EXPERIMENTAL STUDY

MiR-324/SOCS3 Axis Protects Against Hypoxia/Reoxygenation-Induced Cardiomyocyte Injury and Regulates Myocardial Ischemia via TNF/NF-κB Signaling Pathway

Xuefu Han,1,2 PhD, Xi Chen,3 MD, Jiaqi Han,1 PhD, Yu Zhong,4 BD, Qinghua Li,5 MD and Yi An,6,7 PhD

Summary

We aimed at exploring the function of microRNA-324/cytokine signaling 3 (miR-324/SOCS3) axis in hypoxia/reoxygenation (H/R)-induced cardiomyocyte injury and its underlying mechanism. The differential expression genes were analyzed based on the GSE83500 and GSE48060 datasets from the Gene Expression Omnibus (GEO) database. Then, to conduct the function enrichment analysis, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were used. The upstream regulatory microRNAs (miRNAs) of the identified genes were predicted by miRanda, miRWalk, and TargetScan websites. MiR-324 expression was measured with quantitative real-time polymerase chain reaction (qRT-PCR). The target binding of miR-324 and SOCS3 was established by dual-luciferase reporter assay. Cardiomyocyte proliferation was analyzed by cell counting kit-8 (CCK-8) assay, whereas the apoptosis was investigated via flow cytometry. The expression of TNF pathway-related proteins was detected by western blot analysis. SOCS3 was upregulated in patients with myocardial infarction (MI), and function enrichment analyses proved that SOCS3 was enriched in TNF signaling pathway. Moreover, we found that miR-324 was the upstream regulatory miRNA of SOCS3 and negatively regulated SOCS3 expression. MiR-324 was downregulated in cardiomyocytes with H/R-induced injury, inhibiting cell proliferation. In the H/R model, SOCS3 suppresses cardiomyocyte proliferation, which was recovered by miR-324, and induces cell apoptosis, which was repressed by miR-324 via regulating the expression of cleaved caspase-3 and p P38-MAPK. MiR-324 upregulation decreased the protein levels of TNF-α, p-P65, and p-IκBα in cardiomyocytes that suffered from H/R, which was reversed with SOCS3 overexpression. MiR-324/SOCS3 axis could improve the H/R-induced injury of cardiomyocytes via regulating TNF/NF-κB signaling pathway, and this might provide a new therapy strategy for myocardial ischemia.

Key words: Bioinformatics, Cell proliferation, Cell apoptosis, Rescue

Myocardial infarction (MI) is a common type of coronary artery diseases with a high morbidity and mortality.1 Approximately 600,000 people are diagnosed with MI, and in China, 180,000 patients with MI die every year.2,3 The main causes of MI are the stoppage or shrinking of coronary arteries due to the blockage of one of its branches, sudden blood flow stoppage, and insufficient oxygen in the heart muscle.4 MI often causes leukocytosis and recruitment of immune cells to the injured myocardium.5 With the development of percutaneous coronary intervention, undergoing urgent and successful revascularization of the coronary artery becomes the main therapy method for patients with MI.6,7 However, patient outcome is still undesirable. Therefore, seeking for some new therapy strategies for the treatment of MI is necessary.

Cytokine signaling 3 (SOCS3), a vital part of the cytokine signal transduction inhibitory protein (SOCS) family, acts as an inhibitor of JAK-STAT pathway activation.8,9 SOCS3 acts as an important tumor suppressor and regulates tumor progression of numerous cancers.10-13 It also participates in many signaling pathways and serves as a key player in inflammatory diseases.14-16 Previous studies have verified that SOCS3 is one of the differently expressed genes (DEGs) in MI and a diagnostic marker in acute MI, and its cardiac-specific deletion is good at predicting MI.17,18,19 However, the mechanism of SOCS3 in MI remains unclear.

