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NORAD regulates proliferation and apoptosis in cardiomyocytes under high-glucose treatment through miRNA-150-5p/ZEB1 axis

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Abstract. – OBJECTIVE: The purpose of this study was to uncover the potential role of non-coding RNA activated by DNA damage (lncRNA NORAD) in the disease progression of diabetic cardiomyopathy (DCM) and the underlying mechanism.

MATERIALS AND METHODS: Cell viability, 5-Ethynyl-2'-deoxyuridine (EdU)-positive ratio and apoptotic rate in human cardiomyocyte cell line AC16 undergoing treatment of normal-level (NG) or high-level glucose (HG) were assessed at first. NORAD level in HG-induced AC16 cells at different time points was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Subsequently, cell viability, EdU-positive ratio, and apoptotic rate in HG-induced AC16 cells overexpressing NORAD were evaluated. Next, subcellular distribution of NORAD was examined, and Dual-Luciferase reporter gene assay was performed to clarify the interaction among NORAD, miRNA-150-5p, and ZEB1. At last, rescue experiments were conducted to clarify the role of NORAD/miRNA-150-5p/ZEB1 axis in influencing the proliferation and apoptosis in HG-induced AC16 cells.

RESULTS: Results revealed that HG treatment suppressed the proliferative ability and stimulated apoptosis in AC16 cells. Besides, NORAD was time-dependently downregulated in HG-induced AC16 cells, and it was mainly distributed in cytoplasm. In addition, the overexpression of NORAD enhanced proliferative ability, attenuated apoptosis, and increased Bcl-2/Bax ratio in HG-induced AC16 cells. Finally, NORAD/miRNA-150-5p/ZEB1 axis was verified to protect the malignant progression of DCM.

CONCLUSIONS: NORAD is upregulated under high-level glucose treatment. Overexpression of NORAD protects DCM development via miRNA-150-5p/ZEB1 axis.

Key Words:
Diabetic cardiomyopathy (DCM), NORAD, MiRNA-150-5p, ZEB1.

Introduction

Diabetic cardiomyopathy (DCM) is the pathological condition of abnormal heart structure and cardiac functions in DM patients without other cardiac risk factors, such as history of coronary heart disease, hypertension, and significant valvular disease. Clinically, DCM is characterized by diastolic dysfunction, while ejection fraction remains unchanged. The hallmarks of DCM-induced structural changes are interstitial and peripheral vascular fibrosis and left ventricle hypertrophy[1]. Currently, it is believed that oxidative stress[2], inflammation[3], changes in metabolism, and energy production[4] are the major pathophysiological mechanisms of DCM.

Long non-coding RNAs (lncRNAs) are non-coding RNAs with over 200 nucleotides long, which lack the ability to encode proteins and are involved in various disease progressions[5]. With the development of microarray analyses and sequencing technologies, accumulating lncRNAs involved in DCM have been identified[6-8]. These lncRNAs are important mediators in DCM, serving as potential diagnostic and therapeutic targets.

This paper first reported that NORAD was downregulated in HG-induced cardiomyocytes. Besides, its potential regulatory effects on DCM and the molecular mechanisms were further analyzed.

Materials and Methods

Cell Culture and Transfection

Human cardiomyocyte cell line AC16 was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD,

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USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Then, they were treated with NG (5.5 mmol/L glucose) or HG (30 mmol/L glucose) for appointed time periods.

The cells grew to 60% confluence and were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 24 h after transfection, the cells were subjected to NG or HG treatment.

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

The cells were inoculated in a 96-well plate with 1×10^3 cells per well, labeled with EdU solution (RiboBio, Guangzhou, China) in the dark for 30 min, and stained with Hoechst 33342 for another 30 min. Ultimately, images of EdU-labeled cells, Hoechst-labeled nuclei, and the merged one were taken under a fluorescence microscopy (Olympus, Tokyo, Japan).

Apoptosis Determination

The cells were washed in pre-cold phosphate-buffered saline (PBS) twice, re-suspended in 100 μL of binding buffer and dyed in 10 μL of Annexin V-FITC and 5 μL of PI at 4°C, in the dark for 10 min. Apoptosis was determined using flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA, which was quantified using a spectrometer. Then, Nuclear and cytoplasmic RNAs were isolated using the NE-PER kit (Thermo Fisher Scientific, Waltham, MA, USA). Next, RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan), and SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) was utilized for qRT-PCR. With glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6 as the internal reference, the relative level was calculated using $2^{-\Delta\Delta Ct}$ method. NORAD: F: 5'-TGATAGGATACATCTTGGACATGGA-3', R: 5'-AACCTAATGAACAAGTCCTGACATACA-3'; ZEB1: F: 5'-GATGATGAATGCGAGTCAGATGC-3', R: 5'-ACAGCAGTGTCTTGTTGTTGT-3'.

