Long non-coding RNA *Dlx6os1* serves as a potential treatment target for diabetic nephropathy via regulation of apoptosis and inflammation

LI CHENG[1*], JIE CHENG[2*], WENFANG PENG[2], XIAOHONG JIANG[2] and SHAN HUANG[2]

[1]Department of Gynecology, The Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, Guiyang, Guizhou 550003; [2]Department of Endocrinology, Tongren Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200336, P.R. China

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Abstract. The present study investigated the effect of long non-coding RNA (lncRNA) *Dlx6os1* silencing on cell proliferation, apoptosis and fibrosis, and further explored its influence on the mRNA expression profile in mouse mesangial cells (MMCs) of a diabetic nephropathy (DN) cellular model. A DN cellular model was constructed in SV40 MES13 MMCs under high glucose conditions (30 mmol/l glucose culture). lncRNA *Dlx6os1* short hairpin (sh)RNA plasmids and negative control (NC) shRNA plasmids were transfected into the MMCs of the DN cellular model as the sh-lncRNA group and sh-NC group respectively. The mRNA expression profile was determined in the sh-lncRNA and sh-NC groups. Compared with the sh-NC group, the cell proliferation, mRNA and protein expression levels of proliferative markers (cyclin D1 and proliferating cell nuclear antigen) as well as fibrosis markers (fibronectin and collagen I) were suppressed, whereas cell apoptosis was promoted in the sh-lncRNA group. The mRNA expression profile identified 423 upregulated mRNAs and 438 downregulated mRNAs in the sh-lncRNA group compared with the sh-NC group. Additionally, Gene Ontology/Kyoto Encyclopedia of Genes and Genomes enrichment analyses revealed that the differentially expressed mRNAs were enriched in apoptosis and inflammation-related pathways. Further gene-set enrichment analysis of apoptosis and inflammation revealed that lncRNA *Dlx6os1* inhibition promoted apoptosis and suppressed inflammation in MMCs of the DN cellular model. In conclusion, lncRNA *Dlx6os1* may serve as a potential treatment target for DN via regulation of multiple apoptosis- and inflammation-related pathways.

Correspondence to: Dr Shan Huang, Department of Endocrinology, Tongren Hospital, Shanghai Jiao Tong University School of Medicine, 1111 Xianxia Road, Shanghai 200336, P.R. China
E-mail: shi22178331@163.com

*Contributed equally

Abbreviations: DN, diabetic nephropathy; GO, Gene Ontology; GSEA, gene-set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNAs, long non-coding RNAs; MMCs, mouse mesangial cells; PCNA, proliferating cell nuclear antigen; qPCR, quantitative polymerase chain reaction

Key words: long non-coding RNA *Dlx6os1*, diabetic nephropathy, apoptosis, inflammation, mRNA

Introduction

Diabetic nephropathy (DN), which occurs in 20-40% of all diabetes cases, is one of the systemic microvascular complications of diabetes as well as the leading cause of end-stage renal disease (1). DN is a chronic process whose early clinical symptoms are often not evident (2). In addition, there is a large risk of DN patients developing uremia, which is a key contributor to diabetes-related disability or death (2). Hyperglycemia, in the long term, causes hypertrophy, infiltration of inflammatory cells, thickening of the basement membrane, glomerular sclerosis and tubular atrophy, eventually leading to kidney failure (3). Once kidney failure is developed, DN is even more difficult to treat than other kidney diseases (3). Thus, the early prevention of DN is important for diabetes management. However, the current methodology is still insufficient for timely identification of disease onset and prognosis in DN.

Long non-coding RNAs (lncRNAs) are transcripts that measure more than 200 nucleotides with limited protein-coding ability (4). The effects of lncRNAs have been revealed in the pathogenesis of various human diseases including diabetes (5). Several lncRNAs, including lnc-*Rpph1*, lnc-*MEG3* and lnc-*CYP4B1-PS1-001*, are known to regulate fibrosis and inflammation in DN (3,6,7). Our previous study selected five candidate lncRNAs from lncRNA microarray data and found that lncRNA *Dlx6os1* was highly expressed in high glucose-treated mouse mesangial cells (MMCs) (8). The next stage of the investigation observed that inhibition of lncRNA *Dlx6os1* suppressed proliferation and fibrosis but promoted apoptosis in MMCs of the DN cellular model (8). lncRNA *Dlx6os1* is a novel gene that has not been studied in DN, despite the aforementioned evidence from our previous study (8). The present study further analyzed the effect of

lncRNA *Dlx6os1* suppression on the mRNA expression profile in MMCs of a DN cellular model to explore the mechanism of lncRNA *Dlx6os1* inhibition in the pathogenesis of DN.

