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Downregulation of lncRNA MEG3 attenuates high glucose-induced cardiomyocytes injury by inhibiting mitochondria-mediated apoptosis pathway

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Abstract. – OBJECTIVE: The aim of the present work was to investigate the effects and mechanisms of long non-coding RNA (lncRNA) maternally expressed gene 3 (MEG3) in cardiomyocytes injury and apoptosis induced by high glucose (HG) *in vitro*.

MATERIALS AND METHODS: HG-induced rats' cardiomyocytes with si-MEG3 transfection were constructed. Cell viability and lactate dehydrogenase (LDH) level were examined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and LDH assay kits, respectively. Cardiomyocytes apoptosis was detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining. The expressions of Bcl-2, Bax, cleaved caspase-9 and cleaved caspase-3 proteins were determined by Western blot. The expression of lncRNA MEG3 was measured by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

RESULTS: Our results indicated that the expression of MEG3 was significantly upregulated in HG-treated cardiomyocytes. The downregulation of MEG3 could attenuate cardiomyocytes injury and apoptosis by decreasing the Bax/Bcl-2 ratio, cleaved caspase-9 and cleaved caspase-3 expression.

CONCLUSIONS: The downregulation of MEG3 could attenuate cardiomyocytes injury and apoptosis induced by HG. The molecular mechanism was associated with the inhibition of the mitochondria-mediated apoptosis pathway.

Key Words:
Cardiomyocytes, Apoptosis, Long non-coding RNA, Maternally expressed gene 3 (MEG3).

Introduction

Diabetic cardiomyopathy (DCM) is one of the major complications in patients with diabetes mellitus (DM)[1]. It is an independent risk factor for heart failure in DM patients without hypertension and coronary artery disease[2]. The main manifestations of DCM are cardiac dysfunction and energy metabolism disturbance[3]. Mitochondria play important roles in the energy metabolism of cardiomyocytes. Current studies[4,5] have shown that hyperglycemia can lead to mitochondria injury and result in cardiomyocytes apoptosis. However, the molecular mechanisms underlying the development of DCM have not yet been fully elucidated.

Long non-coding RNAs (lncRNAs) are defined as non-protein-coding transcript molecules longer than 200 nucleotides[6]. LncRNAs participate in various biological regulatory processes including embryonic development, cell death, and tumor formation and metastasis[7-9]. The lncRNA maternally expressed gene 3 (MEG3) is now considered as a novel biomarker of multiple tumors, such as hepatocellular carcinoma[10], cervical carcinoma[11] and meningioma[12]. However, the role of MEG3 is very little known in the heart. Recently, Piccoli et al[13] demonstrated that the expression of MEG3 is enriched in cardiac fibroblasts and is poor in cardiomyocytes. In this study, we cultured neonatal rats' cardiomyocyte *in vitro* to investigate the effects of MEG3 on high glucose (HG)-induced cardiomyocytes injury and the potential molecular mechanism.

Materials and Methods

Cell Culture

Neonatal Sprague Dawley (SD) rats (one to two days old) were provided by the Animal Experiment Center of Shandong Province (Jinan, China), and all experimental protocols

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were found to conform with the Guide for the Care and Use of Laboratory Animals (NIH). This study was approved by the Animal Ethics Committee of Dezhou People's Hospital Animal Center. Primary cultures of ventricular myocytes were prepared as described previously[14]. Briefly, after ventricles digestion, cells were pre-plated in a culture dish for 90 min at 37°C to remove non-cardiomyocytes and then incubated in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL Life Technologies, Carlsbad, CA, USA) containing normal glucose concentration (5.5 mM), supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 1% penicillin and streptomycin, and 0.1 mM bromodeoxyuridine. After 2 days, cardiomyocytes were incubated with a normal glucose concentration (5.5 mM) or HG concentration (22, 33 and 55 mM) for 48 h.

