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LncRNA NEAT1 accelerates the occurrence and development of diabetic nephropathy by sponging miR-23c

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Abstract. – OBJECTIVE: LncRNA nuclear enriched abundant transcript 1 (NEAT1) has been reported to play an oncogenic role in the occurrence and development of diabetic nephropathy (DN). The aim of our study was to investigate the potential mechanism by which NEAT1 facilitates the progression of DN.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) was carried out to determine the abundance of NEAT1, kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), proliferating cell nuclear antigen (PCNA), Cyclin D1, P38, apoptosis signal-regulating kinase 1 (ASK1), Fibronectin, α smooth muscle actin (α-SMA) and miR-23c in the serum of DN patients, normal patients and mouse mesangial cells (MMCs). Cell proliferation was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), qRT-PCR and Western blot assays. Flow cytometry and Western blot were applied to measure apoptosis of MMCs. Cell fibrosis and epithelial-to-mesenchymal transition (EMT) were analyzed by qRT-PCR and Western blot. The binding sites between miR-23c and NEAT1 were predicted by starBase bioinformatics software, and the relationship was verified by dual-luciferase reporter assay.

RESULTS: The enrichment of NEAT1 was elevated in the serum of DN patients and MMCs induced by high concentration of glucose. NEAT1 overexpression accelerated proliferation, fibrosis and EMT and restrained apoptosis of MMCs induced by high concentration of glucose. MiR-23c bound to NEAT1, and the inhibition of miR-23c counteracted the suppressive effect of NEAT1 depletion on proliferation, fibrosis and EMT of MMCs induced by high concentration glucose.

CONCLUSIONS: LncRNA NEAT1 promoted proliferation, fibrosis and EMT while impeded apoptosis of MMCs through sponging miR-23c. LncRNA NEAT1 and miR-23c might be underlying therapeutic targets for the treatment of DN.

Key Words:
Diabetic nephropathy, LncRNA NEAT1, MiR-23c, Proliferation, Apoptosis, Fibrosis, EMT.

Abbreviations

NEAT1= LncRNA nuclear enriched abundant transcript 1; DN=diabetic nephropathy; KIM-1=kidney injury molecule-1; NGAL=neutrophil gelatinase-associated lipocalin; PCNA=proliferating cell nuclear antigen; ASK1= apoptosis signal-regulating kinase 1; SMA=smooth muscle actin; MMCs= mouse mesangial cells; EMT=epithelial-to-mesenchymal transition; DM=Diabetes mellitus.

Introduction

Diabetes mellitus (DM) is a common illness caused by abnormal secretion of insulin or the defect on the function of insulin. Diabetic nephropathy (DN) is a common complication of DM [1], and it results in millions of deaths globally. Mesangial cells secrete multiple cytokines under the condition of high concentration of glucose, including transforming growth factor β1 and fibronectin [2]. The pathogenesis of DN is extraordinarily intricate, and uncovering the molecular mechanism of DN is indispensable for the cure of DN.

Long noncoding RNAs (lncRNAs) are a class of RNAs with at least 200 nucleotides[3,4], and they are unable to code any protein in general. LncRNAs could modulate the development of cancer and cellular metabolism, including diabetic nephropathy[5]. Wang et al[6] claimed that lncRNA CYP4B1-PS1-001 was positively related to the progression of DN. Long et al[7] proved that TUG1 was involved in mitochondrial bioenergetics of DN. Herein, we concentrated on the function of lncRNA nuclear enriched abundant transcript 1

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(NEAT1). NEAT1 has been reported to participate in multiple lethal diseases[8-10]. Nevertheless, the biological role of NEAT1 and the potential regulatory network in DN remain largely unknown. Wang et al[11] demonstrated that NEAT1 facilitated the accumulation of extracellular matrix via miR-27b-3p/ZEB1 axis in DN. Huang et al[12] claimed that NEAT1 promoted cell proliferation and fibrosis in DN via Akt/mTOR pathway. Nevertheless, the biological role of lncRNA NEAT1 and the potential signal network are not fully addressed.

MicroRNAs (miRNAs) play a vital role in cellular biological processes through binding to the target messenger RNA and reducing its enrichment[13-15]. Currently, miRNAs have been reported to be involved in the occurrence and development of DN[16,17]. He et al[18] proved that miR-135a facilitated renal fibrosis through TRPC1 in DN. McClelland et al[19] indicated that miR-21 accelerated renal fibrosis via regulating PTEN and SMAD7 in DN. MiR-23c has been indicated to participate in the development of diabetic nephropathy[20]. However, the precise mechanism by which miR-23c regulates the progression of DN remains to be determined.

In this study, we assessed the biological role of NEAT1 in DN and investigated the underlying mechanism by which NEAT1 contributes to the occurrence and progression of DN.

Patients and Methods

Tissue Samples

Blood samples from diabetic nephropathy patients (n=40) and normal patients (n=40) were collected from The Central Hospital of Wuhan. This research was carried out with the permission of the Ethic Committee of The Central Hospital of Wuhan. All patients had provided informed consent. Serum samples were centrifuged and then immediately stored at -80°C until used.

Cell Culture

Mouse mesangial cells (MMCs) and human embryonic kidney cell line HEK293T were purchased from BeNa Culture Collection (Beijing, China). HEK293T cells and MMCs were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NJ, USA) added with 10% fetal bovine serum (FBS; Gibco), 1% penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were cultivated in an incubator at 37°C with 5% CO_2.

Cell Treatment

For glucose treatment in MMCs, MMCs (5×10^4 cells/well) were seeded in 6-well plates and treated with low concentration of glucose (L, 6 mmol) or high concentration of glucose (H, 30 mmol) for 48 h. For TGF-β1 treatment, MMCs were treated with 10 ng/mL TGF-β1 or negative control (NC) for 96 h.

