LncRNA NEAT1/microRNA-124 regulates cell viability, inflammation and fibrosis in high-glucose-treated mesangial cells

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Abstract. Long non-coding RNA (lncRNA) nuclear enriched abundant transcript 1 (NEAT1) has been frequently found to be dysregulated, which contributes to diabetes-related complications. The present study aimed to explore the effect of knockdown on mouse mesangial cell (MMC) viability, apoptosis, inflammation and fibrosis in an *in vitro* model of diabetic nephropathy (DN). The SV40 MES13 MMC cell line was first cultured with high glucose to establish an *in vitro* MMC DN cell model. Lnc-NEAT1 shRNA or the negative control shRNA were transfected into MMC DN cells, followed by the measurement of cell viability, apoptosis, inflammation, fibrosis and microRNA (miR)-124 expression, a known target of lnc-NEAT1, using Cell Counting Kit-8, flow cytometry, ELISA, western blotting [Capain1 (capn1), β-catenin (CTNNB1), cleaved caspase 3, cleaved poly-(ADP ribose) polymerase, fibronectin and Collagen] and reverse transcription-quantitative PCR (Capn1, CTNNB1, lnc-NEAT1, fibronectin, collagen and miR-124), respectively. In rescue experiments, the miR-124 and negative control inhibitor were co-transfected into lnc-NEAT1-downregulated cells, following which cell viability, apoptosis, inflammation, fibrosis, capn1 and CTNNB1 expression were measured. Lnc-NEAT1 expression was increased in high glucose-treated cells compared with that in normal glucose-treated cells and osmotic control cells, suggesting that lnc-NEAT1 is overexpressed in the MMC DN cell model. In the MMC DN cell model, lncRNA-NEAT1 knockdown enhanced cell apoptosis but reduced cell viability and the secretion of inflammatory cytokines in the supernatant (IL-1β, IL-8, monocyte chemotactic protein 1 and TNF-α), in addition to reducing the expression of fibrosis markers fibronectin and collagen I in the lysates. Lnc-NEAT1 knockdown increased miR-124 expression. Furthermore, transfection with the miR-124 inhibitor reduced cell apoptosis but increased cell viability, inflammation and fibrosis in lnc-NEAT1-downregulated MMC DN cells. miR-124 inhibitor transfection also increased the expression levels of Capn1 and CTNNB1. Taken together, the findings of the present study demonstrated that lnc-NEAT1 knockdown was able to attenuate MMC viability, inflammation and fibrosis by regulating miR-124 expression and the Capn1/β-catenin signaling pathway downstream. Therefore, Lnc-NEAT1 may serve as a potential therapeutic target for DN.

Introduction

Diabetic nephropathy (DN) is a common and serious complication of diabetes, which occurs in ~40% patients with diabetes (1,2). DN typically presents with proteinuria followed by a progressive deterioration in renal function (2). In terms of the DN pathology, thickening of the glomerular basement membrane, hypertrophy of glomerular cells and loss of podocytes are the major pathological changes that can be observed (2). Although a number of therapeutic agents are available, including angiotensin-converting enzyme inhibitors and anti-hyperglycemic drugs, the management of DN remains challenging. A deeper understanding in the underlying mechanism of DN pathogenesis may provide guidance for developing novel prevention and treatment strategies for DN.

Accumulating evidence has reported the involvement of epigenetic factors in DN, including long non-coding RNAs (lncRNAs), which are important RNAs that do not enocde proteins but can regulate gene expression on various levels (3-5). Previous studies revealed that the lncRNA nuclear enriched abundant transcript 1 (lnc-NEAT1) may regulate the pathology of several diabetic complications or kidney injuries, including the promotion of hypoxia-induced apoptosis in renal tubular epithelial cells and the aggravation of myocardial ischemia/reperfusion (I/R) injury in diabetic rats (6,7). However, the molecular mechanism of lnc-NEAT1 in DN has not been fully elucidated. According to previous studies, microRNA (miRNA or miR)-124 is a target of lnc-NEAT1 in Aβ-induced

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cellular model of Alzheimer's disease (neurons) and in sepsis (renal cells) (6,7). In addition, miR-124 is predicted to target calpain (Capn)1 in the human neural cell line HCN-2 (8). Capn1 belongs to the Capn family of protease enzymes, the silencing of which can relieve adipose tissue inflammation and fibrosis in a diet-induced mouse model of obesity. Capns are able to regulate the β-catenin (CTNNB) signaling pathway by modifying N-terminal truncation of β-catenin to regulate a multitude of cellular functions, including proliferation and epithelial-mesenchymal transition (9-14). Therefore, it was hypothesized that lnc-NEAT1 is involved in mediating the DN pathology, such that its potential regulatory effects may include miR-124 and the Capn1/CTNNB signaling pathway downstream. Considering that excessive proliferation of glomerular mesangial cells is a main pathological characteristic of DN (2), mouse mesangial cells (MMCs) were treated with high glucose to establish an *in vitro* MMC DN cell model in the present study. To validate the aforementioned hypothesis, the effect of lnc-NEAT1 knockdown on cell viability, inflammation and fibrosis in the MMC DN cell model was explored, where the influence of lnc-NEAT1 knockdown on miR-124 expression and Capn1/CTNNB signaling was investigated.

Materials and methods

Cell line and culture. The MMC cell line SV40 MES13 was purchased from American Type Culture Collection. The SV40 MES13 cells were divided into the following three groups: i) High-glucose-treated cells (HG cells), where SV40 MES13 cells were cultured in 95% DMEM/F-12 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 30 mmol/l glucose, 14 mM HEPES and 5% FBS (Sigma-Aldrich; Merck KGaA); ii) normal-glucose-treated cells (NG cells), where SV40 MES13 cells were grown in 95% DMEM/F-12 medium supplemented with 5.6 mmol/l glucose, 14 mM HEPES and 5% FBS; and iii) osmotic control cells (OC cells), where SV40 MES13 cells were incubated in 95% DMEM/F-12 medium supplemented with 5.6 mmol/l glucose, 24.4 mmol/l 3-O-methyl-D-glucose (Biosynth Carbosynth.), 14 mM HEPES and 5% FBS (15). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. All cell media were renewed every 2 days. After 96 h of culture at 37°C, lnc-NEAT1 expression in the cells was measured by reverse transcription-quantitative PCR (RT-qPCR).

