Inhibition of long non-coding RNA metastasis-associated lung adenocarcinoma transcript 1 attenuates high glucose-induced cardiomyocyte apoptosis via regulation of miR-181a-5p

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Abstract: Diabetic cardiomyopathy (DCM) is one of the cardiovascular complications of diabetes mellitus independent of hypertension, coronary disease, and other heart diseases. The development of DCM is multifactorial and hard to detect at an early stage. Long non-coding RNA metastasis-associated lung adenocarcinoma transcript 1 (Malat1) is emerging as a regulator of DCM, the underlying mechanism of its role in DCM has not been elaborated yet. In this study, we established a mouse DCM model via streptozocin injection as evidenced by cell hypertrophy and cell apoptosis of myocardial tissue, and found that Malat1 expression was upregulated in the myocardium in DCM mice. Meanwhile, elevated expression of pro-apoptotic factors p53, p21, cleaved caspase 3, cleaved caspase 9 and BAX, and down-regulation of anti-apoptotic BCL-2 were observed in DCM myocardium. We further investigated the effect of Malat1 on cardiomyocytes under high glucose condition by silencing Malat1 with its specific short-hairpin RNA. Like in vivo, expression of Malat1 in cardiomyocytes was notably raised, remarkable cell apoptosis and changes in apoptosis-related factors were also observed following high glucose treatment. Besides, we validated that Malat1 acted as a sponge of miR-181a-5p. Inhibition of miR-181a-5p could, at least partially, abolish Malat1 knockdown-induced alteration in cardiomyocytes. In addition, p53, a critical regulator of apoptosis, was validated to be a downstream target of miR-181a-5p. In summary, our findings reveal that Malat1 knockdown attenuates high glucose-induced cardiomyocyte apoptosis via releasing miR-181a-5p, and this mechanism may provide us with new diagnosis target of DCM.

Key words: cardiomyocyte apoptosis, diabetic cardiomyopathy, metastasis-associated lung adenocarcinoma transcript 1, miR-181a-5p, p53
Introduction

Diabetes mellitus (DM) is a metabolic disease featured by a high blood glucose level over a long period. DM is one of the largest global health emergencies of the 21st century owing to the extension of average life and increased obesity rate [5]. The prevalence of DM-related heart disease is in rapid and becomes a serious public health risk. The cardiovascular complications are the leading cause of diabetic patients, and studies show that the rate of heart failure is five times higher in diabetic women and two times higher in diabetic men [15, 36]. There are three major diabetic heart diseases: coronary artery disease (CAD), cardiac autonomic neuropathy (CAN), and diabetic cardiomyopathy (DCM) [29]. The terminology “diabetic cardiomyopathy” was first distinctly proposed in 1972 when heart failure was found in four diabetic patients [32]. DCM is specific cardiomyopathy in diabetic patients which is independent of coronary disease, hypertension, alcohol, and other structural heart diseases. It is characterized by left ventricular hypertrophy, interstitial fibrosis, cardiac microangiopathy and contractile dysfunction [25]. However, as the occurrence of DCM is multifactorial, there is no efficient and specific method for DCM diagnosis. Besides, DCM may remain asymptomatic for many years and accompany with other complications including obesity, hypertension, and vasculopathy, which makes the diagnosis of DCM even harder [19, 23]. Accordingly, exploring the underlying mechanism is of utmost importance for the diagnosis and treatment of DCM.

Long noncoding RNAs (lncRNAs) are defined as non-protein coding transcripts longer than 200 bp [13]. LncRNA metastasis-associated lung adenocarcinoma transcript 1 (Malat1), also known as noncoding nuclear-enriched abundant transcript 2 (Neat2), Linc00047, Ncnr00047 and Hcn, is one of the first identified and most comprehensively studied lncRNAs [12]. Malat1 was first discovered in non-small cell lung cancer and highly conserved among 33 mammalian species [14]. Since then, accumulating studies revealed its pivotal role in a variety of physiological processes. Malat1 is involved in molecular modification such as alternative splicing, transcriptional regulation and post-transcriptional regulation [20, 35, 37]. Malat1 is also implicated in various pathological processes including cancer development and progression, neurological disorders and, particularly, diabetes mellitus-related complications [2, 11, 30]. The expression of Malat1 is significantly up-regulated in rats with DM compared to control. Cardiomyocyte apoptosis is remarkably reduced after Malat1 knockdown and left ventricular function is consequently improved in diabetic rats [39].

