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Suppression of microRNA-101 attenuates hypoxia-induced myocardial H9c2 cell injury by targeting DIMT1-Sp1/survivin pathway

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) are small single-stranded RNAs in eukaryotic cells, which play important regulatory roles in the pathogenesis of various diseases. We aimed to investigate the effects of miRNA-101 (miR-101) on hypoxia-induced myocardial infarction (MI) cell injury model (myocardial H9c2 cell injury model). The possible target gene of miR-101 was also analyzed.

MATERIALS AND METHODS: H9c2 cells were exposed to hypoxia treatment. Cell viability, migration, invasion, apoptosis and the expression of miR-101 were detected using CCK-8 assay, transwell assay, flow cytometry analysis, Western blotting and qRT-PCR, respectively. Then, the effects of miR-101 overexpression or suppression on the cell injury induced by hypoxia were assessed. Dual luciferase reporter assay was used to analyze the possible target gene of miR-101. Finally, the effects of DIMT1 (dimethyladenosine transferase 1 homolog) on the possible target gene of miR-101 in H9c2 cell injury were investigated.

RESULTS: Hypoxia significantly induced H9c2 cell injury. miR-101 was up-regulated after hypoxia induction. Hypoxia-induced cell injury was significantly reversed by miR-101 suppression and exacerbated by miR-101 overexpression. DIMT1 is a direct target gene of miR-101. Knockdown of DIMT1 markedly inhibited the protective effects of miR-101 suppression on hypoxia-induced injury by suppressing specific protein 1 (Sp1)/Survivin pathway.

CONCLUSIONS: We verified the critical roles of miR-101 in regulating myocardial cell injury induced by hypoxia. DIMT1-mediated the Sp1/survivin pathway was also involved in this process. Our findings replenished the understanding of the regulatory roles of miRNAs in hypoxia-induced MI cell injury and provided new molecular target for therapy and diagnosis of MI.

Key words:
Myocardial infarction, Hypoxia injury, MicroRNA-101, Dimethyladenosine transferase 1 homolog, Survivin pathway

Introduction

Myocardial infarction (MI) is a serious heart disease which is caused by acute, persistent ischemia or hypoxia and is responsible for heart failure and sudden death[1,2]. The main clinical symptoms of MI are severe persistent chest pain, dyspnea, fever and syncope[3,4]. Despite the methods of prevention and therapy for MI have been improved in recent years, lots of people still died of this disease all over the world[5,6]. Therefore, it is urgent to explore the critical intracellular regulation factors, intrinsic molecular mechanisms and potential molecular targets for therapy of MI. MicroRNAs (miRNAs) are small single-stranded RNAs in eukaryotic cells, which play important regulatory roles in intracellular signaling pathways by binding to target 3'-untranslated region (3'UTR) of mRNA[7]. Researches have reported that several of miRNAs participant in the pathological conditions of MI. For example, Roy et al[8] demonstrated that miRNA-21 (miR-21) regulated the matrix metalloprotease-2 (MMP-2) expression in cardiac fibroblasts of infarction zone through phosphate and tensin homologue (PTEN) signaling pathway. Fan et al[9] proved that miRNA-34a (miR-34a) was highly expressed in patient with MI, and promoted myocardial cells apoptosis by negatively regulating the expression of aldehyde dehydrogenase 2 (ALDH2). In addition, Janssen et al[10] present-

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ed that miRNA-214 (miR-214) played important regulatory effects on thyroid hormone levels in mouse following MI through thyroid-hormone inactivating enzyme deiodinase type 3. miRNA-101 (miR-101) has been found to play regulatory roles in various cancer formations, pulmonary tuberculosis and angiogenesis[11-14]. In terms of cardiovascular disorders, Pan et al[15] proved that miR-101 suppressed cardiac fibrosis after MI and promoted left ventricular compliance through FBJ osteosarcoma oncogene/transforming growth factor-β1 (FOS/TGFβ1) signaling pathway. However, to our knowledge, it is still unclear whether miR-101 plays a critical regulatory role in myocardial cells injury in MI condition. Hypoxia will disrupt the balance of intracellular microenvironment, leading to the irreparable function injury of myocardial cells and resulting in MI occurrence[16]. In this study, myocardial H9c2 cells were cultured in hypoxia condition to induce MI cell injury model. Then, the effects of miR-101 on hypoxia-induced H9c2 cell viability loss, migration and invasion decreases, and apoptosis increase were investigated, respectively. The possible target gene of miR-101 and the potential intracellular signaling pathways were also analyzed. Our finding will be helpful for understanding the regulatory role of miR-101 in hypoxia-induced MI cell injury and provide a new molecular target for therapy and diagnosis of MI.

Materials and Methods

Cell Culture and Treatment

The rat embryonic ventricular myocardium-derived H9c2 cells were purchased from Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Life Technologies, Carlsbad, CA, USA) containing with 10% (v/v) fetal bovine serum (FBS, HyClone, Southern Logan, UT, USA), 100 U/mL penicillin (Life Technologies, Carlsbad, CA, USA) and 100 μg/mL streptomycin (Life Technologies, Carlsbad, CA, USA) at 37 °C with 5% CO_2 in a humidity incubator (Thermo Fisher Scientific, Waltham, MA, USA). H9c2 cells were cultured in hypoxia incubator with 94% N_2, 5 % CO_2 and 1% O_2 for 24 h to induce injury.

Cell Counting Kit-8 (CCK-8) Assay

Cell viability was measured using Cell Counting Kit-8 (CCK-8, Yeasen, Shanghai, China) assay. Briefly, H9c2 cells were seeded into 96-well plate (Corning Incorporated, Corning, NY, USA) with 5 × 10^3 cells/well and cultured in normal or hypoxia condition for 24 h. Then, CCK-8 (10 μl) was added into the each well following incubation for another 1 h at 37 °C. After that, the absorbance of each well at 450 nm was recorded by a Micro-plate Reader (Bio-Tek Instruments, Winooski, VT, USA).