Construction and transfection of the lnc-NEAT1 and control knockdown plasmids. Lnc-NEAT1 knockdown (lnc-KD) and the negative control knockdown (NC-KD) shRNA plasmids were constructed using the pGPU6 vector (Shanghai GenePharma Co., Ltd.). In total, 0.8 μg lnc-KD plasmids or 0.8 μg NC-KD plasmids were then transfected into HG-treated (96 h at 37°C) SV40 MES13 cells using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.), producing the Lnc-KD and NC-KD groups, respectively. lnc-NEAT1 and miR-124 expression in these two groups was measured using RT-qPCR 24 h after transfection. The shRNA sequences were: lnc-NEAT1 (accession no. NR_003513.3) sense 5'-CAC CGGGCCTGTGAAAGCATTAACGAATTAATGCTTTCA CAGGCCC-3' and antisense, 5'-AAAAGGGCCTGTGAA AGCATTAATTCGTTAATGCTTTCACAGGCCC-3' and

NC sense, 5'-CACCGCTTAAGTTTGTTGTGTTGTTAT GTCGAAACATAACAACACAACAAACTTAAGC-3' and antisense, 5'-AAAAGCTTAAGTTTGTTGTGTTGTTAT GTTTCGACATAACAACACAACAAACTTAAGC-3'. The shRNA was designed by RNAi Designer (https://rnaidesigner. thermofisher.com/rnaiexpress/design.do).

Cell Counting Kit-8 (CCK-8) assay. Cell viability in the different treatment groups (4x10^3 cells per well) was assessed at 0, 24, 48 and 72 h using CCK-8 assay (Sigma-Aldrich; Merck KGaA) by following the protocol of the manufacturer at 37°C. The cells were incubated with 10 μl CCK-8 reagent mixed with 100 μl DMEM/F-12 medium at 37°C for 2 h. Absorbance in each well was measured at 450 nm with the use of a microplate reader (Bio-Rad Laboratories, Inc.). Cell viability was calculated according to the optical density value.

ELISA. Inflammatory cytokines in the supernatant, TNF-α (cat. no. BMS607-2INST), IL-1β (cat. no. BMS6002TEN), IL-8 (CXCL15; cat. no. EMCXCL15) and monocyte chemotactic protein 1 (MCP-1; cat. no. BMS6005) were measured at 48 h after transfection using ELISA kits (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Transfection with the miR-124 inhibitor and NC inhibitor. miR-124-inhibitor (5'-AUCAAGGUCCGCUGUGAACAC G-3'; 50 pM) and NC-inhibitor (5'-AAGAACAACACA AAGAACAG-3'; 50 pM) were purchased from Shanghai GenePharma Co., Ltd., which were respectively transfected into lnc-KD-transfected and HG-treated SV40 MES13 cells with the use of Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.). This produced the lnc-KD + NC-inhibitor and lnc-KD + miR-124-inhibitor groups. Lnc-NEAT1 and miR-124 expression was assessed at 24 h after transfection. In addition, cell viability was evaluated at 0, 24, 48 and 72 h after transfection at 37°C. In addition, cell apoptosis, fibrosis markers and inflammatory cytokines, in these two groups were assessed at 48 h after transfection. The expression of Capn1 and CTNNB1 was also determined by RT-qPCR and western blotting at 24 h after transfection.

Furthermore, 50 pM miR-124-inhibitor alone and 50 pM NC-inhibitor alone were transfected into HG-treated SV40 MES13 cells using the Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.), followed by detection of lnc-NEAT1 and miR-124 expression at 24 h after transfection.

RT-qPCR. Using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.), total RNA was extracted, followed by reverse transcription to form cDNA using PrimeScript™ RT Reagent Kit (Takara Bio, Inc.). The reverse transcription temperature protocol was as follows: 37°C for 20 min and 85°C for 5 sec. qPCR process was then performed using the QuantiNova SYBR Green PCR Kit (Qiagen GmbH). The following thermocycling conditions were conducted: 1 cycle, 95°C for 2 min; 40 cycles of 95°C for 5 sec and 61°C for 20 sec. GAPDH was used as the internal reference for lnc-NEAT1, fibronectin, collagen I, Capn1 and CTNNB1, whilst U6 was used as the internal reference for miR-124. The primer sequences used in RT-qPCR were as follows: miR-124-5p (miRBase: MIMAT0004527; https://www. mirbase.org/cgi-bin/mature.pl?mature_acc=MIMAT0004527)

Table I. Antibodies applied for western blotting in the present study.

Antibody	Supplier	Dilution	Cat. no.
Primary Antibody			
Rabbit monoclonal to Fibronectin	Abcam	1:3,000	ab45688
Rabbit polyclonal to Collagen I	Abcam	1:2,000	ab21286
Rabbit monoclonal to Calpain 1	Abcam	1:2,000	ab108400
Rabbit monoclonal to β-catenin	Abcam	1:5,000	ab32572
Rabbit monoclonal to Cleaved caspase 3	Cell Signaling Technology, Inc.	1:1,000	ab9664
Rabbit monoclonal to Cleaved poly-ADP ribose polymerase	Abcam	1:3,000	ab32064
Rabbit monoclonal to GAPDH	Abcam	1:10,000	ab181602
Secondary Antibody			
Goat Anti-Rabbit IgG H&L (HRP)	Abcam	1:15,000	ab6721

forward, 5'-ACACTCCAGCTGGGCGTGTTCACAGCG GA-3' and reverse, 5'-TGTCGTGGAGTCGGCAATTC-3'; U6 (accession no. NR_003027.2) forward, 5'-CTCGCT TCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGA ATTTGCGT-3'; lnc-NEAT1 (accession no. NR_003513.3) forward, 5'-TTGGGACAGTGGACGTGTGG-3' and reverse, 5'-TCAAGTGCCAGCAGACAGCA-3'; fibronectin (accession no. NM_010233.2) forward, 5'-TCAGTAGAAGGCAGTAGC ACAGA-3' and reverse, 5'-CCTCCACACGGTATCCAGACA -3'; collagen I (accession no. NM_007742.4) forward, 5'-CTC GTGGATTGCCTGGAACA-3' and reverse, 5'-GCACCAACA GCACCATCGT-3'; Capn1 (accession no. NM_001110504.1) forward, 5'-CGGTTGGAGGAGGTGGATGA-3' and reverse, 5'-GCGGATACGGTTGCTGACTT-3'; CTNNB1 (accession no. NM_001165902.1) forward, 5'-GCAGCGACTAAGCAG GAAGG-3' and reverse, 5'-TCAGATGACGAAGAGCACAGA TG-3'; and GAPDH (accession no. NM_008084.3) forward, 5'-AGGTTGTCTCCTGCGACTTCA-3' and reverse, 5'-GGT GGTCCAGGGTTTCTTACTC-3'. The results were calculated by $2^{-\Delta\Delta Cq}$ method (16).