MicroRNAs (miRNAs), a 20-25 nucleotide long cluster of small noncoding RNAs, have been reported to play important regulatory roles in various biological processes in animals, plants, and viruses.20 The abnormal expression

From the 1Department of medicine, Qingdao University, Qingdao, China, 2Department of Cardiology, Weifang People’s Hospital, Weifang, China, 3Department of Stomatology, Weifang Maternal and Child Health Hospital, Weifang, China, 4Department of Personnel, Weifang Maternal and Child Health Hospital, Weifang, China, 5School of Public Health, Weifang Medical University, Weifang, China, 6Department of Cardiology, The Affiliated Hospital of Qingdao University, Qingdao, China and 7Qingdao University, Qingdao, China.

Address for correspondence: Yi An, PhD, Department of Cardiology, The Affiliated Hospital of Qingdao University, No.1677 Wutaishan Road, Yellowdao District, Qingdao, Shandong 266000, China. E-mail: any2018@qdu.edu.cn

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of specific miRNAs has been implicated in the development and progression of diverse cardiovascular diseases, including MI.\(^{25,26}\) miR-324 is located on 17p13.1 and has been confirmed to take effects on various physiological function in many diseases, such as cancers,\(^{27-29}\) Parkinson’s disease,\(^{30}\) polycystic ovarian syndrome,\(^{30}\) and early ectopic pregnancy.\(^{31}\) In addition, miR-324 has been proven to participate in the protection of urocin against myocardial ischemia-reperfusion injury.\(^{32}\) However, details on the regulatory and potential functional mechanisms of miR-324 in myocardial ischemia have never been reported.

MiRNAs could regulate gene expression via inhibition of specific target miRNAs. Besides, in our study, the bioinformatics analysis revealed a targeted binding site between miR-324 and SOCS3 mRNA. Therefore, we investigated the effects of miR-324/SOCS3 axis and its possible mechanism in myocardial ischemia, expecting to identify a new therapy target site for MI.

## Methods

The identification of DEGs, functional enrichment analysis of DEGs, and construction of protein-protein interaction (PPI) network: Microarray gene expression profiles from the datasets of GSE83500 (37 aortic tissue samples including 17 MI tissues and 20 non-MI tissues) and GSE48060 (52 blood samples including 31 MI blood samples and 21 non-MI blood samples) were extracted from the database of Gene Expression Omnibus ( GEO, http://www.ncbi.nlm.nih.gov/geo/geo2r/). The common genes were obtained from the DEGs in GSE83500 and GSE48060 using the online analysis tool GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/).

Next, the functional enrichment analysis of DEGs was implemented by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/). The term/pathway is considered to be significantly enriched when \(P < 0.05\).

Then, the PPI network was constructed with DEGs using search tool for the retrieval of interacting genes/proteins database (http://www.string-db.org/). The hub genes were obtained with degree algorithm.

### Analysis of the differently expressed miRNAs and the prediction of regulatory miRNAs

GSE94605 dataset was used to analyze the differently expressed miRNAs with 13 plasma samples, which included 6 patients with unstable angina pectoris and 7 healthy patients. miRanda, miRWalk, and TargetScan were exploited to predict the upstream regulatory miRNAs of the target genes. Then, the intersection of miRanda, miRWalk, and TargetScan, as well as the downregulated miRNAs from GSE24519, were taken to identify the common elements. And, the expression of the identified miRNAs was analyzed using GSE94605 dataset.

### Cell culture and hypoxia/reoxygenation (H/R) treatment

Human neonatal cardiomyocytes were purchased from Cell Center of Basic Medicine, Institute of Basic Medicine, Chinese Academy of Medical Sciences (Beijing, China). Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics was used to culture the cells. When 70%-80% cell confluence was reached, the cells could be used for the following experiments.

Then, the human neonatal cardiomyocytes were divided into sham and H/R groups. To construct the H/R model, the DMEM was replaced by the DMEM without FBS while the cells were incubated under hypoxia for 6 hours at 37°C with 95% N\(_2\) and 5% CO\(_2\). Subsequently, the cells were transferred to normal incubator for reoxygenation for 24 hours at 37°C with 95% O\(_2\) and 5% CO\(_2\). For the sham group, the cells were not treated with H/R assay, and other procedures were identical with the H/R group.