Cell Transfection

Scrambled siRNA (si-NC), small interfering RNA targeting NEAT1 (si-NEAT1), NEAT1 overexpression vector and control vector were purchased from Genepharma (Shanghai, China). MiR-23c mimics, miR-NC, anti-miR-23c and anti-miR-NC were obtained from Ribobio (Guangzhou, China). Transfection was performed using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

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

TRIzol reagent (Invitrogen) was used for the extraction of RNA. The reverse transcription of NEAT1 was carried out using M-MLV reverse transcriptase kit (Invitrogen), while the cDNA of miR-23c was obtained using All-in-One™ miRNA First stand cDNA Synthesis Kit (GeneCopoeia, Rockville, MD, USA). QRT-PCR was conducted using SYBR green and special primers (GeneCopoeia). The enrichment of NEAT1 and miR-23c was examined with U6 small RNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the control using the $2^{-\Delta\Delta Ct}$ method [21]. The primer sequences were shown as below: NEAT1 (Forward, 5'-TGGCTAGCTCAGGGCTTCAG-3'; Reverse, 5'-TCTCCTTGCCAAGCTTCCTTC-3'), miR-23c (Forward, 5'-CCAGAAGGACGTAGAAG-3'; Reverse, 5'-CTTCACTGTGATGGGCTC-3'), kidney injury molecule-1 (KIM-1; Forward, 5'-TCTGCTTGTCAAAATACACT-3'; Reverse, 5'-TCTTGGAGGACGTGTGGGAA-3'), neutrophil gelatinase-associated lipocalin (NGAL; Forward, 5'-GGCAGGGAATGCAATTCTCA-3'; Reverse, 5'-TTGTAGTTGGTGCTCACCAC-3'), proliferating cell nuclear antigen (PCNA; Forward, 5'-CGCTCCGAAGGCTTCGACAC-3'; Reverse, 5'-CTCCTGTTCTGGGATTCCAA-3'), Cyclin D1 (Forward, 5'-TTACTTCAAGTGCGTGCAGA-3'; Reverse, 5'-TTCTCGGCAGTCAAGGGAAT-3'), P38 (Forward, 5'-ATCGTGTGGCAGTTAAGAAG-3'; Reverse, 5'-GAACGTGGTCGTCGGTCAGC-3'), apoptosis signal-regulating kinase 1 (ASK1; Forward, 5'-CGCCGCCACCGCCGGGCAGT-3'; Reverse, 5'-GGGTGGCGCCCACCGCCTCG-3'),

Fibronectin (Forward, 5'-GGGAAGCACTATCAGATAAA-3'; Reverse, 5'-ACAGCTGATCCTGCCTCTCC-3'), α smooth muscle actin (α-SMA; Forward, 5'-TAATGGTTGGAATGGGCCAA-3'; Reverse, 5'-TAATCTGGGTCATTTTCTCC-3'), U6 (Forward, 5'-CTCGCTTCGGCAGCACA-3'; Reverse, 5'-AACGCTTCACGAATTTGCGT-3'), GAPDH (Forward, 5'-CTGGGCTACACTGAGCACC-3'; Reverse, 5'-AAGTGG TCGTTGAGGGCAATG-3').

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

MMCs were treated with low concentration of glucose (L, 6 mmol) or high concentration of glucose (H, 30 mmol), and MMCs transfected with NEAT1 overexpression vector or empty vector were also treated with high concentration of glucose. MTT (10 μL, 5 mg/mL; Invitrogen) was added to the indicated wells of the cell culture plate after transfection for 24 h, 48 h and 72 h. The cell supernatant was discarded after four-hour incubation. Then 100 μL dimethyl sulfoxide (DMSO) was added to the above wells. The optical density was detected using a microplate reader at 490 nm.

Western Blot Assay

MMCs were disrupted using RIPA lysis solution (Beyotime, Shanghai, China). The total proteins were quantified and were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA). After being blocked the nonspecific binding sites for 1 h, the PVDF membranes were incubated with primary antibodies against proliferating cell nuclear antigen (PCNA; SAB2108448, Sigma-Aldrich, St. Louis, MO, USA), Cyclin D1 (ab134175, Abcam, Cambridge, UK), Bcl-2 (ab692, Abcam), Bcl-2 associated X, apoptosis regulator (Bax; ab32503, Abcam), P38 (ab170099, Abcam), apoptosis signal-regulating kinase 1 (ASK1; ab45178, Abcam), fibronectin (ab2413, Abcam), α smooth muscle actin (α-SMA; ab32575, Abcam), E-cadherin (ab76055, Abcam), Vimentin (ab193555, Abcam) or GAPDH (ab37168, Abcam) for 12 h at 4°C, and incubated with horseradish peroxidase (HRP) combined secondary antibody (ab205719 and ab205718, Abcam) for 2 h. Finally, the protein signal was measured via ECL system (Beyotime). In this experiment, GAPDH was regarded as a control.

Cell Apoptosis Analysis

Flow cytometry was carried out to measure the apoptosis of MMCs. MMCs at a concentration of 5×10^4 cells per well were seeded into 6-well plate and cultured at 37°C. After transfection or treatment with glucose, MMCs were harvested using cold phosphate-buffered saline (PBS) buffer for twice. Then the cells were stained with 5 μL Annexin V combined fluorescein isothiocyanate (FITC) and propidium iodide (PI; Solarbio, Beijing, China) for 10 min at room temperature. Next, the apoptotic cells (FITC+, PI+/-) were identified by the flow cytometer (BD Biosciences, Franklin lakes, NY, USA).

Dual-Luciferase Reporter Assay

The binding sites between miR-23c and lncRNA NEAT1 were predicted by starBase. To verify the combination between miR-23c and lncRNA NEAT1, the sequences of NEAT1 containing wild-type (WT) binding sites or mutant binding sites were amplified and inserted to pmirGLO vector (Promega, Madison, WI, USA), named as WT-NEAT1 and MUT-NEAT1, respectively. WT-NEAT1 or MUT-NEAT1 and miR-23c or miR-NC were co-transfected into HEK293T cells, and the luciferase activity was analyzed after 48-h transfection.