Western blotting. Using RIPA buffer (Sigma-Aldrich; Merck KGaA), total protein was extracted, followed by determination of the protein concentration using a BCA Kit (Sigma-Aldrich; Merck KGaA). Next, 20 μg proteins were loaded onto NuPAGE™ 4-20% Tris-Acetate Midi Protein Gels (Thermo Fisher Scientific, Inc.), before electrophoresis was performed using the Mini-PROTEAN® Tetra Cell system (Bio-Rad Laboratories, Inc.). Subsequently, proteins were transferred onto polyvinylidene fluoride membranes. After being blocked with 5% BSA (Sigma-Aldrich; Merck KGaA) at 37˚C for 1 h, the membranes were incubated with primary antibodies at 4˚C overnight, followed by incubation with secondary antibodies at 37˚C for 1 h. The bands were visualized using Pierce™ ECL Plus Western Blotting Substrate (Invitrogen; Thermo Fisher Scientific, Inc.), X-ray films (Kodak) and Gel Imager (Thermo Fisher Scientific, Inc.). The antibodies used for western blotting are listed in Table I. The grey density evaluation was completed by ImageJ 1.8.0 (National Institutes of Health).

Apoptosis flow cytometry assay. Cell apoptosis was evaluated by Annexin V (AV)/PI assay at 48 h after transfection using the Annexin V-FITC Apoptosis Detection Kit (R&D Systems, Inc.) according to the protocol of the manufacturer. Cells (1x10^6) were suspended in 100 μl binding buffer and 5 μl AV was subsequently added at room temperature. After digestion with pancreatin (Thermo Fisher Scientific, Inc.) at 37˚C for 2 min and washes with PBS, the samples were then incubated at room temperature for 15 min in the dark in the presence of 5 μl PI. Next, flow cytometry was performed to detect cells stained with AV and PI using a BD FACScalibur flow cytometer (BD Biosciences). The total apoptosis rate (early + late) was measured by FlowJo 7.6 (FlowJo LLC) in this study.

Statistical analysis. Data are presented as the mean ± standard deviation. Unpaired Student's t-test was used to determine the differences between two groups, whilst one-way ANOVA followed by Tukey's or Dunnett's post hoc test was used for multiple comparisons. Data analysis was performed with GraphPad 7.01 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference. All assays were repeated three times.

Results

Lnc-NEAT1 expression and the effects of its knockdown on cell viability and apoptosis in the MMC DN cell model. Cell viability was significantly increased in HG cells compared with that of the OC and NG cells (Fig. 1A). In addition, both the mRNA (Fig. 1B) and protein expression levels (Fig. 1D and E) of Capn1 were significantly increased in HG cells compared with those in OC and NG cells. The mRNA (Fig. 1C) and protein expression levels (Fig. 1D and E) of CTNNB1 were also significantly increased in HG cells compared with those in the OC and NG cells. This observed increased viability and expression of fibrotic markers suggests that the establishment of the MMC DN cell model was successful.

Lnc-NEAT1 expression was subsequently detected in the three groups of MMC SV40 MES13 cells (OC, NG and HG cells). As shown in Fig. 1F, lnc-NEAT1 expression was
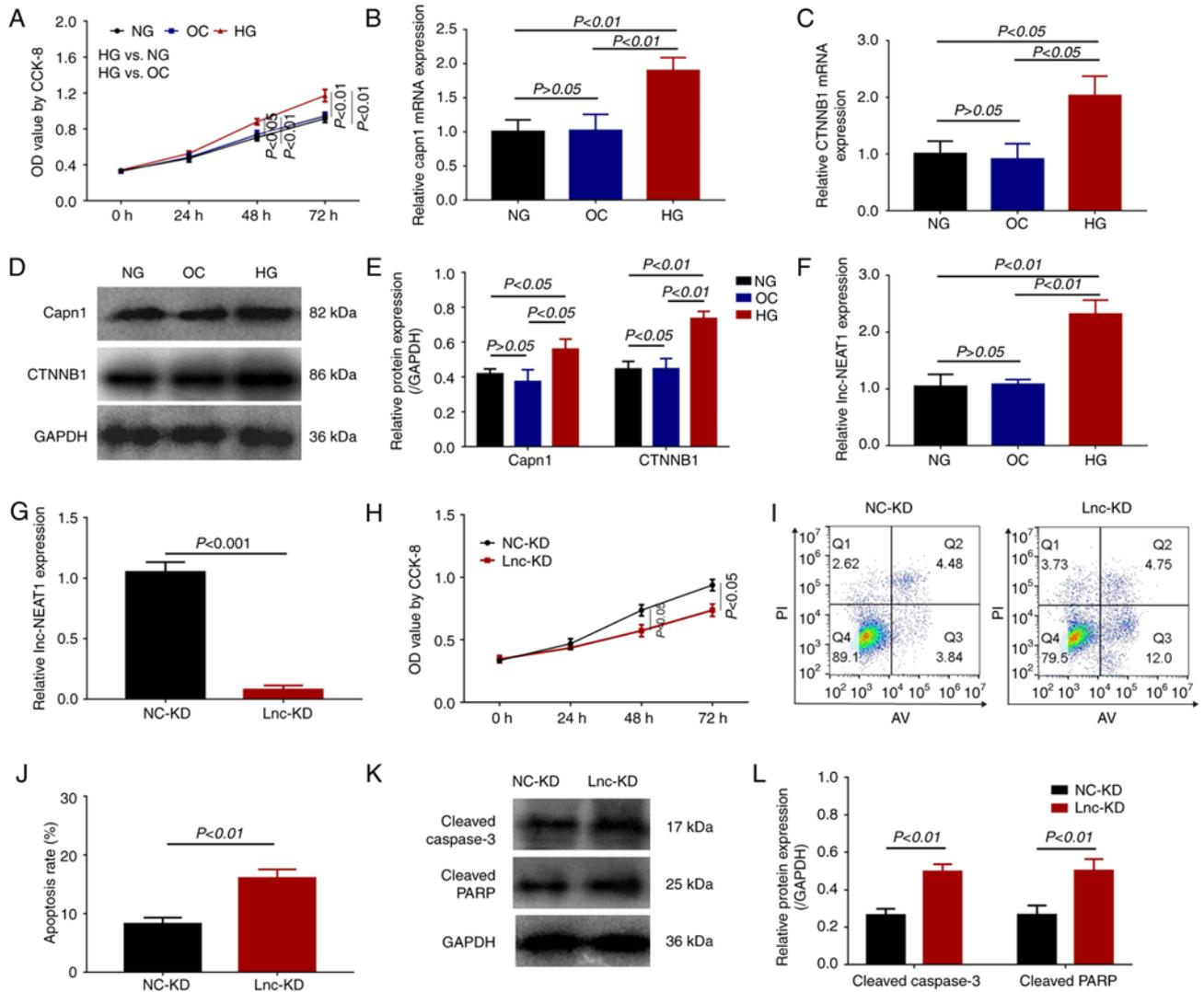
Figure 1. Lnc-NEAT1 expression, cell viability and apoptosis in lnc-NEAT1-downregulated MMC DN cells. (A-F) MMC cells were treated with either HG or NG. (A) Cell viability was measured using CCK-8 assay. (B) Capn1 and (C) CTNNB1 mRNA expression was measured by RT-qPCR. (D) Capn1 and CTNNB1 protein expression was measured by western blotting, (E) which was quantified. (F) Lnc-NEAT1 expression was measured in HG cells by RT-qPCR. (G-L) MMC cells were transfected with either lnc-KD or NC-KD plasmids after HG treatment. (G) Lnc-NEAT1 expression was measured by RT-qPCR after Lnc-NEAT1 knockdown. (H) Cell viability was measured using CCK-8 assay. (I) Cell apoptosis was measured using flow cytometry, (J) which was quantified. (K) Protein expression levels of cleaved caspase 3 and cleaved PARP in the lnc-KD and NC-KD groups were measured by western blotting, (L) which was quantified. Lnc-NEAT1, long non-coding RNA nuclear enriched abundant transcript 1; NG cells, normal glucose-treated cells; HG cells, high glucose-treated cells; lnc-KD, lnc-NEAT1 knockdown; NC-KD, negative control knockdown; MMC, mouse mesangial cells; DN, diabetic nephropathy; RT-qPCR, reverse transcription-quantitative PCR; CCK-8, Cell Counting Kit-8; OD, optical density; OC, osmotic control; capn1, calpain 1; CTNNB1, β-catenin; PARP, poly-ADP ribose polymerase.