#### Cell transfection

- **Si-con/si-SOCS3**: pcDNA3.1/pcDNA 3.1-SOCS3, miR-324 mimic/inhibitor, and miR-324 mimic/inhibitor negative control (NC) were synthesized by GenePharma (Shanghai, China).
- **miR-324 in vitro**: The cells were seeded in 96-well plates at 5 × 10\(^4\) cells/well and were then transfected using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc., California, USA) according to the manufacturer’s instruction. After transfecting for 48 h, the cells were harvested for mRNA and protein analyses.

### Analysis of cell viability using cell counting kit-8 (CCK-8) assay

The routine-cultured cells were digested, counted, and then suspended. Every 100 \(\mu\)L cell suspension was placed into 96-well plates (1000 cells/well) and was then cultured in CO\(_2\) incubator. The cell viability was checked every 24 hours, and 10 \(\mu\)L CCK-8 reagent was placed into each well and hatched at 37°C for 1.5 hours before checking. Eventually, the OD value under a wave of 450 nm was evaluated using a microplate reader, and proliferation curve was finally plotted.

### Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

The total RNA of cells with H/R-induced injury was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). PrimeScript RT Reagent Kit (Takara, Japan) was used to reverse-transcribed mRNA into cDNA, and SYBR Premix Ex Taq II (Takara, Japan) was used for qRT-PCR. Then, miScript Reverse Transcription Kit (Qiagen) was used for the reverse transcription. RT-PCR was implemented using miScript SYBR Green PCR Kit (Qiagen) in 7900HT Real-Time PCR System. Finally, the relative expression of miRNA normalized to U6 was calculated with the method of \(2^{-\Delta\Delta Ct}\). The sequences of the relevant primers were listed below:

- **miR-324**: F: 5’-CATCCCCCTAGGGCCATTG-3’, R: 5’-GAACATGCTGCTATCTC-3’; U6: F: 5’-AGAGAATTGCATGGGCGCCCTG-3’, R: 5’-GCAGGGGGGCTTCAAATC-3’; SOCS3: F: 5’-CATCTCTGTCGGAAGACCGT-3’, R: 5’-GCATCGTACTGGTCCAGGAACT-3’; GAPD: F: 5’-CATCTCTGTCGGAAGACCGT-3’, R: 5’-GCAGGGGCCATGCTAA-3’; U6: F: 5’-AGAAGATTTGGA-3’, R: 5’-TGCTGCCCTCCTGCATG-3’.

### Dual-luciferase reporter assay

The 3’-untranslated region of SOCS3, harboring either the wild-type or mutant miR-324 binding sites, was cloned into the downstream of pmirGLO dual-luciferase vector (Promega, WI, USA) to generate the dual-luciferase reporter plasmid. MiR-324 inhibitor or miR-324 inhibitor NC and miR-324 mimics or...
miR-324 mimic NC miRNAs were co-transfected into cardiomyocytes using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and cultured for 72 hours. Then, luciferase activities were measured using the dual-luciferase reporting kit.

**Cell apoptosis assay**: After cell collection and centrifugation, phosphate-buffered saline (PBS) pre-cooled at 4°C were used for cell resuspension, and cells were centrifuged again. The supernatant was extracted and mixed with 1 X binding buffer while simultaneously regulating the cell density to 1 x 10^5/mL. The suspension liquid (100 μL) was placed into 5-mL flow tube and mixed with 5 μL Annexin V-FITC at room temperature away from light for 5 minutes. Then, 10 μL PI dye liquor and 400 μL PBS were added into the mixture, and the cell apoptotic rate was checked using flow cytometer. The outcomes were analyzed using the FlowJo software.

