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Original Article

Inhibition of lncRNA Dlx6os1 decreases cell proliferation and fibrosis and increases cell apoptosis in diabetic nephropathy

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Abstract: This study aimed to investigate the effect of the long non-coding RNA (lncRNA) Dlx6os1 inhibitor on cell proliferation, apoptosis and fibrosis in mouse mesangial cells (MMCs) under high glucose (HG) conditions. SV40 MES13 cells were cultured under 30 mmol/L glucose (HG group), 5.6 mmol/L glucose (normal glucose group, NG group) and 5.6 mmol/L glucose plus 24.4 mmol/L 3-O-methyl-D-glucose (osmotic control group, OC group), and expressions of lncRNA Dlx6os1, Gm13730, Rdh9, Chrm2 and Bex1 were determined by qPCR. NC-inhibitor plasmids and lncRNA Dlx6os1 inhibitor plasmids were transfected into SV40 MES13 cells cultured under HG conditions, and cell proliferation (at 0 h, 24 h, 48 h and 72 h), and the apoptosis rate (at 72 h), proteins and mRNAs expressions for proliferation and fibrosis markers (at 72 h) were detected by CCK-8, AV-PI, Western blot and qPCR assays, respectively. LncRNA Dlx6os1 was increased in the HG group compared with the OC and NG groups, while no difference of lncRNA Gm13730, Rdh9, Chrm2 or Bex1 was discovered among the three groups. The lncRNA Dlx6os1 inhibitor decreased cell proliferation at 24 h, 48 h and 72 h post plasmids transfection by the CCK-8 assay and reduced the expressions of Cyclin D1 and proliferating cell nuclear antigen (PCNA) mRNA and protein expressions compared with the NC-inhibitor. The cell apoptosis rate at 72 h increased compared with the NC-inhibitor. In addition, protein and mRNA expressions of markers for cell fibrosis (fibronectin and collagen I) were all decreased in the lncRNA Dlx6os1-inhibitor group compared to the NC-inhibitor group. In conclusion, inhibition of lncRNA Dlx6os1 decreases cell proliferation and fibrosis and increases cell apoptosis in diabetic nephropathy.

Keywords: LncRNA Dlx6os1, mouse mesangial cells, high glucose, diabetic nephropathy

Introduction

Diabetic nephropathy (DN), also known as diabetic kidney disease (DKD), is a progressive kidney disease caused by diabetes mellitus (DM) which is characterized by podocyte loss, epithelial dysfunction, inflammation, hypertrophy, and dedifferentiation via the activation of various classic pathways of regeneration [1-3]. Although many improvements have been realized such as novel drugs, advanced treatment strategies, individual patient care and so on, DN is still the leading cause of kidney failure around the world, and it's also the single strongest predictive factor for mortality in DM patients [4-6]. Thus, exploration of the novel mechanisms of DN and its corresponding treatment targets are of great importance to further improve the prognosis of DN patients.

Long non-coding RNA (lncRNA), as a critical component of non-coding RNA (ncRNA) consisting of more than 200 nucleotides, is involved in lots of essential biological phenomena including imprinting genomic loci, shaping chromosome conformation, regulating enzymatic activity and so on [7]. Accumulating evidence has revealed that lncRNAs are implicated in the pathology of numerous diseases, such as cancers, diabetes, and inflammatory diseases [8-10]. As to DN, only several studies have disclosed that dysregulated lncRNAs might be involved in the development or progression of DN [11, 12]. Thus, in this study, we selected 5

candidate lncRNAs by analyzing a previous lncRNAs microarray data to determine the influence of high glucose (HG) on their expressions in mouse mesangial cells (MMCs) and, most importantly, to investigate the effect of the lncRNA Dlx6os1 inhibitor on cell proliferation, apoptosis and fibrosis in MMCs under HG conditions.