significantly higher in HG cells compared with that in the NG and OC cells. However, the levels of lnc-NEAT1 expression was similar between OC and NG cells, suggesting that lnc-NEAT1 was overexpressed in the MMC DN cell model.

At 24 h post-transfection of lnc-KD and NC-KD plasmids into the MMC DN cell model, lnc-NEAT1 expression was significantly reduced in the lnc-KD group compared with that in the NC-KD group (Fig. 1G). Cell viability was also significantly decreased in the lnc-KD group compared with that in the NC-KD group at both 48 and 72 h (Fig. 1H). In terms of cell apoptosis, the apoptosis rate was significantly enhanced in the lnc-KD group compared with that in the NC-KD group at 48 h (Fig. 1I and J). The protein expression levels of cleaved caspase 3 and cleaved PARP were significantly increased in the lnc-KD group compared with that in the NC-KD group (Fig. 1K and L).

These data suggest that lnc-NEAT1 knockdown can inhibit cell viability and promote cell apoptosis in the MMC DN cell model.

Effect of lnc-NEAT1 knockdown on inflammation and fibrosis in the MMC DN cell model. The expression levels of inflammatory cytokines IL-1β (Fig. 2A), IL-8 (Fig. 2B), MCP-1 (Fig. 2C) and TNF-α (Fig. 2D) were all significantly decreased in the lnc-KD group compared with those in the NC-KD group, suggesting that lnc-NEAT1 knockdown was able to reduce the inflammation levels in the MMC DN cell model.