Cell Transwell Assay

Cell migration was evaluated using a two-chamber transwell (Bio-Rad, H9c2 cells (1×10^3) were seeded in the chamber of Corning (Corning Incorporated, Corning, NY, USA) with 200 μl serum free medium and 600 μl medium containing 10% FBS was added into the lower chamber of Corning chamber. After Corning chambers incubation at normal or hypoxia condition for 48 h, migrated cells were fixed with 20% methanol and stained with 0.2 % crystal violet (Beyotime Biotechnology, China). The relative rate of cell migration (%) was quantified by numbers of migrated cells in treated group/numbers of migrated cells in control group × 100%. Cell invasion was detected similarly with the cell migration except that the two-chamber transwell membrane was pre-incubated with Matrigel (BD Bioscience, Franklin Lakes, NJ, USA). The relative rate of cell invasion (%) was quantified by numbers of invaded cells in treated group/numbers of invaded cells in control group × 100%.

Apoptosis Assay

Quantification of cell apoptosis was performed using Annexin V-FITC/PI apoptosis detection kit and flow cytometry (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Briefly, H9c2 cells were seeded into 6-well plate (Corning Incorporated, Corning, NY, USA) with 1× 10^5 cells per well and cultured in normal or hypoxia condition for 24 h. After that, cells were harvested and washed with phosphate-buffered saline (PBS, Beyotime Biotechnology, Shanghai, China) for three times. Next, cells were stained with Annexin V-FITC (10 μl) and propidium iodide (PI) (5 μl) for 20 min at room temperature in the dark. The rate of cell apoptosis was determined by flow cytometry (Millipore, Billerica, MA, USA).

Quantitative Reverse Transcription PCR (qRT-PCR)

H9c2 cells were treated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNAs were extracted in line with the manufacturer's instructions. For test of miR-101 levels, the Taqman™ MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and Taqman Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) were used according to the manufacturer's protocol. RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Biotechnology, Dalian, China) was performed to detect the expression levels of dimethyl adenosine transferase 1 homolog (DIMT1). The expression levels were quantified using $2^{-\Delta\Delta Ct}$ method described previously[17]. U6 acted as the internal control for test the expression of miR-101 and GAPDH acted as the internal control for test the expression of DIMT1. The information of primer sequences was as follows: miR-101 forward primer: 5'-CGGCGGTACAGTACTGTGATAA-3'; miR-101 reverse primer: 5'-CTGGTGT CGTGGAGTCGGCAATTC-3'; DIMT1 forward primer: 5'-GGCTGCCTTAAG ACCAACTG-3'; DIMT1 reverse primer: 5'-CGTGCCCTGAACTCTTT[illegible]T-3'; U6 forward primer: 5'-TGGGGTTATACA[illegible]T-GAGAGGA-3'; U6 reverse primer: 5'-GTGT[illegible]TACGGAGTTCAGAGGTT-3'; GAPDH forward primer: 5'-ACCAGG AAATGA[illegible]TGACA-3'; GAPDH reverse primer: [illegible]CAGTC-CATGC CATC-3'.

miRNA Transfection

miR-101 mimic, miR-101 inhibitor and si-DIMT1 were designed and synthesized by GenePharma Co., Ltd. (Shanghai, China). Cells without any vector transfection were regarded as control group. Cell transfection was performed using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Dual-Luciferase Reporter Assay

The fragment of DIMT1 containing the predicted binding site of miR-101 was amplified by PCR and cloned into a pmirGLO vector (Promega, Madison, WI, USA) to from DIMT1-wild type (DIMT1-wt). The fragment of putative binding site was replaced, cloned into a pmirGLO vector (Promega, Madison, WI, USA), which was referred as DIMT-mutated-type (DIMT1-mt). DIMT1-wt (DIMT1-mt) and miR-101 mimic were co-transfected using lipofectamine 3000 reagent. Reporter assay was performed using the Dual-luciferase system (Promega, Madison, WI, USA) in line with the manufacturer's instruction.

Western Blotting

Total proteins in H9c2 cells were extracted using RIA lysis buffer (Beyotime Biotechnology, Shanghai, China) and electrophoresed in polyacrylamide gels. Western blot methods as described previously[18]. Then the proteins in polyacrylamide gels were transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA), which co-incubated with primary antibodies. The following antibodies were used in this study: Bcl2 associated X (Bax, Cat. No.: [illegible]), Caspase 3 (Cat. No.: 9662), Caspase 9 (Cat. No.: 9508), GAPDH (Cat. No.: 2118), Proliferating Cell Nuclear Antigen (PCNA, Cat. No.: 2586), and Survivin (Cat No.: 2808) were obtained from Cell Signaling Technology (Danvers, MA, USA). B-cell lymphoma-2 (Bcl-2, Cat. No.: ab59348), Cyclin A (Cat. No. ab181591), Cyclin E1 (Cat. No.: ab71535), Cyclin D1 (Cat. No.: ab134175), Cyclin-dependent kinase 2 (CDK2, Cat. No.: ab32147), CDK4 (Cat. No.: ab199728), DIMT1 (Cat. No.: ab69434) and Specific protein 1 (Sp1, Cat. No.: ab124804) were all purchased from Abcam Biotechnology (Cambridge, MA, USA). After that, the nitrocellulose membranes were co-incubated with horseradish peroxidase (HRP)-marked secondary antibodies (Cat. No.: 14708 and 14709, Cell Signaling Technology, Danvers, MA, USA) and the protein bands were captured using Bio-Rad ChemiDoc™ XRS system (Bio-Rad Laboratories, Hercules, CA, USA), followed by adding 200 µl Immobilon Western Chemiluminescent HRP Substrate (Pierce, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Statistical Analysis