Materials and methods

Construction of DN cellular model. lncRNA *Dlx6os1* exhibits good homology between mice and humans. A DN cellular model was constructed according to the method described in our previous study (8). In brief, SV40 MES13 cells (MMCs) were purchased from Shanghai Institutes for Biological Sciences and were cultured in Dulbecco modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator with 5% CO_2 at 37˚C. Then 30 mmol/l glucose was added to treat SV40 MES13 cells for 96 h to construct the DN cellular model.

Construction and transfection of short hairpin (sh)RNA plasmids. Negative control (NC) shRNA and lncRNA *Dlx6os1* shRNA plasmids were constructed by Genewiz, Inc. and transfected into a DN cellular model (SV40 MES13 cells under 30 mmol/l glucose culture), then termed the sh-NC group and sh-lncRNA group. Subsequently, i) lncRNA *Dlx6os1* expression in the two groups was detected by reverse transcription-quantitative (RT-q) PCR at 48 h; ii) proliferation ability was detected by Cell Counting Kit-8 (Sigma-Aldrich; Merck KGaA) at 0, 24, 48 and 72 h according to the manufacturers' instructions; iii) the mRNA and protein expression levels of proliferative markers (cyclin D1 and proliferating cell nuclear antigen) as well as fibrosis markers (fibronectin and collagen I) were detected by RT-qPCR and western blotting at 48 h; iv) the cell apoptosis rate was detected by FITC Annexin V Apoptosis Detection kit II with propidium iodide (AV/PI; BD Biosciences) at 48 h according to the manufacturer's protocol (Fig. S1). Data were analyzed using a flow cytometer (BD FACSCalibur) with FlowJo 7.6 software (FlowJo, LLC). A wavelength of 488/535 nm was used.

mRNA sequencing. mRNA sequencing was performed by Genergy Bio Company (http://www.genenergy.cn/). In brief, cells were harvested at 48 h after transfection, and total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), then RNA concentration, purity and integrity were assessed. Subsequently, mRNA was captured by Dynabeads Oligo $(dT)_{25}$ (Thermo Fisher Scientific, Inc.), then first and second strands of the cDNA were synthesized and library fragments were purified using AMPure XP system (Beckman Coulter, Inc.). PCR assay and assessment of library quality were conducted using Bioanalyzer 2100 system (Agilent Technologies, Inc.), then clustering of index-coded samples was performed using HiSeq PE Cluster kit v4 cBot (Illumina, Inc.), and the libraries were sequenced on Illumina Hiseq X10 platform (Illumina, Inc.). A total of 150 bp paired-end reads was produced after cluster generation for mRNA. Automated quality control and adapter trimming were conducted using Trim Galore, Cutadapt and FastQC (9). Then the trimmed reads were mapped to the mouse genome mm10 by Hisat2 (Version 2.1.0; http://ccb.jhu.edu/software/hisat2/manual.shtml), and mapping quality control was conducted using RSeQC (Version 3.0.0; http://rseqc.sourceforge.net/). Finally, the read counts of mRNA were then calculated using FeatureCounts (Rsubread; http://www.bioconductor.org). The top 20 dysregulated mRNAs are presented in Table I as a reference for future studies that investigate specific mRNA targeted by lncRNA *D1x6os1* in DN.

Bioinformatics. The mRNAs which were identified in ≥50% samples were included in the analysis and raw read counts were normalized and logarithmically transformed for bioinformatics analysis. The bioinformatics analysis was conducted using R software (Version 3.3.3; http://www.R-project.org/) (10). In brief, i) differentially expressed mRNAs were detected using DeSeq2 package (Version 1.12.3; http://www.bioconductor.org/), and statistical significance was defined as adjusted P-value <0.05; the biological significance was defined as a difference of at least abs [$\log_2$ (fold change)]>2, which was presented as a Volcano plot; ii) Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed using Database for Annotation, Visualization and Integrated Discovery web servers; iii) gene-set enrichment analysis (GSEA) on apoptosis and inflammation pathways was performed using the phenoTest package (11). The datasets of the present study were uploaded on Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) for public availability. The GSE number is GSE145301.

