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LncRNA MEG3 accelerates apoptosis of hypoxic myocardial cells *via* FoxO1 signaling pathway

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Abstract. – OBJECTIVE: To explore the effects of long non-coding ribonucleic acid (lncRNA) maternally expressed gene 3 (MEG3) on the proliferation and apoptosis of hypoxic myocardial cells by regulating the expression of forkhead box O1 (FoxO1).

MATERIALS AND METHODS: The myocardial A10 cell lines were divided into myocardial cell group (group A), hypoxic myocardial cell group (group B), and hypoxic myocardial cell + transfection with lncRNA MEG3 mimic group (group C). The correlations of the adenosine triphosphate (ATP) concentration, the degree of apoptosis, and the proliferation with FoxO1 and FoxO3a proteins in the cells were observed *via* ATP assay, Cell Counting Kit-8 (CCK-8) assay, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, Western blotting, and quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR), respectively.

RESULTS: The ATP concentration in myocardial cells was the highest in group A ($p<0.05$), and it was higher in group B than that in group C ($p<0.05$). The results of the CCK-8 assay showed that the proliferation rate of the myocardial cells was the highest in group A and the lowest in group C ($p<0.05$), and it was significantly increased in group B compared with that in group C ($p<0.05$). The results of the TUNEL assay revealed that the normal cells displayed the purple color, and apoptotic cells displayed the green color. The myocardial cells were arranged orderly, and the number of apoptotic cells was smaller in group A, the number of apoptotic cells was significantly larger in group B than that in group A, and it was the largest in group C ($p<0.05$). Moreover, the results of Western blotting manifested that the concentrations of FoxO1 and FoxO3 proteins in myocardial cells were the lowest in group A ($p<0.05$), and they were significantly higher in group C than those in group B ($p<0.05$). According to the results of qRT-PCR, the mRNA expressions of FoxO1 and FoxO3 in myocardial cells were the lowest in group A ($p<0.05$), and they were remarkably lower in group B than those in group C ($p<0.05$).

CONCLUSIONS: LncRNA MEG3 can increase the activity of FoxO1 to promote myocardial apoptosis in a hypoxic environment.

Key Words:
Hypoxic myocardial cells, FoxO1, LncRNA MEG3.

Introduction

Oxygen is one of the necessary factors to maintain the normal vital movement of the human body, which can convert such nutrients as glucose and protein into energy. However, the heart, brain, kidney, and other organs will be damaged in various degrees in the case of severe hypoxia. When the oxygen is insufficient to satisfy the demand of oxygen supply in myocardial cells, the myocardial necrosis and apoptosis will occur, and the weakened oxygen metabolism enhanced anaerobic glycolysis and decreased cardiac output are the main characteristics of myocardial hypoxia[1,2]. These characteristics will, if they persist, aggravate the body's damage and lead to common clinical complications, such as angina, coronary heart disease, and myocardial infarction. Therefore, it is extremely important to deeply understand and explore the causes and therapeutic mechanism of apoptosis of hypoxic myocardial cells, and it is also of great significance to control the myocardial apoptosis and increase the viability and survival time of myocardial cells to the maximum degree in the increase of the survival rate of the myocardial cells in hypoxic environment[3]. The forkhead box O (FoxO) family widely existing in organisms is expressed in different organs, such as the brain, heart, and kidney. FoxO1 can move

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freely inside and outside the cells and plays an important role in myocardial cell growth, apoptosis, and oxidative stress[4]. In the hypoxic environment, FoxO1 can promote apoptosis, lead to massive autophagy, and induce the functional changes in the myocardial cells. Moreover, the increased activity of FoxO1 will aggravate the oxygen free radical injury in myocardial cells, resulting in the decline in the tolerance of them. The complete opposite is the case in which the activity of FoxO1 declines. Long non-coding ribonucleic acid (lncRNA) maternally expressed gene 3 (MEG3) is a cancer suppressor gene constantly expressed in many normal organs and tissues, but its expression is deleted or declines in a variety of tumor tissues[5]. Studies have demonstrated that lncRNA MEG3 can increase the activity of FoxO1 to promote the apoptosis and destroy the cells under hypoxia-ischemia of myocardial cells. Besides, the decreased expression of lncRNA MEG3 can reduce the apoptosis of hypoxic myocardial cells[6]. In this paper, the effects of lncRNA MEG3 on the proliferation and apoptosis of hypoxic myocardial cells by regulating the expression of FoxO1 were explored in the hypoxic environment.