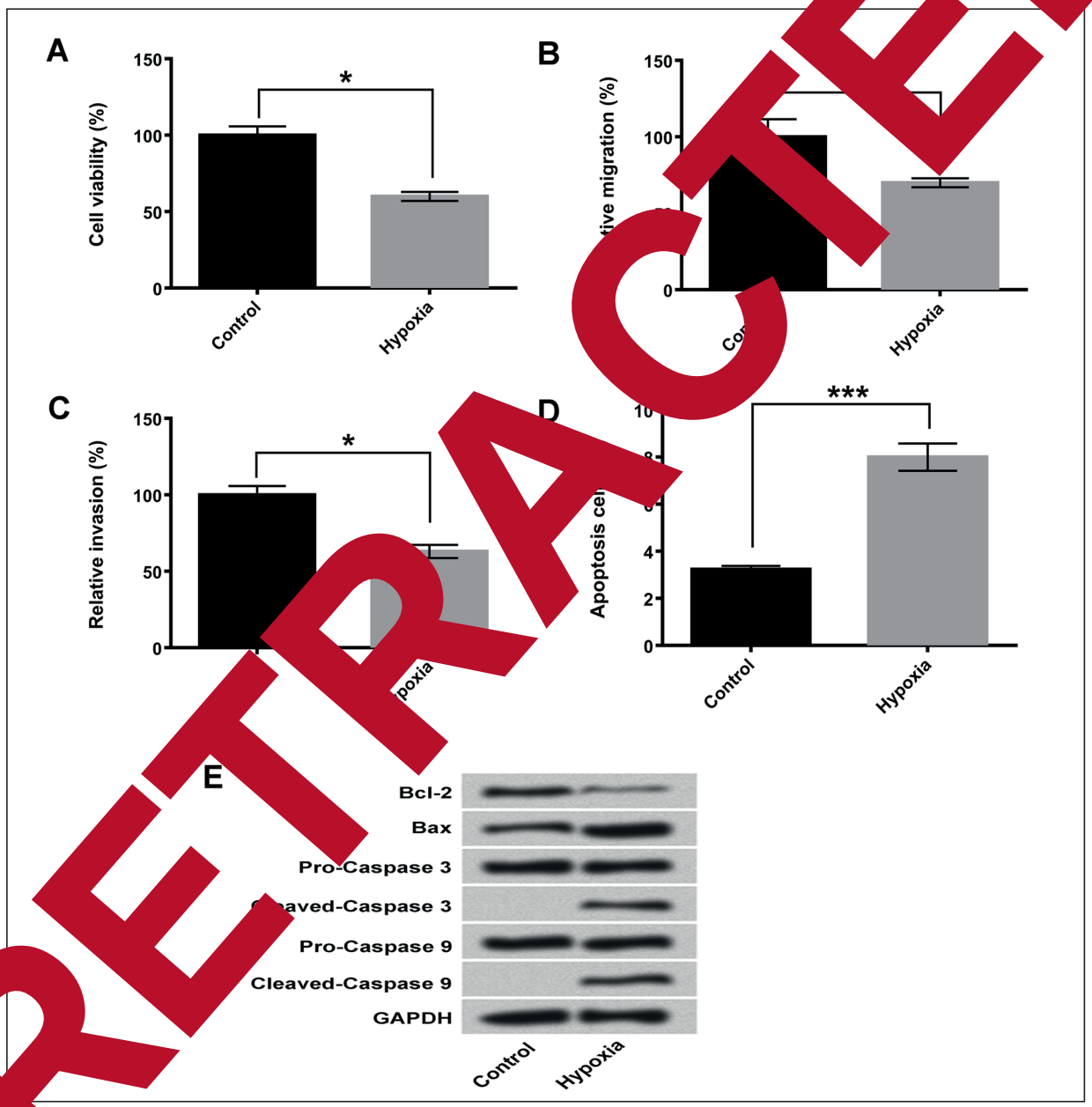
All experiments in this research were repeated at least three times. The results of multiple experiments were presented as means ± standard deviation (SD). SPSS 19.0 statistical software (IBM, Armonk, NY, USA) was performed for statistical analyses. Student's t-test was used for calculation the *p*-value of two different groups and One-way analysis of variance (ANOVA) with Sidak post-hoc test was utilized for calculation the *p*-value

of more than three different groups. $p < 0.05$ was considered to be statistically significant.

Results

Cell Injury was Induced by Hypoxia

The effects of hypoxia induction on H9c2 cell viability, migration, invasion and apoptosis were detected. Figure 1A showed that the viability of H9c2 cells was notably decreased after hypoxia treatment ($p < 0.05$). Relative migration and invasion of H9c2 cells were both significantly reduced in hypoxia treatment grou... 1B and Figure 1C, $p < 0.05$). In ...tion, a... ter hypoxia treatment, the rate ... apoptotic cells was remarkably increased ...re 1D, $p < 0.001$). Western blotting ...presen... ...at the



...e 1. H9c2 cell injury was induced by hypoxia. Cell viability ***(A)***, migration ***(B)***, invasion ***(C)*** and apoptosis ***(D)*** after hy... induction were detected using Cell Counting Kit-8 (CCK-8) assay, cell transwell assay, Annexin V-FITC/PI staining and f... w cytometer analysis, respectively. ***(E)*** The expression levels of Bcl-2, Bax, Caspase 3, Caspase 9 in H9c2 cells after hypoxia treatment were measured using Western blotting. Results of multiple experiments were expressed as means ± standard deviation (SD). Bcl-2: B-cell lymphoma-2; Bax: Bcl2-associated X. *$p < 0.05$, ***$p < 0.001$.

expression levels of pro-apoptotic protein Bax, cleaved-Caspase 3, and cleaved-Caspase 9 were all up-regulated and the expression level of anti-apoptotic protein Bcl-2 was down-regulated in hypoxia induction group (Figure 1E). These results indicated that hypoxia treatment significantly induced H9c2 cell injury.

miR-101 Was Up-Regulated in H9c2 Cells After Hypoxia Treatment

The relative expression level of miR-101 in H9c2 cells after hypoxia treatment was measured using qRT-PCR. As shown in Figure 2, after hypoxia treatment, the relative miR-101 expression level was significantly increased in H9c2 cells ($p < 0.05$), which suggested that hypoxia treatment up-regulated the expression of miR-101 in H9c2 cells.

