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Knockdown of long non-coding RNA LUCAT1 reverses high glucose-induced cardiomyocyte injury *via* targeting CYP11B2

Y. YIN[1,2,3], Z.-F. YANG[4], X.-H. LI[5], L.-Q. ZHOU[5], Y.-J. ZHANG[5], B. YAN[1,2,3]

[1]Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan, China
[2]Cardiovascular Research Institute, Wuhan University, Wuhan, China
[3]Hubei Key Laboratory of Cardiology, Wuhan University, Wuhan, China
[4]Department of Pathology, Affiliated People's Hospital of Inner Mongolia University, Hohhot, China
[5]Department of Emergency Medicine, First Hospital of Hohhot, Hohhot, China

Ying Yin and *Zhen Fang Yang* contributed equally to this work

Abstract. – OBJECTIVE: Diabetic cardiomyopathy (DCM) is one of the major complications in patients with diabetes mellitus. Recently, long noncoding RNAs (lncRNAs) have been well concerned for their roles in the progression of multiple diseases, including DCM. In this res we aimed to explore the role of lncRNA L in cardiomyocyte injury and apoptosis ind by high glucose (HG) *in vitro*.

MATERIALS AND MATHODS: High glucose duced (HG-induced) AC16 cardio ytes tran fected with LUCAT1 shRNA structed LUCAT1 expression was d ed eal-time quantitative polymerase ch eaction -qPCR). Subsequently, cell prolife and were detected after LU duced AC16 cells. M over, R and Western blot assay we rformed t ore the potential underlyin nism of LU in DCM.

RESULTS: T xp on of LUCA was significantly u gulated G-treated AC16 cardiomyocy Moreover, down of LUCAT1 could r e cardiomyocyt ry and apoptosis th n dow egulating C P11B2.

C LUSIO We first demonstrated that kno yn LUCAT could reverse HG-induced yocyt ry by down-regulating 11B2 findi might offer a new direc- r int he mechanism of DCM development.

K oding RNA, Diabetic cardiomyopathy CM), LUCAT1, CYP11B2.

Introduction

Diabetic cardiomyopathy (DCM) is the most prevalent complication in patients with diabetes

itus worldwi[1]. DCM exacerbates cardiac unction and rgy metabolism disturbance, w is a crucia ntributor to cardiac morbidity a rtali Meanwhile, hyperglycemia has been re to induce cardiomyocyte apoptosis[3]. Therefore, it is urgent to uncover the under- olecular mechanisms of DCM.

N oding RNAs have been identified to exert important functions in many diseases[4]. For example, long non-coding RNA (lncRNA) NEAT1 is upregulated in the development of Huntington's disease[5]. LncRNA HOTAIR facilities the development of Parkinson's disease through LRRK2[6]. Recent studies have demonstrated that non-coding RNA participates in the progression of DCM. For instance, lncRNA H19 inhibits autophagy of DCM *via* down-regulating DIRAS3[7]. Furthermore, lncRNA MIAT acts a sponge of miR-22-3p, further up-regulating DAPK2 in DCM[8].

In this study, knockdown of LUCAT1 reversed high glucose-induced (HG-induced) cardiomyocyte injury *in vitro*. In addition, the possible underlying mechanism of LUCAT1 function in HG-induced cardiomyocyte injury was explored.

Materials and Methods

Cell Culture

Human adult ventricular cardiomyocyte cell line (AC16) was bought from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville,

Corresponding Author: Bo Yang, MD; e-mail: yy18689019355@126.com

MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and penicillin, and maintained in humidified incubator with 5% CO_2 at 37°C.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA in cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted total RNA was reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) according to the instructions of Reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Primers used in this study were as follows: LUCAT1, forward 5′-CCTATCCCTTTCTCTAAGAA-3′ and reverse 5′-ACTTCTGCAAAAACGTGCTG-3′; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5′-CCAAAATCAGATGGGGCAATGCTGG-3′ and reverse 5′-TGATGGCATGGACTGTGGTCATTCA-3′. Thermal cycle was as follows: 30 s at 95°C, 5 s at 95°C for a total of 40 cycles, and 35 s at 60°C.