Transfection of Small Interfering RNAs (siRNAs)

The constructed plasmids si-MEG3 were provided by GenePharma Co., Ltd. (Shanghai, China). Non-targeting siRNA (si-NC) was used as a negative control for si-MEG3. All siRNAs (si-MEG3 and si-NC) were transfected into HG-induced cardiomyocytes by using the Lipofectamine 3000 reagent according to the manufacturer's instructions (Life Technologies Corporation, Gaithersburg, MD, USA). The transfection efficiency was detected by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA in cardiomyocytes was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary deoxyribose nucleic acid (cDNA) synthesis was using the reverse transcription (RT) reagent according to kit instructions (TaKaRa Biotechnology, Otsu, Shiga, Japan) and a Real Time-PCR reaction system was performed by the Eppendorf Mastercycler ep realplex. The primer sequences were as follows: MEG3 (forward) 5'-CTGCCCATCTACACCTCACG-3' and (reverse) 5'-CTCTCCGCCGTCTGCGCTAGGGGCT-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward) 5'-TGCACCACCAACTGCTTAGC-3' and (reverse) 5'-GGCATGCACTGTGGTCATGAG-3'. The relative expression levels of MEG3 were normalized to GAPDH and was calculated using the $2^{-\Delta\Delta CT}$ method.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) and Lactate Dehydrogenase (LDH) Assay

Cell damage was measured using MTT and LDH assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Collected cells were plated in a 96-well plate and incubated with MTT and LDH solution, respectively. The OD value at 490 nm was detected by a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The degree of cell damage was calculated as a percentage.

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP Nick-End Labeling (TUNEL) Staining

Cardiomyocytes apoptosis was detected using an in situ cell death detection kit according to the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique (Roche Applied Science, Mannheim, Germany). Briefly, cultured cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 h at room temperature (RT) and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. After incubating with the TUNEL reaction mixture for 1 h at 37°C, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min and visualized under a fluorescence microscope. The number of TUNEL-positive nuclei (green fluorescence) was expressed as a percentage of total nuclei (blue fluorescence).

Western Blot

Cytoplasmic proteins were isolated from cardiomyocytes using a cytoplasmic proteins extraction kit (Keygen Biotechnology, Nanjing, China) according to the protocol. Briefly, proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% (v/v) non-fat dry milk, membranes were then incubated with Bcl-2, Bax, cleaved caspase 9 and cleaved caspase 3 primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After incubated with these primary antibodies, the membranes were washed in Tris-Buffered Saline and Tween (TBS-T) (Beyotime, Shanghai, China) and then incubated with the HRP-conjugated secondary antibody at room temperature for another 2 h.

Western Blot Detection kit and Image J software (NIH) were used to measure the blot signal and density.

Statistical Analysis

All results were presented as the means ± SD. Statistical comparisons between different groups were measured using one-way ANOVA followed by post-hoc test (Least Significant Difference) and the Student-Newman-Keuls test. The level of significance was set at $p<0.05$.

Results

The Degree of Cell Damage Induced by HG

We first evaluated the cytotoxicity of HG on cardiomyocytes. Cells were treated with glucose (5.5, 22, 33, 55 mM) for 48 h. Cell viability was significantly decreased in the HG (22, 33 and 55 mM) groups compared with the control group (5.5 mM) (Figure 1A). The levels of LDH were significantly increased in the HG groups compared with the control group (Figure 1B).

Downregulation of MEG3 Attenuated the Degree of Cell Damage Induced by HG

As shown in Figure 2A, the expression of MEG3 was significantly increased in the HG (33 mM) group compared with the control group. After si-MEG3 was transfected into HG-induced cardiomyocytes, the expression of MEG3 was significantly decreased compared with the HG group. The downregulation of MEG3 also increased the cell viability and decreased the level of LDH in HG-induced cardiomyocytes (Figure 2B and 2C).

Downregulation of MEG3 Inhibited HG-Induced Cardiomyocytes Apoptosis

The TUNEL staining was used to evaluate cardiomyocytes apoptosis. The apoptotic nuclei (blue) was remarkably increased in the HG group compared with the control group. In contrast, the downregulation of MEG3 significantly reduced the TUNEL-positive nuclei induced by HG (Figure 3).

Downregulation of MEG3 Inhibited Mitochondria-Mediated Apoptosis Activation

As shown in Figure 4, there was a significant increase in the ratio of Bax/Bcl-2 and the expressions of cleaved caspase 9 and cleaved caspase 3 in the HG group compared with the control group. The downregulation of MEG3 substantially decreased the ratio of Bax/Bcl-2 and the expressions of cleaved caspase 9 and cleaved caspase 3 in HG-induced cardiomyocytes.