Transfection Efficiencies of miR-101 Mimic and miR-101 Inhibitor

To examine the role of miR-101 in hypoxia-induced H9c2 cell injury, miR-101 mimic and [illegible] 101 inhibitor were transfected into H9c[illegible] respectively. qRT-PCR was used to confi[illegible]he transfection efficiency. Results in Figure 3 [illegible] played that the relative expression of miR-[illegible] was dramatically increased a[illegible]ansfecti[illegible]

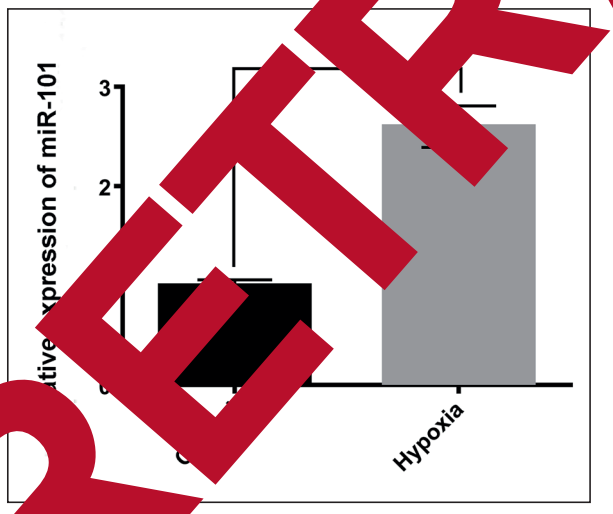


Figure 2. miR-101 was up-regulated in H9c2 cells after hypoxia treatment. The relative expression level of miR-101 in H9c2 cells after hypoxia treatment was quantified using quantitative Reverse Transcription PCR (qRT-PCR). Results of multiple experiments were expressed as means ± standard deviation (SD). miR-101: microRNA-101. *$p < 0.05$.

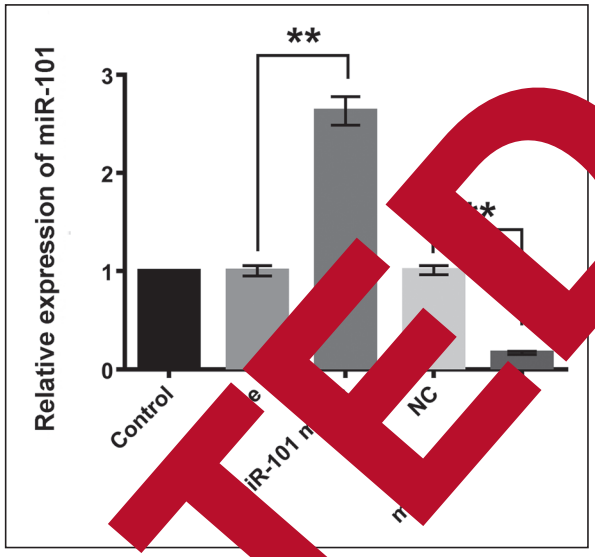


Figure 3. Transfection efficiencies of miR-101 mimic and miR-101 inhibitor in H9c2 cells. The relative expression levels of miR-101 in H9c2 cells after transfection with miR-101 mimic or miR-101 inhibitor were detected using quantitative Reverse Transcription PCR (qRT-PCR). Results of multiple experiments were expressed as means ± standard deviation (SD). miR-101: microRNA-101; NC: Negative [illegible] $p < 0.01$.

with miR-101 mimic ($p < 0.01$) and remarkably decreased after transfection with miR-101 inhibitor ($p < 0.01$). These results demonstrated that the transfection was efficiency and could be used for further experiments.

Effects of miR-101 on Hypoxia-Induced H9c2 Cell Injury

miR-101 mimic and miR-101 inhibitor were transfected into H9c2 cells respectively to explore the role of miR-101 in hypoxia-induced H9c2 cell injury. As shown in Figure 4A, compared to hypoxia single treatment group, the viability of H9c2 cells was remarkably decreased in hypoxia+miR-101 mimic transfection treatment group ($p < 0.05$) and significantly increased in hypoxia+miR-101 inhibitor transfection treatment group ($p < 0.05$). The relative migration and invasion of H9c2 cells after transfection with miR-101 mimic were significantly reduced compared to single hypoxia treatment (Figure 4B and Figure 4C, $p < 0.05$). Transfection with miR-101 inhibitor had an opposite effect ($p < 0.05$). Moreover, hypoxia-induced H9c2 cell apoptosis was markedly increased by miR-101 mimic transfection ($p < 0.05$) and notably in-