Cell Transfection

After synthesized, lentiviral small h[illegible]in RNA (shRNA) targeting LUCAT1 was cl[illegible] into pGPH1/Neo vector (GenePharma, Shangh[illegible] China). AC16 cells were then [illegible]ted wit[illegible] LUCAT1 shRNA (sh-LUCA[illegible] vecto[illegible] (sh-ctrl) according to relev[illegible]nstruc[illegible]s.

Cell Counting kit-8 (CCK-8), Lactate Dehydrogenase (LDH) Assay

Transfected ce[illegible]re first seed[illegible]o 96-well plates. Cell vi[illegible] LDH relea[illegible]were determined acc[illegible]ng to [illegible]structions of CCK-8 assay (D[illegible]o Laborato[illegible]Kumamoto, Japan) and LD[illegible]ssay (Nanjing Jia[illegible]ng Bioengineering I[illegible]ute, N[illegible]g, China) at 24, 48, and 72 h, res[illegible]ely. [illegible]rbance at 450 nm was measured by a s[illegible]otomet[illegible]hermo-Fisher Scientific Walth[illegible]MA, [illegible]). Finally, the degree of [illegible] image[illegible]ulated as a percentage.

Cell Apoptosis

[illegible]-APC/7-AAD Apoptosis Detection [illegible]t II (KeyGEN Biotech. Co., Ltd, Nanjing, China) [illegible]used to evaluate the apoptosis of transfected ce[illegible]. Briefly, 1×10^6 of cells were collected and washed twice with pre-cooled phosphate-buffered saline (PBS). These cells were then dissolved in 1,000 mL of binding buffer. 100 µL of the solution containing 1×10^5 cells were replaced to a fresh tube with 5 µL 7-AAD 5 µL and Annexin V-APC. After culture at 37°C for 15 minutes in dark, 400 µL binding buffer was added to each tube. Flow cytometry (FACScan, BD Biosciences, [illegible] CA, USA) programmed with CellQ[illegible] software (BD Biosciences, San Diego, CA, [illegible]) was used to discriminate dead, viable, late [illegible]tic cells and early apoptotic. Finally, the perc[illegible]s of cells in different phases w[illegible]sed for com[illegible] between experimental a[illegible]ntrol g[illegible]ups. Th[illegible] was repeated for at le[illegible]ree ti[illegible]

Western Blot Analysis

Transfected [illegible]s were fi[illegible]ch with ice-cold PBS [illegible]d using lys[illegible]fier. Radioimmunop[illegible]pitat[illegible]ssay (RIPA) (Beyotime, Shanghai, China) w[illegible]ilized to extract total prot[illegible]lls. Concent[illegible] of extracted protein w[illegible]etermined by the bicinchoninic acid (BCA) p[illegible]ein assay kit (TaKaRa, Dalian, China). After separation by [illegible]dium dodecyl sulphate-polyacr[illegible]mide gel [illegible]trophoresis (SDS-PAGE), the prot[illegible]were [illegible]sferred onto polyvinylidene difluoride [illegible]) membranes (Millipore, Billerica, MA, USA). Next, the membranes were blocked [illegible]% fat-free milk in Tris-buffered saline and [illegible]0 (TBST) buffer containing Tris-HCl (50 mM), NaCl (150 mM) and Tween 20 (0.05%) at 4°C for 1 hour. Subsequently, the membranes were incubated with primary antibodies at 4°C overnight and corresponding secondary antibodies at room temperature for 2 hours. Abcam (Cambridge, MA, USA) provided us rabbit anti-GAPDH and rabbit anti-CYP11B2, as well as goat anti-rabbit secondary antibody. Enhanced chemiluminescence (ECL) (Millipore, Billerica, MA, USA) was applied for assessment of protein expression.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Student *t*-test was performed to compare the intergroup differences. Data were presented as mean ± SD (standard deviation). $p<0.05$ was considered statistically significant.