The mRNA expression of the fibrosis markers fibronectin and collagen I was significantly reduced in the lnc-KD group compared with that in the NC-KD group (Fig. 2E and F). In addition, the protein expression of these fibrosis makers displayed similar trends as those of their mRNA counterparts
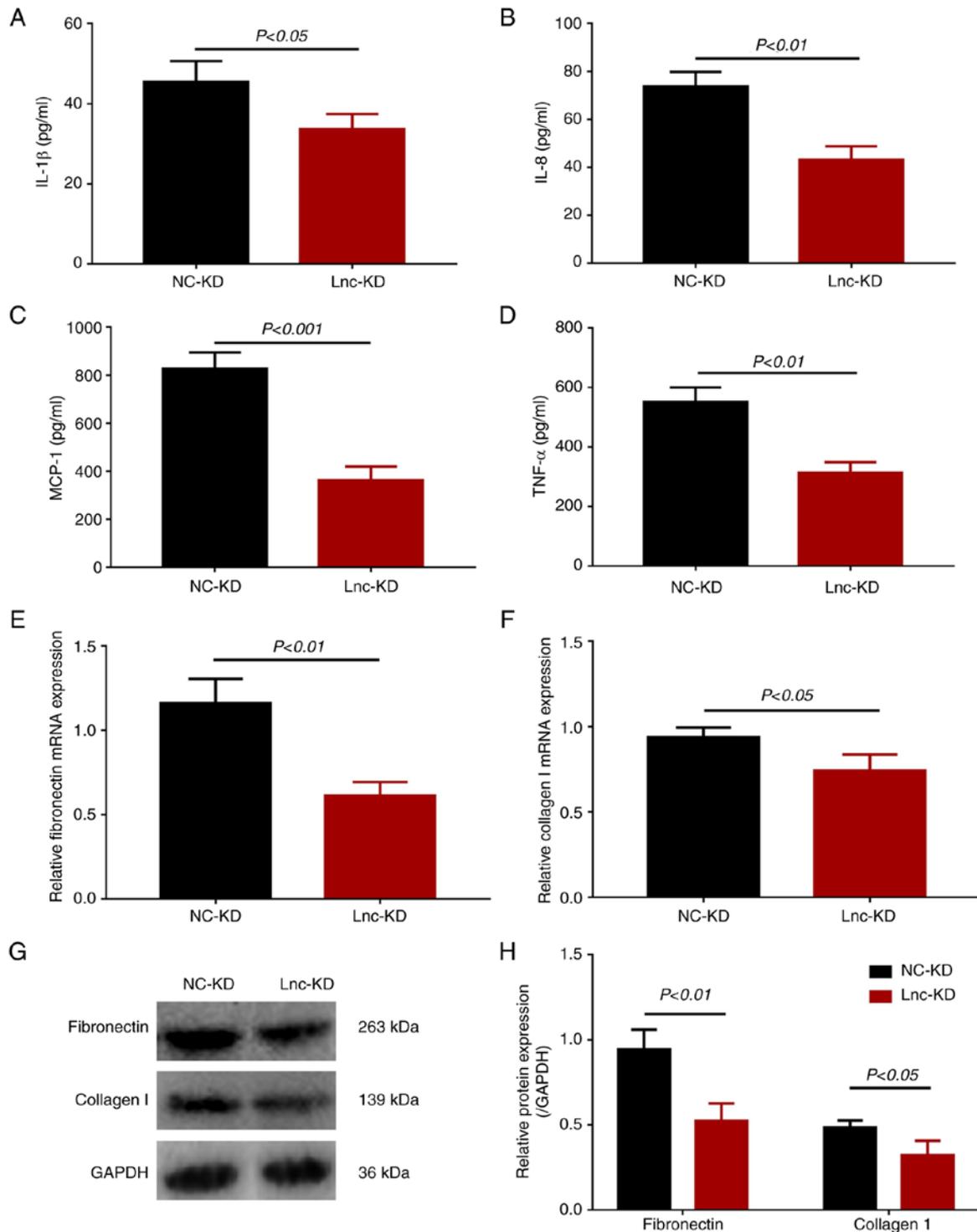
Figure 2. Secretion levels of inflammatory cytokines and expression of fibrosis markers in lnc-NEAT1-downregulated MMC DN cells. (A) IL-1β, (B) IL-8, (C) MCP-1, (D) TNF-α secretion into the cell supernatant of lnc-KD and NC-KD groups were measured by ELISA. (E) Fibronectin and (F) collagen I mRNA expression in the lnc-KD and NC-KD groups were measured by reverse transcription-quantitative PCR. (G) Fibronectin and collagen I protein expression was measured by western blotting, (H) which was quantified. lnc-NEAT1, long non-coding RNA nuclear enriched abundant transcript 1; lnc-KD, lnc-NEAT1 knockdown; NC-KD, negative control knockdown; MMC, mouse mesangial cell; DN, diabetic nephropathy; MCP-1, monocyte chemotactic protein 1.

(Fig. 2G and H). These results revealed that lnc-NEAT1 knockdown decreased fibrosis in the MMC DN cell model.

Cell viability, apoptosis, inflammation and fibrosis in miR-124 inhibitor rescue experiments. miR-124 was previously reported to be a target of lnc-NEAT1 (6,7). Therefore, its expression

levels were subsequently measured. miR-124 expression was found be significantly higher in the lnc-KD group compared with that in the NC-KD group (Fig. 3A), suggesting that miR-124 expression may be negatively regulated by lnc-NEAT1 in the MMC DN cell model. In subsequent assays, the miR-124 inhibitor was transfected into the lnc-NEAT1-downregulated
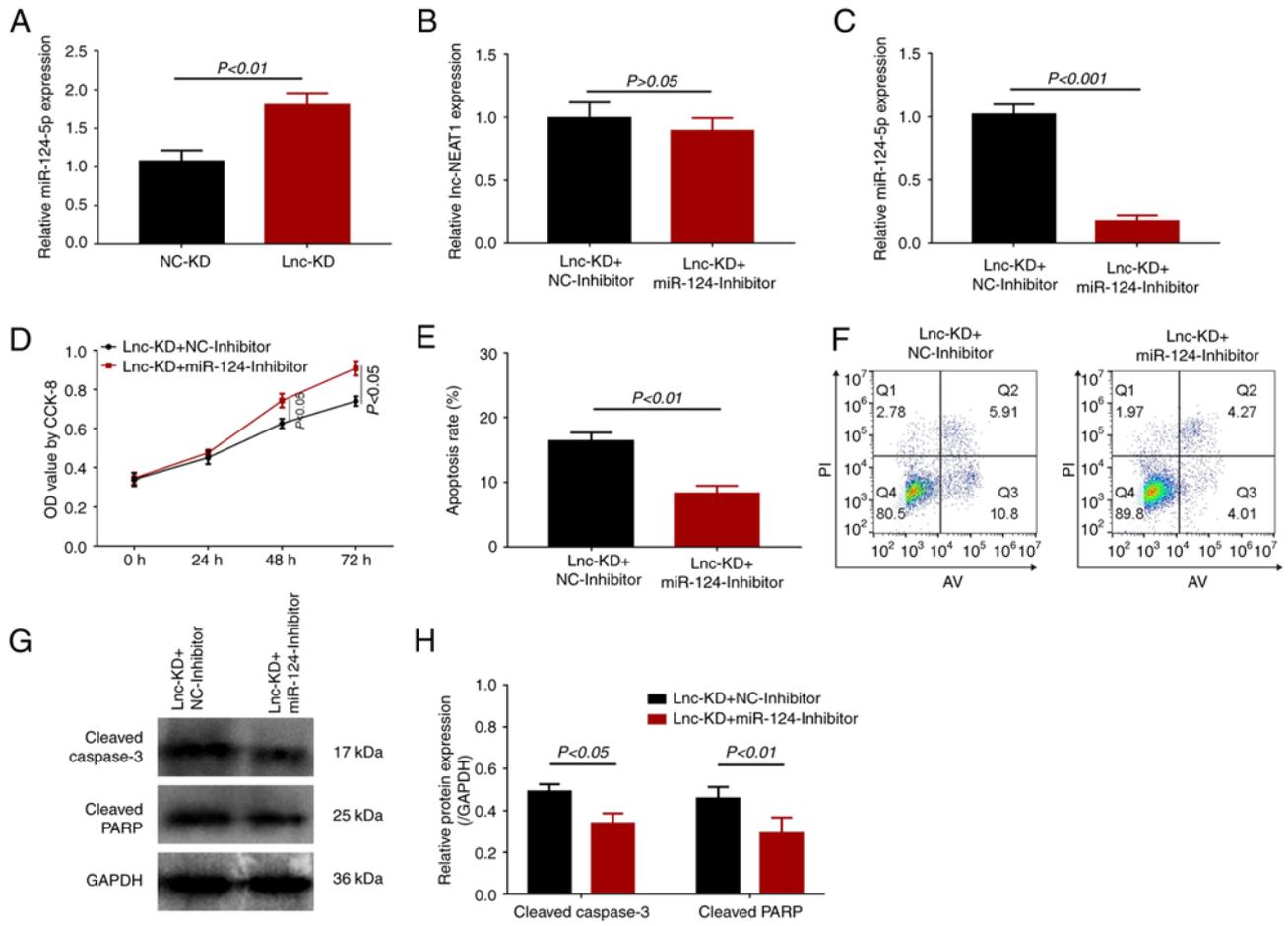
Figure 3. miR-124 expression and effect of miR-124 inhibition on cell viability and apoptosis in a lnc-NEAT1-downregulated MMC DN cell model. (A) miR-124 expression in the lnc-KD and NC-KD groups was measured by RT-qPCR. (B-H) The MMC DN cell model were transfected with the Lnc-KD or NC-KD plasmid and miR-124 inhibitor or NC-inhibitor. (B) Lnc-NEAT1 and (C) miR-124 expression was measured by RT-qPCR. (D) Cell viability was measured using CCK-8 assay. (E) Cell apoptosis was measured using flow cytometry, (F) which was quantified. (G) Cleaved caspase 3 and cleaved PARP protein expression was measured by western blotting, (H) which was quantified. miR, microRNA; lnc-NEAT1, long non-coding RNA nuclear enriched abundant transcript 1; MMC, mouse mesangial cell; DN, diabetic nephropathy; lnc-KD, lnc-NEAT1 knockdown; NC-KD, negative control knockdown; RT-qPCR, reverse transcription-quantitative PCR; PARP, poly-ADP ribose polymerase; CCK-8, Cell Counting Kit-8; OD, optical density.