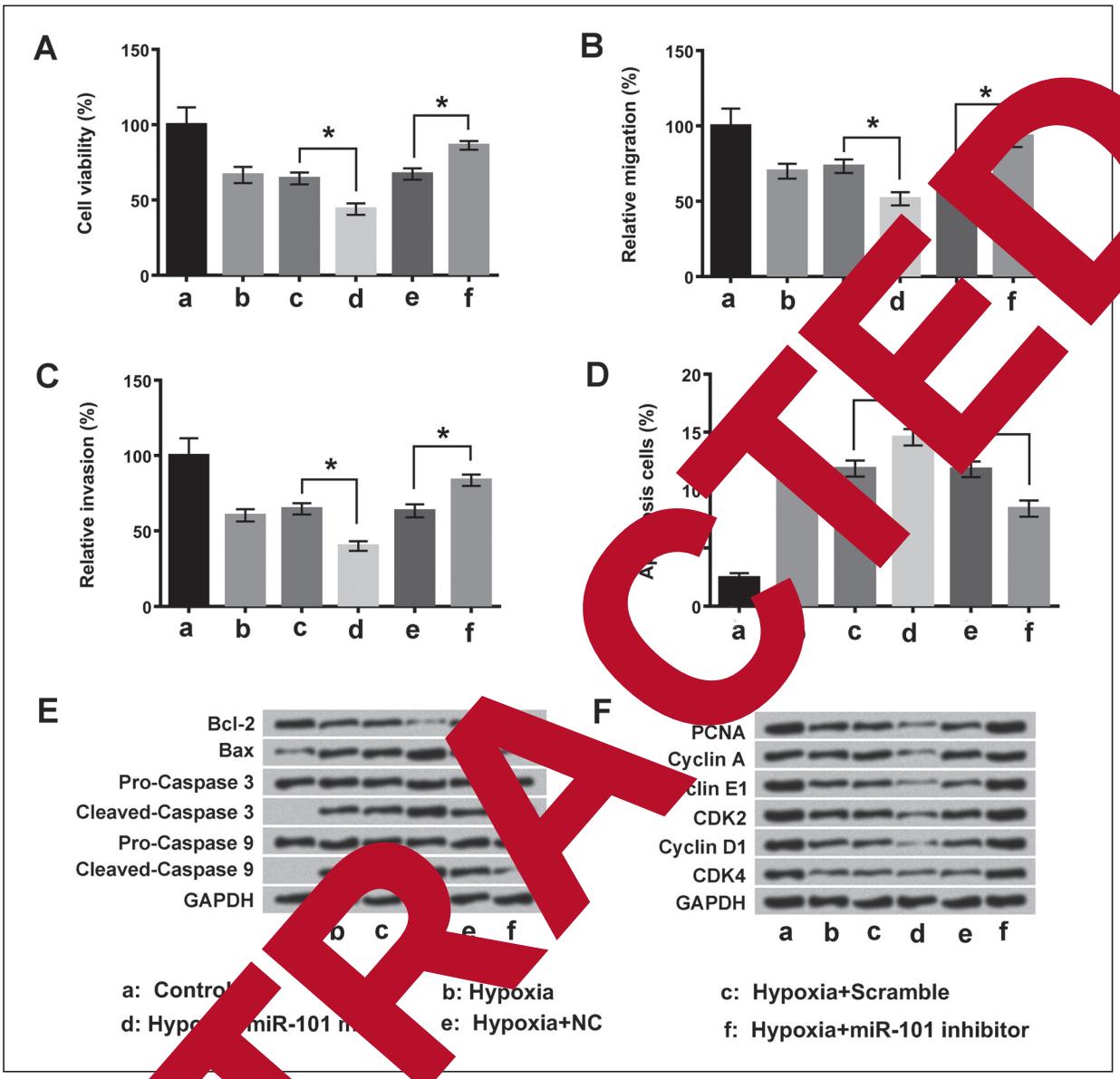


Figure 4. Effects of miR-10[illegible] hypoxia-induced H9c2 cell injury. The effects of miR-101 mimic or miR-101 inhibitor on hypoxia-ind[illegible] cell viability [illegible] migration decrease ***(B)***, invasion inhibition ***(C)***, apoptosis increase ***(D)*** were analyzed using Ce[illegible]unting Kit-8 (CCK-8) [illegible]y, cell transwell assay, Annexin V-FITC/PI staining and flow cytometer analysis, respect[illegible] The ef[illegible]ts of miR-101 [illegible]mic or miR-101 inhibitor on the expression levels of Bcl-2, Bax, Caspase 3, Caspase 9 ***(E)***, PCNA, [illegible]n A, Cyclin E1, Cyclin D1, CDK2, CDK4 ***(F)*** after hypoxia induction were measured using Western blot[illegible] sult[illegible] ultiple [illegible]eriments were expressed as means ± standard deviation (SD). Bcl-2: B-cell lymphoma-2; Bax: Bcl2-as[illegible], PCNA [illegible]iferating cell nuclear antigen; CDK: Cyclin-dependent kinase; miR-101: microRNA-101; NC: [illegible]tive c[illegible] $^*p < 0$[illegible]

hib[illegible] by miR-[illegible]01 inhibitor transfection (Fig-u[illegible] 0.05). The expression levels of [illegible], Cleaved-caspase 3, Cleaved-caspase 9 in [illegible]c2 cells were all up-regulated after hypox-[illegible]iR-101 mimic treatment and down-regulat-ed [illegible]er hypoxia+miR-101 inhibitor treatment (Figure 4E). The expression levels of Bcl-2 were decreased in hypoxia+miR-101 mimic treatment group and increased in hypoxia+miR-101 inhibitor treatment group. In addition, Western blotting also showed that the expression levels of PCNA, cyclin A, cyclin E1, cyclin D1, CDK 2, CDK 4, which exerted indispensable roles in cell cycle transition, were all down-regulated after hypoxia+miR-101 mimic treatment and up-regulated after hypoxia+miR-101 inhibitor

treatment (Figure 4F). These above experiments suggested that the hypoxia-induced H9c2 cell injury was significantly exacerbated by miR-101 overexpression and remarkably inhibited by miR-101 suppression.