Materials and Methods

Materials, Reagents, and Instruments

The myocardial A10 cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA), adenosine triphosphate (ATP) antibody, FoxO1 and FoxO3a antibodies, phosphorylated FoxO1 and FoxO3a and GAPDH antibodies from BD (Franklin Lakes, NJ, USA), trypsin and Dulbecco's Modified Eagle's Medium (DMEM) from Yiheng (Shanghai, China), RNA-iMAX from IKa (Staufen, Germany), and Cell Counting Kit-8 (CCK-8) kit from Proteintech (Wuhan, China).

Cell Culture and Grouping in Three Groups

The myocardial A10 cell lines were added with erythromycin and penicillin (80 mg/mL) and incubated in the complete medium containing 12% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37.5°C. The medium was replaced once every 24 h. When 90% of A10 cells were fused, a certain number of myocardial cells in the growth stage were taken and cultured under hypoxia, according to the literature[3]. The cells were divided into myocardial cell group (group A), hypoxic myocardial cell group (group B), and hypoxic myocardial cell + transfection with lncRNA MEG3 mimic (according to the literature[7]) group (group C).

ATP Assay

A small number of myocardial cells were taken, added with 150 μL of lysis buffer, fully stirred, and centrifuged for 10 min, and the supernatant was retained for later use. A small amount of ATP reagent was diluted at 5-fold to be the ATP assay working solution and stored under low temperature. Then, 50 μL of ATP assay working solution was added into a 96-well plate and placed at 37°C for 10 min, and 30 μL of supernatant was added. After 10 s, the relative light unit (RLU) value was detected and recorded using a multifunctional microplate reader, based on which the ATP concentration was calculated.

CCK-8 Assay of Cell Proliferation

The myocardial cells were inoculated into the 96-well plate (100 μL/well) and pre-incubated in the incubator with 8% CO_2 at 37°C. After that, 15 μL of lncRNA MEG3 solution was added into each well, followed by incubation in the incubator for 1-4 h. Then, the optical density (OD) value was measured at 450 nm using the microplate reader. If the OD value was not measured temporarily, 10 μL of 0.1 M HCl solution or 1% (w/v) SDS solution was added into each well, and the culture plate was stored at 37°C in a dark place. The OD value will not change if measured within 24 h.

TUNEL Assay of Myocardial Apoptosis

The myocardial cells were centrifuged and re-paved into the 96-well plate. After adherent growth overnight, the cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with Triton X-100 for 15 min and stained with TUNEL to detect myocardial apoptosis in each group in strict accordance with the instructions. The cells in each group were observed in five non-crossed and non-repeated fields randomly selected under a microscope. The myocardial apoptosis rate = apoptotic cells/total cells × 100%.

Analysis of FoxO1 and FoxO3a Protein Expressions Via Western Blotting

The cells in a 6-well plate were digested with 100 μg of trypsin extract, and then the digestion was terminated with 2 mL of medium. The myocardial cell extract was placed into the Eppendorf

Table I. Primer sequences.

Gene		Primer sequence
FoxO1	Forward	5'-GGCTGAGGGlTrAGTGAGCA-3'
	Reverse	5'-AGGGAGTTGGTGAAAGACATC-3'
FoxO3a	Forward	5'-CGGGCAGAATCATGAGCAAGT-3'
	Reverse	5'-AGGGTCTGCATrGGATGGCAT-3'
GAPDH	Forward	5'-CACCATIGGCAATGAGGGGTFC-3'
	Reverse	5'-AGGTCTTTGCGGATGTCCACGT-3'

(EP) tube, mixed with trypsin extract at 1:100, and frozen in a refrigerator for 10 min. After complete lysis of cells, the E solution was obtained. The myocardial cells were placed into the EP tube, mixed with 2 mL of trypsin extract at 1:100. After complete lysis of cells, the F solution was obtained. Then, E solution and F solution were mixed evenly at 80:1, prepared into a working solution and placed in the incubator at 37.5°C for 20 min. After cooling, the protein concentration was calculated.