Statistical analysis. Statistical analysis and figures were performed using GraphPad Prism 7.00 (GraphPad Software, Inc.). Comparison between two groups was determined by paired t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

lncRNA D1x6os1 expression following transfection. In order to detect the relative expression of lncRNA *D1x6os1* after transfection, qPCR was conducted at 48 h. lncRNA *D1x6os1* expression was decreased in the sh-lncRNA group compared with the sh-NC group following transfection (P<0.001), which indicated successful transfection under high glucose exposure (Fig. 1).

Effect of lncRNA D1x6os1 silencing on cell proliferation, fibrosis and apoptosis in MMCs of a DN cellular model. Cell proliferation, fibrosis and apoptosis were determined to assess the influence of lncRNA *D1x6os1* silencing. Cell proliferation was reduced in the sh-lncRNA group compared with the sh-NC group at 24 (P<0.05), 48 (P<0.05), and 72 h (P<0.01; Fig. 2A). The relative expression levels of cyclin D1 (P<0.05), PCNA (P<0.01), FN (P<0.05) and collagen I (P<0.05) were suppressed in the sh-lncRNA group compared with the sh-NC group (Fig. 2B and C). In addition, the cell apoptosis rate was increased in the sh-lncRNA group compared with the sh-NC group (P<0.001; Fig. 2D).

Volcano plot of differentially expressed mRNAs. A Volcano plot is an intuitive plot that screens out the differentially expressed genes between the two group of samples. In the

Table I. Top 20 dysregulated mRNAs (10 upregulated and 10 downregulated) in sh-lncRNA cells vs. sh-NC cells.

Gene symbol	ID	Chromosome	Log_2 FC	P-value	Trend
Ramp2	ENSMUSG00000001240	11	2.078303	$4.52x10^{-6}$	Up
Tagln	ENSMUSG00000032085	9	2.016348	$6.7x10^{-6}$	Up
Krt7	ENSMUSG00000023039	15	1.749297	$2.09x10^{-5}$	Up
Aifm3	ENSMUSG00000022763	16	1.757504	$3.1x10^{-5}$	Up
Sult1c2	ENSMUSG00000023122	17	1.781638	$3.4x10^{-5}$	Up
1700001P01Rik	ENSMUSG00000018543	11	5.796657	$3.42x10^{-5}$	Up
Foxp2	ENSMUSG00000029563	6	2.688633	$4.09x10^{-5}$	Up
Sirt2	ENSMUSG00000015149	7	2.458172	$4.93x10^{-5}$	Up
Sep15	ENSMUSG00000037072	3	2.153584	$7.67x10^{-5}$	Up
Slc39a4	ENSMUSG00000063354	15	3.04629	$8.55x10^{-5}$	Up
Ccdc160	ENSMUSG00000073207	X	-6.79158	$4.98x10^{-7}$	Down
Myom1	ENSMUSG00000024049	17	-2.09334	$6.97x10^{-6}$	Down
Lmbr1l	ENSMUSG00000022999	15	-2.19777	$8.64x10^{-6}$	Down
Ddx43	ENSMUSG00000070291	9	-1.72587	$1.94x10^{-5}$	Down
Vwa5b2	ENSMUSG00000046613	16	-2.63226	$2.39x10^{-5}$	Down
Slc11a1	ENSMUSG00000026177	1	-5.77347	$2.84x10^{-5}$	Down
Ccdc7b	ENSMUSG00000056018	8	-4.83542	$4.45x10^{-5}$	Down
Fmn1	ENSMUSG00000044042	2	-1.85445	$4.85x10^{-5}$	Down
Fer1l4	ENSMUSG00000013338	2	-5.69906	$5.37x10^{-5}$	Down
As3mt	ENSMUSG00000003559	19	-1.94909	$5.43x10^{-5}$	Down

Ten upregulated and ten downregulated mRNAs were selected according to the rank of the P-value. sh, short hairpin; lnc, long non-coding; NC, negative control; FC, fold change.