Results

Cell Viability and LDH Level in HG-induced AC16 Cell Site

In this study, we first evaluated the cytotoxicity of HG in AC16 cardiomyocytes. Cells were

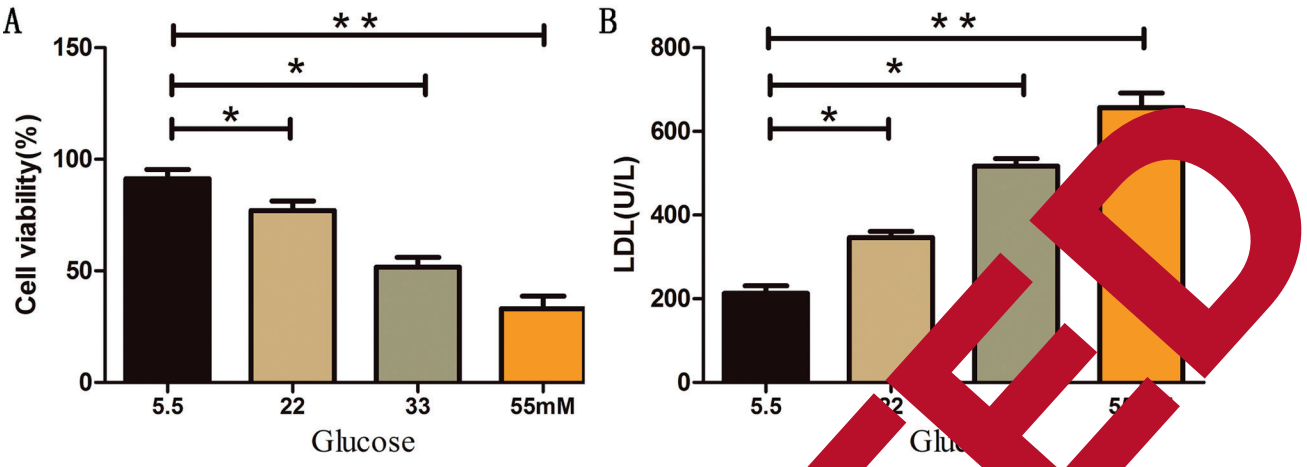

Figure 1. Cytotoxicity of HG in AC16 cardiomyocytes. ***A***, Cell viability was de[illegible] by CCK-8 as[illegible]DH level was determined by LDH assay kit. The results represented the average of three in[illegible]de[illegible]eriments. Data[illegible]re presented as mean ± standard error of the mean. *$p<0.05$.

treated with different doses of glucose (5.5, 22, 33, 55 mM) for 48 hours. Results showed that cell viability significantly decreased in HG (22, 33 and 55 mM) groups when compared with control group (5.5 mM) (Figure 1A). LDH level incr[illegible]d significantly in HG groups compared with[illegible] group (Figure 1B).

Knockdown of LUCAT1 Reversed HG-Induced Inhibition of C[illegible]bility

HG-induced AC16 cells w[illegible]rst[illegible]tructed. The expression of LUCA[illegible] was s[illegible]ficantly higher in HG group tha[illegible]t of c[illegible]n (Figure 2A). To expl[illegible]th[illegible]al fun[illegible]n of LUCAT1, LUCA[illegible]shRNA[illegible]constructed. Transfection effi[illegible]y of LUCA[illegible]hRNA in HG-induced A[illegible]c[illegible]as verified b[illegible]T-qPCR (Figure 2B). R[illegible]sults i[illegible]ted that knockdown of LUCA[illegible]bviously in[illegible]d the viability of HG-ind[illegible]d AC16 cells (F[illegible]e 2C). Furthermore, knockdo[illegible]of LUCAT1 remarkably decre[illegible]the l[illegible]of LDH in HG-induced AC16 cells a[illegible]igure 2

Knockdown of LUCAT1 Reversed HG-Induced Apoptosis

To further explore the interaction between LUCAT1 and HG-induced apoptosis, the apoptosis of treated AC16 cells was detected. Results demonstrated that cell apoptotic rate increased remarkably in HG group when compared with control group (Figure 3A). Moreover, knockdown of LUCAT1 significantly reduced the apoptosis of HG-induced cells (Figure 3B).