DIMT1 was a Direct Target Gene of miR-101

The relative mRNA expression levels of DIMT1 after transfection with miR-101 mimic or miR-101 inhibitor were assessed using qRT-PCR. Results showed that miR-101 mimic transfection significantly down-regulated the expression level of DIMT1 in H9c2 cells and miR-101 inhibitor transfection remarkably up-regulated the expression level of DIMT1 in H9c2 cells (Figure 5A, $p < 0.05$ or $p < 0.01$). Dual luciferase reporter assay presented that the relative luciferase activity was significantly decreased in cells co-transfected with miR-101 mimic and DIMT1-wt (Figure 5B, $p < 0.05$). No significant change was observed in co-transfection with miR-101 mimic and DIMT1-mt. These results suggested that the DIMT1 was a direct target gene of miR-101.

DIMT1 was Involved in the Regulatory Roles of miR-101 in Hypoxia-Induced H9c2 Cell Injury

si-DIMT1 was transfected into H9c2 cells to further explore the regulatory role of miR-101 in hypoxia-induced cell injury. The results indicated that the protective role of miR-101 inhibitor for cell viability was markedly reversed by DIMT1

transfection ($p < 0.01$). Similar results were found in migration and invasion assay (Figure 6B and 6C, $p < 0.05$). In addition, si-DIMT1 transfection significantly aggravated the apoptosis of cells (Figure 6D, $p < 0.05$). Western blotting displayed that compared to hypoxia+miR-101 inhibitor treatment group, the expression levels of Bax, Cleaved-caspase 3, Cleaved-caspase 9 in hypoxia+miR-101 inhibitor+DIMT1 treatment group were increased (Figure 6E). Moreover, protein levels of DIMT1 and Bcl-2 were both decreased after transfection with DIMT1. These above results implied that the role of DIMT1 was involved in the protective role of miR-101 inhibitor in hypoxia-induced H9c2 cell injury. Knockdown of DIMT1 reversed the protective effects of miR-101 inhibitor.

Knockdown of DIMT1 Inhibited Sp1/Survivin Pathway in Hypoxia-Induced H9c2 Cell Injury

As shown in Figure 7, after si-DIMT1 transfection, the expression levels of Bcl-2, Sp1 and Survivin increased, accompanied with the expression level of Cleaved-caspase 3 which was decreased, indicating that knockdown of DIMT1 reversed the protective effects of miR-101 inhibitor by down-regulating the expression of anti-apoptosis proteins and up-regulating the expression of pro-apoptosis protein. These findings suggested that DIMT1-mediated Sp1/Survivin pathway was involved in the regulatory effects of miR-101 in H9c2 cells.

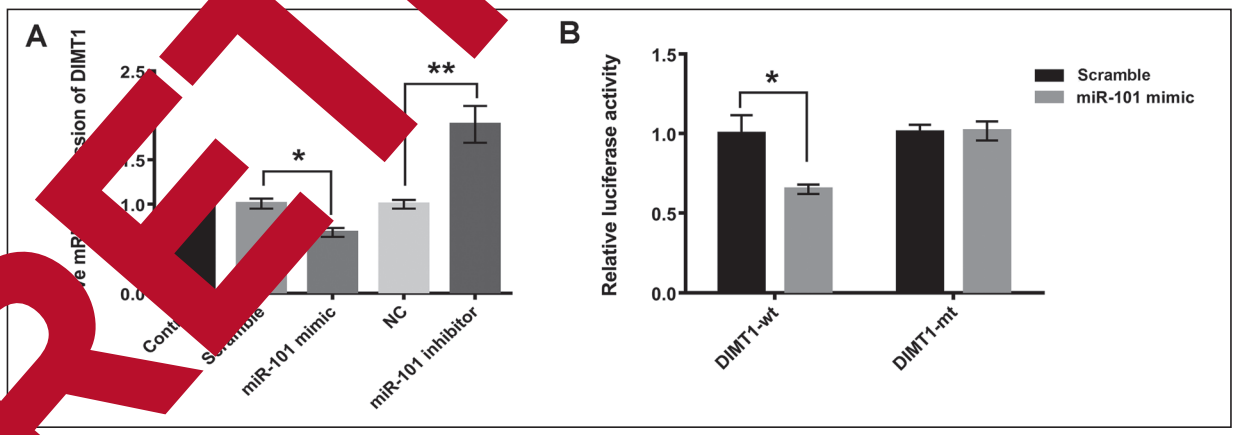


Figure 5. DIMT1 was a direct target gene of miR-101. ***(A)*** The relative mRNA expressions of DIMT1 in H9c2 cells after transfection with miR-101 mimic or miR-101 inhibitor were assessed using Quantitative Reverse Transcription PCR (qRT-PCR). ***(B)*** Dual luciferase reporter assay was used to define the targeting relationship between miR-101 and DIMT1. Results of multiple experiments were expressed as means ± standard deviation (SD). DIMT1: Dimethyladenosine transferase 1 homolog; wt: wide type; mt: mutant type; miR-101: microRNA-101; NC: Negative control. *$p < 0.05$, **$p < 0.01$.