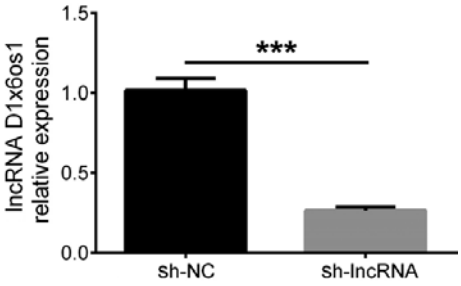

Figure 1. Silencing of lncRNA *D1x6os1*. The relative expression of lncRNA *D1x6os1* was reduced after lncRNA *Dlx6os1* silencing. Silencing was performed by lncRNA *Dlx6os1* shRNA plasmid transfection to SV40 MES13 cells under 30 mmol/l glucose culture. ***P<0.001; lnc, long non-coding; sh, short hairpin; NC, negative control.

present study, it revealed 423 upregulated mRNAs and 438 downregulated mRNAs in the sh-lncRNA group compared with the sh-NC group in MMCs of a DN cellular model (absolute value of FC>2, P_{adj}<0.05; Fig. 3). The top 20 dysregulated mRNAs are presented in Table I.

GO and KEGG enrichment analysis of differentially expressed mRNAs. To evaluate the possible mechanisms of dysregulated mRNAs in response to lncRNA *D1x6os1* silencing, GO and KEGG enrichment analyses were conducted. GO enrichment analysis revealed that the differentially expressed mRNAs were mainly enriched in biological processes including 'apoptotic process', 'inflammatory response' and 'cytoskeleton organization'; and were mainly present in cellular components such as 'integral component of membrane', 'cell surface' and 'extracellular space'; in addition, they were involved in molecular functions including 'cytokine receptor activity', 'phospholipase binding' and 'cysteine-type endopeptidase activity' (Fig. 4A). According to KEGG enrichment analysis, the dysregulated mRNAs were mainly enriched in inflammation and apoptosis-related pathways such as 'NF-κB signaling pathway', 'cytokine-cytokine receptor interaction' and apoptosis pathways (Fig. 4B).

GSEA. Since apoptosis and inflammation are the two main pathological mechanisms of DN, GSEA of apoptosis and inflammation was performed. In GSEA analysis of apoptosis, the leading-edge subset of mRNAs were overexpressed in sh-lncRNA cells compared with sh-NC cells according to the location of the maximum enrichment score (Fig. 5A). While in GSEA analysis of inflammation, the leading-edge subset of mRNAs were downregulated in sh-lncRNA cells compared with sh-NC cells (Fig. 5B). These results indicated that lncRNA *D1x6os1* inhibition promoted cell apoptosis and suppressed inflammation in MMCs of a DN cellular model.

Discussion

As a progressive microvascular complication, DN is heralded by a subsequent reduction in glomerular filtration rate and marked by microalbuminuria (12). The renal inflammation underlies the pathogenic mechanism of DN (13). Principle