MMC DN cell model. No difference in lnc-NEAT1 expression could be observed between the lnc-KD + miR-124-inhibitor and the lnc-KD + NC-inhibitor groups (Fig. 3B), but significantly reduced miR-124 expression was found in the lnc-KD + miR-124-inhibitor group compared with that in the lnc-KD + NC-inhibitor group (Fig. 3C). Compared with those in the lnc-KD + NC-inhibitor group, cell viability was also significantly enhanced (Fig. 3D), whilst the apoptosis rate was significantly reduced, in the lnc-KD + miR-124-inhibitor group (Fig. 3E and F). In addition, cleaved caspase 3 and cleaved PARP protein expression were significantly decreased in the lnc-KD + miR-124-inhibitor group compared with that in the lnc-KD + NC-inhibitor group (Fig. 3G and H).

In terms of inflammation, the expression levels of inflammatory cytokines IL-1β, IL-8, MCP-1 and TNF-α were significantly increased in the lnc-KD + miR-124-inhibitor group compared with those in the lnc-KD + NC-inhibitor group (Fig. 4A-D). The expression levels of fibronectin and collagen I were also significantly enhanced in the lnc-KD + miR-124-inhibitor group compared with those in the lnc-KD + NC-inhibitor group (Fig. 4E-H). In addition, either the miR-124 inhibitor alone or NC miRNA inhibitor alone were transfected

into the HG-treated cells, where it was observed miR-124 expression was significantly downregulated in miR-124-inhibitor group compared with that in NC-inhibitor group, whilst lnc-NEAT1 expression remained unchanged (Fig. S1). These data suggest that lnc-NEAT1 can regulate cell viability, apoptosis, inflammation and fibrosis by negatively regulating miR-124 expression in the MMC DN cell model.

Effect of miR-124 knockdown on Capn1 and CTNNB1 expression in the MMC DN cell model after lnc-NEAT1 knockdown. miR-124 has been reported to target Capn1, which in turn regulates CTNNB1 (8). Both mRNA and protein expression of Capn1 were significantly increased in the lnc-KD + miR-124-inhibitor group compared with that in the lnc-KD + NC-inhibitor group (Fig. 5). The mRNA and protein expression of CTNNB1 were also increased in the lnc-KD + miR-124-inhibitor group compared with that in the lnc-KD + NC-inhibitor group (Fig. 5). Taken together, these findings suggest that lnc-NEAT1 can negatively regulate miR-124 expression, which then upregulates the Capn1/β-catenin pathway upstream of increasing cell viability, inflammation and fibrosis whilst decreasing apoptosis in this *in vitro* MMC DN cell model.
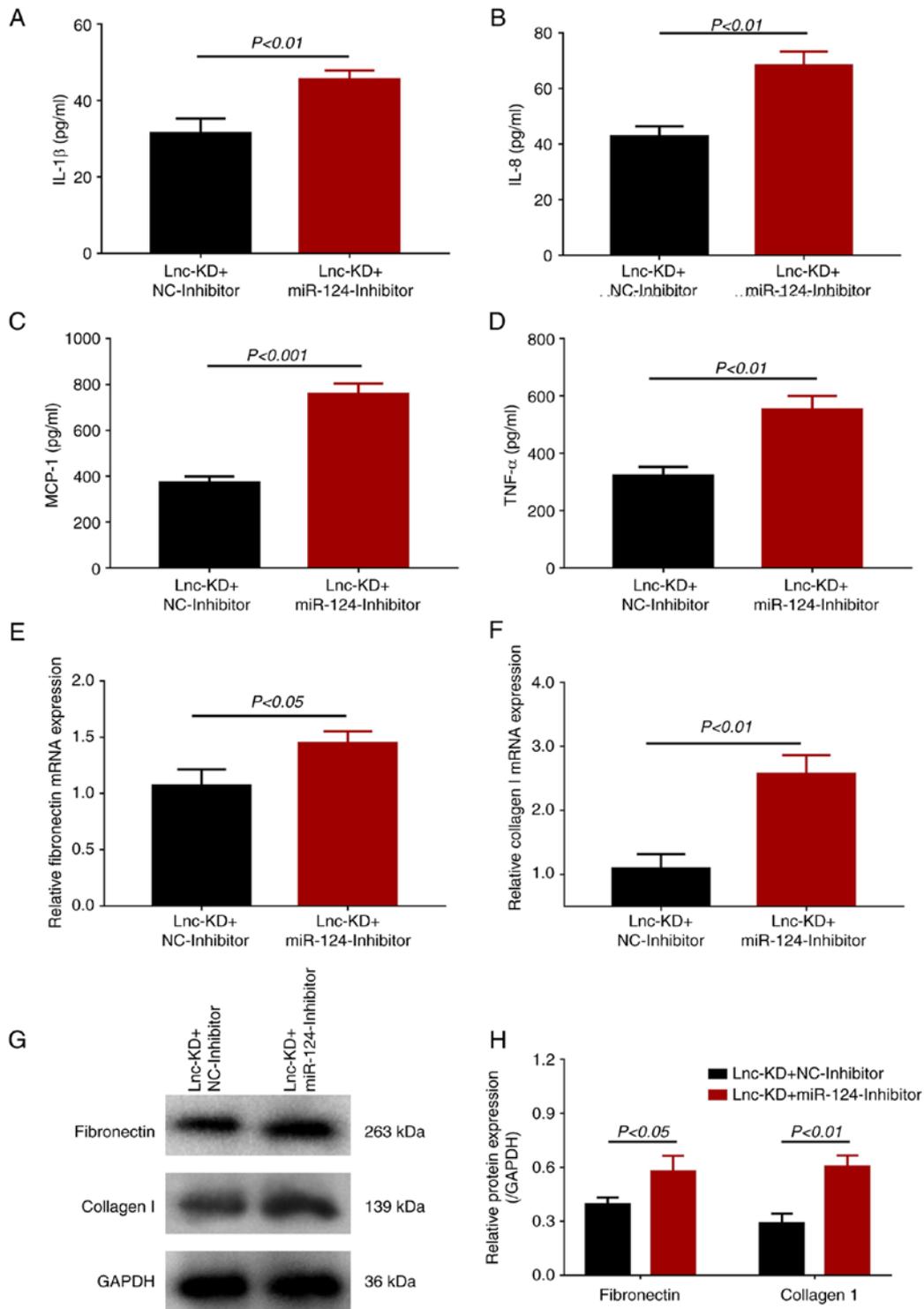
Figure 4. Effect of miR-124 inhibition on the secretion of inflammatory cytokines and expression of fibrosis markers in a lnc-NEAT1-downregulated MMC DN cell model. Secretion of (A) IL-1β, (B) IL-8, (C) MCP-1 and (D) TNF-α into the supernatant of cells from lnc-KD + miR-124-inhibitor and lnc-KD + NC-inhibitor groups were measured using ELISA. (E) Fibronectin and (F) collagen I mRNA expression was measured using reverse transcription-quantitative PCR. (G) Fibronectin and collagen I protein expression in the lnc-KD + miR-124-inhibitor and lnc-KD + NC-inhibitor groups was measured by western blotting, (H) which was quantified. miR, microRNA; lnc-NEAT1, long non-coding RNA nuclear enriched abundant transcript 1; MMC, mouse mesangial cell; DN, diabetic nephropathy; lnc-KD, lnc-NEAT1 knockdown; NC-KD, negative control knockdown; MCP-1, monocyte chemotactic protein 1; TNF-α, tumor necrosis factor α; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Discussion