**Western blot analysis**: After transfecting for 48 hours, the cells cultured in 6-well plates were placed on ice. Then, the total protein was isolated using RIPA lysis with protease inhibitor, and its content was measured with bicinchoninic acid protein assay kit (Sigma). Next, 20 μg protein was added into each well of vertical electrophoresis tank and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subsequently, the protein was transferred onto the polyvinylidene fluoride membrane. After blocking with 5% skim milk for 1 hour, the membranes were respectively mingled with the primary antibodies, including SOCS3 (ab16030; 1:100; Abcam), TNF-α (ab6671, 1:1000, Abcam), P65 (ab16502, 2000, Abcam), p-P65 (S536, ab86299, 2000, Abcam), IκBα (ab32518, 1:500, Abcam), p-IκBα (S36, ab133462, 1:500, Abcam), caspase-3 (ab4051, 1:500, Abcam), cleaved caspase-3 (ab2302, 1:500, Abcam), p38 MAPK (8690S, 1:1000, CST), and phosphorylated p38 MAPK (9216S, 1:2000, CST), at 4°C overnight. The membranes were then washed with TBST for three times (each time for 5 minutes), added into the second antibody, and incubated for 1 hour at ambient temperature. Finally, the membranes were rinsed and developed using ECL. The gray value was estimated by QUANTTTY ONE software, and the relative protein expression was normalized to GAPDH.

**ELISA assay**: Cells were grown to about 80%-90% confluence in 6-well plates. Then, the culture supernatant of cardiomyocytes was collected for use in the detection of TNF-α concentrations. TNF-α was determined using mouse TNF-α (Cat. No.: CSB-EQ023955MO) ELISA kits (Cusabio, Wuhan, China), according to the manufacturer’s instructions. The OD450 was detected using the iMark microplate reader (Bio-Rad, Hercules, CA, USA).

**Statistical analysis**: SPSS 22.0 was used for data analysis, whereas GraphPad Prism 6.0 was employed for plotting line figures. The differences between the two groups and three or above groups were compared using Student’s t-test or one-way ANOVA analysis with a Dunnett’s or Tukey’s post hoc test. P < 0.05 meant that the difference was significantly different.

**Results**

SOCS3 is selected as the key regulatory gene of myocardial ischemia: To identify the DEGs in MI and normal tissues, we used two datasets, GSE83500 and GSE48060, which obtained 2820 and 5068, respectively. Then, we took the intersection of these two groups of DEGs, and 697 common DEGs were acquired (Figure 1A). Next, the function enrichment analyses of the DEGs were conducted with GO and KEGG databases using DAVID website. The result showed that 6 and 10 most enriched GO and KEGG terms were presented, respectively (Figure 1B, C). Through PPI network analysis of DEGs, CDC20, EGFR, SOCS3, MAPK8, and CREBBP were identified as the hub genes (Figure 1D, F). According to the data from GSE83500 dataset, SOCS3 was proven to be dramatically elevated in the MI group compared with that in the non-MI group (Figure 1G). Moreover, SOCS3 were enriched in TNF signal pathway, which was one of the enriched KEGG terms in MI. Therefore, SOCS3 was considered to be the key regulatory molecule of myocardial ischemia and was selected for further analysis.

**MiR-324 is the upstream miRNA of SOCS3 and was downregulated in myocardial ischemia**: To predict the upstream miRNA of SOCS3, we further explored the differently expressed miRNAs in the plasma from healthy subjects and patients with unstable angina pectoris using the dataset of GSE94605. As a consequence, 477 differently expressed miRNAs, including 358 upregulated miRNAs and 119 downregulated miRNAs, were obtained. Then, we predicted the upstream regulating miRNAs using the websites miRanda, miRWalk, and TargetScan. According to the intersection of the prediction website of the miRNA target genes and the downregulated miRNAs in GSE94605, 20 common miRNAs were obtained (Figure 2A). Among them, miR-324 was notably reduced in patients with unstable angina pectoris compared to that in healthy subjects (Figure 2B). Besides, qRT-PCR analysis was utilized to monitor the expression level of miR-324 in cardiomyocytes with H/R-induced injury. The result displayed that miR-324 was significantly lower in the H/R-induced injury group than that in the control group (Figure 2C). Notably, Diaz et al. had verified that miR-324 could mediate the improvement of urocortins in myocardial ischemia reperfusion.21 Hence, miR-324 was selected as the upstream regulatory miRNA of SOCS3 for the next investigation.

