important apoptotic genes in the network should also be emphasized, including IGF-1, HSP90B1, and CASP2.

The miRNA-gene network associated with angiogenesis

It has been well established that a pathophysiological role of angiogenesis exists in the process of MI [28]. The network provided valuable data elucidating relationships between angiogenic gene targets and miRNAs (Fig. 2.). We noticed that miR-15b, miR-195, and miR-16 showed their significant roles in angiogenic modulation, by regulating more gene targets than other miRNAs. Current evidence suggests that Smad7 promotes vascular formation [29]. In the network, eight miRNAs targeted SMAD7 gene, including miR-15b, miR-16, miR-195, miR-

181a, miR-181c, miR-21, miR-25, and miR-92a. HIF-1 is well known to induce expression of several growth factors required for the angiogenic response [30]. Thus, it was interesting that miR-18a, miR-18b, and miR-424 targeted HIF1A gene. Slit-Robo signaling plays an important role in angiogenic process. We noticed that miR-218 targeted ROBO1 gene in the network.

Hypoxia-induced miR-106b exhibited anti-apoptosis property

In the apoptotic miRNA-gene network, miR-106b was a hub node. To date, no experimental data report its regulatory role in cardiac apoptosis. In the present study, hypoxia treatment caused a significant increase in the expression of miR-106b in H9c2 cells, which reached a peak at 2 h and declined to near basal levels at 8 h (Fig.

Fig. 3. Hypoxia-induced miR-106b exhibits anti-apoptosis property. (A) H9c2 cells were treated with hypoxia for the indicated time points. The relative expression level of miR-106b was detected by q-PT-PCR ($n=5$, means $\pm$ S.E.M). * $P<0.05$; ** $P<0.01$. (B) H9c2 cells were transfected with miR-106b inhibitor (100 nM) or miR inhibitor scramble (100 nM). After 18 h, cells were treated with hypoxia for 6 h. The relative expression level of miR-106b (top) was detected as in A ($n=5$, means $\pm$ S.E.M). ** $P<0.01$; ## $P<0.01$. The protein levels of cleaved caspase-8, cleaved caspase-9, cleaved caspase-3, and β -actin were detected by immunoblot analysis and a representative blot was shown (middle). Quantitative analysis of protein levels (bottom) was expressed as fold change above normoxic miR inhibitor scramble group ($n=5$, means $\pm$ S.E.M). # $P<0.05$. (C) H9c2 cells were treated as in B, and apoptosis were determined using ELISA assay ($n=5$, means $\pm$ S.E.M). ** $P<0.01$; ## $P<0.01$.

the pGL3 construct with either miR-106b mimic or inhibitor into H9c2 cells. As expected, the miR-106b inhibitor and mimic upregulated or downregulated the luciferase activity, respectively (Fig. 4D). Taken together, these results indicated that miR-106b could bind to 3'-UTR of p21 and inhibit its expression in H9c2 cells.

miR-15b displayed anti-angiogenesis activity

In the angiogenic miRNA-gene network, miR-15b was a focus. However, few investigations reported its functional role in angiogenesis after MI. In the present experiment, hypoxia treatment caused a reduction in the expression of miR-15b in HUVECs, significantly at time points of 4 and 8 h (Fig. 5A). To further investigate the role of miR-15b in angiogenic process, we transfected HUVECs with miR-15b mimic to abolish hypoxia-caused decrease of miR-15b expression. A significant increase of miR-15b expression was observed in cells transfected with miR-15b mimic compared with scramble group (Fig. 5B). Further, functional assays showed that miR-15b mimic markedly attenuated hypoxia-induced tube formation (Fig. 5C) and cell migration (Fig. 5D). However, HIF-1 α was not affected obviously by miR-15b mimic

transfection (Fig. 6A). Notably, hypoxia-induced VEGF and Ang2 expression was significantly suppressed after transfection of miR-15b mimic (Fig. 6B). Thus, it is possible that miR-15b inhibited expression of VEGF and Ang2 and displayed anti-angiogenesis activity following heart attack.