Detection of FoxO1 and FoxO3a Activity Via Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The myocardial cells were digested with trypsin, washed with Phosphate-Buffered Saline (PBS), and added with 1.5 mL of lncRNA MEG3 vector reagent to extract RNA. Then, 30 mg of cell suspension was placed into the EP tube, and the total mRNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into cDNA, followed by RT-PCR amplification with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference (98°C for 6 min, 98°C for 28 s, 75°C for 30 s, and 80°C for 4 min, a total of 55 cycles). The primer sequences are shown in Table I.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis of the experimental data. The *t*-test was performed for the differences in the myocardial cell viability, cell proliferation rate, degree of apoptosis, FoxO1, and FoxO3a activity among group A, B, and C. The measurement data were expressed as mean ± standard deviation. The comparison between groups was done using the One-way ANOVA test followed by the post-hoc test (Least Significant Difference). Spearman rank correlation coefficient was adopted for the correlation analysis. $p<0.01$ suggested statistically significant differences.

Results

ATP Concentration in Myocardial Cells in Each Group

The higher ATP concentration corresponds to the higher myocardial cell viability. The detection results of ATP concentration in myocardial cells showed that the ATP concentration in myocardial cells was the highest in group A ($p<0.05$), and it was higher in group B than that in group C ($p<0.05$) (Figure 1).

Myocardial Cell Proliferation Rate Detected Via CCK-8 Assay

The results of the CCK-8 assay showed that the proliferation rate of the myocardial cells was the highest in group A and the lowest in group C ($p<0.05$), and it was significantly increased in group B, compared with that in group C ($p<0.05$) (Figure 2).

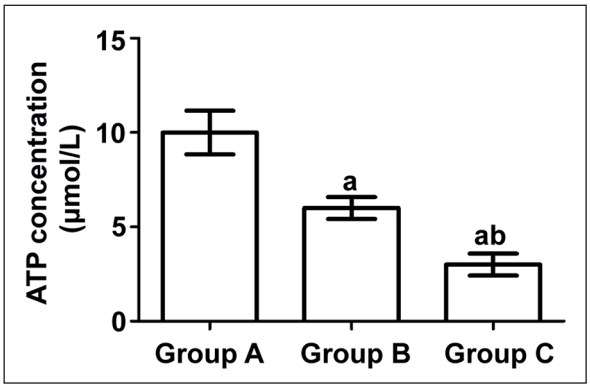

Figure 1. Comparison of ATP concentration in myocardial cells among the three groups. Note: a: $p<0.05$ *vs.* group A, b: $p<0.05$ *vs.* group B.

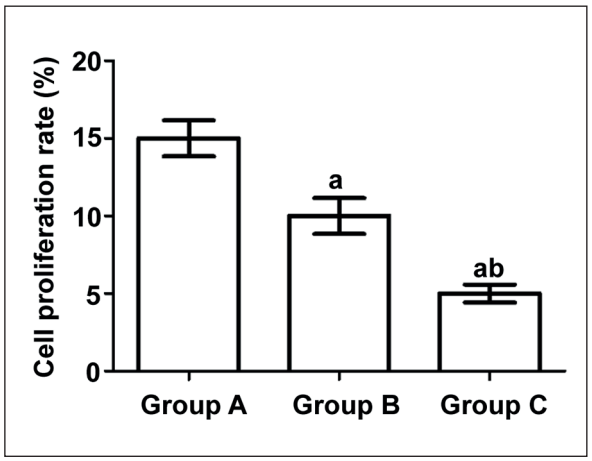

Figure 2. Myocardial cell proliferation rate in the three groups. Note: a: $p<0.05$ *vs.* group A, b: $p<0.05$ *vs.* group B.

Myocardial Apoptosis Detected Via TUNEL Assay

The results of the TUNEL assay revealed that apoptotic cells displayed the green color, and the blue color indicated the nuclei. The number of apoptotic cells was smaller in group A, the number of apoptotic cells was significantly larger in group B than that in group A, and it was the largest in group C ($p<0.05$) (Figure 3).

FoxO1 and FoxO3a Protein Expressions in Myocardial Cells Detected Via Western Blotting

The results of the Western blotting manifested that the concentrations of FoxO1 and FoxO3a proteins in myocardial cells were the lowest in group A ($p<0.05$), and they were significantly higher in group C than those in group B ($p<0.05$) (Figure 4).