Cell Counting Kit-8 (CCK-8) Assay

Cells were inoculated into a 96-well plate with 5×10^3 cells per well. At the appointed time points, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added in each well. Finally, the absorbance at 450 nm of each sample was recorded.

Dual-Luciferase Reporter Gene Assay

The cells were inoculated in 24-well plates and co-transfected with wild-type/mutant-type vectors and NC/miRNA-150-5p mimics for 24 h. Subsequently, cells were lysed, and the supernatant was collected for measuring the relative Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 6.0 statistical software (La Jolla, CA, USA) was used for data analysis. All data were expressed as mean ± SD (standard deviation). The paired two-tailed *t*-test was used for comparing differences between two groups. $p<0.05$ was considered to be statistically significant.

Results

NORAD was Downregulated in Cardiomyocytes Undergoing High-Level Glucose Treatment

AC16 cells were treated with NG (5.5 mmol/L glucose) or HG (30 mmol/L glucose) for 24, 48, and 72 h, respectively. It was shown that cell viability (Figure 1A) and EdU-positive ratio (Figure 1B) time-dependently decreased in the HG group. Meanwhile, the apoptotic rate was time-dependently elevated (Figure 1C). In HG-induced AC16 cells, NORAD level was time-dependently downregulated (Figure 1D).

Overexpression of NORAD Enhanced Proliferative Ability and Suppressed Apoptosis in HG-Induced Cardiomyocytes

Transfection of pcDNA3.1-NORAD effectively upregulated NORAD in HG-induced AC16 cells (Figure 2A). Compared with those of controls, overexpression of NORAD remarkably increased viability (Figure 2B) and EdU-positive ratio (Figure 2C), suggesting the promotive effect of NORAD on cardiomyocyte proliferation. Besides, transfection of pcDNA3.1-NORAD reduced apoptotic rate (Figure 2D) and elevated Bcl-2/Bax ratio (Figure 2E) in HG-induced AC16 cells. In addition, NORAD was confirmed to suppress apoptosis in HG-induced cardiomyocytes.