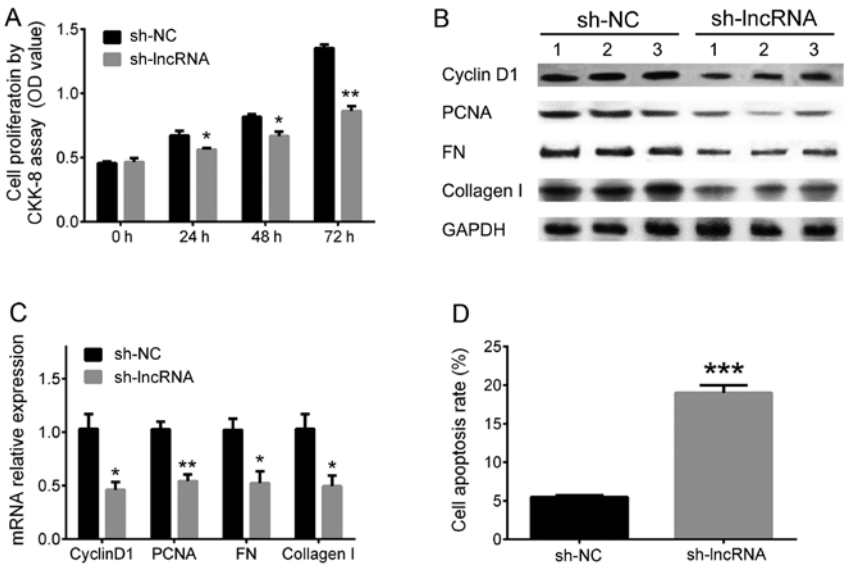

Figure 2. Effect of lncRNA *D1x6os1* silencing on cell proliferation, fibrosis and apoptosis in MMCs of a DN cellular model. lncRNA *D1x6os1* silencing (A) inhibited cell proliferation, (B and C) reduced proliferative markers and fibrosis markers, and (D) promoted cell apoptosis. Silencing was performed by lncRNA *Dlx6os1* shRNA plasmid transfection to SV40 MES13 cells under 30 mmol/l glucose culture. *P<0.05, **P<0.01 and ***P<0.001 vs. sh-NC. lnc, long non-coding; MMCs, mouse mesangial cells; DN, diabetic nephropathy; sh, short hairpin; NC, negative control.

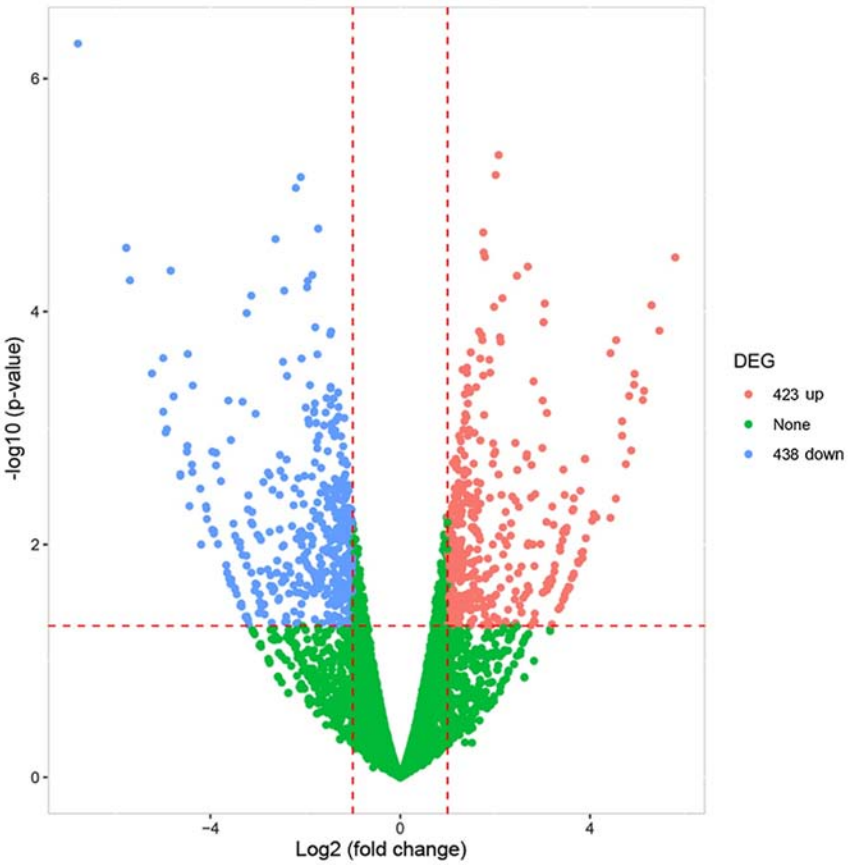

Figure 3. Volcano plot of mRNA expression profile. The blue dots represent downregulated mRNAs, red dots represent upregulated mRNAs and green dots represent mRNAs that were not differentially expressed in the sh-lncRNA group vs. the sh-NC group in MMCs of the DN cellular model. There were 438 downregulated and 423 upregulated mRNAs in the sh-lncRNA group vs. the sh-NC group. sh, short hairpin; lnc, long non-coding; MMCs, mouse mesangial cells; DN, diabetic nephropathy; NC, negative control; DEG, differentially expressed genes.