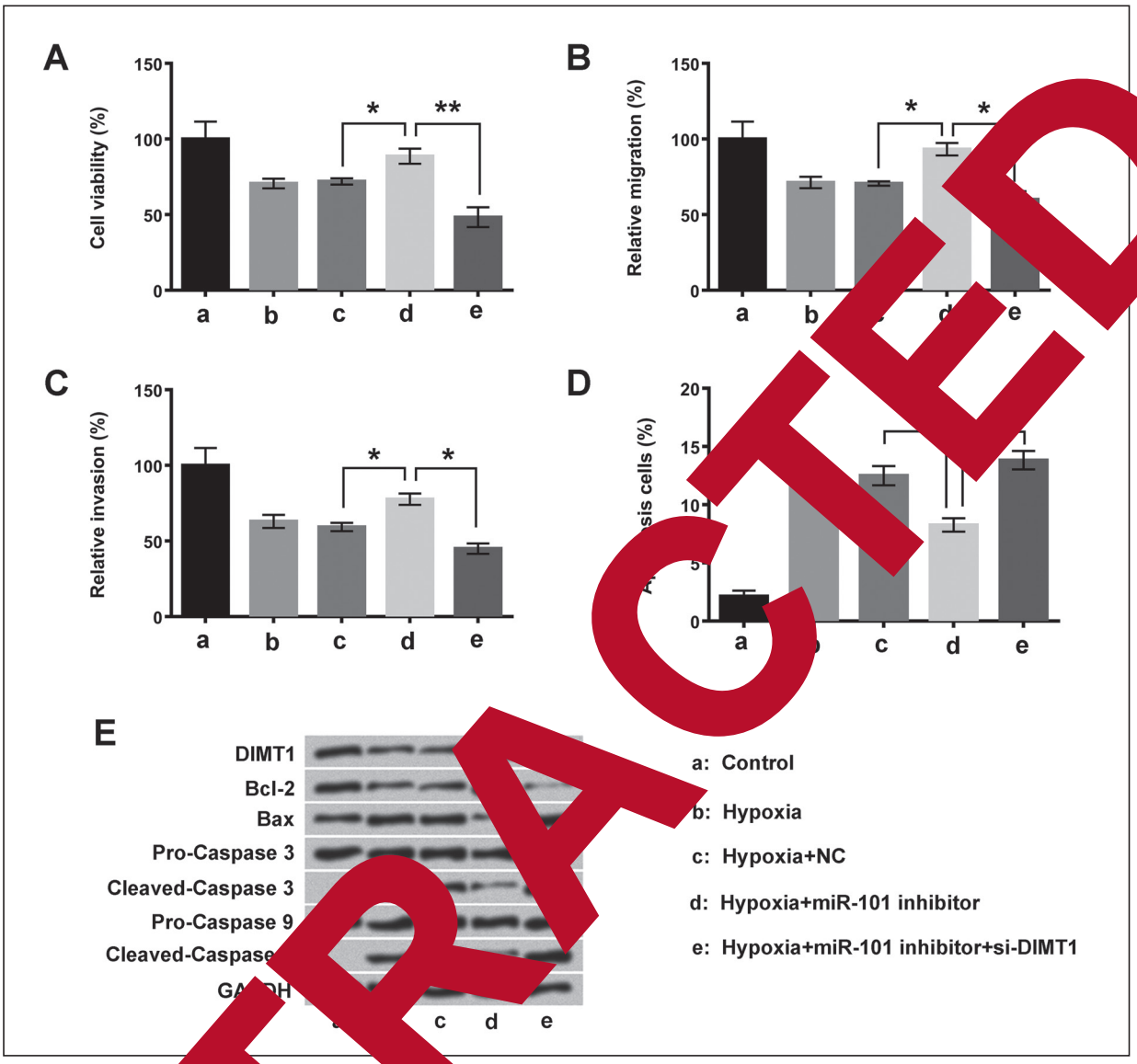


Figure 6. DIMT in the regula roles of miR-101 in hypoxia-induced H9c2 cell injury. The effects of DIMT1 on the regulatory es of mi for cell viability ***(A)***, migration ***(B)***, invasion ***(C)***, apoptosis ***(D)*** after hypoxia induction were measured us Cell Counting (CCK-8) assay, cell transwell assay, Annexin V-FITC/PI staining and flow cytometer analysis, r tively. ***(E)*** The ef DIMT1 on the regulatory roles of miR-101 for expression levels of DIMT1, Bcl-2, Bax, Ca 3 and Caspase 9 after h oxia induction in H9c2 cells were detected using Western blotting. Results of multiple exper s were ssed as means ± standard deviation (SD). DIMT1: Dimethyladenosine transferase 1 homolog; Bcl-2: B-c phom ax: Bcl2-associated X; miR-101: microRNA-101; NC: Negative control. *$p < 0.05$, **$p < 0.01$.

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ays play critical roles in many ological processes of various diseases, in- ding MI[19, 20]. In this study, we investigated the tory roles of miR-101 in the hypoxia-in- duc MI cell injury model (myocardial H9c2 cell injury model). The possible target gene of miR-101 and the potential intracellular signaling pathways were also detected. Our results presented that hypoxia treatment remarkably led to myocardial H9c2 cell injury. The expression level of miR-101 was significantly up-regulated in hypoxia-induced H9c2 cells. Knockdown of miR-101 significantly reversed the H9c2 cell injury induced by hypoxia as evidenced by the cell viability, migration and invasion increases, as well as the rate of apoptotic cells decrease.

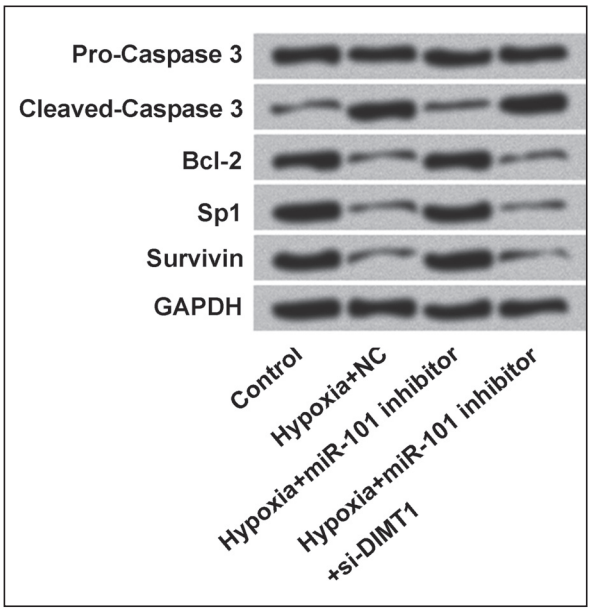

Figure 7. Knockdown of DIMT1 inhibited the Sp1/Survivin pathway in hypoxia-induced H9c2 cell injury. The effects of DIMT1 on the regulatory roles of miR-101 for Caspase 3, Bcl-2, Sp1 and Survivin expression levels in H9c2 cells were analyzed using Western blotting. DIMT1: Dimethyl adenosine transferase 1 homolog; Bcl-2: B-cell lymph 2; Sp1: Specific protein 1; miR-101: microRNA-101.

