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LncRNA UCA1 stimulates the repair of hyperglycemic vascular smooth muscle cells through targeting miR-582-5p

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Abstract. – OBJECTIVE: The purpose of this study was to elucidate the role of long non-coding RNA (lncRNA) UCA1 in inducing the repair of hyperglycemic vascular smooth muscle cells (VSMCs) by targeting microRNA-582-5p (miR-582-5p), thus alleviating diabetic angiopathy.

PATIENTS AND METHODS: Arterial vessels and serum exosomes were collected from 40 type 2 diabetes mellitus (T2DM) patients and 40 non-T2DM patients. Relative levels of UCA1 and miR-582-5p in collected samples were detected. Then, the interaction between UCA1 and miR-582-5p was assessed by Dual-Luciferase reporter assay. Moreover, the regulatory effects of UCA1 and miR-582-5p on VSMCs phenotypes were determined.

RESULTS: Results showed that compared with non-T2DM patients, UCA1 was markedly downregulated, while miR-582-5p was upregulated in VSMCs and serum exosomes of T2DM patients. They exerted a negative expression correlation between each other. Besides, miR-582-5p was the direct target of UCA1. Under the induction of increased doses of glucose, UCA1 stimulated proliferative and invasive abilities in VSMCs. MiR-582-5p was responsible for the repairability of UCA1 in VSMCs under the hyperglycemia state.

CONCLUSIONS: LncRNA UCA1 induces the repair of hyperglycemic VSMCs via negatively regulating miR-582-5p. UCA1 may be a novel target for T2DM diagnosis and treatment.

Key Words:
LncRNA UCA1, MiR-582-5p, T2DM, VSMCs, Repair.

Introduction

Morbidity and mortality of T2DM (type 2 diabetes mellitus) are relatively high nowadays, and T2DM has been a major chronic disease threating human health[1,2]. It is well known that chronic vascular complications are the leading causes of disability and death of T2DM. Diabetic microangiopathy attributes to T2DM-induced blindness and kidney failure[3,4]. Therefore, chronic vascular complications of diabetes (kidney, retina, cardiovascular and cerebrovascular system) have been well concerned in clinical practice[5]. Natural immunity and chronic, low-level inflammation are considered as pathogenic factors of T2DM and its complications[6,7]. This research focused on the role of differentially expressed lncRNAs in T2DM that regulated repair of vascular smooth muscle cells (VSMCs) under the hyperglycemia state[8,9].

Long non-coding RNAs (lncRNAs) are non-coding RNAs that lack of protein-encoding ability[10,11]. They used to be considered as byproducts during the process of transcription[11,12]. Later, vital functions of lncRNAs in embryo development, gene expression regulation and disease progression have been identified[13,14]. MicroRNAs (miRNAs) are vital factors involved in vascular repair process[15,16]. They exert typical features of high conservation, cell-specificity, location-specificity, and time-specificity, which are able to regulate gene expressions[16,17]. In this paper, relative levels of UCA1 and miR-582-5p in aorta samples and serum exosomes of T2DM patients and controls were detected, and the functions of UCA1 and miR-582-5p in the repair of VSMCs were mainly explored.

Patients and Methods

Patients

T2DM patients (n=40) with over 10 years of disease course and insulin control management were enrolled. Non-T2DM subjects (n=40)

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in the same period were enrolled as controls. Their aorta and serum samples were collected and stored in liquid nitrogen. Thereafter, exosome extraction kit (Invitrogen, Carlsbad, CA, USA) was used for extracting serum exosomes. Patients and their families in this study have been fully informed. This study was approved by the Ethics Committee of Affiliated Hospital of Shandong University of Traditional Chinese Medicine.

Cell Culture

VSMCs were 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, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a 5% CO_2 incubator at 37°C. The original medium was replaced with a fresh one every 2-3 days. Next, cell passage was conducted in trypsin until 80% confluence. Finally, serum starvation for 24 h was performed when VSMCs were grown to 70% confluence, followed by induction with 5 mM or 45 mM glucose for 12 h.

Transfection

The cells were inoculated in a 6-well plate and cultured to 30-40% confluence. Using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), sh-NC or sh-UCA1 (GenePharma, Shanghai, China) was transfected into cells, which were collected at 48 h.