MicroRNAs (miRNAs) are another group of endogenous non-coding RNA that usually containing approximately 22 nucleotides [1]. MiRNAs participate in multiple pathways and processes owing to its low complementarity requirement between the sequences of miRNAs and their targets. MicroRNA miR-181a-5p was first found to be involved in carcinogenesis, and aberrant miR-181a-5p expression was responsible for abnormal cellular functions in cancers [18, 33]. A recent study revealed that miR-181a-5p was significantly down-regulated in diabetic patients, diabetic rats, and high-glucose treated cardiomyocytes and that p53 and p21 acted as its target genes [31]. Besides, lncRNA could specifically bind to miR-181a-5p predicted on Starbase (http://starbase.sysu.edu.cn/index.php), which implies that Malat1 may serve as a sponge of miR-181a-5p. Thus we hypothesize that Malat1 is involved in DCM by regulating miR-181a-5p.

In this study, we examined the expression of Malat1 and miR-181a-5p in mice with DCM. Then we investigated the effect of MLAT1 and miR-181a-5p on cardiomyocyte apoptosis. Furthermore, we validated the correlation between Malat1 and miR-181a-5p.

Materials and Methods

Animal model establishment

Healthy C57BL/6J mice, 6–8 weeks old, were purchased from HKF (Shanghai, China). Mice were randomly divided into four groups: Control-4 weeks, DCM-4 weeks, Control-8 weeks, and DCM-8 weeks (n=6/group). Mice were intraperitoneally injected with 100 mg/kg streptozocin (STZ) (Aladdin, Shanghai, China) or the same volume normal saline (Dubang, Shenzhen, China) once a day for two days. A blood glucose level higher than 300 mg/dl demonstrated that the mouse diabetic model was successfully established. Mice were euthanized at week 4 and 8 following the STZ injection, and myocardial tissues were ultra-cryopreserved for subsequent detection. All animal experiments were carried out following the guideline for the care and use of laboratory animals and approved by Mudanjiang Medical College.

Cardiomyocyte isolation and viral infection

Cardiomyocytes were isolated as previously described [24]. In short, the heart of C57BL/6J mouse was carefully removed from thorax under a sterile environment and washed with PBS twice. Cardiac tissue was minced using curved scissors and digested with digestion buffer containing 1 mg/ml collagenase II, 1 mg/ml albumin and

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25 μM CaCl₂. Digested tissues were then filtered with 140 μm screen mesh, cardiomyocytes were then cultured with serum-free Dulbecco’s modified Eagle medium (DMEM) containing 0.2% albumin, 2 mM L-carnitine, 5 mM creatine, 5mM taurine, 0.1 μM insulin, 0.1 mM triiodothyronine and 10 mM 2,3-butanedione monoxime in a humidified incubator with 5% CO₂ at 37°C. The Malat1 short hairpin RNA (shMalat1) was inserted into lentivirus shuttle plasmid Tet-pLKO-puro between AgeI and EcoRI. Anti-miR-181a-5p sequence was also inserted into Tet-pLKO-puro. In order to investigate the effect of Malat1 silencing on cardiomyocyte apoptosis, cardiomyocytes were infected with LV-shMalat1 (virus titer = 1 × 10⁸ Tu/ml) and treated with 30 mM glucose for 48 h after infection. To further investigate the effect of miR-181a-5p inhibition on cardiomyocyte apoptosis, cardiomyocytes were co-infected with LV-shMalat1 and LV-anti-miR-181a-5p, and treated with 30 mM glucose for 48 h after infection. Sequences used in this study were as follow:

Malat1 shRNA: 5′-ccggcccGAATTCTGAAGCTCGACTTCGACTTTTT-3′ (sense); NC shRNA: 5′-ccggcccTTTGATGTAGCTTCACGCACGTTTACGCAGAATTTTT-3′ (sense).