inflammatory cells such as macrophages are responsible for inflammation response via the release of cytokines, chemokines and reactive oxygen species, which eventually increase the production of extracellular matrix in glomeruli as well as progressive tubulointerstitial fibrosis (13). In addition, T-lymphocyte activation induced by hyperglycemia induces

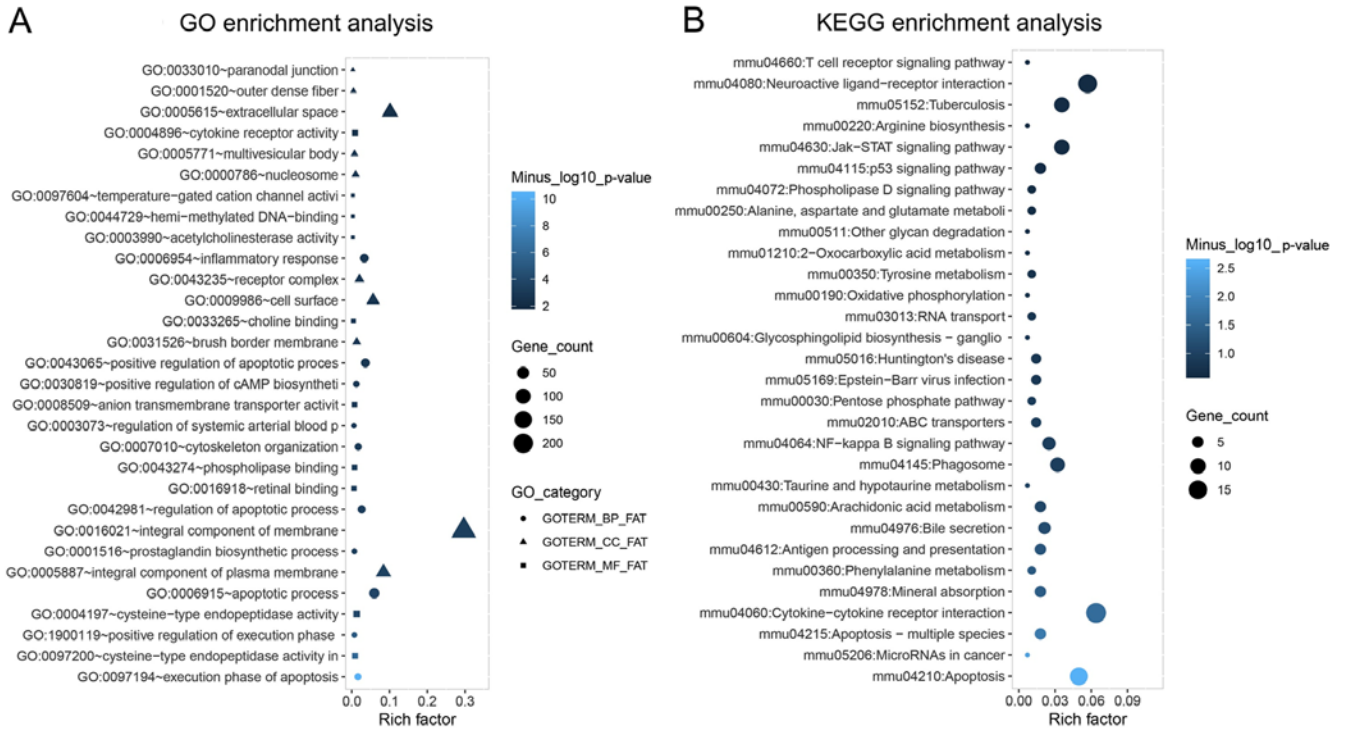

Figure 4. GO and KEGG enrichment analysis of differentially expressed mRNAs. (A) GO enrichment analysis of differentially expressed mRNAs revealed the BP, CC and MF in which these mRNAs were enriched. (B) KEGG enrichment analysis of differentially expressed mRNAs revealed the possible pathways in which these mRNAs were enriched. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological processes; CC, cellular components; MF, molecular functions.