Cell Counting Kit-8 (CCK-8) Assay

The cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell Invasion Assay

The cells were inoculated in a 24-well plate with 5.0×10^5 /mL. 200 μL of suspension was applied in the upper side of transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate. On the bottom, 500 μL of medium containing 10% FBS was applied. After 48 h of incubation, cells invading to the bottom were fixed in methanol for 15 min, dyed with crystal violet for 20 min, and counted using a microscope (Olympus, Tokyo, Japan). At last, invasive cell number was counted in 5 randomly selected fields per sample (magnification 40×).

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

Cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNAs were purified and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). Then, the obtained cDNA was subjected to qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). β-actin and U6 were used as the internal references. Each sample was performed in triplicate, and the relative level was calculated by $2^{-\Delta\Delta Ct}$. LncRNA UCA1: forward: 5'-CTCTCCATTGGGTTCACCATTC-3', reverse: 5'-GCGGCAGGTCTTAAGAGATGAG-3', β-actin: forward: 5›-CCTGGCACCCAGCACAAT-3›, reverse: 5›-TGCCGTAGGTGTCCCTTTG-3›, MiR-582-5p: forward: 5'-GCGGTTACAGTTGTTCAACC-3', reverse: 5'-CTCAACTGGTGTCGTGGA-3', U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3.

Dual-Luciferase Reporter Assay

The predicted sequences were inserted into pMIR to construct wild-type and mutant-type UCA1 vectors. HEK293 cells inoculated in 24-well plates were co-transfected with WT-UCA1 (5'-AACAGATAACCACCTTTAACTGTAA-3') or MUT-UCA1 (5'-AACAGATAACCACCTTTTTGACATT-3') and miR-582-5p mimics/NC, respectively. 48 hours later, cells were lysed for determining relative Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The differences between two groups were analyzed by the *t*-test. Pearson correlation test was performed to assess the expression relationship between two genes. $p<0.05$ was considered as statistically significant.

Results

Relative Levels of UCA1 and MiR-582-5p in T2DM Patients

Compared with non-T2DM subjects, UCA1 levels in serum exosomes and VSMCs of T2DM patients were lower (Figure 1A, 1C). Conversely, miR-582-5p was upregulated in serum exosomes and VSMCs of T2DM patients (Figure 1B, 1D).

Differentiation Expressions of UCA1 and MiR-582-5p in VSMCs Induced with Different Doses of Glucose

VSMCs were treated with 5, 15, 30, 45 mM glucose for 12 h, or 25 mM glucose for 0, 1, 6 or 12 h, respectively. As qRT-PCR data shown, UCA1 was dose-dependently and time-dependently downregulated, while miR-582-5p presented the opposite trends (Figure 2A, 2B). Treatment of 45 mM glucose for 12 h resulted in the most pronounced changes of UCA1 and miR-582-5p levels. CCK-8 results illustrated the dose-dependent and time-dependent declines in VSMCs viability following glucose treatment (Figure 2C). Similarly, invasiveness of VSMCs was also reduced with the extended treatment of increased doses of glucose (Figure 2D).

UCA1 Stimulated Hyperglycemia-Induced Proliferation and Invasiveness

Transfection of sh-UCA1 markedly downregulated UCA1 level in VSMCs treated with 45 mM glucose for 12 h, suggesting the effective transfection (Figure 3A). Besides, knockdown of UCA1 remarkably reduced viability (Figure 3B) and invasive cell number (Figure 3C) in hyperglycemic VSMCs.

MiR-582-5p was the Target Gene of UCA1

Bioinformatics analysis uncovered the binding sequences in 3'UTR of UCA1 and miR-582-5p (Figure 4A). Luciferase vectors were constructed based on these sequences. It was discovered that the overexpression of miR-582-5p greatly decreased Luciferase activity in wild-