FoxO1 and FoxO3a mRNA Expressions in Myocardial Cells Detected Via qRT-PCR

According to the results of qRT-PCR, the mRNA expressions of FoxO1 and FoxO3 in myocardial cells were the lowest in group A ($p<0.05$), and they were significantly lower in group B than those in group C ($p<0.05$) (Figure 5).

Discussion

Myocardial hypoxia refers to the pathological process of the myocardial tissues caused by the abnormal changes in tissue metabolism, cell function, and morphological structure in a hypoxic environment due to the decline in oxygen supply in cells[8,9]. Hypoxia is not an independent disease but exists in a variety of diseases, whose pathological process is similar in the body. In the clinic, hypoxia is the direct cause of many diseases[10], which may lead to death in severe cases. Myocardial hypoxia is one of the important causes of long-term progressive apoptosis of myocardial cells. According to the previous analysis, the persistent myocardial hypoxia will only result in cell necrosis, but there are many experimental data indicating that the long-term myocardial hypoxia is a major cause of myocardial apoptosis[11]. Studies have demonstrated that the mitochondria are one of the most important elements in myocardial cells to maintain the body function, and they participate in the growth and metabolism of myocardial cells. However, the mitochondrial structure is susceptible to damage under hypoxic conditions, manifested as the rupture and disorder inside, accelerating apoptosis[12,13]. Therefore, it is a priority to ensure the sufficient oxygen supply to the heart in time during treatment.

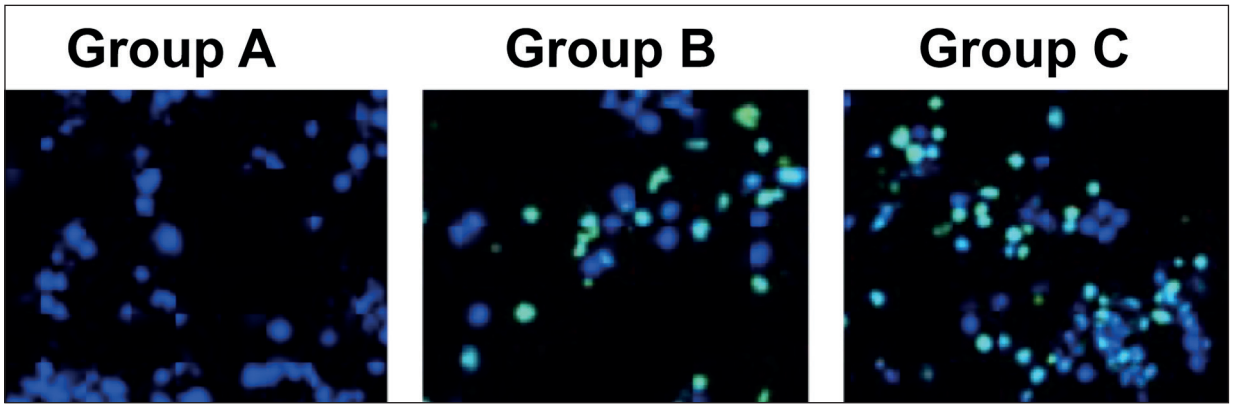

Figure 3. Myocardial apoptosis in the three groups (magnification: 100×).

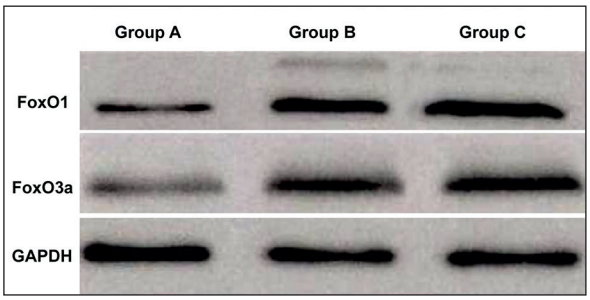

Figure 4. FoxO1 and FoxO3a protein expressions in myocardial cells in the three groups.