Quantitative real-time PCR

Total RNAs of myocardial tissues and cardiomyocytes were extracted and reversely transcribed into cDNA using M-MLV reverse transcriptase (Tiangen, Beijing, China) following the manufacturer’s manual. Quantitative real-time PCR was performed using SYBR Green mix (Solarbio, Beijing, China) and data were analyzed using the 2⁻∆∆CT method. U6 snRNA was used as an internal control for miR-181a-5p and Gapdh was used as a control for Malat1 and p53. Stem-loop RT primers for miRNAs and real-time PCR primers used in this study were as follows:

Mmu-miR-181a-5p specific stem-loop primer: 5′-GGTGCGCTCTCGGAGGCTCGAGGTATTCG-CACAGAGCAACACTC-3′; 
U6 snRNA specific stem-loop primer: 5′-GGTGCGCTCTCGGAGGCTCGAGGTATTCG-CACAGAGCAACACTC-3′; 
Mmu-miR-181a-5p Forward: 5′-CGCACTCATCAACGCTGTG-3′; Mmu-miR-181a-5p Reverse: 5′-GGTGCGCTCTCGGAGGCTCGAGGTATTC-C3′; 
U6 snRNA-Forward: 5′-CGCACTCATCAACGCTGTG-3′; U6 snRNA-Reverse: 5′-GGTGCGCTCTCGGAGGCTCGAGGTATTC-3′; 
LncRNA Malat1-Forward: 5′-TTTGGCTGTTTGTGTTAGGT-3′; LncRNA Malat1-Reverse: 5′-ACAGGAGT-GAGGTGTGCTGCTG-3′; 
P53-Forward: 5′-GGGCATGAAAGCGCCACCTA-3′; p53-Reverse: 5′-GGCGATGAAACCAACACGAGAACC-3′; 
GAPDH-Forward: 5′-TGTTCTGACCCCTTCAATGTGCACGCT-3′; GAPDH-Reverse: 5′-CTGGTCCTCAGTG- TAGGATGAGC-3′.

Western blot

Tissues and cells were lysed with RIPA lysis buffer containing 1 mM PMSF (Solarbio) and protein concentration was quantified using a BCA kit (Solarbio). Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). PVDF membranes were then blocked with 5% skim milk (Sangon Biotech, Shanghai, China) and incubated with one of the primary antibodies overnight at 4°C. After three-times PBS washing, PVDF membranes were incubated with the corresponding secondary antibody for 60 min at 37°C. Primary antibodies used in this study were as follows: p53 antibody (1:2,000, Proteintech, Hangzhou, China); p21 antibody (1:1,000, Abcam, Cambridge, UK), cleaved caspase 3 antibody (1:1,000, CST, Framingham, MA, USA), cleaved caspase 9 antibody (1:1,000, CST), BCL-2 antibody (1:2,000, Proteintech), BAX antibody (1:5,000, Proteintech), and GAPDH antibody (1:10,000, Proteintech). HRP-conjugated goat anti-rabbit IgG (1:3,000, Solarbio) and HRP-conjugated goat anti-mouse IgG (1:3,000, Solarbio) were used as secondary antibodies.

Hematoxylin-eosin staining

Mouse myocardial tissues were embedded with paraffin and sliced into 5-μm sections, the paraffin sections were then deparaffinized and stained with hematoxylin and eosin successively. The morphological change of mouse myocardial tissues was photographed under a microscope (×400 magnification).

TdT mediated X-dUTP nicked labeling (TUNEL) assay

Cell apoptosis was detected via TUNEL staining. For mouse myocardial tissues, paraffin sections of 5 μm were deparaffinized and permeated with 0.1% Triton X-100. Afterward, sections were labeled with In Situ Cell Death Detection Kit (Roche, Basel, Switzerland), stained with hematoxylin and washed with gradient ethanol. The apoptosis of myocardial tissues was observed under a microscope (×400 magnification). For mouse cardiomyocytes, cell slides were permeated with 0.1% Triton X-100 and labeled with In Situ Cell Death Detection Kit at 37°C for 1 h. Then cell slides were stained with DAPI.
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(Beyotime, Shanghai, China) for 5 min at room temperature and sealed with anti-fluorescence quenching reagent (Solarbio), typical pictures were captured under a microscope (×200 magnification).