**MiR-324 promotes cell proliferation of cardiomyocytes that suffered from H/R injury**: Next, CCK-8 assay was implemented to study the function of miR-324 on the approach of cardiomyocyte proliferation after the cardiomyocytes in the sham and H/R groups were transfected with miR-324 mimic/miR-324 mimic NC and miR-324 inhibitor/miR-324 inhibitor NC, respectively. As shown in Figure 3, in the H/R group, the proliferative ability of cardiomyocytes was decreased after being treated with H/R, compared to that in the sham group. Moreover, compared to H/R group, cardiomyocyte proliferation in the H/R+miR-324 mimic group was promoted, whereas that of cardiomyocytes in the H/R+miR-324 inhibitor group was significantly suppressed. Furthermore, the proliferative ability of cardiomyocytes in the H/R+miR-324 inhibitor NC, H/R+miR-324 mimic NC, and H/R groups had no significant difference. All data suggested that miR-324
could be a promoter of cell proliferation of cardiomyocytes that suffered from H/R-induced injury.

The confirmation of the sponging effect between miR-324 and SOCS3: As miR-324 was considered as the regulatory miRNA of SOCS3 in bioinformatics analysis, dual-luciferase reporter assay was carried out to check the association between miR-324 and SOCS3. The binding sites of miR-324 and SOCS3 were presented in Figure 4 A. The luciferase activity of pmirGLO-SOCS3-WT in cardiomyocytes transfected with miR-324 inhibitor was prominently higher and was lower in those transfected with miR-324 mimics than that in those transfected with
miR-324 NC, whereas that of pmirGLO-SOCS3-MUT exhibited no obvious difference (Figure 4B). These results indicated that SOCS3 was targeted by miR-324.

**MiR-324 suppresses SOCS3 expression in H/R-induced cardiomyocyte injury:** To validate the effect of miR-324 on SOCS3 expression, western blot analysis was performed. First, according to qRT-PCR and western blot analysis, we found that the relative mRNA and protein expressions of SOCS3 were remarkably increased once cardiomyocytes were subjected to H/R-induced injury, respectively (Figure 4C, D). Then, we compared the expression of SOCS3 in cells severally transfected with miR-324 mimic, miR-324 mimic+pcDNA3.1-SOCS3, miR-324 inhibitor, and miR-324 inhibitor+si-SOCS3. The outcomes indicated that the expression of SOCS3 was clearly inhibited after cardiomyocytes were transfected with miR-324 mimic, whereas SOCS3 expression was increased in cardiomyocytes transfected with miR-324 inhibitor+pcDNA3.1-SOCS3 compared to that in controls (Figure 4E). Meanwhile, SOCS3 expression in cardiomyocytes transfected with miR-324 inhibitor was notably elevated but decreased in those transfected with miR-324 inhibitor+si-SOCS3 in comparison to that in controls (Figure 4F). Hence, the expression of SOCS3 was negatively associated with miR-324 in H/R-induced cardiomyocyte injury.

**MiR-324/SOCS3 axis accelerates the proliferation and reduces the apoptosis of cardiomyocytes induced by H/R:** The functions of miR-324/SOCS3 on cardiomyocyte proliferation and apoptosis were analyzed, respectively. MiR-324 mimic or pcDNA3.1-SOCS3 was used to raise the expression of miR-324 or SOCS3, respectively. The result of CCK-8 assay indicated that the proliferation of OGD/R-treated cardiomyocytes was significantly promoted when miR-324 was upregulated and SOCS3 attenuated this effect. Concurrently, the inhibitory effect of the silenced miR-324 on cell proliferation was weakened by the knockdown of SOCS3 (Figure 5A). To evaluate the effects of miR-324/SOCS3 on apoptosis, flow cytometry was performed. Indeed, the auxo-action of the downregulation of miR-324 on cell apoptosis was similarly suppressed by the decrease of SOCS3, whereas the overexpression of SOCS3 could abate the inhibitory effect of the
enhanced miR-324 on cardiomyocyte apoptosis induced by H/R (Figure 5B).