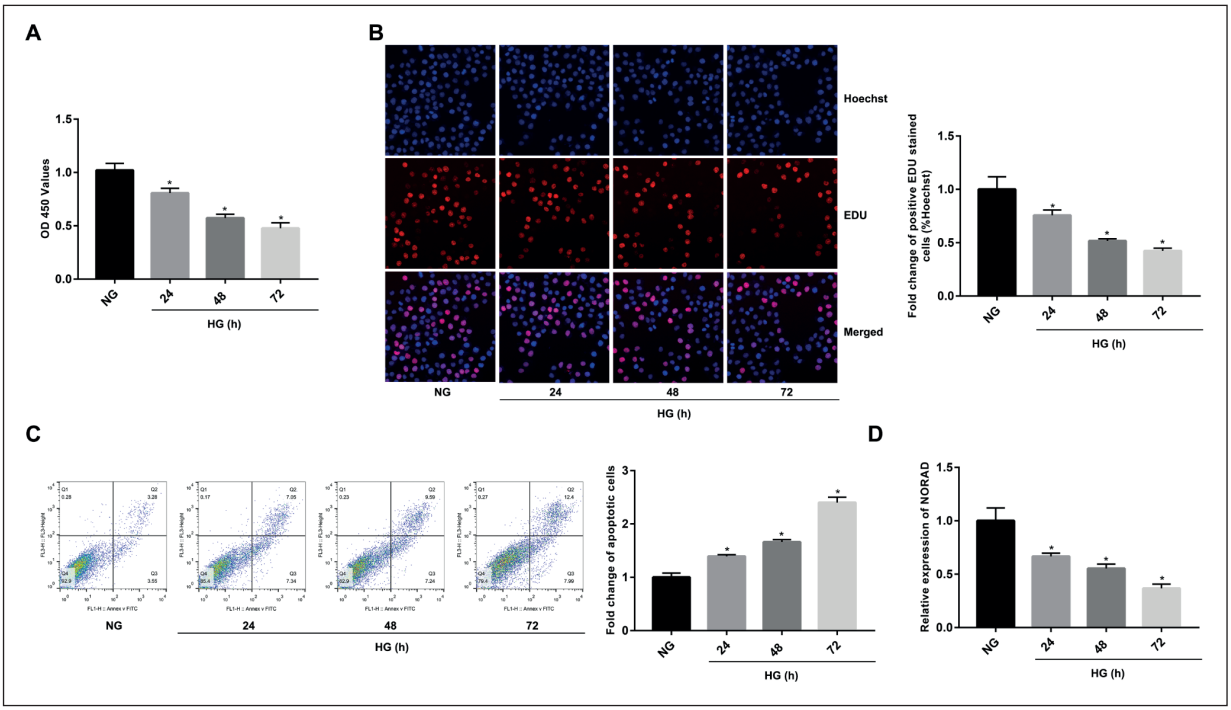

Figure 1. NORAD is downregulated in cardiomyocytes undergoing high-level glucose treatment. **A**, Viability in NG-induced or HG-induced AC16 cells for 24, 48 or 72 h. **B**, EdU-positive ratio in NG-induced or HG-induced AC16 cells for 24, 48 or 72 h (magnification: 400×). **C**, Apoptotic rate in NG-induced or HG-induced AC16 cells for 24, 48 or 72 h. **D**, NORAD level in NG-induced or HG-induced AC16 cells for 24, 48 or 72 h. NG: Normal-level glucose treatment (5.5 mmol/L glucose), HG: High-level glucose treatment (30 mmol/L glucose).

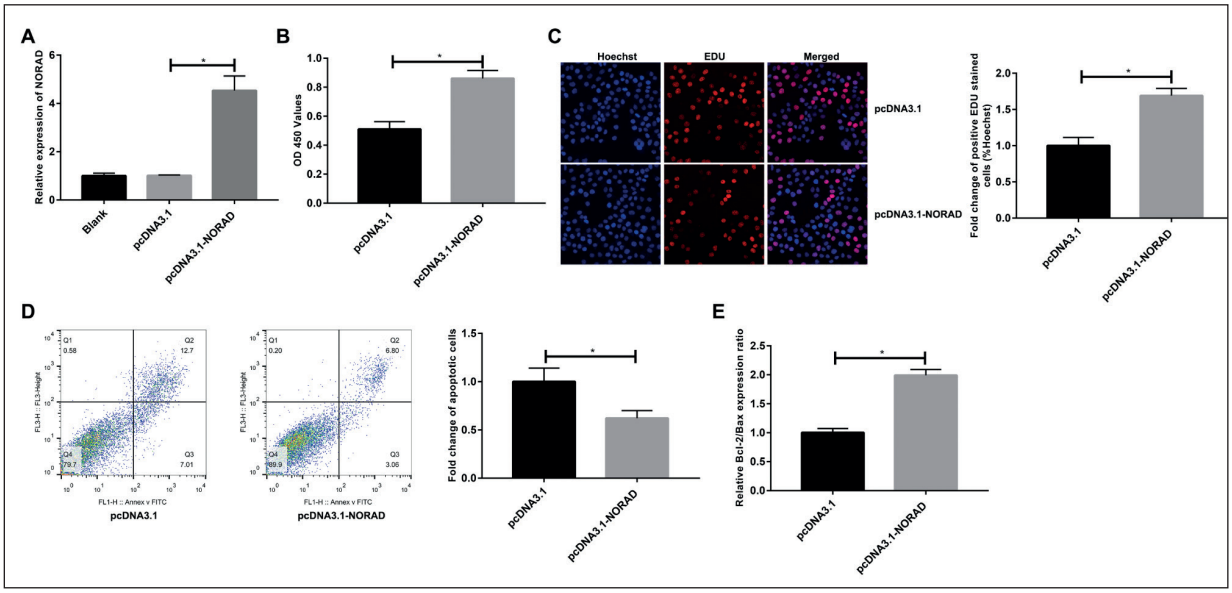

Figure 2. Overexpression of NORAD enhances proliferative ability and suppresses apoptosis in HG-induced cardiomyocytes. **A**, Transfection efficacy of pcDNA3.1-NORAD in HG-induced AC16 cells. **B**, Viability in HG-induced AC16 cells transfected with pcDNA3.1 or pcDNA3.1-NORAD. **C**, EdU-positive ratio in HG-induced AC16 cells transfected with pcDNA3.1 or pcDNA3.1-NORAD (magnification: 400×). **D**, Apoptotic rate in HG-induced AC16 cells transfected with pcDNA3.1 or pcDNA3.1-NORAD. **E**, Bcl-2/Bax ratio in HG-induced AC16 cells transfected with pcDNA3.1 or pcDNA3.1-NORAD.