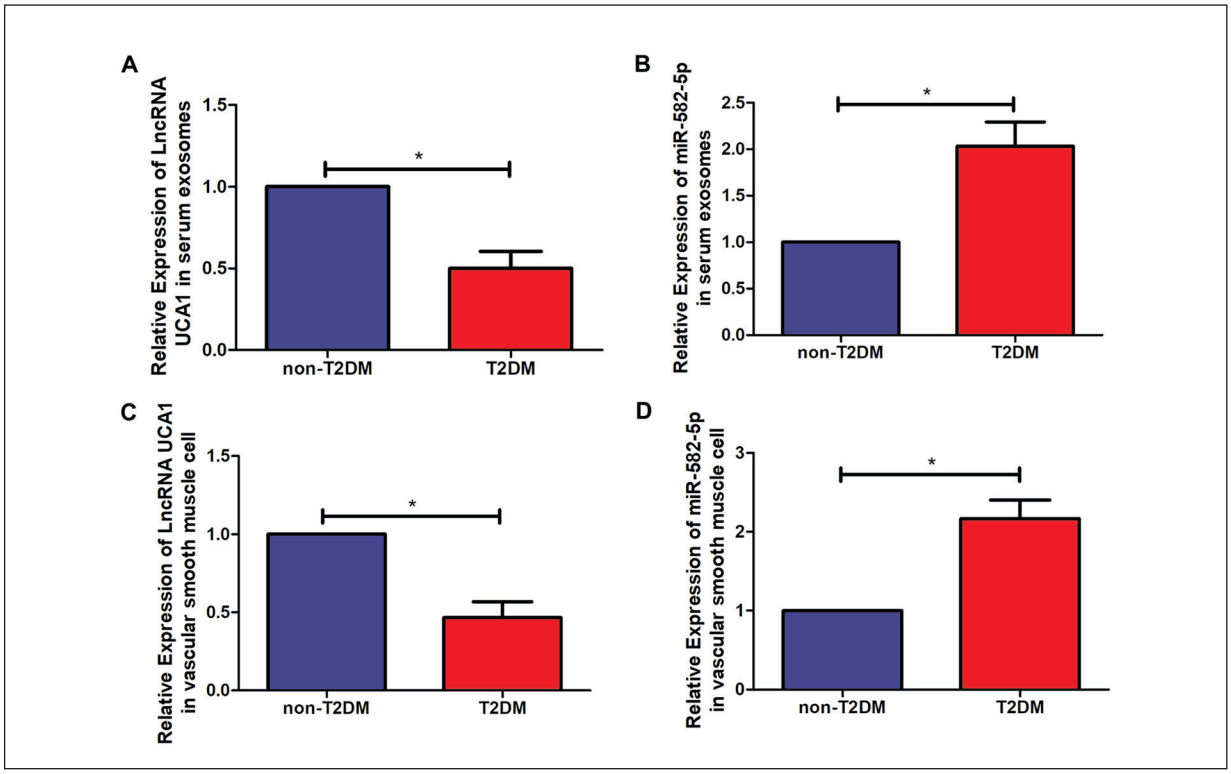

Figure 1. Relative levels of UCA1 and miR-582-5p in T2DM patients. **A, B,** Relative levels of UCA1 (**A**) and miR-582-5p (**B**) in serum exosomes of non-T2DM subjects and T2DM patients. **C, D,** Relative levels of UCA1 (**C**) and miR-582-5p (**D**) in VSMCs of non-T2DM subjects and T2DM patients. Data are expressed as mean ± S.D. *$p<0.05$ *vs.* non-T2DM group.