**Dual-luciferase assay**

To investigate the correlation between lncRNA Malat1 and miR-181a-5p, the wildtype and mutant type of Malat1 fragments were inserted into pmirGLO (Promega, Madison, WI, USA) between Nhe I and Sal I, abbreviated as Malat1-WT and Malat1-MUT. 293T cells were co-transfected with Malat1 plasmids and miR-181a-5p/NC mimics. To investigate the regulation of miR-181a-5p on p53, the wildtype and mutant type of p53 3'-UTR were inserted into pmirGLO between Nhe I and Sal I, abbreviated as p53-WT and p53-MUT. 293T cells were co-transfected with p53 3'-UTR and miR-181a-5p/Nonspecific control microRNA mimic, respectively. Cells were lysed and the binding activities were evaluated by firefly luciferase activity/renilla luciferase activity.

**Statistical analysis**

GraphPad Prism 7 was used to perform the data analysis. All results were presented as means ± SD and compared using Student’s *t*-test (two groups) or One-way ANOVA (three or more groups). A *P* value < 0.05 was considered as statistically significant.

**Results**

**Mouse DCM model was successfully established**

By hematoxylin-eosin staining, we found that myocardial fibers in control mice were arranged neatly, and...
showed no cell swelling. Myocardial fibers of mice 4 weeks after STZ injection showed obvious disordered arrangement, cell hypertrophy and hyperchromatic nucleus. These characteristics were more notable at week 8, accompanied by cardiomyocyte necrosis and a large area of light staining (Fig. 1A). Then we detected the apoptosis in myocardial tissues using TUNEL assay. Cardiomyocytes in the control heart were arranged orderly and cell apoptosis was hardly detected. Four weeks post-STZ injection, cardiomyocytes were arranged disorderly, partial apoptotic cells were detected, which was more significant at week 8 (Fig. 1B). These results suggest that we successfully established the mouse diabetic cardiomyopathy model.

The expression of Malat1, miR-181a-5p and apoptosis-related factors in mice with DCM

We analyzed the expression changes of lncRNA Malat1 and miR-181a-5p in mouse myocardial tissues by real-time PCR. Malat1 was up-regulated at week 4 following STZ injection compared to control, which was even more significant at week 8. On the contrary, the RNA level of miR-181a-5p was remarkably decreased at week 4 after STZ treatment and the decrease was exacerbated at week 8 (Fig. 2A). Then we evaluated the expression of apoptosis-related factors by western blot. We found that the protein levels of p53, p21, cleaved caspase 3, cleaved caspase 9 and BAX were remarkably elevated at week 4 post-STZ injection in contrast with control, which were more significant at week 8. On the contrary, the expression of BCL-2 was significantly suppressed at week 8 after STZ injection in contrast with control (Fig. 2B). These results indicate that STZ injection induces cardiomyocyte apoptosis.

Fig. 2. Expression of long noncoding RNA Malat1, miR-181a-5p and apoptosis-related factors in mouse myocardial tissue. (A) The level of lncRNA Malat1 and miR-181a-5p were measured by quantitative real-time PCR. (B) Protein levels of apoptosis-related factors were evaluated by western blot assay. All data were presented as mean ± SD. ns: not significant; *P<0.05; **P<0.01; ***P<0.001.
**Malat1 acts as a sponge of miR-181a-5p**

Mouse cardiomyocyte treated with high glucose (HG) showed significant higher Malat1 expression compared to mannitol group, and the expression of Malat1 was successfully knocked down by shMalat1. Like in vivo, the expression of miR-181a-5p showed the contrary trend to that of Malat1. The level of miR-181a-5p was downregulated following HG treatment but elevated after Malat1 knockdown (Fig. 3A). These results inspired us to investigate the relationship between Malat1 and miR-181a-5p. LncRNA Malat1 could specifically bind to miR-181a-5p predicted on StarBase (http://starbase.sysu.edu.cn/index.php), and we validated their interaction by performing the dual-luciferase assay. The binding site of miR-181a-5p on Malat1 was shown in Fig. 3B.