In this paper, the detection results of ATP concentration in myocardial cells showed that the ATP concentration in myocardial cells was the highest in group A ($p<0.05$), and it was higher in group B than that in group C ($p<0.05$). The results of CCK-8 assay showed that the proliferation rate of myocardial cells was the highest in group A and the lowest in group C ($p<0.05$), and it was significantly increased in group B, compared with that in group C ($p<0.05$). The results of the TUNEL assay revealed that the myocardial cells were arranged orderly and the number of apoptotic cells was smaller in group A, the number of apoptotic cells was significantly larger in group B than that in group A, and it was the largest in group C ($p<0.05$). The expression of lncRNA is important in the human body, and there are many downstream members, including lncRNA MEG3. LncRNA MEG3 is able to inhibit cancer and plays a role in the form of RNA in essence[14-16]. It was found in previous studies that lncRNA MEG3 is significantly expressed in brain and kidney diseases, and some investigations[17,18] have also shown that lncRNA MEG3 can be involved in the growth and differentiation of myocardial cells and activate the activity of FoxO1 in myocardial cells in hypoxic conditions, thereby damaging the myocardial cells. Moreover, lncRNA MEG3 plays an important role in hypoxic myocardial cells, reduces the regeneration and antioxidant ability of myocardial cells, and damages the myocardial cells, which is similar to the results in this paper.

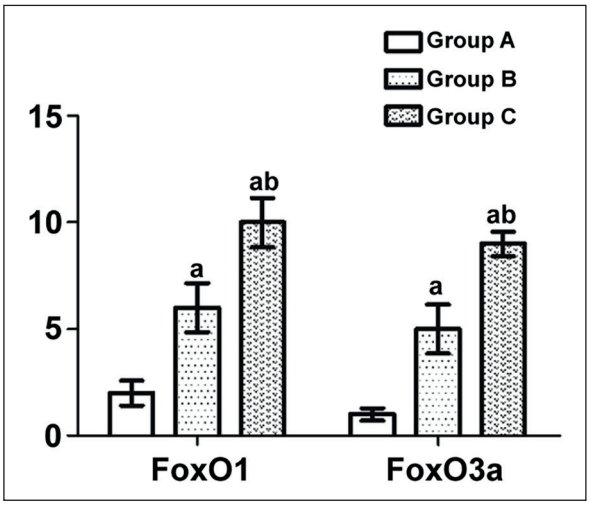

Figure 5. FoxO1 and FoxO3a activity in the three groups. Note: a: $p<0.05$ *vs.* group A, b: $p<0.05$ *vs.* group B.

In the present study, the results of Western blotting manifested that the concentrations of FoxO1 and FoxO3a proteins in myocardial cells were the lowest in group A ($p<0.05$), and they were significantly higher in group C than those in group B ($p<0.05$). According to the results of qRT-PCR, the mRNA expressions of FoxO1 and FoxO3 in myocardial cells were the lowest in group A ($p<0.05$), and they were remarkably lower in group B than those in group C ($p<0.05$). The Fox transcription factor family includes such downstream members as FoxO1 and FoxO3a, which play a role in body growth and development, and their characteristics affect cell growth, differentiation, and apoptosis[19,20]. FoxO1, a transcriptional regulator widely distributed in multiple tissues *in vivo*, is mainly involved in the transcription process of the target protein in the cells[21,22], and its activation in the form of phosphorylation is mainly regulated by a variety of factors. The phosphorylated FoxO1 transfers from the nucleus to the cytoplasm and promotes the myocardial apoptosis under hypoxic conditions. FoxO3a, an important nuclear transcription factor in the FoxO family, is active in various cells, tissues and organs, whose role is like that of FoxO1 in myocardial cells in hypoxic environment[23]. However, studies have revealed that the expressions of FoxO1 and FoxO3a are affected by lncRNA MEG3 during myocardial hypoxia, and their binding will enhance the autophagy and aggravate the damage in myocardial cells. In addition, the enhanced activity of FoxO1 and FoxO3a can be observed in hypoxic myocardial cells, and their activity is further strengthened after that hypoxic myocardial cells are transfected with lncRNA MEG3[24-26]. The changes in FoxO1 activity will affect cellular stability. For example, the activation of FoxO1 can lead to cell disorder and facilitate apoptosis of hypoxic myocardial cells, which is similar to the results in this paper.

Conclusions

We demonstrated that lncRNA MEG3 can increase the activity of FoxO1 to promote myocardial apoptosis in a hypoxic environment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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