Materials and methods

Cells culture

SV40 MES13 cells (MMCs) were purchased from Shanghai Institutes for Biological Science (Shanghai, China). After the resuscitation, SV40 MES13 cells were cultured in Dulbecco modified Eagle medium (DMEM) (Gibco, USA) completed with 10% fetal bovine serum (FBS) (Gibco, USA), and then the cells were incubated in a humidified incubator with 5% CO_2 at 37°C.

Measurement of candidate lncRNAs expressions under high glucose and normal glucose

1×10^5 SV40 MES13 cells were then cultured in DMEM (Gibco, USA) with 10% FBS (Gibco, USA) and 30 mmol/L glucose (Higher glucose group, HG group), DMEM (Gibco, USA) with 10% FBS (Gibco, USA) and 5.6 mmol/L glucose (normal glucose group, NG group), and DMEM (Gibco, USA) with 10% FBS (Gibco, USA) and 5.6 mmol/L glucose plus 24.4 mmol/L 3-O-methyl-D-glucose (osmotic control group, OC group), respectively. After 96 h culturing, the expressions of five candidate lncRNAs in three groups were determined by quantitative polymerase chain reaction (qPCR) assay. Candidate lncRNAs were selected by analyzing the previous lncRNAs microarray data of lncRNAs profiles in DN mouse model as follows: ENSMUST00000159113 (Dlx6os1), ENSMUST00000121036 (Gm13730), ENSMUST00000128133 (Rdh9), ENSMUST00000169004 (Chrm2) and ENSMUST00000142172 (Bex1) [12]. The primers were designed by Shanghai QeeJen BioTech Co., Ltd (Shanghai, China) as follows: lncRNA Dlx6os1, F: GTTCTTATTCCAGCCATTGACCTT, R: AACTCCTAATTCCTTCTTCCTACCT; lncRNA Gm13730, F: GAAGCATCCAAGCAGGCAGAT, R: CGATCATAGTAGGCAGCATCACA; lncRNA Rdh9, F: ATCGTCAATGCCTACACCTTCC, R: GCCTGTCAGATGTCTTGTTCCT; lncRNA Chrm2, F: CCAGCCAGACTCCACCAGAT, R: CACTTGTGCCAGTAATCCTTCAG; lncRNA Bex1, F: AAAGAGGAGAAGGCAAGGATAGG, R: CCACCGTGTTTCCTGCAGATATA; U6, F: CTCGCTTCGGCAGCACA, R: AACGCTTCACGAATTTGCGT.

Plasmids transfection

1×10^5 SV40 MES13 cells were plated in 24-well plates, and NC-inhibitor plasmids and lncRNA Dlx6os1 inhibitor plasmids were transfected into SV40 MES13 cells, and then the cells were cultured in DMEM (Gibco, USA) with 10% FBS (Gibco, USA) and 30 mmol/L glucose (under HG conditions). Plasmids were constructed by the GENEWIZ Company (Suzhou, China). At 24 h after the transfection, the lncRNA Dlx6os1 expression in each group was determined by a qPCR assay.

Cell proliferation detection

A CCK-8 assay was performed at 0 h, 24 h, 48 h and 72 h post transfection to determine the cell proliferation as follows: CCK-8 was added to each plate of cells, and then the cells were incubated under 5% CO_2 at 37°C; subsequently the optical density (OD) value was determined using a microplate reader (Biotek, USA). In addition, the protein and mRNA expressions of the proliferation indicators (Cyclin D1 and proliferating cell nuclear antigen (PCNA)) at 72 h were determined by Western blot and qPCR assays respectively.

Cells apoptosis detection

At 72 h after transfection, cells were digested by pancreatin and washed by phosphate buffer saline (PBS). After the cells were suspended in 100 μl Blinding Buffer, 5 μl Annexin V-FITC (AV) was added and the cells were placed in darkness for 15 mins at room temperature, then 5 μl Propidium Iodide (PI) was added and flow cytometry was used to analyze the results.