Knockdown of LUCAT1 Reversed HG-Induced Injury Via Down-Regulating CYP11B2

Previous results suggested that knockdown of LUCAT1 reversed HG-induced cardiomyocyte injury. Recently, CYP11B2 has been reported to participate in the progression of DCM. In the present study, we discovered that CYP11B2 level was significantly higher in HG group than control group by RT-qPCR (Figure 4A). Western blot assay also showed that the protein expression of CYP11B2 in HG group was remarkably up-regulated when compared with control group (Figure 4B). Meanwhile, CYP11B2 level in HG-induced AC16 cells of LUCAT1 shRNA group was significantly lower when compared with that of control group (Figure 4C). Western blot assay also showed that the protein expression of CYP11B2 in HG-induced AC16 cells was significantly down-regulated in LUCAT1 shRNA group than control group (Figure 4D).

Discussion

DCM is one of the most common complications in diabetes mellitus patients, bringing huge burden to both patients and society. During DCM progression, cardiomyocyte injury and apoptosis further attenuate the disease. Multiple studies have shown that non-coding RNAs participate in the development of DCM through regulating cardiomyocyte injury and apoptosis. For example, lncRNA MALAT1 participates in the pathogene-

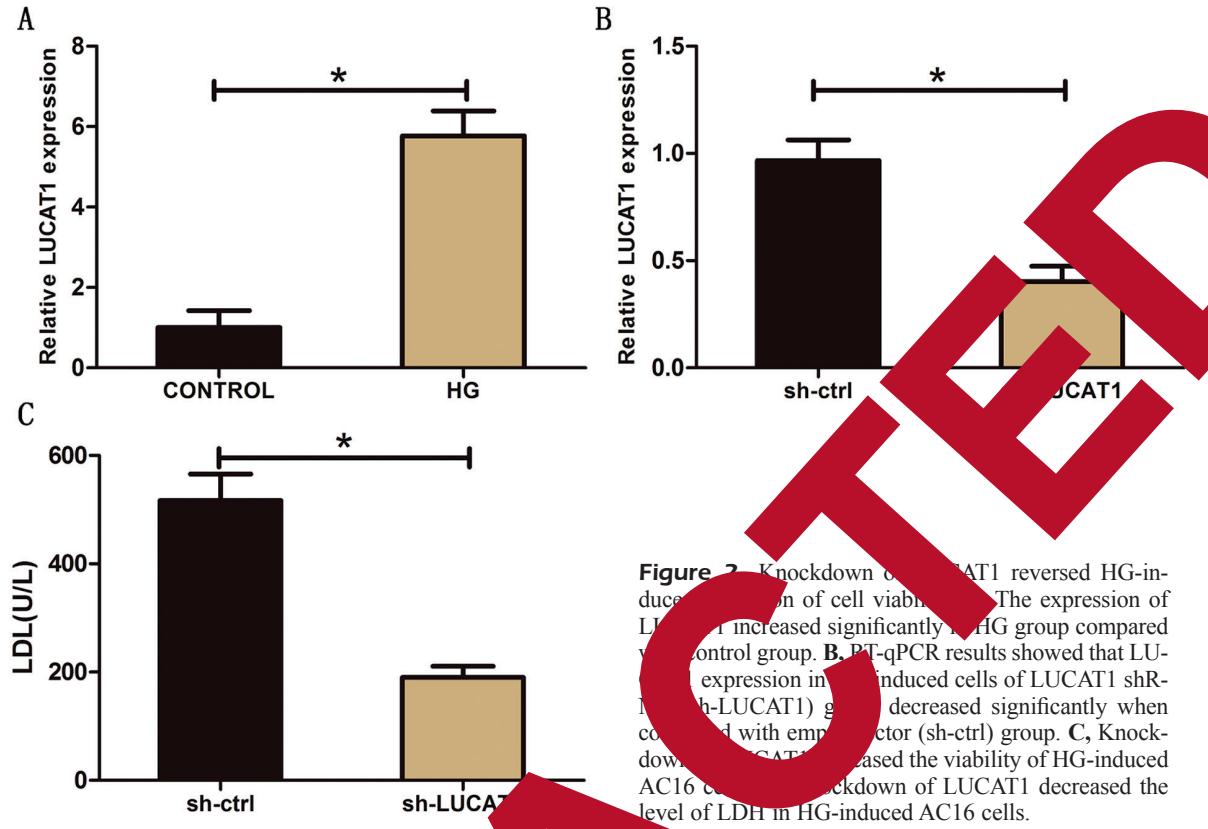

Figure 2. Knockdown of LUCAT1 reversed HG-induced reduction of cell viability. The expression of LUCAT1 increased significantly in HG group compared with control group. **B,** RT-qPCR results showed that LUCAT1 expression in HG-induced cells of LUCAT1 shRNA (sh-LUCAT1) group decreased significantly when compared with empty vector (sh-ctrl) group. **C,** Knockdown of LUCAT1 increased the viability of HG-induced AC16 cells. Knockdown of LUCAT1 decreased the level of LDH in HG-induced AC16 cells.