NORAD Directly Bound MiRNA-150-5p

The ceRNA theory proposes that lncRNA sponges target miRNAs to further regulate downstream genes. Hence, subcellular distribution of a lncRNA is of significance. The findings of this study uncovered that NORAD was mainly distributed in cytoplasm, suggesting its potential post-transcriptional regulatory role (Figure 3A). Through prediction on StarBase (http://starbase.sysu.edu.cn/index.php), the binding sequences in 3'UTR of NORAD and miRNA-150-5p were depicted (Figure 3B). Decreased Luciferase activity after co-transfection of NORAD-WT and miRNA-150-5p mimics proved the binding between NORAD and miRNA-150-5p (Figure 3C). In addition, miRNA-150-5p level was reduced after transfection of pcDNA3.1-NORAD (Figure 3D). In a similar way, ZEB1 was indicated to be the downstream gene binding miRNA-150-5p (Figure 3E, 3F). With the prolongation of HG treatment, ZEB1 was gradually downregulated in AC16 cells (Figure 4A).

Overexpression of MiRNA-150-5p Partially Reversed the Promotive effect of NORAD on Cardiomyocyte Proliferation

Of note, the increased EdU-positive ratio (Figure 4B) and viability (Figure 4C) in HG-induced AC16 cells overexpressing NORAD were partially reversed by co-overexpression of miRNA-150-5p. Besides, the overexpression of NORAD reduced apoptotic rate in HG-induced AC16 cells, and the decreased trend was reversed by overexpression of miRNA-150-5p (Figure 4D).

Discussion

DCM is a chronic disease that is popular in the world, posing a great burden on medical system. It is also a risk factor for cardiovascular diseases[9]. Currently, specific target strategies for DCM treatment are lacked. Standard palliative heart failure intervention is applied for advanced DCM as an assistant method. So far, gene therapy and non-cod-