The possible crosstalk between lncRNAs and the DN pathology has been garnering attention over the past decade (3-5).

LncRNAs have been reported to regulate the progression of DN by regulating a number of molecular events underlying the pathological processes of fibrogenesis, mesangial cells proliferation and accumulation of extracellular matrix, which
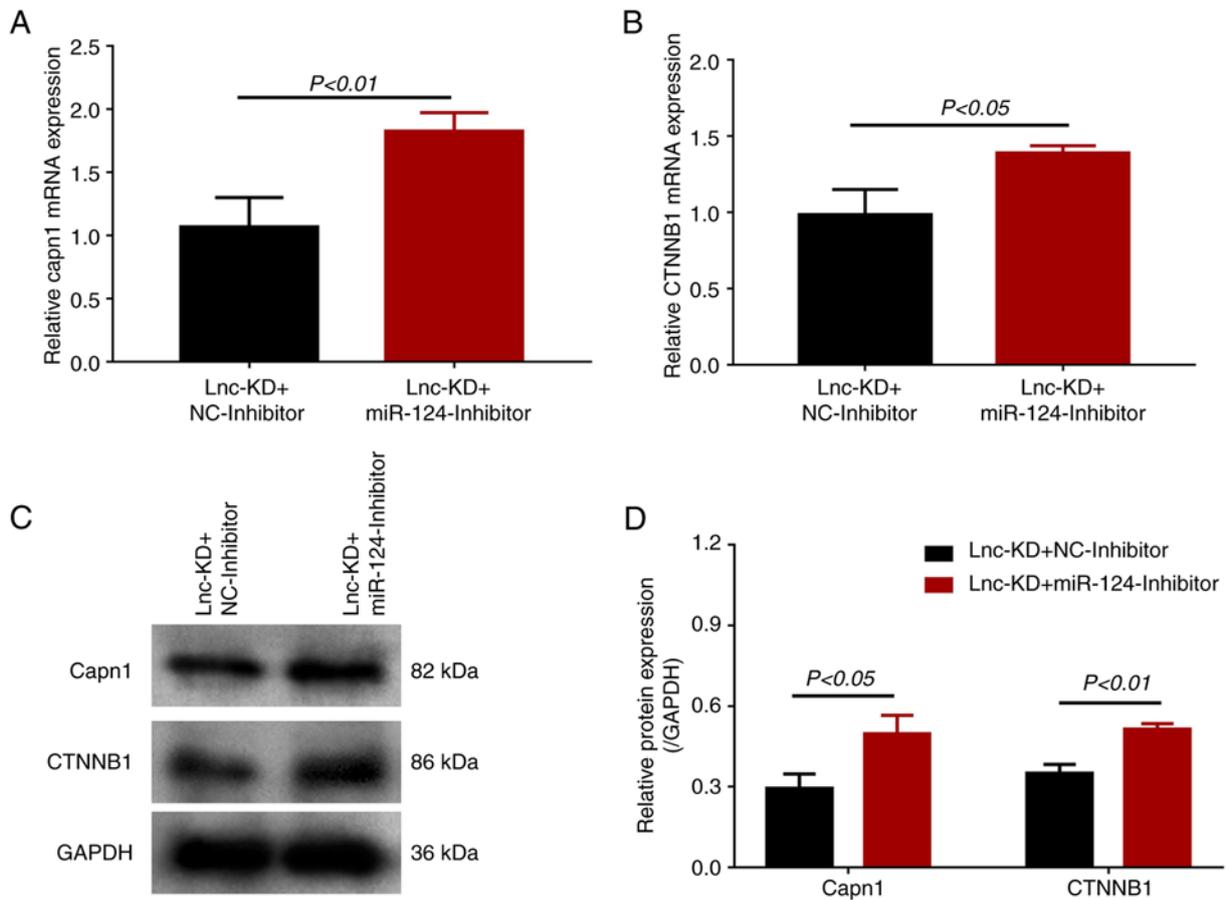
Figure 5. miR-124 inhibition increases Capn1 and CTNNB1 expression the lnc-NEAT1-downregulated MMC DN cell model. The MMC DN cell model was transfected with either lnc-KD + miR-124-inhibitor or lnc-KD + NC-inhibitor. (A) Capn1 and (B) CTNNB1 mRNA expression was measured by reverse transcription-quantitative PCR. (C) Capn1 and CTNNB1 protein expression was measured by western blotting, (D) which was quantified. miR, microRNA; Capn1, calpain 1; CTNNB1, catenin β1; lnc-NEAT1, long non-coding RNA nuclear enriched abundant transcript 1; MMC, mouse mesangial cell; DN, diabetic nephropathy; lnc-KD, lnc-NEAT1 knockdown; NC-KD, negative control knockdown.