Measurement of markers for fibrosis

Markers for fibrosis including fibronectin (FN) and collagen I were detected by qPCR and Western blot assays to determine the influence of the lncRNA Dlx6os1 inhibitor on fibrosis of SV40 MES13 cells.

qPCR assay

Total RNA was extracted using the TRIzol reagent (R&D, USA) according to the manufacturer's instructions. After quantification of RNA by

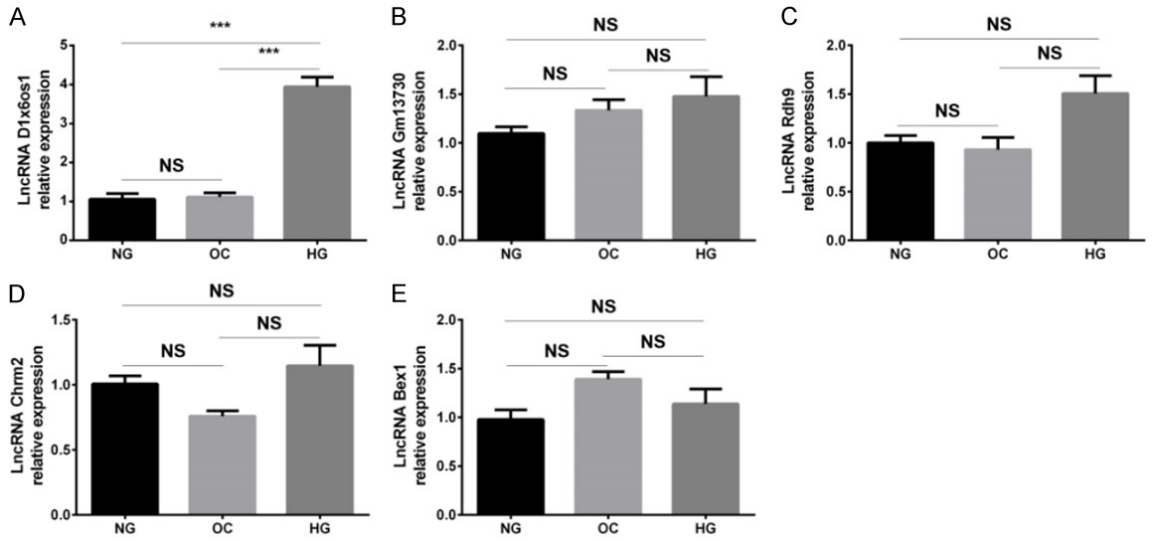

Figure 1. Expressions of candidate lncRNAs expressions in MMCs under HG, OC and NG conditions. lncRNA Dlx6os1 was elevated in MMCs under HG conditions compared with OC and NG conditions (A). While lncRNA Gm13730 (B), lncRNA Rdh9 (C), lncRNA Chrm2 (D) or lncRNA Bex1 (E) presented with no difference among three groups. Comparison was determined by a t test. *P<0.05, **P<0.01, ***P<0.001. MMCs, mouse mesangial cells; HG, high glucose; OC, osmotic control; NG, normal glucose.

OD 260, 1 μg of total RNA from each sample was used for cDNA synthesis using a reverse transcription kit (TaKaRa, Japan), and then the cDNA product was subjected to qPCR with a SYBR Green kit (TaKaRa, Japan) according to the manufacturer's instructions. The PCR amplification was performed as follows: 95°C for 5 mins, followed by 40 cycles of 95°C for 5 s, 61°C for 30 s. GAPDH was used as a reference gene for the mRNAs, while U6 was used as reference gene for the lncRNAs. The qPCR result was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers of lncRNA Dlx6os1 and mRNAs were designed by Shanghai QeeJen Bio-Tech Co., Ltd (Shanghai, China) as follows: lncRNA Dlx6os1, F: GTTCTTATTCCAGCCATTGACCTT, R: AACTCCTAATTCCTTCTTCCTACCT; U6, F: CTCGCTTCGGCAGCACA, R: AACGCTTCACGAATTTGCGT; FN, F: TCAGTAGAAGGCAGTAGCACAGA, R: CCTCCACACGGTATCCAGACA; PCNA, F: GCCGAGACCTTAGCCACATTG, R: ATGGTTACCGCCTCCTCTTCTT; Collagen I, F: CTGACTGGAAGAGCGGAGAG, R: CTGTAGGTGAAGCGACTGTTG; Cyclin D1, F: CCAGAGGCGGATGAGAACAAG, R: GCGGTAGCAGGAGAGGAAGT; GAPDH, F: AATGGTGAAGGTCGGTGTGAAC, R: TCGCTCCTGGAAGATGGTGAT.