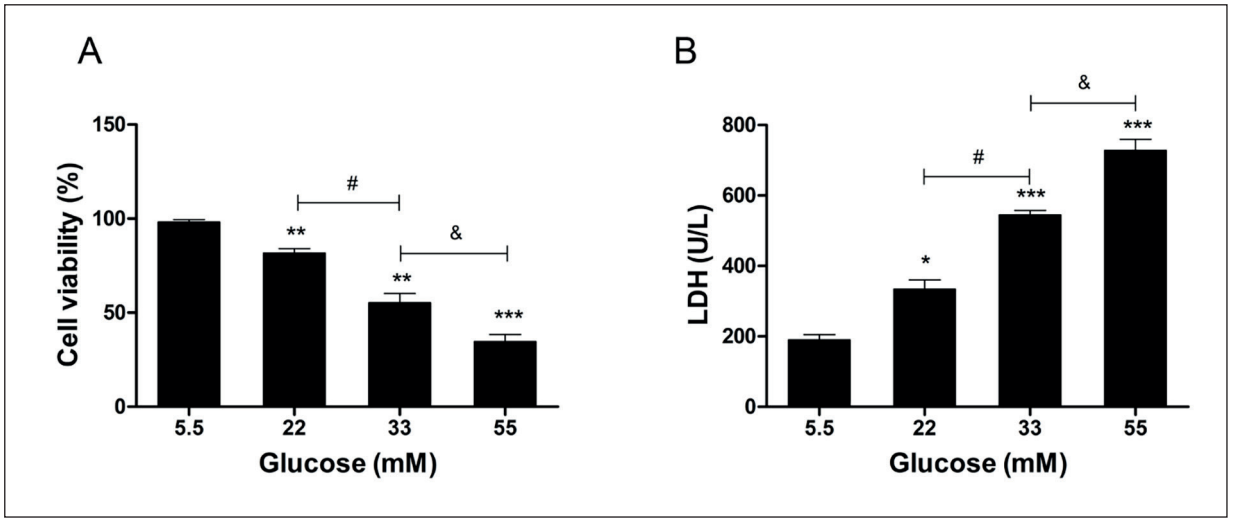

Figure 1. The cytotoxicity of HG on cardiomyocytes. ***A,*** Cell viability was determined by MTT. ***B,*** LDH level was determined by LDH assay kit. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ *vs* control; $^{\#}p<0.05$ *vs* HG (22 mM)-treated cells; $^{\&}p<0.05$ *vs* HG (55 mM)-treated cells.

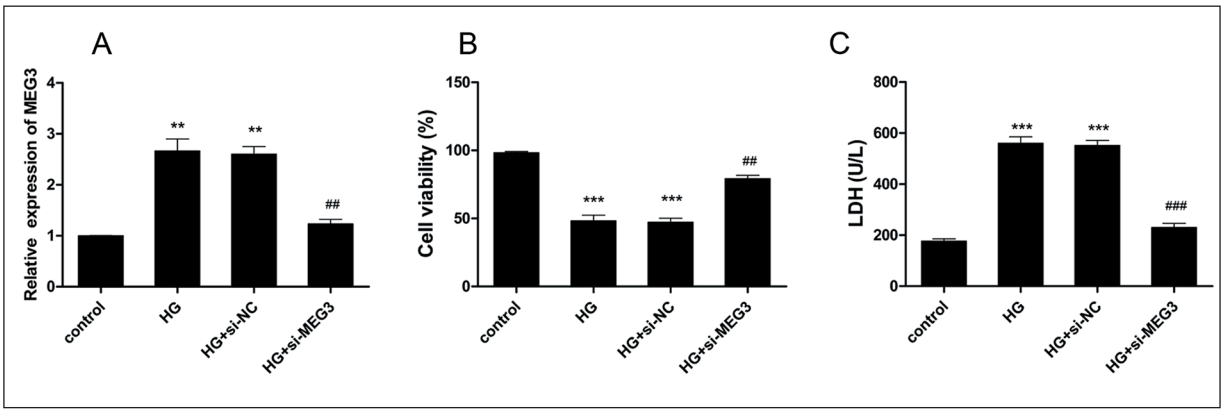

Figure 2. Downregulation of MEG3 attenuated the degree of cell damage induced by HG. ***A,*** Relative gene expression of MEG3. ***B,*** Cell viability. ***C,*** LDH level. $^{**}p<0.01$ and $^{***}p<0.001$ *vs* control; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ *vs* HG group.