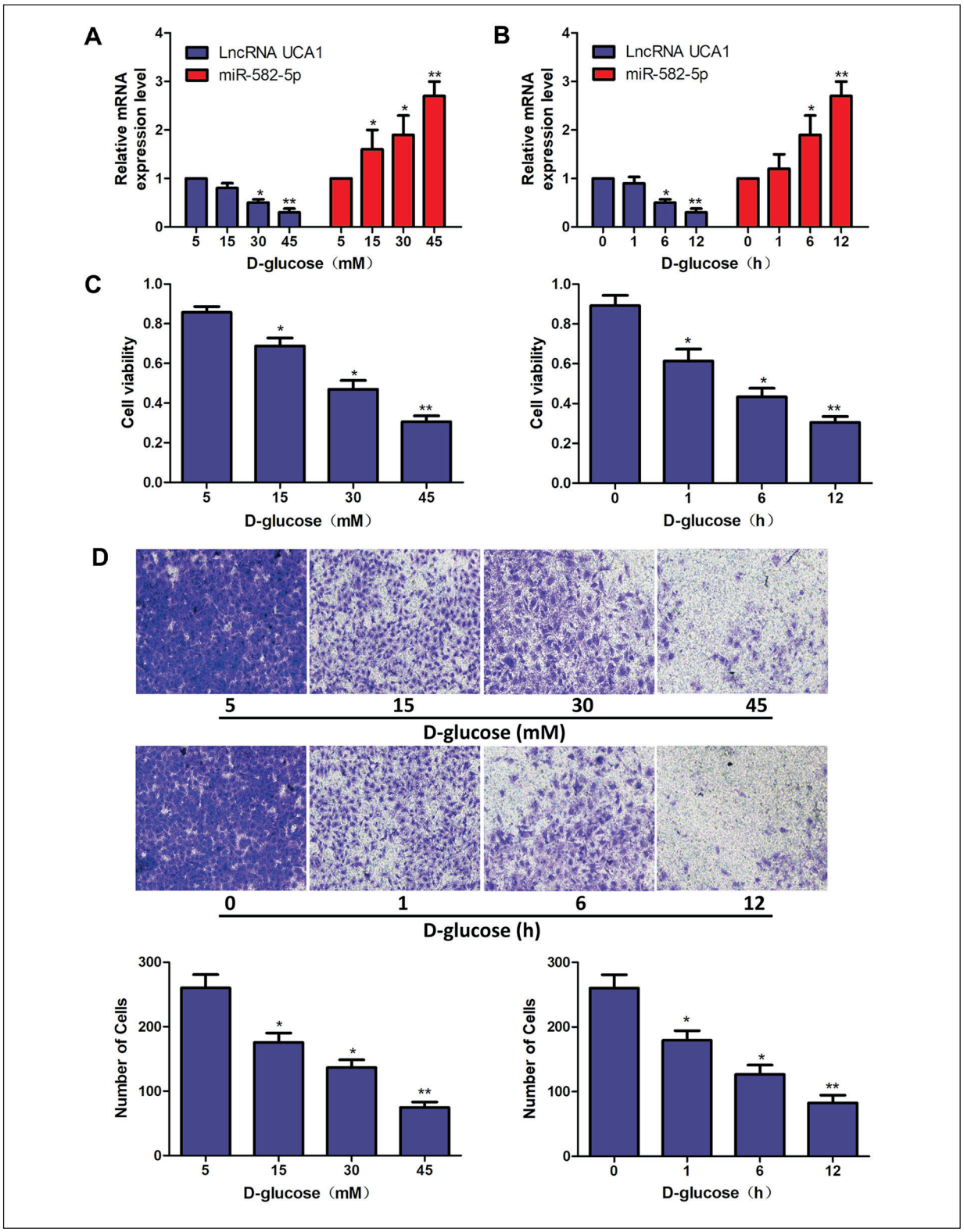

Figure 2. Differentiation expressions of UCA1 and miR-582-5p in VSMCs induced with different doses of glucose. **A,** Relative levels of UCA1 and miR-582-5p in VSMCs treated with 5, 15, 30, 45 mM glucose for 12 h, respectively, **B,** Relative levels of UCA1 and miR-582-5p in VSMCs treated with 25 mM glucose for 0, 1, 6 or 12 h, respectively. **C,** Viability in VSMCs treated with 5, 15, 30, 45 mM glucose for 12 h, or 25 mM glucose for 0, 1, 6 or 12 h, respectively. **D,** Invasion in VSMCs treated with 5, 15, 30, 45mM glucose for 12 h, or 25 mM glucose for 0, 1, 6 or 12 h, respectively (magnification 40×). Data are expressed as mean ± S.D. *p<0.05.