Western blot assay

Total protein was extracted from cells with 1 ml radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, USA), and then the protein concentration was determined using a bicinchoninic acid (BCA) kit (Pierce Biotechnology, USA) and evaluated according to the standard curve. Then 20 μg protein samples with equal concentrations were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Millipore, USA). After blocking with 5 % skim milk for 2 h, the membranes were incubated with the primary antibody overnight at 4°C. Then, the membranes were incubated with the corresponding secondary antibody for 1 h at room temperature. The bands were visualized using an enhanced chemiluminescence (ECL) kit (Millipore, USA) followed by exposure to X-ray film. The primary antibodies used in this study were as follows: Rabbit Anti-Collagen I antibody (1:1000 dilution, ab34710, Abcam, USA); Rabbit Anti-Cyclin D1 antibody (1:10000 diluti-on, ab134175, Abcam, USA); Rabbit Anti-PCNA antibody (1:1000 dilution, ab92552, Abcam, USA); Rabbit Anti-Fibronectin antibody (1:1000 dilution, ab199056, Abcam, USA); Rabbit Anti-GAPDH antibody (1:10000 dilution, ab181602, Abcam, USA). The secondary antibody used in this study was Goat Anti-Rabbit IgG H&L (HRP) (1:2000 dilution, ab205718, Abcam, USA).

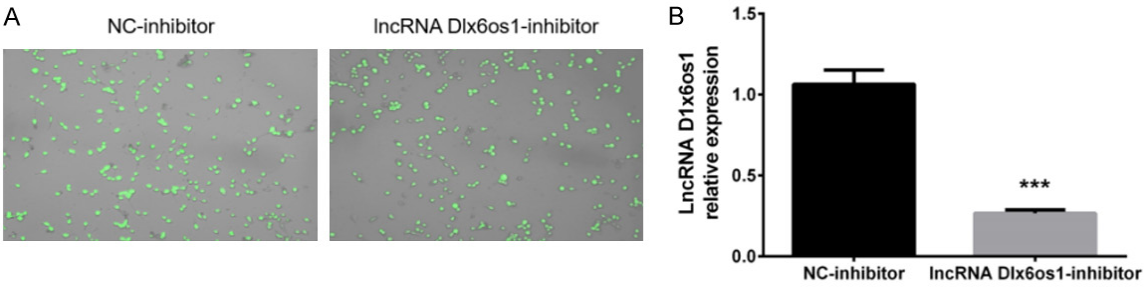

Figure 2. lncRNA Dlx6os1 expressions after plasmids transfection. A. Transfection efficiencies were all above 90% in both the NC-inhibitor and lncRNA Dlx6os1-inhibitor groups. B. lncRNA Dlx6os1 expression was greatly lower after lncRNA Dlx6os1-inhibitor transfection than it was after NC-inhibitor transfection. Comparison was determined by a t test. *P<0.05, **P<0.01, ***P<0.001.