Statistical Analysis

All data were displayed in the form of means±SD from three independent experiments. The liner relationship between the abundance of NEAT1 and the enrichment of KIM-1 or NGAL was assessed by Spearman's correlation coefficient. The differences between two groups were analyzed using Student's *t*-test, whereas the comparison among multiple groups was examined using one-way analysis of variance (ANOVA) followed by Tukey's test. $p<0.05$ was considered as statistically significant.

Results

LncRNA NEAT1 is Dramatically Elevated in the Serum of Diabetic Nephropathy (DN) Patients

To investigate the pivotal role of NEAT1 in DN, the expression of NEAT1 was measured in the serum of normal patients (n=40) and DN patients (n=40). QRT-PCR proved that the abundance of NEAT1 was enhanced in the serum of DN patients compared with that in the serum of normal

patients (Figure 1A). Kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) were recognized as the markers of DN. The enrichment of the two proteins was increased in the serum of DN patients compared with that in the serum of normal patients (Figure 1B and 1C). We wondered whether lncRNA NEAT1 serves as a diagnostic marker of DN. The correlation analysis displayed a positive connection between the expression of NEAT1 and the enrichment of KIM-1 or the level of NGAL (Figure 1D and 1E). The abnormal level of NEAT1 might imply its crucial role in DN.

LncRNA NEAT1 Facilitates Cell Proliferation and Suppresses Apoptosis of MMCs Treated with High Concentration of Glucose

To explore the role of NEAT1 in proliferation and apoptosis of MMCs, MMCs were treated with low concentration of glucose (L, 6 mmol) or high concentration of glucose (H, 30 mmol), and MMCs transfected with control or NEAT1 were also treated with high concentration of glucose. The abundance of NEAT1 was determined by qRT-PCR. As indicated in Figure 2A, the enrichment of NEAT1 was enhanced with the treatment of high concentration of glucose (H), and the transfection of NEAT1 further increased the expression of NEAT1. MTT assay was conducted to analyze cell proliferation of the above MMCs. Cell proliferation was facilitated under the condition of high concentration of glucose (H) compared with that in low concentration of glucose (L), and the accumulation of NEAT1 further accelerated the proliferation of MMCs (Figure 2B). Besides, we also measured the abundance of proliferation-associated proteins PCNA and Cyclin D1 by qRT-PCR and Western blot. Compared with low concentration of glucose (L) group, the mRNA levels of PCNA and Cyclin D1 were notably elevated with the treatment of high concentration of glucose (H), and the transfection of NEAT1 further increased the mRNA enrichment of PCNA and Cyclin D1 (Figure 2C and 2D). Meanwhile, Western blot assay demonstrated that the protein levels of PCNA and Cyclin D1 were elevated under high concentration of glucose (H); the overexpression of NEAT1 further enhanced the enrichment of PCNA and Cyclin D1 (Figure 2E and 2F). Cell apoptosis of treated MMCs was assessed by flow cytometry. As shown in Figure 2G, compared with the low concentration of glucose (L) group, the cell apoptosis rate of MMCs was prominently declined with the treatment of

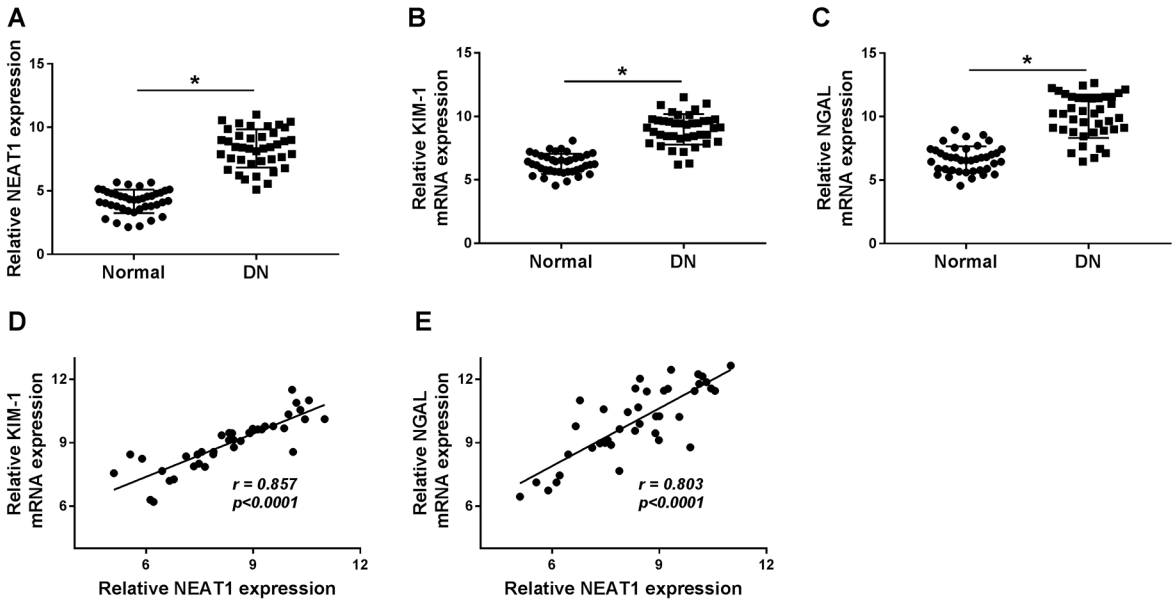

Figure 1. LncRNA NEAT1 is dramatically elevated in the serum of diabetic nephropathy (DN) patients**. (A)** The expression of lncRNA NEAT1 was determined in the serum of normal patients (n=40) and DN patients (n=40) by qRT-PCR. **(B)** The abundance of KIM-1 was measured in the serum of normal patients (n=40) and DN patients (n=40) by qRT-PCR. **(C)** The enrichment of NGAL was detected in the serum of normal patients (n=40) and DN patients (n=40) by qRT-PCR. **(D)** The correlation analysis was conducted between the mRNA level of KIM-1 and the abundance of NEAT1 in the serum of NC patients (n=40). **(E)** The relationship between the enrichment of NGAL mRNA and the expression of NEAT1 in the serum of NC patients (n=40) was analyzed. *$p<0.05$.