The regulation of miR-324/SOCS3 axis on the expression of total caspase, cleaved caspase-3, p-P38 MAPK, and P38 MAPK: Next, to explore the possible underlying mechanism of the effects of miR-324/SOCS3 axis on the biological behaviors of H/R, the expression of relative proteins total caspase, cleaved caspase-3, p-P38 MAPK, and P38 MAPK were checked. The data indicated that, once the cells were injured by H/R, cleaved caspase-3 was distintively upregulated, whereas p-P38 MAPK was significantly downregulated (Figure 6). Moreover, miR-324 mimic inhibited the expression trend of cleaved caspase-3 and p-P38 MAPK compared to those in the H/R group. Furthermore, rescue assay displayed that SOCS3 overexpression restrained the effects of miR-324 mimic on the expression of cleaved caspase-3 and p-P38 MAPK. On the contrary, cleaved caspase-3 was notably increased, while p-P38 MAPK was decreased in the H/R+miR-324 inhibitor group in comparison with the H/R group, while the influences of miR-324 inhibitor on these two proteins were weakened by si-SOCS3. Furthermore, the expression of total caspase and P38 MAPK had no significant difference among each groups. These data revealed that miR-324/SOCS3 was good for the cell proliferation, but not for cell apoptosis, in cardiomyocytes damaged by H/R via modulating the expression of cleaved caspase-3 and p-P38 MAPK.

The influence of miR-324/SOCS3 on cardiomyocytes treated by H/R is mediated by TNF signaling pathway:

Based on the researches above, we further explored the underlying mechanism of miR-324/SOCS3 on cardiomyocytes induced by H/R. As SOCS3 was enriched in the TNF signaling pathway, we inferred that the function of miR-324/SOCS3 might be associated with this signaling pathway. Therefore, we detected the expression of related proteins, including TNF-α, p-P65, P65, p-IκBα, and IκBα, using western blot analysis. As displayed in Figure 7A, compared to the sham group, the expressions of TNF-α, p-P65, and p-IκBα were all remarkably enhanced in the H/R group, while the expression of P65 and IκBα had no significant difference. However, TNF-α, p-P65, and p-IκBα were all downregulated in the H/R+miR-324 mimic group but elevated in the H/R+miR-324 mimic+pcDNA3.1-SOCS3 group compared to those in the H/R group. Furthermore, the expressions of P65 and IκBα in the H/R+miR-324 mimic and H/R+miR-324 mimic+pcDNA3.1-SOCS3 groups exhibited no noteworthy differences. Conversely, TNF-α, p-P65, and p-IκBα were all notably increased in the H/R+miR-324 inhibitor group but attenuated in the H/R+miR-324 inhibitor+si-SOCS3 group, whereas the expressions of P65 and IκBα did not changed much (Figure 7B). In addition, the concentration of TNF-α was also elevated after cardiomyocytes were injured by H/R; meanwhile, miR-324 mimic suppressed but miR-324 inhibitor induced TNF-α expression (Figure 7C). Furthermore, SOCS3/si-SOCS3 could cripple the effects of miR-324 mimic/inhibitor on the expression of TNF-α. These data might revealed that miR-324/SOCS3 could notably strengthen the expressions of TNF-α, p-P65, and p-
Figure 5. The effects of miR-324/SOCS3 on cardiomyocyte proliferation and apoptosis with H/R-induced injury. A: The effect of miR-324/SOCS3 on cardiomyocyte proliferation with H/R-induced injury. B: The effect of miR-324/SOCS3 on cardiomyocyte apoptosis with H/R-induced injury. **P < 0.001, ***P < 0.0001, versus sham. ##P < 0.001, ###P < 0.001, versus H/R.

Discussion

MI is an ischemic heart disease that causes loss of cardiomyocytes. MI has become a leading cause of morbidity and mortality worldwide. Although having standard therapy and reperfusion strategies, MI often caused I/R injury. I/R injury is a pathological process characterized by the reduction of blood supply to tissues and the restoration of perfusion and concomitant reoxygenation. Myocardial I/R can not only restore the blood supply to ischemic area but also cause further damage such as oxidative stress, arrhythmia, cell apoptosis, and cell death. Therefore, exploring effective preventive and therapeutic measures of myocardial I/R injury is imminent and of significant clinical value.