DIMT1 was a direct target gene of miR- which was involved in the re roles miR-101 in hypoxia-induced injury. Knockdown of DIMT1 m dly in ted the protective effects of miR inhibit ia-induced cell injury by vivin pathway. Ap ate supplement is critical for m ning effic yocardial cell function[21] stress, al with the insufficient o en w d to the irreversible function i ry for euk c cells, including in myo al cells[22, 23]. H lls are a kind of ca yocyt like cell line derived from rat emb ic hea in which L-type Ca_2^+ channels and ward channels are present[24]. Investig ,26] ha ased the H9c2 cells to re no ic target or mechanism of M r exam ou et al[27] demonstrated that sup ssion of Frizzled-2 attenuated hypox- i accumulation in rat H9c2 cells. ng et a proved that cyclosporine A protect- H9c2 cells against chemical hypoxia-induced via inhibition of mitogen-activated protein kin (MAPK) signaling pathway. These above researches indicate that H9c2 was a good cell model for explores the newly therapeutic target or mechanism of MI. In this research, the viability, migration and invasion of H9c2 cells were dramatically reduced after hypoxia treatment. The rate of apoptotic cells and the levels of pro-apoptotic proteins w both creased after hypoxia treatment, indicated that the myocardial cell injury induced by hypoxia was established succ y. The different miRNAs have dif t funct les in regulating various ce logical proc Scholars[30] have unvei the cr effect miRNAs on the pr s o and everal of myocardial alt tio ng ar mia, cardiac fibrosi d myo l ch trophia. miR-101 was d to inhibit brosis after MI and eft ventricu ompliance[15]. In this st , miR as up-regulated in H9c2 cells after hypoxia i on. Transfection with mi mic or miR-1 hibitor significantly i ased or decreased the expression levels of -101 in H9c ells. Hypoxia-induced H9c2 injury was kedly exacerbated by miR- 1 erexpress and remarkably inhibited by mi ion. These results were quite consist the previous studies, which point- d out that miR-208, miR-34a, miR-24, miR-16 regulated in patients with MI and played p t regulation effects on various biological processes in myocardial cells[9,31-34]. However, miR-1, miR-133a, and miR-29 were down-regulated in patients with MI[32,35]. These findings implied that numerous miRNAs participated in the regulation of MI pathological process, which worked together to from a complicated regulatory network in myocardial cells. To further analyze the potential molecular mechanism of miR-101 on hypoxia-induced myocardial cell injury, we defined DIMT1 to be the direct target gene of miR-101 as evidenced by the negative relationship between miR-101 and DIMT1 in H9c2 cells and the relative luciferase activity decrease after co-transfection with miR-101 mimic and DIMT1-wt. As 18S rRNA base methyltransferase, DIMT1 participates in the regulation of the expression levels of ribosomal proteins in cells[36]. Ikeda et al[37] presented that DIMT1 was involved in the regulation effects of miR-210 on multiple myeloma in hypoxia condition. Liu et al[38] demonstrated that DIMT1 overexpression correlated with the progression and prognosis in gastric carcinoma. In our research, compared to hypoxia+miR-101 inhibitor treatment, the H9c2 cell viability, migration and invasion were markedly decreased but cell apoptosis was significantly increased

after hyooxia+miR-101 inhibitor+si-DIMT1 treatment, which indicated that DIMT1 was involved in the regulation effects of miR-101 on hypoxia-induced myocardial H9c2 cell injury and knockdown of DIMT1 significantly reversed the protective effects of miR-101 suppression. Sp1 is a nuclear transcription factor, which regulates the expression of diverse genes in cells[39]. Authors[40-42] presented that Sp1 participates in the regulation of cell apoptosis, fibrosis and other biological processes. Survivin, one of the target genes of Sp1, is an apoptosis inhibitor in eukaryotic cells[43]. Yang et al[44] displayed that overexpression of Survivin remarkably reduced the levels of apoptosis promoting protein in myocardial cells of the rats with MI, and decreased the apoptosis rate of myocardial cells and the MI zone. In our researches, suppression of DIMT1 down-regulated the expression levels of Sp1 and Survivin, which accompanied with the levels of Cleaved-caspase 3 increase and Bcl-2 decrease, indicating that DIMT1-mediated the Sp1/Survivin pathway was involved in the regulation effects of miR-101 in H9c2 cells. In addition, our results were consistent with the previous studies, which prove[d] that miR-7a/b protected myocardial H9c2 cell[s] hypoxia-induced apoptosis via Sp1 and PA[K][42].

Conclusions

We showed the critical r[ole] of miR[-101] in regulating myocardial cell i[njury] induc[ed by] hypoxia. DIMT1-mediated [Sp1/Survivin] pa[thway] was also involved i[n] this pr[ocess]. We propose that miR-101 cou[ld] as an effi[cient] molecular target for thera[py] diagnosis of [MI]. Further investigations [sh]ould be performed to confirm this propos[al].

Conflict of Interest

The [authors] d[eclare] that the[y] have no conflict of interests.

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