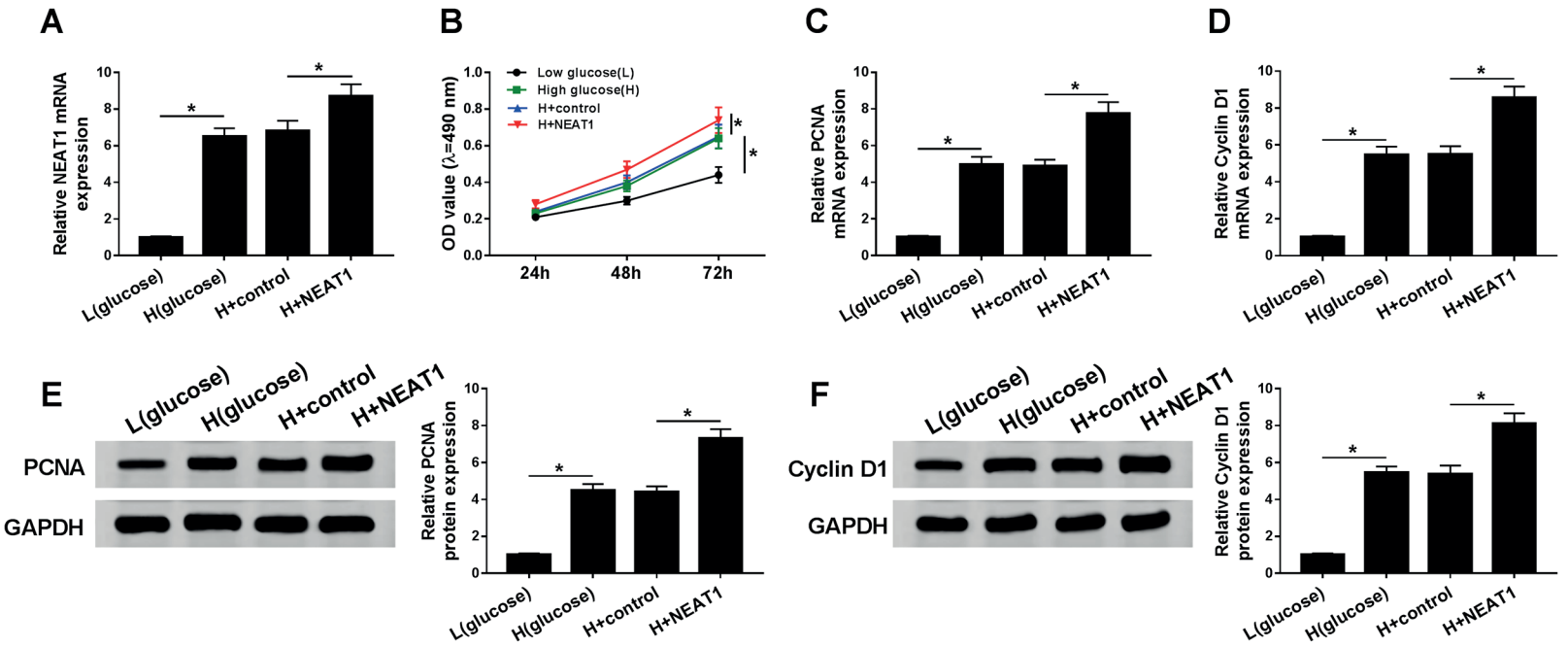

Figure 2. LncRNA NEAT1 facilitates cell proliferation and suppresses apoptosis of MMCs treated with high concentration of glucose. MMCs were treated with low concentration of glucose (L, 6 mmol) or high concentration of glucose (H, 30 mmol), and MMCs transfected with NEAT1 or control were also treated with high concentration of glucose. The four different treatment groups were used for the following experiments. **(A)** The expression of NEAT1 was determined in the above MMCs by qRT-PCR. **(B)** Cell proliferation was measured in the above MMCs by MTT assay. **(C** and **D)** The mRNA levels of proliferation-related proteins (PCNA and Cyclin D1) were detected by qRT-PCR in the above MMCs. **(E** and **F)** The protein expression of PCNA and Cyclin D1 was determined by Western blot in MMCs.