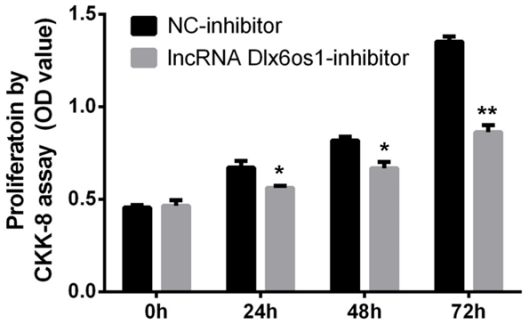

Figure 3. MMCs proliferation. Cell proliferation was decreased in lncRNA Dlx6os1-inhibitor group compared to the NC-inhibitor group at 24 h, 48 h and 72 h post plasmids transfection in MMCs under HG condition. Comparison was determined by a t test. *P<0.05, **P<0.01, ***P<0.001. MMCs, mouse mesangial cells; HG, high glucose.

Statistics

SPSS 22.0 software (IBM, USA) and GraphPad 6.01 software (GraphPad, USA) were used for statistical analysis. Data was mainly presented as mean ± standard error (SEM). Comparison between two groups was determined by a t test. P<0.05 was considered significant.

Results

Expression of candidate lncRNAs in NG, OC and HG groups

In order to investigate the effect of glucose on candidate lncRNAs' expressions in SV40 MES13 cells, we detected their expressions in NG, OC and HG groups, which showed that lncRNA Dlx6os1 was increased in the HG group compared with that in the OC and NG groups (**Figure 1A**), while no difference of lncRNA Gm13730 (**Figure 1B**), lncRNA Rdh9 (**Figure 1C**), lncRNA Chrm2 (**Figure 1D**) or lncRNA Bex1 (**Figure 1E**) was discovered among the three groups.

LncRNA Dlx6os1 expression after plasmids transfection

A microscope revealed that the plasmid transfection rates were all above 90% in both the NC-inhibitor and lncRNA Dlx6os1-inhibitor groups (**Figure 2A**). And at 24 h after plasmid transfection, the lncRNA Dlx6os1 expression was observed to be greatly reduced in the lncRNA Dlx6os1-inhibitor group compared to the NC-inhibitor group (**Figure 2B**).

Cells proliferation after plasmids transfection

A CCK-8 assay revealed that lncRNA Dlx6os1 inhibitor decreased cell proliferation at 24 h, 48 h and 72 h post plasmid transfection into SV40 MES13 cells under HG conditions (**Figure 3**). In addition, protein and mRNA expressions of the proliferation indicators (Cyclin D1 and PCNA) were both lower in the lncRNA Dlx6os1-inhibitor group than in the NC-inhibitor group (**Figure 4A-C**).

Cells apoptosis after plasmids transfection

An AV-PI assay disclosed that the cell apoptosis rate was enhanced in the lncRNA Dlx6os1-inhibitor group compared to the NC-inhibitor group (**Figure 5A**, **5B**) at 72 h after the plasmids transfection into SV40 MES13 cells under HG condition.

Expressions of markers for cells fibrosis

Protein and mRNA expressions of markers for cells fibrosis were subsequently detected by

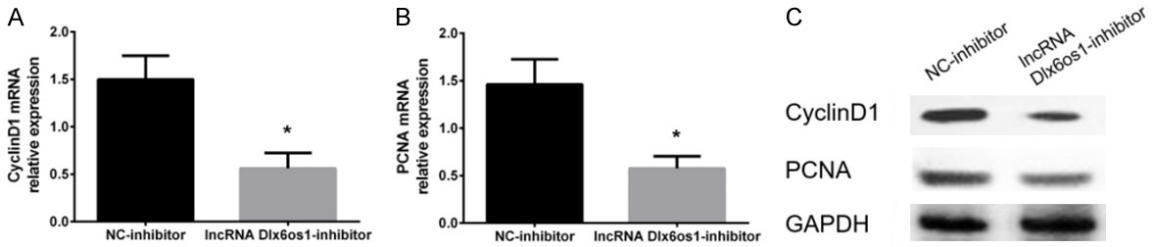

Figure 4. Proliferation marker expressions. mRNAs expressions of proliferation markers Cyclin D1 (A) and PCNA (B) were decreased in lncRNA Dlx6os1-inhibitor group compared to the NC-inhibitor group at 72 h. And their protein expressions presented with similar treads (C). Comparison was determined by a t test. *P<0.05, **P<0.01, ***P<0.001. PCNA, proliferating cell nuclear antigen.