**Malat1 knockdown abrogates cardiomyocyte apoptosis induced by high glucose**

We investigated the effect of Malat1 on cardiomyocyte apoptosis via TUNEL assay. Obvious cardiomyocyte apoptosis was observed post the high glucose treatment. It was noteworthy that Malat1 silencing abolished the apoptosis induced by high glucose (Fig. 4A). High glucose treatment significantly up-regulated the pro-apoptosis proteins including p53, p21, cleaved caspase 3, cleaved caspase 9 and BAX, yet inhibited the expression of BCL-2. Malat1 silencing could alleviate the expression changes of these proteins induced by high glucose (Fig. 4B). These results indicate that Malat1 silencing can suppress cardiomyocyte apoptosis.

**The effect of miR-181a-5p on cardiomyocyte apoptosis**

Owing to the interaction between Malat1 and miR-181a-5p, we further investigated the effect of miR-181a-5p on cardiomyocyte apoptosis. Like we previously described, Malat1 knockdown abrogated the high glucose-induced cardiomyocyte apoptosis. Then we sup-

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**Fig. 3.** Long noncoding RNA Malat1 acts as a sponge of miR-181a-5p. (A) The levels of lncRNA Malat1 and miR-181a-5p in mouse cardiomyocytes after Malat1 silencing were measured by quantitative real-time PCR. (B) The specific binding site of miR-181a-5p on Malat1 was shown, and the correlation between Malat1 and miR-181a-5p was analyzed by dual-luciferase assay, ns: not significant; *P<0.05; ***P<0.001.
pressed the miR-181a-5p expression with anti-miR-181a-5p viral particles, and apoptotic cells re-appeared (Fig. 5a). Malat1 silencing down-regulated the expression of p53, p21, cleaved caspase 3, cleaved caspase 9 and Bax, but elevated the protein level of BCL-2. All these expression changes were remarkably reversed after miR-181a-5p inhibition (Fig. 5B). These results suggest that miR-181a-5p is involved in Malat1-mediated cardiomyocyte apoptosis.

**P53 is a downstream target of miR-181a-5p**

The binding site of miR-181a-5p and p53 was shown in Fig. 6A. Cells co-transfected with p53-WT and miR-181a-5p mimics showed notably weaker luciferase activity compared to that co-transfected with p53-WT and NC mimics. There was no difference of luciferase activity between cells co-transfected with p53-MUT + NC mimics and that co-transfected with p53-MUT + miR-181a-5p mimics (Fig. 6A). Furthermore, the mRNA level of p53 was remarkably elevated after miR-181a-5p inhibition, demonstrating that p53 is a downstream target of miR-181a-5p.

**Discussion**

Cardiovascular-related complications are responsible for approximately 65% of diabetic death [29]. Among which DCM leads to diastolic dysfunction and further systolic dysfunction, and eventually greatly increases the risk of heart failure [10]. Myocardial fibrosis and
hypertrophy, as well as cardiomyocyte apoptosis, are vital contributors in the onset and progression of DCM [9]. In this study, we established a mouse DCM model via intraperitoneal injection of STZ according to a previous study [34]. Clear hypertrophy of myocardial fibers and myocardial necrosis were detectable at week 8 following STZ injection. TUNEL assay further showed that STZ treatment led to substantial cardiomyocyte apoptosis. Taken all these together, we built an appropriate model for investigations of DCM.