Figure continued

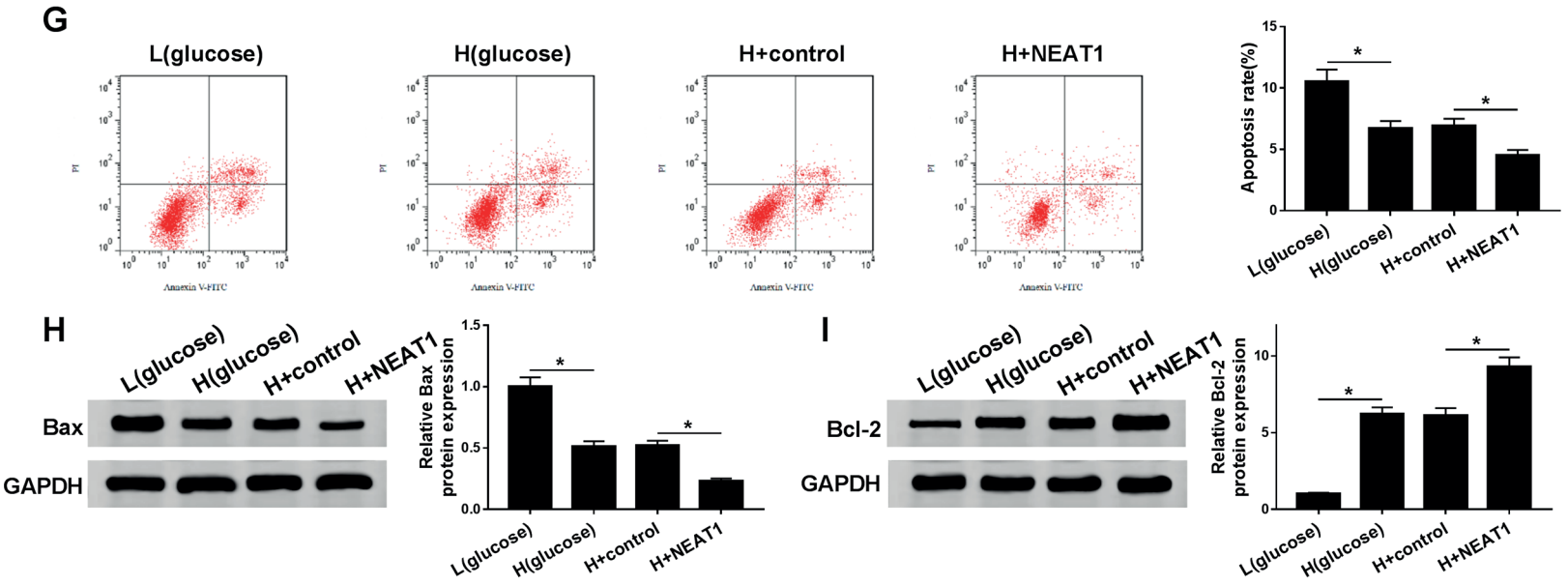

Figure 2. *(Continued).* **(G)** Flow cytometry was performed to detect the apoptosis of the above MMCs. **(H** and **I)** Western blot was conducted to measure the protein abundance of apoptosis-related proteins (Bax and Bcl-2) in the above MMCs. *$p<0.05$.

high concentration of glucose (H). And the transfection of NEAT1 further down-regulated the apoptosis rate of MMCs. Western blot assay was performed to detect the abundance of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 in four groups to assess the effect of NEAT1 accumulation on cell apoptosis. The expression of Bax was decreased under high concentration of glucose, and the overexpression of NEAT1 further reduced its abundance (Figure 2H). The level of anti-apoptotic protein Bcl-2 displayed an opposite trend to Bax (Figure 2I). Therefore, NEAT1 promoted cell proliferation and restrained cell apoptosis of MMCs treated with high concentration of glucose.

NEAT1 Accelerates Fibrosis of MMCs

To investigate the effect of NEAT1 accumulation on cell fibrosis, MMCs were treated with low concentration of glucose (L) or high concentration of glucose (H), and MMCs transfected with NEAT1 or control were also treated with H. QRT-PCR and Western blot were carried out to determine the mRNA and protein levels of fibrosis-related proteins, including P38, ASK1, Fibronectin and α-SMA. The mRNA levels of fibrosis-related proteins were markedly elevated with the treatment of high concentration of glucose (H), which were further enhanced with the accumulation of NEAT1 (Figure 3A-3D). The effects of high concentration of glucose and NEAT1 accumulation on the protein expression of fibrosis-related proteins were similar to the mRNA level (Figure 3E-3H). Collectively, lncRNA NEAT1 facilitated fibrosis of MMCs.

NEAT1 Promotes EMT of MMCs

MMCs were transfected with NEAT1 or control, and the NEAT1 transfection group was further treated with 10 ng/mL EMT inducer TGF-β1 for 96 h. Western blot assay was applied to measure the abundance of the marker of the epithelial cells, named as E-cadherin, and the marker of the mesenchymal cells, named as Vimentin. NEAT1 overexpression decreased the level of E-cadherin and the addition of TGF-β1 further down-regulated the abundance of E-cadherin (Figure 4A). On the contrary, the enrichment of Vimentin was enhanced with the accumulation of NEAT1, and it was further elevated with the addition of TGF-β1 (Figure 4B). Therefore, NEAT1 accelerated EMT of MMCs, and TGF-β1 further promoted NEAT1-mediated EMT. Simultaneously, MMCs were treated with 10 ng/mL TGF-β1 or negative control, and TGF-β1 treatment group was further transfected with NEAT1 or control. The addition of TGF-β1 reduced the abundance of E-cadherin, and the overexpression of NEAT1 further decreased the level of E-cadherin (Figure 4C). The enrichment of Vimentin displayed an inverse trend to E-cadherin (Figure 4D). These results demonstrated that TGF-β1 induced EMT of MMCs and NEAT1 overexpression accelerated TGF-β1-induced EMT. Collectively, NEAT1 facilitated EMT of MMCs.

NEAT1 Accelerates Proliferation and Restrains Apoptosis of MMCs by Sponging miR-23c

To illustrate the potential mechanism by which NEAT1 promotes cell proliferation, fibrosis and EMT and suppresses cell apoptosis, bioinformatics analysis was applied to screen the target of NEAT1. As indicated in Figure 5A, miR-23c was predicted to be a functional target of NEAT1, and the putative binding sites between lncRNA NEAT1 and miR-23c were predicted by starBase online software. The sequences of NEAT1 containing wild-type or mutant binding sites were amplified and inserted to the luciferase reporter vector, named as WT-NEAT1 or MUT-NEAT1. Luciferase activity was assessed in HEK293T cells co-transfected with miR-23c or miR-NC and WT-NEAT1 or MUT-NEAT1. The transfection of miR-23c dramatically declined luciferase activity in WT-NEAT1 group compared with that in MUT-NEAT1 group (Figure 5B). Compared with the serum of normal patients, the enrichment of miR-23c was significantly decreased in the serum of DN patients (Figure 5C). A significant inverse correlation was found between the expression of miR-23c and the abundance of NEAT1 (Figure 5D).