Discussion

MI is caused by sudden insufficient blood supply to the heart, resulting in a loss of viable cardiac tissue. The miRNAs are small, non-coding RNAs that regulate gene expression in a sequence-dependent manner. Recently, miRNAs have been shown to play essential roles in MI. However, evidence is limited about how the pathogenetic processes are caused by the malexpressed miRNAs, and the overall correlations are to be elicited between miRNAs and the gene targets which contribute to the specific cellular phenotype after MI. For these reasons, the present study was performed. The MI-related miRNAs were retrieved from publications. The gene targets were predicted and analyzed bioinformatically to detect how

Fig. 4. p21 was a functional target of miR-106b during hypoxia. (A) Bioinformatic analysis indicated the p21 might be a target gene of miR-106b. (B) H9c2 cells were infected with adenovirus vector (Ad-control) or p21 (Ad-p21) and exposed to normoxia or hypoxia for 6 h. The protein levels of p21, cleaved caspase-8, cleaved caspase-9, cleaved caspase-3, and β -actin were detected by immunoblot analysis and a representative blot was shown (Top). Apoptosis were determined using ELISA assay ($n=4$, means $\pm$ S.E.M). $\#P<0.05$ (Bottom). (C) H9c2 cells were transfected with miR-106b inhibitor (100 nM) or mimic (50 nM). After 18 h, cells were treated with hypoxia for 6 h. The protein levels of p21 and β -actin were detected by immunoblot analysis and a representative blot was shown. (D) A construct containing a fragment of the 3'-UTR of p21 mRNA with the putative miR-106b binding sequence was cloned into a luciferase reporter construct (pGL3 construct) and transfected into H9c2 cells with miR-106b inhibitor (100 nM) or miR-106b (50 nM) and 100 ng of luciferase vector and 25 ng of Renilla vector (pRL-TK) ($n=3$, means $\pm$ S.E.M). $\#P<0.05$.

these miRNAs functioned in MI, and the roles of miRNAs in apoptosis and angiogenesis were especially examined.

From the manual retrieval of the publications, 119 miRNAs were obtained (Table 1.). Current understanding of the functions of miRNAs is mainly derived from the computational target prediction [33-34]. Thus, identifying the gene targets of miRNAs by bioinformatic analysis will give us more insight into the molecular mechanisms involved in MI. Nowadays, PicTar, TargetScanS, and miRanda are the most common target prediction tools. These three target prediction programs were used in our study to acquire gene targets of the retrieved miRNAs, and only the target predicted by all the three programs was considered as a target gene for a specific miRNA. The acquired gene targets were collected and subjected to GO analysis which was applied in order to organize genes into BP categories and uncover functional roles of miRNAs. We observed that the regulation of cellular processes was the most enriched BP in the gene targets with the score of 22.5, and the processes associated closely with MI were also significantly, such as regulation of apoptosis and angiogenesis (Table 2.). Additionally,

through analysis of biological pathways, it seemed that the gene targets were involved in many pathways associated with cardiovascular system, such as apoptosis, angiogenesis, fibrosis, and cardiac hypertrophy (data not shown). Collectively, our results suggested that the differentially regulated miRNAs were enriched in multiple biological processes involved in the response to MI, which confirmed the report from earlier study that the miRNAs were functionally important in different pathological phenotypes [35-36].

Independent of the animal or other reasons, there are some typical pathological features in MI, including cellular apoptosis and angiogenesis. According to the GO functional annotations and pathway analysis, gene targets associated with apoptosis or angiogenesis were identified to illustrate the miRNA-gene network.