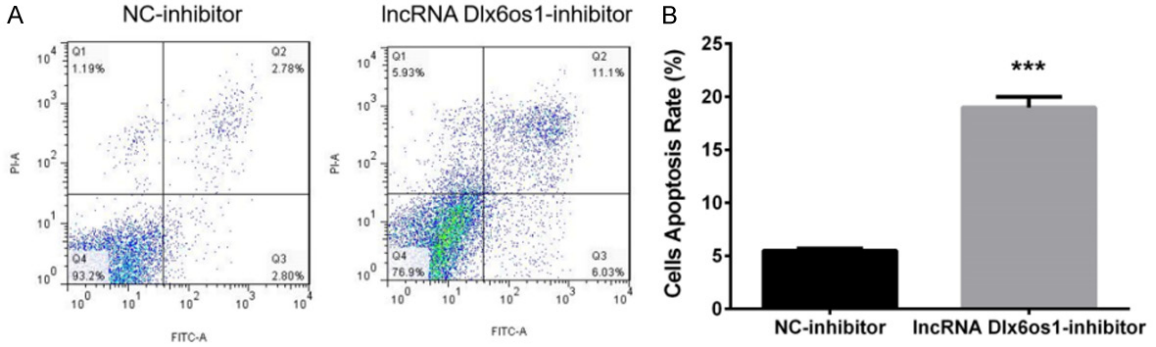

Figure 5. MMCs apoptosis. The cell apoptosis rate was increased in the lncRNA Dlx6os1-inhibitor group compared to the NC-inhibitor group at 72 h post plasmids transfection in MMCs under HG conditions. Comparison was determined by a t test. *P<0.05, **P<0.01, ***P<0.001. MMCs, mouse mesangial cells; HG, high glucose.

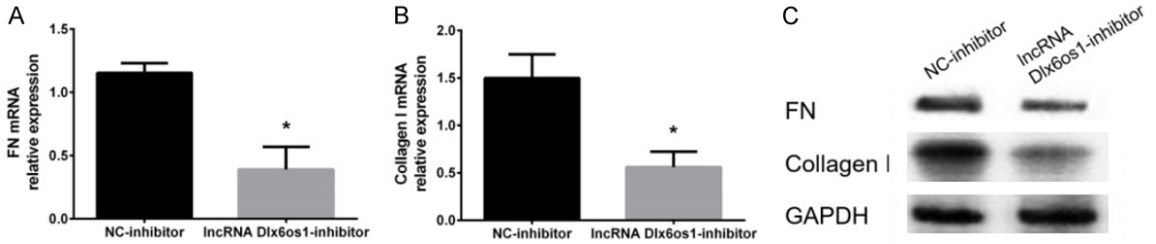

Figure 6. Fibrosis markers expressions. The mRNAs expressions of cell fibrosis markers FN (A) and collagen I (B) were decreased in the lncRNA Dlx6os1-inhibitor group compared to the NC-inhibitor group at 72 h. And their protein expressions presented with similar treads (C). Comparison was determined by a t test. *P<0.05, **P<0.01, ***P<0.001. FN, fibronectin.

qPCR and Western blot, which showed that protein and mRNA expressions of FN and collagen I were both decreased in lncRNA Dlx6os1-inhibitor group compared to the NC-inhibitor group (**Figure 6A-C**).

Discussion

In this study, we found that the lncRNA Dlx6os1 inhibitor suppressed cell proliferation and fibrosis while it improved cell apoptosis in MMCs under HG conditions, which indicates that the inhibition of lncRNA Dlx6os1 might be a potential option for delaying DN progression.