To investigate whether miR-23c was involved in NEAT1-mediated cell proliferation, apoptosis, fibrosis and EMT, MMCs were treated with high concentration of glucose (H) and respectively transfected with si-NC, si-NEAT1, si-NEAT1 and anti-miR-NC or anti-miR-23c. The depletion of NEAT1 restrained cell proliferation and the addition of anti-miR-23c reversed the inhibition impact of NEAT1 intervention on cell proliferation (Figure 5E). Flow cytometry was performed to determine the changes in the apoptosis rate in different groups. Cell apoptosis rate was notably increased with the interference of NEAT1, and the inhibition of miR-23c counteracted the promoting effect of NEAT1 depletion on cell apoptosis (Figure 5F). Accordingly, miR-23c was a direct target of NEAT1, and it acted as the downstream gene of NEAT1 to regulate cell apoptosis, proliferation, EMT and fibrosis.

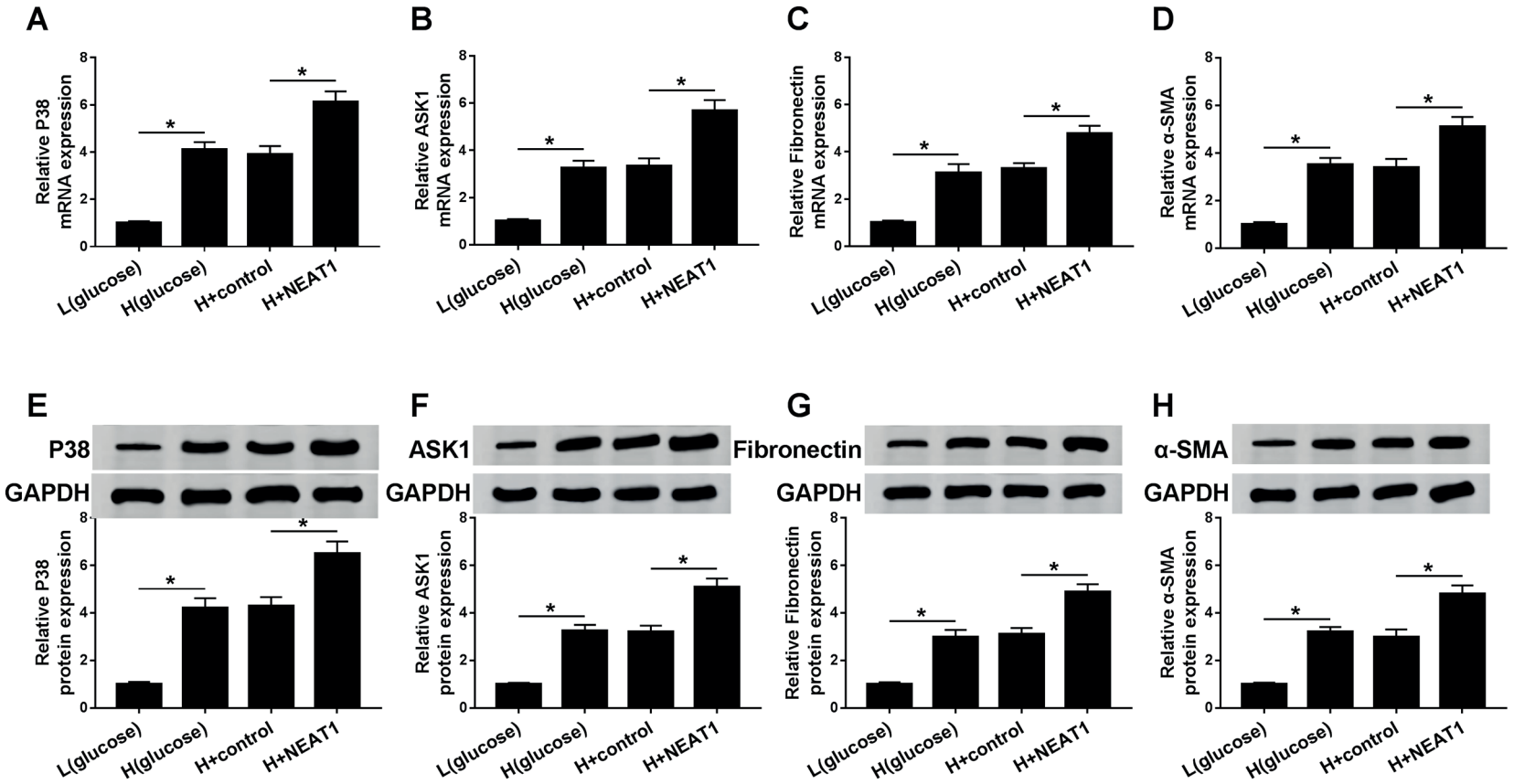

Figure 3. NEAT1 accelerates fibrosis of MMCs. **(A-D)** The mRNA abundance of fibrosis-associated proteins (P38, ASK1, Fibronectin and α-SMA) was analyzed in MMCs treated with low concentration of glucose (L, 6 mmol), high concentration of glucose (H, 30 mmol), H and transfection with empty vector or transfection with NEAT1 overexpression vector by qRT-PCR. **(E-H)** The above cells were also used for the detection of the protein abundance of P38, ASK1, Fibronectin and α-SMA by Western blot. *$p<0.05$.