SOCS3 is induced by JAK-STAT-activating cytokines and has been proven to be a novel therapeutic target for cardioprotection. In prior studies, SOCS3 was confirmed to be abnormally expressed in acute MI. However, its potential role and mechanism in myocardial I/R injury were never reported. Currently, we screened the DEGs between patients with MI and non-MI using datasets GSE83500 and GSE48060, and found that SOCS3 was overexpressed in MI. Furthermore, the PPI network was constructed with...
Figure 6. The expression of total caspase, cleaved caspase-3, p-P38 MAPK, and P38 MAPK in cardiomyocytes with H/R-induced injury. **A:** Compared to H/R group, miR-324 mimic suppressed cleaved caspase-3 expression and elevated p-P38 MAPK expression, which was separately restored by SOCS3 and si-SOCS3, whereas total caspase and P38 MAPK expressions had no obvious difference among every groups. **B:** Cleaved caspase-3 expression was increased, whereas p-P38 MAPK expression was reduced by miR-324 inhibitor in comparison with H/R group. Moreover, total caspase and P38 MAPK still showed no significant difference in each group. **P < 0.001, ***P < 0.0001, versus sham. ##P < 0.001, ###P < 0.001, versus H/R.

the DEGs, which indicated that SOCS3 was the hub gene and thus was selected as the key regulatory gene in our study.