Discussion

In this study, we explored the effects and mechanisms of lncRNA MEG3 on HG-induced cardiomyocytes injury. Our results indicated that the expression of MEG3 was significantly upregulated in the HG-treated cardiomyocytes, which was closely associated with cardiomyocytes injury and apoptosis. Furthermore, we found that downregulation of MEG3 could attenuate cardiomyocytes injury and apoptosis by inhibiting mitochondria-mediated apoptosis pathway.

MEG3 is a conserved lncRNA and has been identified as a transcriptional regulator of various diseases[15]. Many studies have demonstrated that the upregulation of MEG3 could induce cells apoptosis of hepatocellular carcinoma[10], bladder cancer[16], and cervical carcinoma[11]. Xu et al[17] reported that knockdown of MEG3 inhibited chondrocytes apoptosis and promoted osteoarthritis by regulating miR-16. Recently, Piccoli et al[13] identified that downregulation of MEG3 in cardiac fibroblasts could prevent cardiac fibrosis and dysfunction induced by pathological hypertrophy by blocking the TGF-β1/MMP-2 signal. Moreover, Gong et al[18] found that the downregulation of MEG3 could attenuate hypoxia-induced H9c2 cells injury

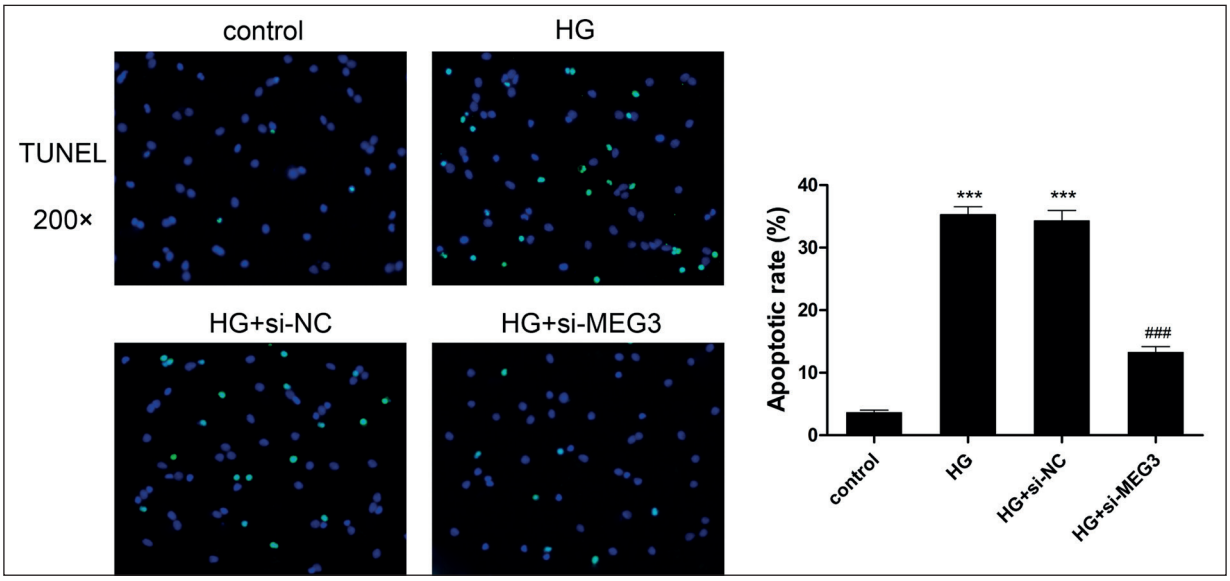

Figure 3. Downregulation of MEG3 inhibited HG-induced cardiomyocytes apoptosis. Cardiomyocytes apoptosis were stained by TUNEL. Scale bar = 50 μm. $^{***}p<0.001$ *vs* control; $^{\#\#\#}p<0.001$ *vs* HG group.