The major pathological characteristics of DM patients include hyperglycemia, hyperlipidemia, and inflammation, among which hyperglycemia is widely believed to be the leading cause of DCM pathogenesis [28]. Sustained hyperglycemia leads to a variety of metabolic changes in cardiomyocytes including oxidative stress through the development of reactive oxygen species (ROS), which could further induce the apoptosis and DNA damage of cardiomyocytes [3, 8]. Hyperglycemia-induced cardiomyocytes apoptosis is regulated by
the mitochondrial apoptotic pathway, as evidenced by the release of cytochrome c and activation of caspase 3 [3]. Furthermore, p53, a well-known tumor suppressor, is involved in the development of oxidative damage-induced cardiomyopathy through regulating p21 [26]. P53 also interacts with p21 to inhibit cell proliferation and induce cell apoptosis via activating BAX [16]. In particular, the expression of p53 is significantly up-regulated in diabetic mice and leads to cardiac dysfunction, whereas cardiac impairment is abolished in p53 knock-out mice [27]. In this study, the elevated expression of p53, p21, cleaved caspase 3, cleaved caspase 9, and pro-apoptotic BAX, and down-regulation of anti-apoptotic BCL-2 were detected in the myocardium of STZ-induced DCM mice, indicating that mitochondrial apoptotic pathway mediated apoptosis occurred in diabetic myocardial tissue. Similar cell apoptosis was also observed in high glucose-treated cardiomyocytes, indicating that hyperglycemia could result in cardiomyocyte apoptosis in vitro.

LncRNAs are involved in various physiological processes via binding to DNA/RNA/protein or changing the localization/affinity of protein [6]. They also regulate gene expression via decoying specific microRNAs. Increasing evidence indicates that abnormal expression of lncRNAs is implicated in cardiovascular complications of diabetes [7]. Malt1, highly conserved among various mammals, is emerging as a regulator of DCM. For instance, Malt1 is notably up-regulated in myocardial tissue of diabetic rats. Knockdown of Malt1 significantly improved the left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD), indicating that Malt1 silencing could abrogate the cardiac impairment of diabetic rats [38]. Besides, cardiomyocyte apoptosis was reduced and cardiac function was improved in diabetic rats after Malt1 knockdown [39]. Our results, in accord with previous studies, demonstrated that Malt1 was up-regulated in DCM mice and high glucose-treated cardiomyocytes. Knockdown of Malt1 via shMalt1 significantly alleviated apoptosis induced by high glucose as evidenced by expression changes of apoptosis-related proteins. MiR-181a-5p was found to be involved in regulating apoptosis of endothelial cells [4]. MiR-181a-5p also promotes invasion of hepatocellular carcinoma by p53 signaling pathway [21]. Our study first validated the interaction between Malt1 and miR-181a-5p, whose expression levels were opposite in both diabetic myocardial tissues and high glucose-treated cardiomyocytes. Inhibition of miR-181a-5p could partially reverse the anti-apoptotic effect of shMalt1. In particular, miR-181a-5p is down-regulated in DCM rats developed by high-fat diet and
low-dose STZ, and decreased miR-181a-5p promotes cell apoptosis via regulating the p53-p21 pathway [31, 40]. Increasing evidence revealed that aberrant microRNA expression is associated with diabetic cardiovascular complications. For instance, cardiomyocyte hypertrophy induced by diabetes showed close relationship with down-regulation of miR-150, miR-133a and miR-30c, among which elevated miR-30c expression could alleviate the cardiomyocyte hypertrophy [22]. MiR-30d, miR-133a, miR-206, and miR-1 are involved in diabetes-induced cardiomyocyte apoptosis as well as mitochondrial dysfunction[22]. For clinical application, a previous clinical study showed that the expression of seven microRNAs is remarkably up-regulated in diabetic patients in contrast with pre-diabetes patients and susceptible individuals [17]. DCM is hard to detect at the early stage of the disease, thus the serum levels of microRNAs could act as a sensitive and efficient indicator for the cardiovascular complication of diabetes [17].

In conclusion, our study demonstrates that Malat1 promotes cardiomyocyte apoptosis by preventing miR-181a-5p from binding to p53. Malat1-miR-181a-5p-p53 might serve as a therapeutic target for DCM.

Conflict of Interest
None.

Acknowledgments
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