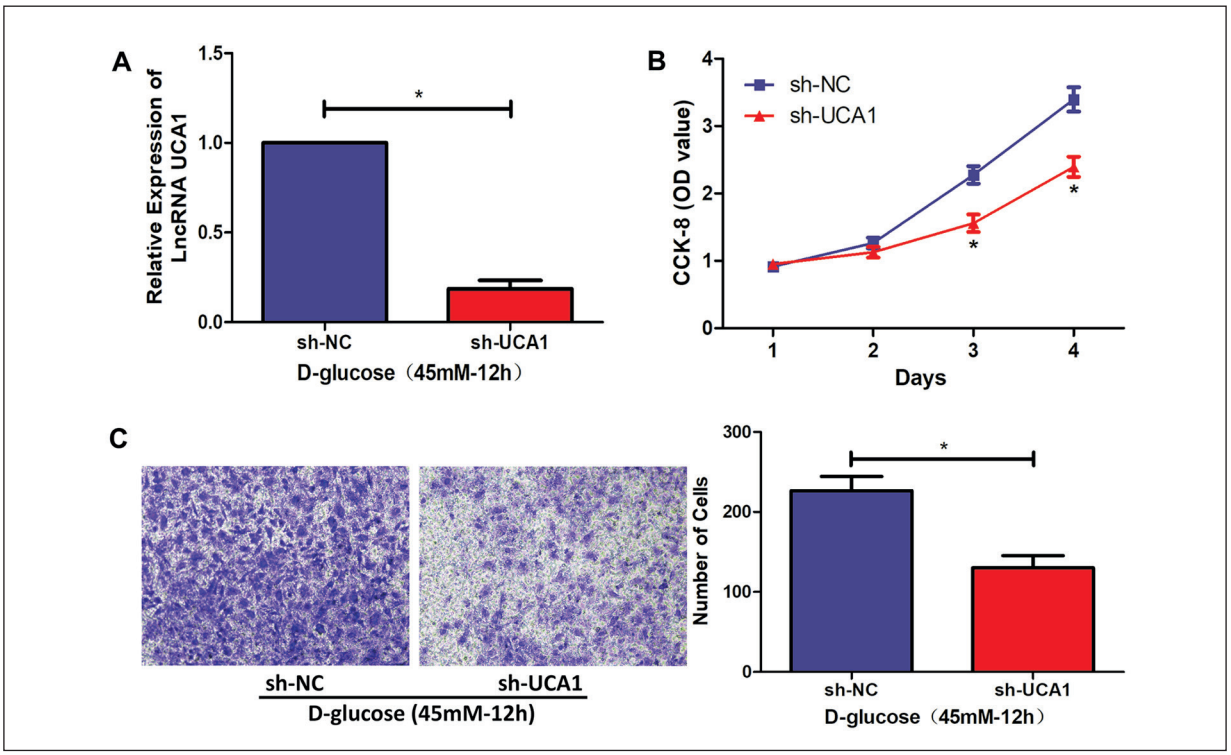

Figure 3. UCA1 stimulates hyperglycemia-induced proliferation and invasiveness. **A,** UCA1 level in VSMCs treated with 45 mM glucose for 12 h, followed by transfection of sh-NC or sh-UCA1. **B,** Viability in VSMCs treated with 45 mM glucose for 12 h, followed by transfection of sh-NC or sh-UCA1. **C,** Invasion in VSMCs treated with 45 mM glucose for 12 h, followed by transfection of sh-NC or sh-UCA1 (magnification 40×). Data are expressed as mean ± S.D. *p<0.05 *vs.* sh-NC group.