DN, as the leading cause of DM-related mortality worldwide, is a critical life-threatening disease [13]. Intensification of glycemic control and good blood pressure control are the main treatment options for DN currently, while rapid disease progression and the disease's high mortality rate are still critical issues [14, 15].

Thus, it's essential to further investigate the underlying pathophysiological processes implicated in DN and explore novel treatment targets for DN.

LncRNAs, a diverse class of molecules lacking the potential of coding proteins which are composed of longer than 200 nucleotides, are characterized by their unique regulatory mechanisms, alternative forms of biogenesis, cis-regulatory activities and functional structured RNA domains [7, 16]. Accumulating evidences reveal that dysregulated lncRNAs are involved in the pathology of DM and stimulate glucose homeostasis by regulating numerous signaling pathways [9, 17, 18].

As to DN, a lncRNA microarray experiment was performed and 221 upregulated lncRNAs as well as 797 downregulated lncRNAs were identified in kidney tissues obtained from db/db mice compared to the controls [12]. Another study comparing the lncRNA expression patterns between DN mice models and the controls using a microarray showed that 311 lncRNAs were dysregulated in DN mice, and a bioinformatics analysis suggests that most of the differentially expressed lncRNAs are enriched in glutathione metabolism signaling and other DN-related pathways [19]. These suggest lncRNA profiles play important role in DN development and progression. A recent experimental study found that lncRNA taurine-upregulated gene 1 (TUG1) reduces extracellular matrix accumulation by sponging microRNA-377 and subsequently increasing the peroxisome proliferator-activated receptor γ (PPARγ) in MMCs under HG conditions [20]. And a similar study also shows that lncRNA TUG1 modulates mitochondrial bioenergetics by targeting PPARγ in podocytes of the diabetic mileu [21]. Another experiment found that the knockdown of the lncRNA plasmacytoma variant translocation 1 gene (PVT1) decreases expressions of major extracellular matrix (ECM) related proteins and mRNAs including FN1, collagen type IV alpha 1 (COL4A1), transforming growth factor beta 1 (TGFB1) and plasminogen activator Inhibitor-1 (PAI-1) in MMCs [22]. And lncRNA ENSMUST00000147869 is observed to protect MMCs from proliferation and fibrosis induced by DN [23]. These studies indicate lncRNAs are greatly involved in the pathogenesis of DN as well as its progression.

LncRNA Dlx6os1 (also called as Evf2), with Ensemble ID ENSMUST00000159113, is located on Chromosome 6: 6,820,189-6,871,592. A previous study discloses that lncRNA Dlx6os1 interacts with the Brahma-related gene 1 (BRG1) and transcriptional activators to present with the RNA-dependent inhibition of chromatin remodeling [24]. While only a few studies have reported on its role in DN pathogenesis, a recent study found that lncRNA Dlx6os1 is upregulated in the kidneys of db/db mice compared to the controls using a lncRNA microarray analysis [12]. In this present study, first we detected the expression of five candidate lncRNAs expressions (selected by analyzing published microarray data) in MMCs under different glucose concentrations, and we found lncRNA Dlx6os1 was greatly increased in MMCs under HG conditions compared with concentrations under OC and NG conditions, indicating that HG induced the upregulation of lncRNA Dlx6os1 in MMCs. Subsequently we investigated the effect of the lncRNA Dlx6os1 inhibitor on cells proliferation, apoptosis and fibrosis using the CCK-8 assay, the AV-PI assay and the measurement of corresponding markers, and we observed that inhibition of lncRNA Dlx6os1 decreased the MMCs proliferation and fibrosis-related markers expressions, while it increased the MMCs apoptosis, which indicated lncRNA Dlx6os1 was involved in the pathology of DN progression, and its inhibitor might be a potential target for DN treatment.

In conclusion, inhibition of lncRNA Dlx6os1 decreases cell proliferation and fibrosis and increases cell apoptosis in DN.

Acknowledgements

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Disclosure of conflict of interest

None.

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