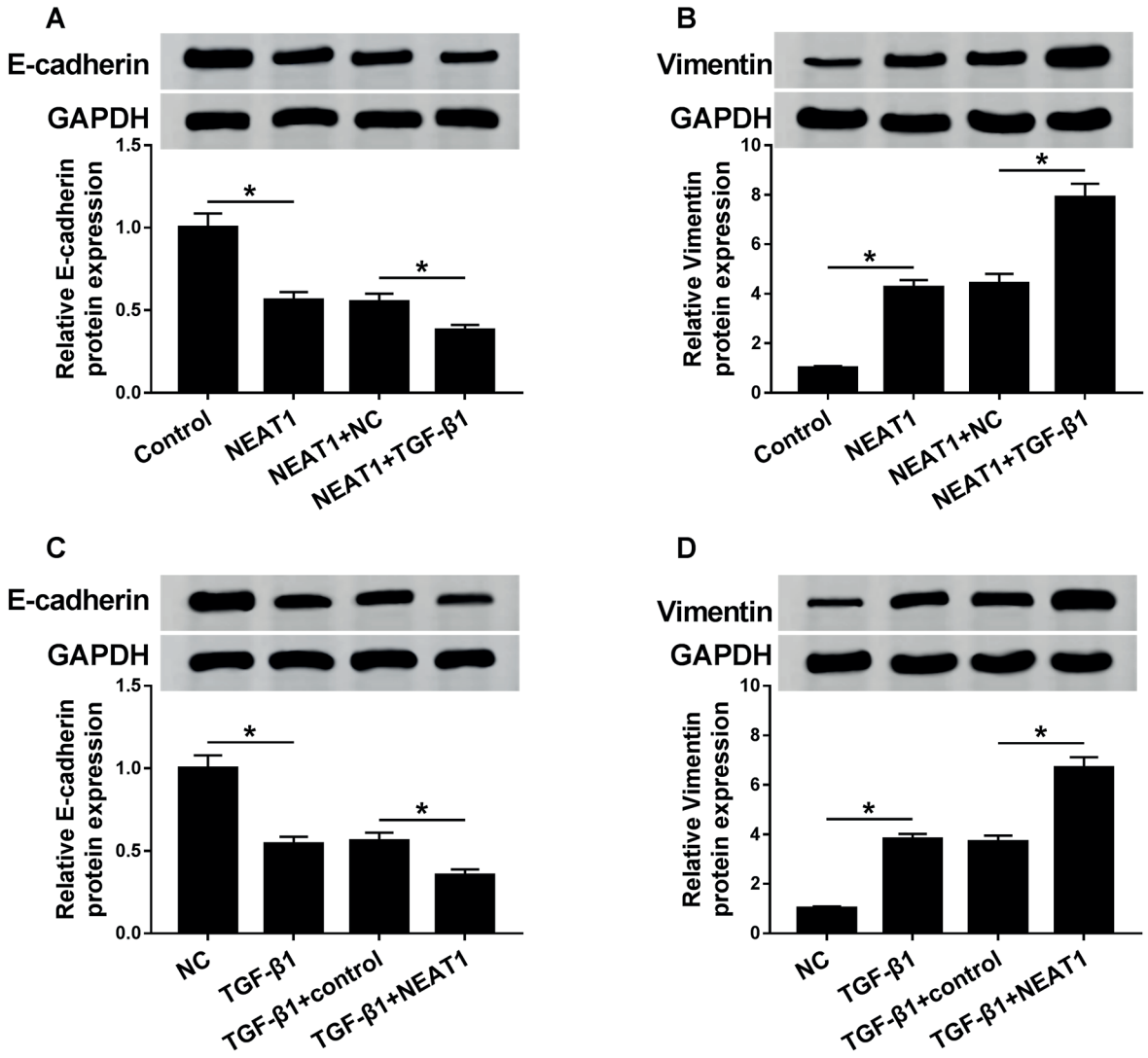

Figure 4. NEAT1 promotes EMT of MMCs. **(A** and **B)** MMCs were transfected with NEAT1 or control, and the NEAT1 group was further treated with negative control (NC) or 10 ng/mL TGF-β1 for 96 h. The abundance of EMT-related proteins, including E-cadherin and Vimentin, was determined in the above MMCs by Western blot. **(C** and **D)** MMCs were treated with NC or 10 ng/mL TGF-β1, and MCCs transfected with NEAT1 or control were also treated with 10 ng/mL TGF-β1. Western blot was performed to detect the enrichment of E-cadherin and Vimentin in the above MMCs. *$p<0.05$.

NEAT1 Facilitates Fibrosis and EMT of MMCs by Serving as a Competing Endogenous RNA (ceRNA) of miR-23c

MMCs were treated with high concentration of glucose (H) and respectively transfected with si-NC, si-NEAT1, si-NEAT1 and anti-miR-NC or anti-miR-23c. Western blot assay was carried out to detect the abundance of fibrosis-related proteins (P38, ASK1, Fibronectin and α-SMA) and EMT-associated proteins (E-cadherin and Vimentin). As mentioned in Figure 6A, the knockdown of NEAT1 reduced the enrichment of P38, ASK1, Fibronectin and α-SMA, and the depletion of miR-23c abrogated the down-regulation of the four proteins caused by NEAT1 interference. Meanwhile, the co-transfection of si-NEAT1 and anti-miR-23c alleviated the declination of Vimentin and the up-regulation of E-cadherin caused by NEAT1 inhibition (Figure 6B). Thus, NEAT1 promoted fibrosis and EMT of MMCs at least partly through sponging miR-23c.