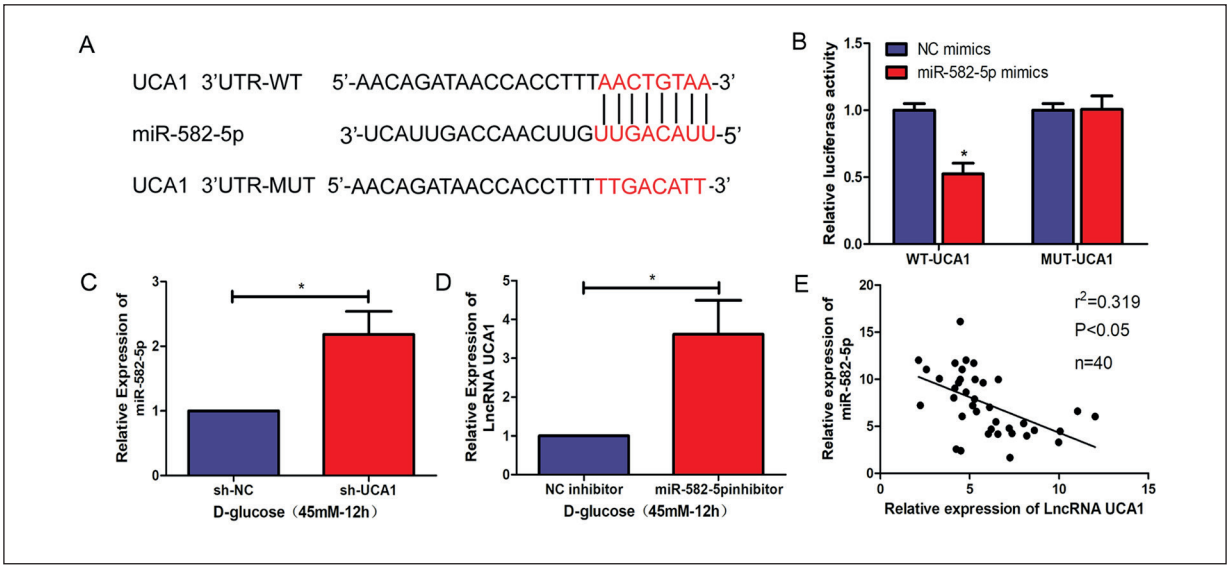

Figure 4. MiR-582-5p is the target gene of UCA1. **A,** Binding sequences in 3'UTR of UCA1 and miR-582-5p. **B,** Luciferase activity in HEK293 cells co-transfected with NC/miR-582-5p mimics and WT-UCA1/MUT-UCA1. **C,** MiR-582-5p level in VSMCs treated with 45 mM glucose for 12 h, followed by transfection of sh-NC or sh-UCA1. **D,** UCA1 level in VSMCs treated with 45 mM glucose for 12 h, followed by transfection of NC or miR-582-5p inhibitor. **E,** A negative correlation between expression levels of UCA1 and miR-582-5p. Data are expressed as mean ± S.D. *p<0.05.

type UCA1 vector, suggesting the binding relationship between UCA1 and miR-582-5p (Figure 4B). Interestingly, miR-582-5p was upregulated in hyperglycemic VSMCs transfected with sh-UCA1 (Figure 4C) and UCA1 was upregulated in VSMCs with miR-582-5p knockdown (Figure 4D). Furthermore, a negative correlation was identified between expression levels of UCA1 and miR-582-5p (Figure 4E).

UCA1 Stimulated Proliferative Ability and Invasiveness in Hyperglycemia-Induced VSMCs Through Targeting MiR-582-5p

Relative levels of UCA1 and miR-582-5p in co-transfected VSMCs were first examined (Figure 5A, 5B). Previous results have demonstrated that the knockdown of UCA1 inhibited viability and invasiveness in hyperglycemic VSMCs. However, these inhibited trends were partially abolished by co-transfection of miR-582-5p inhibitor (Figure 5C, 5D). Therefore, it was believed that miR-582-5p was involved in UCA1-induced VSMCs repair under the hyperglycemic state.

Discussion

Chronic vascular complications are the leading causes of the death in T2DM patients. Their incidences become higher and the disease onset is younger[3-5]. A retrospective analysis involving 24,496 T2DM patients with chronic complications in Chinese metropolis showed the occurrence of diabetic vascular complications: cardiovascular disease (17.1%), cerebrovascular disease (12.6%), lower extremity vascular disease (5.2%), retinopathy (35.7%), nephropathy (34.7%), and neuropathy (61.8%)[6-8]. The pathogenesis of diabetic vascular complications is complicated, and many factors could be involved, such as long-term hyperglycemia, glycosylated protein end products, polyol pathway, oxidative stress, lipid metabolism disorders, hemorheology changes, and endothelial cell dysfunction[8,9].

LncRNAs are vital regulators during disease progression, and lncRNAs in body fluids are stable, and accurately reflect the condition of T2DM[18]. VSMCs' functions are speculated to be regulated by