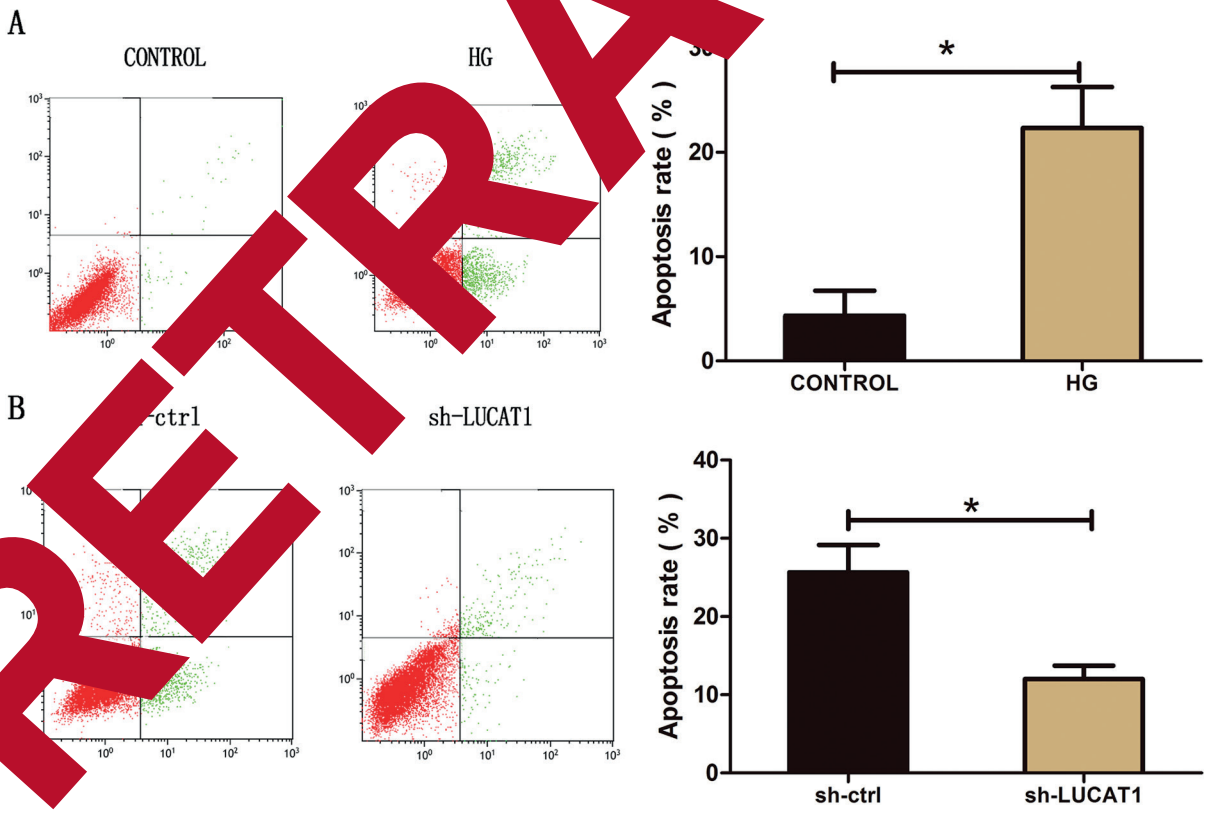

Figure 3. Knockdown of LUCAT1 inhibited HG-induced cardiomyocyte apoptosis. **A,** The apoptotic rate increased remarkably in HG group compared with control group. **B,** Knockdown of LUCAT1 significantly reduced HG-induced apoptosis. *p<0.05.

Figure 4. Interaction between LUCAT1 and CYP11B2 [illegible] duced AC16 cells. **A,** RT-qPCR results showed that CYP11B2 expression increased significantly in H[illegible] compared [illegible] control group. **B,** Western blot assay results showed that the protein expression of CYP11B2 incr[illegible]ntly in H[illegible]up compared with control group. **C,** RT-qPCR results showed that CYP11B2 expression decrea[illegible] LUC[illegible] shRNA (s[illegible]CAT1) group when compared with empty vector (sh-ctrl) group. **D,** Western blot assay re[illegible] showed t[illegible]he protein e[illegible]pression of CYP11B2 decreased remarkably in LUCAT1 shRNA (sh-LUCAT1) group compa[illegible]th em[illegible]) group. The results represented the average of three independent experiments. Data were pr[illegible]ted [illegible]tandard [illegible] of the mean. *$p<0.05$.

sis of DCM[9]. [illegible]cRNA [illegible]nhibits autophagy of DCM by [illegible]n-regulating [illegible]AS3[7]. Meanwhile, lncRN[illegible]AT induces DCM [illegible]sponging miR-22-3[illegible] addi[illegible] up-regulated MG53 induces DC[illegible] acti[illegible]ng peroxisome proliferation-activate[illegible] alpha[illegible]