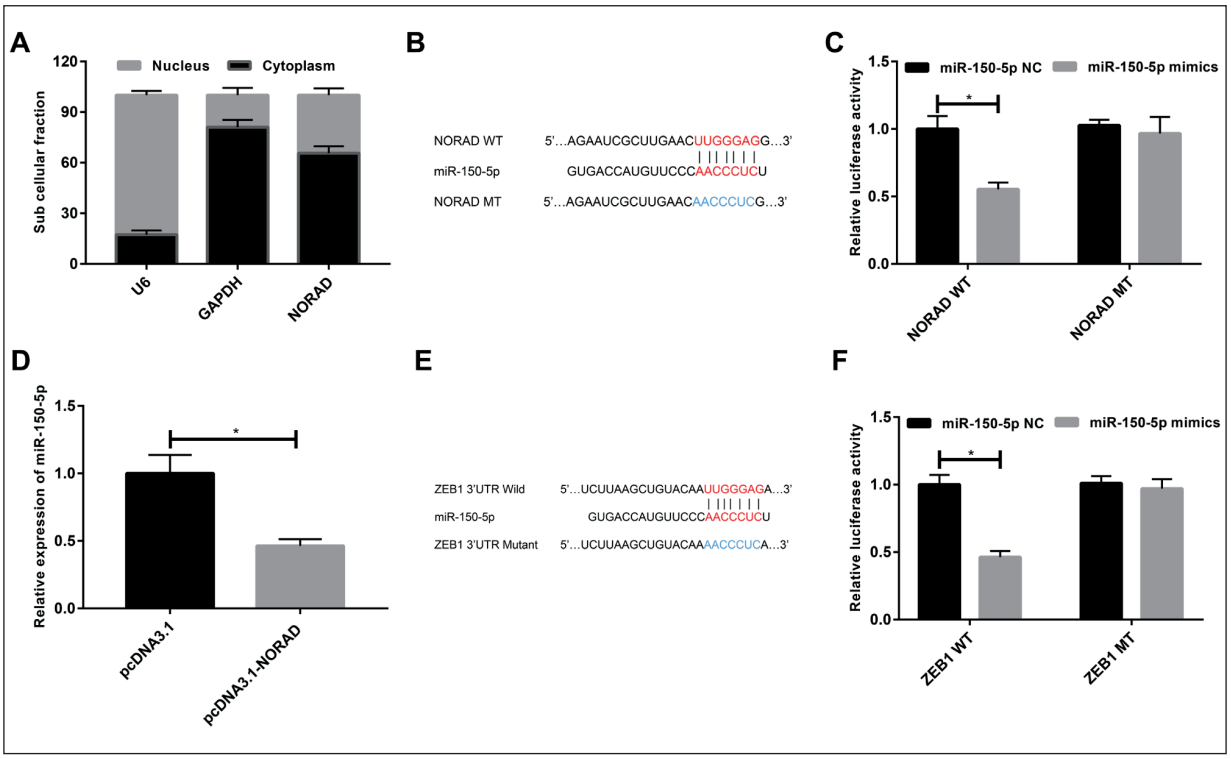

Figure 3. NORAD directly binds to miRNA-150-5p. **A**, Subcellular distribution of NORAD in AC16 cells. **B**, Binding sequences in 3'UTR of NORAD and miRNA-150-5p. **C**, Luciferase activity in AC16 cells co-transfected with NORAD-WT/NORAD-MUT and miRNA-150-5p mimics/NC. **D**, MiRNA-150-5p level in HG-induced AC16 cells transfected with pcDNA3.1 or pcDNA3.1-NORAD. **E**, Binding sequences in 3'UTR of miRNA-150-5p and ZEB1. **F**, Luciferase activity in AC16 cells co-transfected with ZEB1-WT/ZEB1-MUT and miRNA-150-5p mimics/NC.