Several miRNAs have been described to be associated with apoptosis after MI, including miR-1 [11], miR-206 [11], miR-21 [7], miR-210 [24], miR-214 [37], miR-24 [38], miR-29 [39], and miR-494 [21], all of which were incorporated into the apoptotic miRNA-gene network (Fig. 1). Recently, miR-214 was suggested to

Fig. 5. miR-15b displays anti-angiogenesis activity. (A) HUVECs were treated with hypoxia for the indicated time points. The relative expression level of miR-15b was detected by q-PT-PCR (n=5, means+S.E.M). *P<0.05. (B) HUVECs were transfected with miR-15b mimic (25 nM, 50 nM) or miR mimic scramble (25 nM, 50 nM). After 16 h, cells were treated with hypoxia for 8 h. The relative expression level of miR-15b was detected as in A (n=5, means+S.E.M). *P<0.05; #P<0.05; ##P<0.01. (C) HUVECs were transfected with miR-15b mimic (50 nM) or miR mimic scramble (50 nM). After 16 h, cells were treated with hypoxia for 16 h. The tube formation assay was performed as described in Material and Methods. The photomicrographs (Left) were taken (Scale bar: 25 μ m), and the length of the tubes was quantified (n=4, means+S.E.M). *P<0.05; #P<0.05. (D) HUVECs were transfected as in C, then cells were treated with hypoxia for 24 h. The cell migration assay was performed as described in Material and Methods. A representative field (Left) was shown (scale bars: 50 μ m). The histogram represents the quantification of cells that migrated (n=4, means+S.E.M). *P<0.05; #P<0.05.

protect the mouse heart from ischemic-reperfusion injury by controlling Ca^{2+} overload and cell apoptosis [37]. In our result, miR-214 targeted CADM1 and PPP3CB in apoptotic process. The miR-1 and miR-206 are involved in apoptotic cell death in MI by post-transcriptional repression of IGF-1 [11]. In the miRNA-gene network, miR-1 and miR-206 did target IGF-1, and interestingly, these two miRNAs targeted PREX as well (Fig. 1). Furthermore, some studies suggest that miR-21 protect against cardiac myocyte damage by suppressing PDCD4 expression in the border and infarcted areas [7]. In the present results, miR-21, as an anti-apoptotic regulator, would also target TIMP3 (Fig. 1).

Previous researches demonstrate key roles of miR-106b in various types of human cancer [40-42]. Besides this, some other biological functions of miR-106b are also reported concerning cholesterol homeostasis [43] and spermatogonial development [44]. However, its regulatory roles in apoptosis, especially in cardiac tissue are largely unknown. In our study, we noticed that miR-106b was a hub node in the network (Fig. 1). So the present experiment

was performed to test its predicted activity towards regulation of apoptosis. Hypoxia treatment caused a significant increase in the expression of miR-106b in H9c2 cells (Fig. 3A). Furthermore, suppression of miR-106b markedly promoted the hypoxia-induced cell apoptosis (Fig. 3B and C). These results thus suggested that hypoxia-induced miR-106b might protect cardiomyocytes from apoptosis. The miRNAs perform their biological functions through inhibition of multiple target gene expression. Target gene must be experimentally verified, as the miRNA targets and functions are cell specific [45]. Among the putative targets of miR-106b, CDKN1A (encoding the protein p21) is of importance. Current literatures report importance of p21 in cardiomyocyte apoptosis [32, 46]. However, few investigation is conducted to determine whether miR-106b modulate p21 expression in cardiomyocytes under hypoxic condition. In our study, overexpression of p21 resulted in upregulation of hypoxia-induced apoptosis in H9c2 cells (Fig. 4B). In addition, using both gain-of-function and loss-of-function experiments, we observed that the expression of p21 in

H9c2 cells would be modulated negatively by miR-106b (Fig. 4C). Finally, we detected that miR-106b was able to decrease p21 expression directly by using a construct which included the putative miR-106b binding sequence in the 3'-UTR of p21 mRNA (Fig. 4D). Thus, these results convincingly suggested that p21 was most likely a functional target gene of miR-106b, which performed as an anti-apoptotic regulator in cardiomyocytes under hypoxic condition.