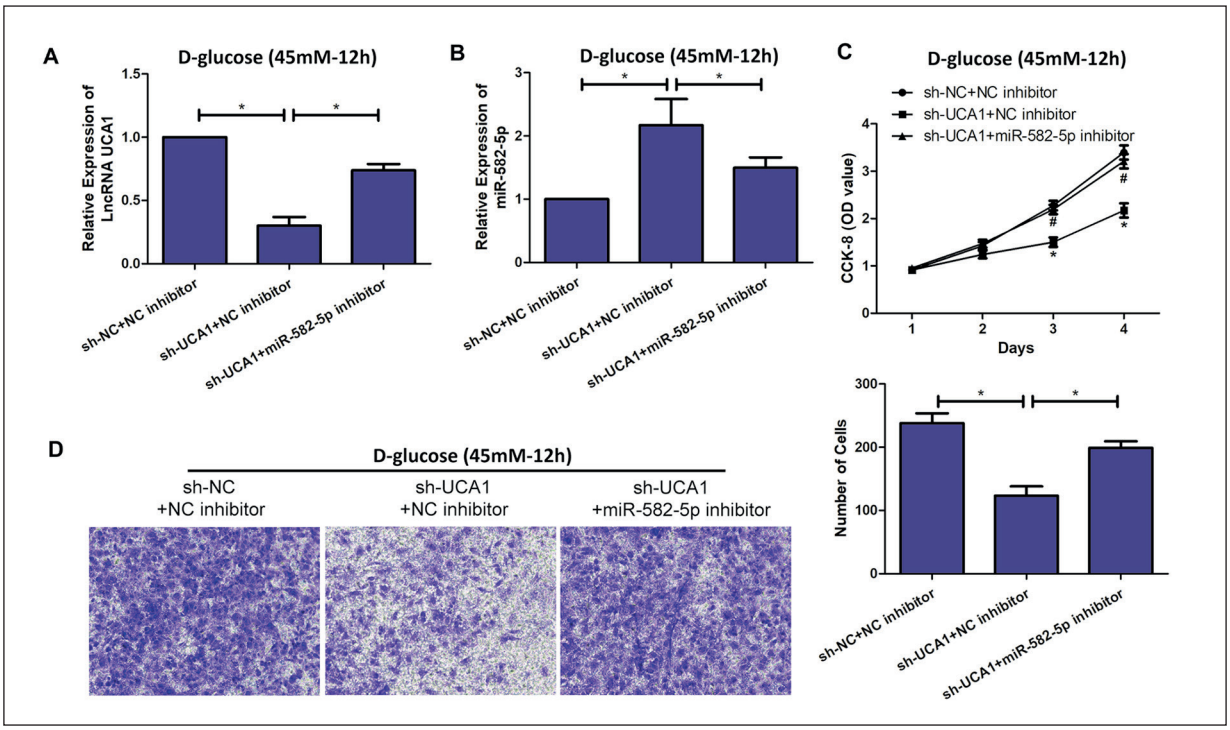

Figure 5. UCA1 stimulates proliferative ability and invasiveness in hyperglycemia-induced VSMCs through targeting miR-582-5p. VSMCs are treated with 45 mM glucose for 12 h, followed by transfection of sh-NC+NC inhibitor, sh-UCA1+NC inhibitor or sh-UCA1+miR-582-5p inhibitor. **A,** UCA1 level. **B,** MiR-582-5p level. **C,** Viability. **D,** Invasion (magnification 40×). Data are expressed as mean ± S.D. *p<0.05.

lncRNAs, thereafter influencing the development of T2DM[18,19]. Therefore, the objective of this study was firstly to elucidate the role of lncRNAs in inducing the repair of hyperglycemic VSMCs by targeting miRNA thus alleviating diabetic angiopathy. As a novel lncRNA, UCA1 was found in a series of ischemic and toxic diseases[20,21]. Lu et al[20] suggested that reduction of UCA1 levels plays a pro-apoptotic role in primary cardiomyocytes partially through stimulation of p27 protein expression[20]. In addition, Geng et al[21] revealed that lncRNA UCA1 suppressed pilocarpine-induced epilepsy by inhibiting apoptosis of hippocampal neurons through miR-495/Nrf2-ARE pathway, and thereby inhibiting brain injury induced by seizure. In this paper, lncRNA UCA1 was found to be downregulated in VSMCs and serum exosomes of T2DM patients. Knockdown of UCA1 inhibited viability and invasiveness in VSMCs under the hyperglycemia state, suggesting that UCA1 induces VSMCs repair.

LncRNAs exert multi-level regulations by interacting with miRNAs, proteins, etc. They have the potential to be therapeutic targets of T2DM[22]. Here, miR-582-5p was detected to be the direct target binding to UCA1. Previous studies[23,24] have shown that UCA1 activation triggers multiple factors to protect the body from external stimuli. The results of this study identified that miR-582-5p was responsible for VSMCs repair induced by UCA1 under the high-glucose treatment.

Conclusions

The above data demonstrated that lncRNA UCA1 induces VSMC repair under the hyperglycemia state *via* negatively regulating miR-582-5p, and it may be a novel target for T2DM diagnosis and treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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