In addition, miRNAs have not only been reported to participate in various cellular and biological processes including ischemic cardiomyopathy but also have been affirmed to control gene expression via blocking mRNA translation or inducing mRNA degradation. The accumulated evidences have suggested that various miRNAs have been abnormally expressed in myocardial I/R injury of various models, demonstrating that miRNAs take a vital part in myocardial ischemic injury. Therefore, we investigated the upstream miRNAs of SOCS3 using TargetScan, miRanda, and miRWalk in our study. Among the candidate miRNAs, miRNA-324 was fund to be downregulated in cardiomyocytes with H/R-induced injury. However, beyond that, miR-324 had been verified to play an important role in mediating urocortins to improve myocardial I/R injury. Hence, miR-324 was selected as a potential regulatory miRNA. Furthermore, the function of miR-324 on myocardial ischemia was never uncovered, let alone its mechanism. Generally speaking, the proliferation of adult cardiomyocytes is “locked,” and studies have shown that the cardiomyocyte numbers increase from 1 to 4 billion from birth to an age of 20 years, after which the total number of cardiomyocytes remain constant throughout life in human. However, about 45% of cardiomyocytes is exchanged during the entire life time of a person, and this regenerative ability is important. Moreover, studies have confirmed that it is the proliferation of preexisting cardiomyocytes, not the endogenous progenitor or stem cells, that contributes to cardiac regeneration. Cardiomyocyte proliferation is a potential source for heart repair after injury, and cardiomyocytes in injured myocardium are derived from preexisting cardiomyocytes. Therefore, we researched the expression of miR-324 and its effects on the proliferation and apoptosis of cardiomyocytes with H/R-induced injury. The outcomes showed that miR-324 had a poor expression in cardiomyocytes with H/R-induced injury and that its overexpression could promote proliferation of cardiomyocytes with H/R injury. Then, we discovered that cell proliferation of cardiomyocytes with H/R-induced injury was dramatically inhibited/promoted after miR-324 mimic+pcDNA3.1-SOCS3/miR-324 inhibitor+si-SOCS3 treatment, and this was contrary to the apoptosis of cardiomyocytes with H/R-induced injury. Moreover, the apoptosis-related protein cleaved caspase-3 was significantly suppressed/elevated, while p-
P38 MAPK was induced/reduced by miR-324 mimic/inhibitor in cardiomyocytes with H/R-induced injury, which also could be restored by SOCS3/si-SOCS3. These consequences might revealed that the auxo-action of miR-324 on the proliferation of cardiomyocytes with H/R-induced injury could be attenuated by SOCS3. Similarly, the weakening effects of miR-324 on the apoptosis of cardiomyocytes with H/R-induced injury could be dropped off by SOCS3: miR-324 and SOCS3 presented a negative correlation, and miR-324 mediated cardiomyocyte proliferation and apoptosis via regulating the expression of cleaved caspase-3 and p-P38 MAPK.
As an intracellular negative regulator of cytokine signaling pathway, SOCS3 has two main transcription factor response elements: STAT3 and NF-κB. STAT3 is a vital member of the STAT family and plays a protective role in the heart, which is the ingredient of JAK/STAT pathway. JAK/STAT pathway has been reported to be activated in MI, plays a pivotal role in cytoprotective signaling, and contributed to the pathogenesis of myocardial ischemia. Besides, the JAK/STAT pathway was proven to be involved in the promotion of proliferation rate and inhibition of apoptosis of cardiomyocytes. NF-κB is one of the key transcription factors. Chhabra et al. considered NF-κB activation an early event, while SOCS3 expression was a delayed event following TNF-α signaling in granulocytes in asthma. Besides, in previous studies, SOCS3 has been verified to be induced in animal models by pro-inflammatory stimuli like cytokines, TNF-α, IL-6, and IL-1β. Moreover, SOCS3 expression may be increased with the intake of glucose and saturated fat (cream), which makes SOCS3 becoming a part of macronutrient-induced inflammatory stress simultaneously increasing the activation of the pro-inflammatory transcription factor, NF-κB, TNF-α, IL-6, and IL-1β expression. Above all, TNF-α is the main activator of NF-κB, is produced by cardiomyocytes following ischemia, and is the major inflammatory response mechanism that contributes to ischemic brain injury. Upon the activation by TNF-α, NF-κB can initiate transcription of many target genes and the presence of NF-κB motifs. TNF-α/NF-κB pathway has been recognized as one of the key pathways in the development of inflammation in many diseases, including MI. In addition, an increase TNF-α expression has been reported to induce cardiomyocyte apoptosis, thus exacerbating the myocardial damage. Interestingly, we discovered that TNF-α expression was highly upregulated in H/R model, which was in line with previous research. However, the research on NF-κB in cardiomyocytes is not well defined. Some researchers pointed that NF-κB promotes inflammatory events and mediates adverse cardiac remodeling following I/R. Conversely, others have suggested the beneficial effect of NF-κB in I/R injury in relation to its antiapoptotic effects. Beyond that, Bergmann and colleagues uncovered that the inhibition of NF-κB in cardiomyocytes promotes TNF-α-induced apoptosis by regulating the expression of anti-apoptosis proteins. Based on previous studies and the discovery that SOCS3 was enriched in TNF signaling pathway, we explored the possible influences of miR-324/SOCS3 on the activation of TNF-α/NF-κB pathway via detecting the pathway-associated protein expressions of TNF-α, p-P65, P65, p-IkBα, and IκBα. Consequently, the high expression of miR-324 alone could signally decrease the expressions of TNF-α, p-P65, and p-IkBα in cardiomyocytes with H/R-induced injury. However, the overexpression of miR-324+ pcDNA3.1-SOCS3 could significantly induce the expressions of TNF-α, p-P65, and p-IκBα. In line with the observations from Chandrasekar et al., our results suggest that the inhibitory effects of miR-324 on TNF signaling pathway could be attenuated by the upregulation of SOCS3, and it also could concurrently activate the NF-κB pathway. Hence, our study preliminarily demonstrated that miR-324/SOCS3 axis exerted effects in cardiomyocytes with H/R-induced injury through mediating TNF-α/NF-κB pathway. However, the detail mechanism of miR-324/SOCS3 still needs further study and relative in vivo experiments are also suggested for future explore.

In conclusion, miR-324 was downregulated, whereas SOCS3 was upregulated in MI, and SOCS3 was targeted by miR-324. MiR-324/SOCS3 could promote proliferation but could reduce apoptosis of cardiomyocytes with H/R-induced injury via regulating the TNF signal pathway.

Disclosure

Conflicts of interest: The authors declare no conflict of interest.

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