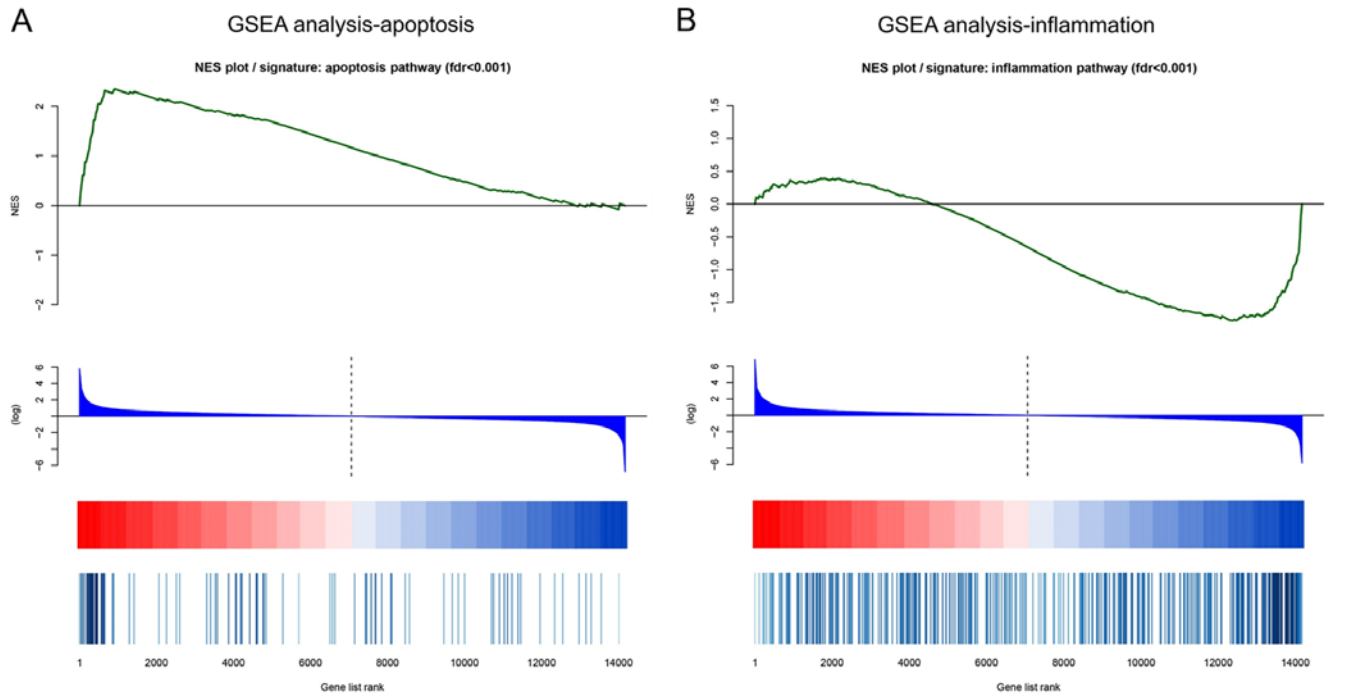

Figure 5. GSEA. GSEA of (A) apoptosis pathway and (B) inflammation pathway revealed that lncRNA D1x6os1 inhibition promoted cell apoptosis and suppressed inflammation. GSEA, gene-set enrichment analysis; fdr, false discovery rate.

kidney damage in the early stages by disturbing albumin glomerular excretion as well as renal infiltration (14). Apoptosis-induced dysfunction of podocytes and damage to mesangial cells and renal tubular epithelial cells are indicators of glomerular filtration dysfunction (15-18). Although the pathology and pathophysiology of DN have been investigated for some time and there has been progress, the complex molecular network is still not fully understood and identification of

genes or molecules by conventional tools remains deficient. Therefore, screening of genes and molecules by novel strategies, such as high-throughput platforms are necessary for determining valuable information on the numerous molecules involved in DN pathology.