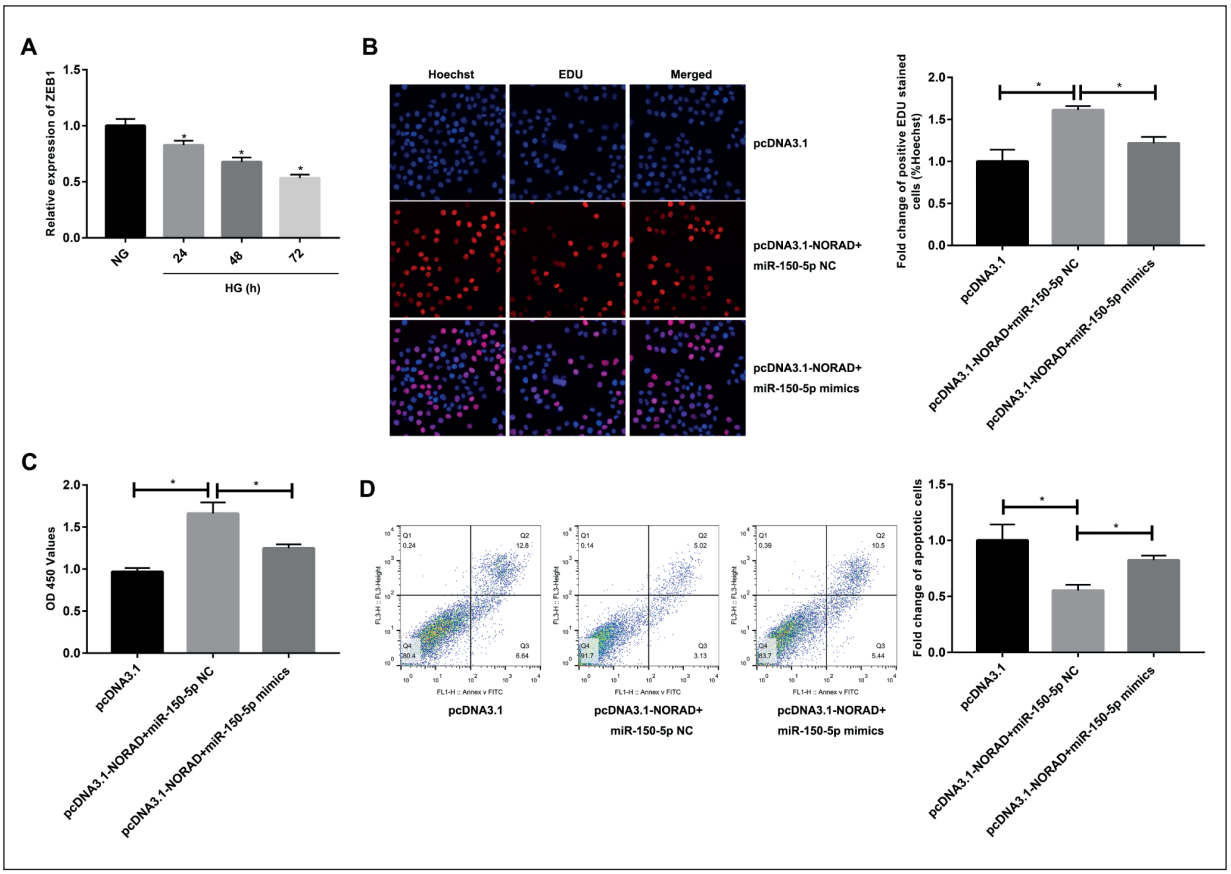

Figure 4. Overexpression of miRNA-150-5p partially reverses the promotive effect of NORAD on cardiomyocyte proliferation. ***A***, ZEB1 level in NG-induced or HG-induced AC16 cells for 24, 48 or 72 h. ***B***, EdU-positive ratio in HG-induced AC16 cells transfected with pcDNA3.1, pcDNA3.1-NORAD+NC, or pcDNA3.1-NORAD+miRNA-150-5p mimics (magnification: 400×). ***C***, Viability in HG-induced AC16 cells transfected with pcDNA3.1, pcDNA3.1-NORAD+NC, or pcDNA3.1-NORAD+miRNA-150-5p mimics. ***D***, Apoptotic rate in HG-induced AC16 cells transfected with pcDNA3.1, pcDNA3.1-NORAD+NC, or pcDNA3.1-NORAD+miRNA-150-5p mimics.

ing RNA intervention in DCM models have shown a promising application, which are still required to be further validated in clinical trials[10,11].

LncRNAs are a type of novel regulators in epigenetics. They are responsible for modifying DNAs and regulating histones and chromatin remodeling[12]. LncRNA Kcnq1ot1 is reported to be upregulated in HG-induced cardiomyocytes and cardiac tissues of DM mice. Knockdown of Kcnq1ot1 alleviates apoptosis and cell death, and improves cytoskeletal structure abnormalities and calcium overload, thus improving cardiac function and morphology[13,14]. As a result, Kcnq1ot1 may be a new target for clinical treatment of DCM. HOTAIR level is markedly declined in myocardial tissues and serum of DCM patients. Furthermore, ROC curves demonstrated the diagnostic value of HOTAIR in DCM. The overexpression of HOTAIR in AC16 cells greatly enhances cell viability and stimulates Akt phosphorylation, which are reversed by applying the PI3K/Akt inhibitor. It is suggested[15-17] that HOTAIR enhances cardiomyocyte viability by activating the PI3K/Akt pathway, thereafter improving DCM. Analyses in this study manifested that NORAD was markedly downregulated in HG-induced AC16 cells, and its overexpression in HG-induced AC16 cells promoted the proliferative ability. Bcl-2/Bax ratio determines caspase activation and thus the initiation of cell apoptosis[18]. Here, the overexpression of NORAD attenuated apoptosis, and decreased Bcl-2/Bax ratio.

ZEB protein family includes ZEB1 and ZEB2. Structurally, both ZEB1 and ZEB2 contain C2H2 zinc finger at N-terminal and C-terminal, and Smad domain in their middle[19]. ZEB1 is of significance during tumor progression and EMT[20-22]. Zhou et al[23] pointed out that miR-200c suppresses TGF-β-induced EMT *via* targeting ZEB1 and

ZEB2, thereafter improving Trastuzumab-sensitivity in gastric cancer. Yuan et al[24] showed that lncRNA CAT104 promotes gastric cancer to proliferate and metastasize through miR-381/ZEB1 axis. In this paper, ZEB1 was time-dependently downregulated in HG-induced AC16 cells, and it was identified to be the downstream gene of miRNA-150-5p. Of note, the findings of this study uncovered that NORAD/miRNA-150-5p/ZEB1 axis protected the malignant progression of DCM.

Conclusions

Shortly, the above mentioned results disclosed that NORAD is upregulated under the high-level glucose treatment. Overexpression of NORAD protects DCM development *via* miRNA-150-5p/ZEB1 axis, so NORAD can be a promising target in clinical prevention and treatment of DCM.

Conflict of Interests

The authors declare that they have no conflict of interest.

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