[illegible]ncR[illegible]UCA[illegible] (lung cancer associated [illegible]ript 1)[illegible]on 5q14.3, has been proved to [illegible] an imp[illegible]ant role in many diseases[11-14]. H[illegible]he role of LUCAT1 in DCM develop[illegible] unknown. In this study, we revealed [illegible]at LUCAT1 was significantly up-regulated in [illegible]treated cardiomyocytes. Meanwhile, it was c[illegible] associated with cardiomyocyte injury and apoptosis. Furthermore, knockdown of LUCAT1 could reverse HG-induced cardiomyocyte injury and apoptosis.

Recent reports have revealed that non-coding RNAs function in cardiac diseases by targeting related genes. Cytochrome P450 family 11 subfamily B member 2 (CYP11B2) has been researched for its important role in diseases including DCM[15,16]. CYP11B2 is abnormally expressed in patients with coronary heart disease[17]. CYP11B2 is associated with essential hypertension in Chinese population[18]. Moreover, CYP11B2 acts as a promoter in chronic kidney disease[19]. Torasemide prevents the development of atrial fibrosis through down-regulating CYP11B2 in mice[20]. In the present study, CYP11B2 was significantly up-regulated in HG-treated cardiomyocytes and its expression was down-regulated *via* knockdown of LUCAT1 in HG-treated cardiomyocytes.

Conclusions

We identified that LUCAT1 could reverse cardiomyocyte injury and apoptosis induced by HG through targeting CYP11B2 *in vitro*. LUCAT1/CYP11B2 axis played a vital role in cardiomyocyte apoptosis. Our findings might help to understand the molecular mechanisms of DCM progression.

Conflicts of interest

The authors declare no conflicts of interest.

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