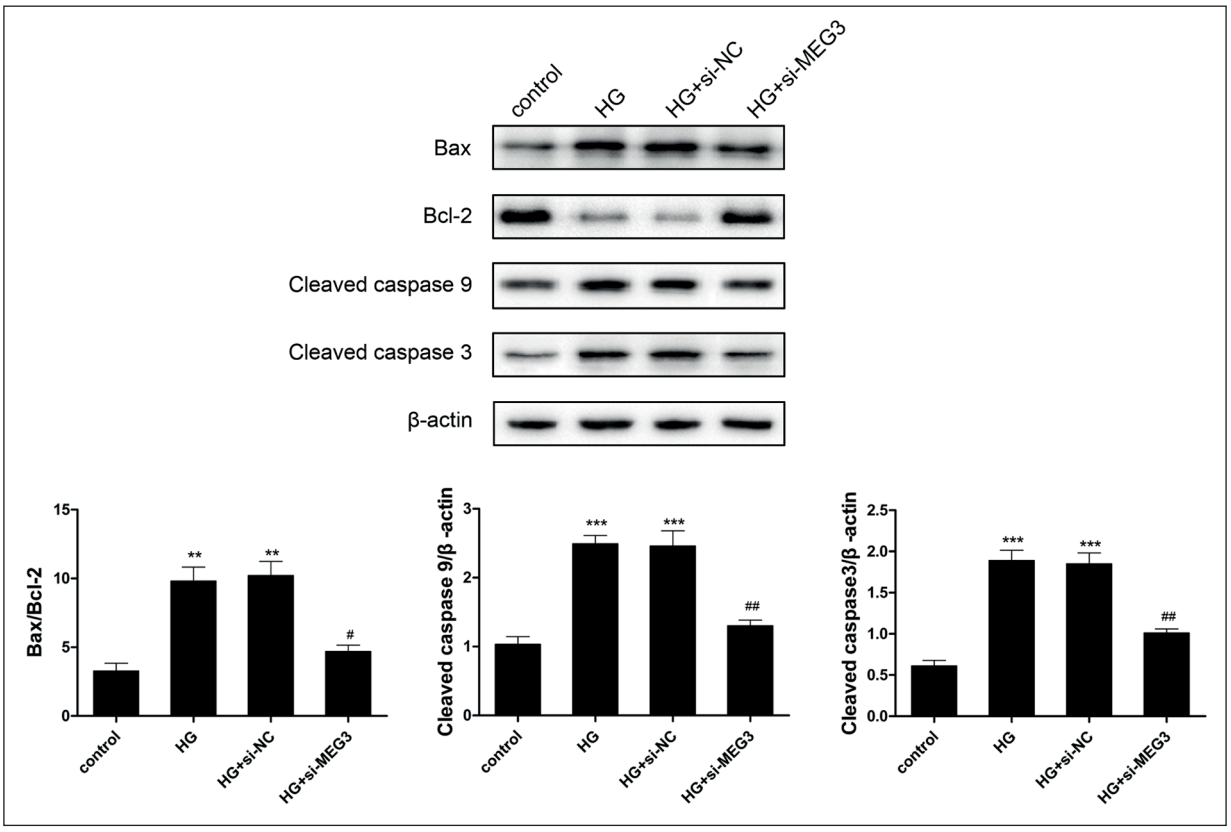

Figure 4. Downregulation of MEG3 inhibited mitochondria-mediated apoptosis activation. The expression of Bax, Bcl-2, cleaved caspase-9 and cleaved caspase-3. $^{**}p<0.01$ and $^{***}p<0.001$ *vs* control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ *vs* HG group.

and apoptosis by upregulating miR-183. In line with these previous reports, our results indicated that downregulation of MEG3 attenuated cell injury and apoptosis of HG-treated cardiomyocytes.

Cardiomyocyte apoptosis plays an important role in the development of DCM[19] and is closely associated with cardiac hypertrophy, fibrosis and remodeling[20]. Mitochondria are the central organelles of cardiomyocyte apoptosis and the decrease of the mitochondrial membrane potential initiates the activation of mitochondrial-mediated apoptosis, which is mainly regulated by the Bcl-2 family proteins, including the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax. The upregulation of Bax/Bcl-2 ratio leads to the release of cytochrome c into the cytoplasm and activates caspase-9 and caspase-3, thereby leading to apoptosis[21,22]. In the present study, the results of TUNEL staining revealed that the downregulation of MEG3 significantly inhibited cardiomyocytes apoptosis in HG condition by decreasing the Bax/Bcl-2 ratio and cleaved caspase-9 and cleaved caspase-3 expression.

Conclusions

This is the first study reporting that downregulation of MEG3 could attenuate cardiomyocytes injury and apoptosis induced by HG *in vitro*. The molecular mechanism was associated with the inhibition of mitochondria-mediated apoptosis pathway. Our results provide new insights in understanding the molecular mechanisms of cardiomyocytes apoptosis. Further research is needed to investigate *in vivo*.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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