was found to be mediated through interplay with miRNAs or other associated pathways, including the NF-κB and β-catenin pathways (17-20). Lnc-NEAT1 is commonly expressed in mammalian cells and has been found to be dysregulated in the pathology of various diabetic complications and kidney diseases (6,7,21). A previous study revealed that lnc-NEAT1 expression was upregulated by high glucose in mouse cardiomyocytes, which in turn downregulated miR-140-5p expression and upregulated the expression of histone deacetylase 4 to enhance myocardial apoptosis (21). In addition, another previous study reported that lnc-NEAT1 is overexpressed in the myocardial tissues of I/R-treated diabetic mice, which promoted apoptosis and autophagy in the cardiomyocytes to aggravate myocardial I/R injury (7). Lnc-NEAT1 was also reported to be overexpressed and enhances sepsis-induced acute kidney injury in rats by sponging miR-204 and activating the NF-κB signaling pathway (6). These previous studies indicate the role of lnc-NEAT1 in aggravating functional deterioration in animal or cell models of diabetic complications or kidney injuries. Therefore, it was speculated that lnc-NEAT1 may also mediate the DN pathophysiology. The present study treated MMCs with high glucose to establish the MMC DN cell model before investigating the influence of lnc-NEAT1 knockdown on MMC viability and apoptosis. The results

revealed that lnc-NEAT1 knockdown reduced cell viability whilst enhancing cell apoptosis in this *in vitro* MMC DN cell model. Lnc-NEAT1 may have modulated the Akt/mTOR or NF-κB signaling pathways to promote mesangial cell viability and reduce mesangial cell apoptosis in sepsis-induced acute kidney injury and DN models, respectively, which would explain these findings in the present MMC DN cell model (6,22). In addition, lnc-NEAT1 may have sponged miRNAs, such as miR-124, which was suggested by the data in the present study, resulting in the aggravated DN injury.

These aforementioned previous studies indicated that lnc-NEAT1 may serve as a target for the treatment of excess mesangial cell proliferation during DN (6,7,21,22). In addition to strategies for reducing the number of mesangial cells, measures to suppress renal fibrosis and inhibit inflammation may also assist in alleviating functional deterioration during DN (2). Regarding the influence of lnc-NEAT1 on fibrosis, silencing lnc-NEAT1 expression was found to repress liver fibrosis in a carbon tetrachloride-induced mouse liver fibrosis model (23). In terms of the role of lnc-NEAT1 in inflammation, lnc-NEAT1 knockdown decreased cytokine levels, including TNF-α, IL-6, IL-8 and IL-1β, in a lipopolysaccharide-induced sepsis renal cell model (6). These data suggest that lnc-NEAT1 knockdown may exert anti-fibrotic and

anti-inflammatory effects in these specific diseases. However, to the best of our knowledge, the effects of lnc-NEAT1 on DN pathology have not been previously studied. The present study assessed the influence of lnc-NEAT1 knockdown on inflammatory cytokine secretion and fibrosis marker expression in this MMC DN cell model. It was found that lnc-NEAT1 knockdown significantly decreased the secretion of inflammatory cytokines and the expression of fibrosis markers, suggesting that lnc-NEAT1 also has the potential to be a target in reducing inflammation and fibrosis observed in the mesangial cells during DN.

miR-124 is an miRNA that has been reported to be a tumor suppressor and serve a role in the pathophysiology of a number of diabetic or inflammatory diseases (24-26). miR-124 expression has been shown to be reduced in high glucose-treated vascular smooth muscle cells and in human retinal endothelial cells under hyperglycemia exposure in a time-dependent manner (24,25). Additionally, miR-124 upregulation can reduce the inflammatory response and renal lesions in mice with sepsis, where it has been suggested to be a treatment approach for attenuating sepsis induced-acute kidney injury (26). These data suggest that miR-124 expression may be dysregulated under high-glucose conditions, where it serves a favorable role in attenuating inflammation or kidney injury. Data from these two previous studies also showed that miR-124 expression could be negatively regulated by lnc-NEAT1 (6,7), such that miR-124 can in turn directly bind to the Capn1 mRNA, which is known to modulate cell physiology through the Wnt/β-catenin pathway (8-14,27,28). Capn1 has been reported to increase the plasma glucose level, where the Wnt/β-catenin pathway can affect renal tubulointerstitial transdifferentiation during DN (29,30). This suggests that the Capn1/β-catenin pathway may participate in the DN pathophysiology. These data also support the hypothesis that the regulatory effects of lnc-NEAT1 in DN may be mediated by regulating miR-124 expression and the Capn1/β-catenin signaling pathway downstream. The present study found that miR-124 knockdown reversed the influence of lnc-NEAT1 knockdown on the MMC DN cell model, whilst increasing the expression levels of Capn1 and CTNNB1.

In conclusion, data from the present study suggest that lnc-NEAT1 knockdown may inhibit mesangial cell viability, inflammation and fibrosis by regulating miR-124 expression and the Capn1/β-catenin signaling pathway in DN, implicating that lnc-NEAT1 can serve as a potential therapeutic target for DN.

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Availability of data and materials

Authors' contributions

NZ and JM confirm the authenticity of all the raw data. NZ and JM designed the experiments. LD, YW and YM performed the experiments. NZ and ZF analyzed the data. 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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