From the published microarray data, lncRNA expression profile is critical in the pathogenesis and progression of DN (3,19). Previous studies report that several lncRNAs regulate inflammation and cell proliferation in DN (3,19). For instance, lncRNA *Rpph1* was demonstrated to facilitate inflammation and promote proliferation of MMCs by interacting with DN-related factor galectin-3 in a DN cellular model (3). Knockdown of lncRNA *Gm4419* was revealed to inhibit pro-inflammatory cytokine expression and renal fibrosis biomarkers and reduce cell proliferation of MMCs under high glucose conditions (19). lncRNA *Dlx6os1* was upregulated in the kidneys of diabetic mice and its expression was induced under high glucose conditions (20). Therefore, in order to mimic the DN environment, SV40 MES13 cells (MMCs) cultured under high glucose conditions were used for the transfection of lncRNA *Dlx6os1* inhibitor plasmids in our previous study (8). In the present study, the same DN cellular model derived from MMCs was constructed, and it was confirmed that lncRNA *Dlx6os1* silencing suppressed cell proliferation and fibrosis and promoted cell apoptosis in MMCs of the DN cellular model. The following theories may account for these effects: i) lncRNA *Dlx6os1* silencing may influence the expression of target mRNAs and attenuate the cell cycle to suppress cell proliferation and facilitate apoptosis. It was demonstrated in the following GSEA analysis of apoptosis that lncRNA *Dlx6os1* silencing promoted cell apoptosis in MMCs of the DN cellular model. The reduced cell apoptosis may attenuate the mesangial expansion as well as formation of glomerulosclerosis in DN (21). ii) Inhibition of lncRNA *Dlx6os1* may suppress inflammation by blocking signaling pathways such as the NF-κB pathway, interfering with cytokine-cytokine receptor interaction (as demonstrated in the KEGG enrichment analysis) and reducing the inflammatory cytokines, as well as reactive oxygen species, leading to reduced secretion of the extracellular matrix and subsequent fibrosis.

Since the pathogenic mechanism of DN is complex and involves multiple molecular pathways, a more refined screening of the genetic background of lncRNA *Dlx6os1* will certainly provide a greater insight into the disease. Therefore, additional RNA sequencing for the mRNA expression profile in lncRNA *Dlx6os1*-silenced MMCs of the DN cellular model was performed. The mRNA expression profile revealed that lncRNA *Dlx6os1* silencing led to 423 upregulated mRNAs and 438 downregulated mRNAs in MMCs of the DN cellular model. The KEGG enrichment analyses revealed that the differentially expressed mRNAs were enriched in apoptotic and inflammatory responses/pathways. GSEA analysis of apoptosis and inflammation pathways was also performed and suggested that lncRNA *Dlx6os1* silencing promoted cell apoptosis and inhibited inflammation in MMCs of the DN cellular model. These observations supplemented the potential molecular mechanism of lncRNA *Dlx6os1* in the pathogenesis of DN to some extent. However, deeper exploration of downstream mRNAs of lncRNA *Dlx6os1* in regulating cell proliferation, fibrosis and cell apoptosis in DN is still required.

There were several limitations to the present study: i) The effect of lncRNA *Dlx6os1* silencing on the mRNA expression profile was evaluated by RNA sequencing, which is high-throughput and lacks accuracy (22). Therefore, detecting individual mRNA expression using qPCR and corresponding protein expression using western blotting may be helpful to validate the influence of lncRNA *Dlx6os1* on mRNA expression in MMCs of the DN cellular model. ii) Although commonly used in DN research, knocking down an lncRNA in a short-term as well as an acute kidney damage cellular model does not substantially represent the real DN condition as a chronic disease. Therefore, further animal study is required to validate the results of the present study. iii) lncRNA *Dlx6os1* was revealed to regulate apoptosis and inflammation in MMCs of the DN cellular model, whereas the clinical implications, including its correlation with disease risk, disease progression or prognosis of DN patients, remains to be elucidated, which may be a valuable research direction for diabetic diseases in the future. iv) The present study only reported general information concerning lncRNA Dlx6os1 silencing on biological processes and molecular functions such as apoptosis and inflammation in DN, whereas the detailed pathways or the protein markers were not investigated.

In conclusion, lncRNA *Dlx6os1* silencing attenuated disease progression by regulating cell apoptosis and inflammation-related pathways in MMCs of a DN cellular model, suggesting that it could assist with developing a novel treatment target for DN.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SH conceived and designed the study. LC and JC collected the data. WP and XJ performed the experiments and drafted the manuscript. All authors reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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