Angiogenesis, induced by secretion of growth factors such as VEGF and Ang2, is an important response of the myocardium to ischemic injury. Rapid formation of collateral vessels that bypass the infarcted coronary artery is necessary for the subsequent cardiac repair. Abnormal blood vessel formation and embryonic lethality in mice with genetic deletion of Dicer further the notion that miRNAs might play important roles in angiogenesis in response to ischemic injury [47]. The miR-92a is a crucial node in angiogenic network (Fig. 2), which was reported as an endogenous repressor of the angiogenic process [48]. The inhibition of miR-92a enhances the functional recovery of ischemic tissue both in a mouse model of hindlimb ischemia and following MI [49]. Indeed, miR-92a inhibition significantly augmented the number of vessels, particularly in the infarct border zone. A important target of the antiangiogenic effect of miR-92a is Integrin- α 5 (ITGAV) [48], which was in accordance with our bioinformatic result (Fig. 2). Further, current studies suggested angiogenic action of miR-126 following MI. The miR-126 null mice show reduced survival and defective cardiac and hindlimb neo-vascularization following ischemia, suggesting a critical function of miR-126 in angiogenesis post ischemia [25, 50]. In addition, wang report that the endothelial cell-restricted miR-126 promotes angiogenesis via direct suppression of Sprouty-related protein 1 (Spred-1, encoded by SPRED1 gene), a negative regulator of VEGF signaling [25]. Indeed, the present results showed that SPRED1 was targeted by miR-126. However, some other miRNAs, including miR-1, miR-101, miR-15b, miR-16, miR-195, and miR-206, were also involved in regulation of SPRED1 expression (Fig. 2). In the angiogenic miRNA-gene network, miR-15b was a focus. In a previous investigation of tumor angiogenesis, miR-15b has been shown to be repressed under hypoxia in human carcinoma cell line, resulting in an increased level of VEGF [51]. In the present experiment, hypoxia treatment inhibited the expression of miR-15b in HUVECs (Fig. 5A). After transfection of HUVECs with miR-15b mimic, hypoxia-induced tube formation and cell migration were markedly attenuated

Fig. 6. The effect of miR-15b on the expression of HIF-1 α , VEGF, and Ang2 during hypoxia. (A) HUVECs were transfected with miR-15b mimic (50 nM) or miR mimic scramble (50 nM). After 16 h, cells were treated with hypoxia for 8 h. The protein levels of HIF-1 α and β -actin were detected by immunoblot analysis and a representative blot was shown. (B) HUVECs were treated as in A, and the levels of VEGF and Ang2 were determined using q-PT-PCR (n=5, means+S.E.M) (upper) and ELISA assay (n=6, means+S.E.M) (lower). *P<0.05; **P<0.01; #P<0.05.

(Fig. 5C and D). Thus, the results suggested that miR-15b might serve as a negative modulator in angiogenesis. As shown in Figure 2, HIF-1 α , VEGF, and Ang2 were not direct targets of miR-15b. Interestingly, our results showed that miR-15b had no effect on HIF-1 α ; however, negative correlations existed between miR-15b and expression of VEGF and Ang2 (Fig. 6). So, these results raised a new issue to further study the molecular

mechanisms involve in modulatory activity of miR-15b on VEGF and Ang2 expression. Further, BCL2 is not a mediator in the angiogenic process of BP classification in GO; however, many studies suggested its functional role in promoting angiogenesis [52, 53]. In our results, BCL2 gene is potentially suppressed by miR-15b through bioinformatic prediction. Maybe these provided another point to demonstrate the anti-angiogenesis activity performed by miR-15b. Additionally, some other correlations in the angiogenic miRNA-gene network might be of significance as well, such as HIF1A being targeted by miR-18a, miR-18b, and miR-424.

In summary, the present study analyzed the recently reported miRNAs which were suggested to be correlated with MI. Our bioinformatic investigation yielded a large amount of data and demonstrated essential roles of miRNAs in pathological processes of MI. Moreover, validation experiments offered novel functional demonstrations of miR-106b and miR-15b in apoptosis or angiogenesis, respectively.

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