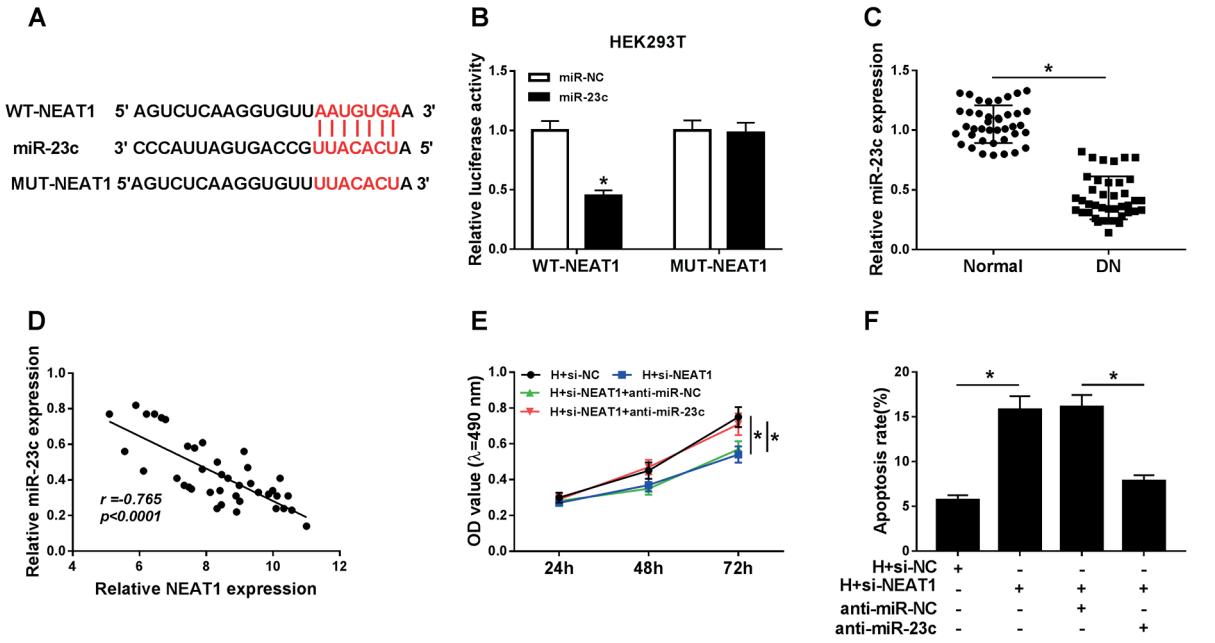

Figure 5. NEAT1 accelerates proliferation and restrains apoptosis of MMCs by sponging miR-23c. **(A)** The complementary sites between lncRNA NEAT1 and miR-23c were predicted by starBase bioinformatics software. **(B)** Dual-Luciferase reporter assay was conducted in HEK293T cells co-transfected with WT-NEAT1 or MUT-NEAT1 and miR-NC or miR-23c to detect the changes of Luciferase activity in multiple groups. **(C)** The abundance of miR-23c was measured in the serum of normal patients and DN patients by qRT-PCR. **(D)** The correlation between the expression of miR-23c and the level of NEAT1 in the serum of DN patients was analyzed. **(E** and **F)** Cell proliferation and apoptosis were examined in MMCs treated with high concentration of glucose (H) + si-NC transfection, H + NEAT1 interference, H + NEAT1 interference + anti-miR-NC transfection and H + NEAT1 interference + miR-23c inhibition by MTT assay or flow cytometry, respectively. *$p<0.05$.

Discussion

Diabetic nephropathy (DN) is a common renal disease of patients with DM, which results in renal failure. Accumulating evidence suggested that lncRNAs and miRNAs are involved in the initiation and development of DN[22,23]. Our study first identified miR-23c as a direct target of lncRNA NEAT1 and illustrated that lncRNA NEAT1 facilitated proliferation, fibrosis and EMT through sponging miR-23c in DN.

Researches have proved that lncRNAs could regulate multiple biological processes, including cellular metabolism[5,24]. LncRNA NEAT1 has been reported to play an oncogenic role in esophageal squamous cell carcinoma (ESCC) and gastric cancer (GC)[9,25]. Chen et al[9] reported that NEAT1 was dysregulated in ESCC, and the abundance of NEAT1 was positively related to tumor volume, lymph node metastasis and clinical staging of ESCC patients and was positively correlated with proliferation, migration and invasion of ESCC cells. Fu et al[25] demonstrated that lncRNA NEAT1 promoted metastasis of GC cells. Recent investigations claimed that NEAT1 could modulate extracellular matrix accumulation, EMT, proliferation and fibrosis in DN[11,12]. Consistent with the above findings, qRT-PCR results showed that NEAT1 was markedly enhanced in the serum of DN patients and high-concentration-glucose-induced MMCs compared with that in the serum of normal patients and low-concentration-glucose-induced MMCs. To explore the biological role of NEAT1 in high-concentration-glucose-induced MMCs, MTT assay and flow cytometry were conducted to measure cell proliferation and apoptosis, and qRT-PCR and Western blot assays were performed to examine the mRNA and protein levels of proteins associated with cell growth, apoptosis, fibrosis and EMT. EMT is a crucial process during DN progression. During the EMT process, epithelial cells undergo a series of changes, including loss of epithelial markers, the acquisition of mesenchymal markers and ECM deposition. The overexpression of NEAT1 facilitated proliferation, fibrosis and EMT, while restrained apoptosis of high-concentration-glucose-induced MMCs.

We aimed to find the downstream component of NEAT1 to better understand the regulatory mechanism of NEAT1 on diabetic nephropathy. MiR-23c was screened to be a potential target of NEAT1 by bioinformatics analysis, and the combination between miR-23c and NEAT1 was validated by dual-luciferase reporter assay. MiR-23c has been proved to play a suppressive role in human hepatocellular carcinoma (HCC)[26]. The potential mechanism by which miR-23c modulates DN remains barely known. Li et al[20] demonstrated that miR-23c was reduced in high-glucose-treated HK-2 cells, and lncRNA MALAT1 promoted the development of DN via miR-23c/ELAVL1 axis. Consistent with these findings, the abundance of miR-23c was declined in the serum of DN patients compared with that in the serum of normal patients. As mentioned above, the enrichment of NEAT1 was higher in the serum of DN patients than that in the serum of normal patients, showing an inverse trend with the abundance of miR-23c. Thus, there was a significantly negative relationship between the enrichment of miR-23c and the level of NEAT1.

We wondered whether miR-23c was involved in NEAT1-mediated cell proliferation, apoptosis, fibrosis and EMT. MMCs treated with high concentration of glucose were respectively transfected

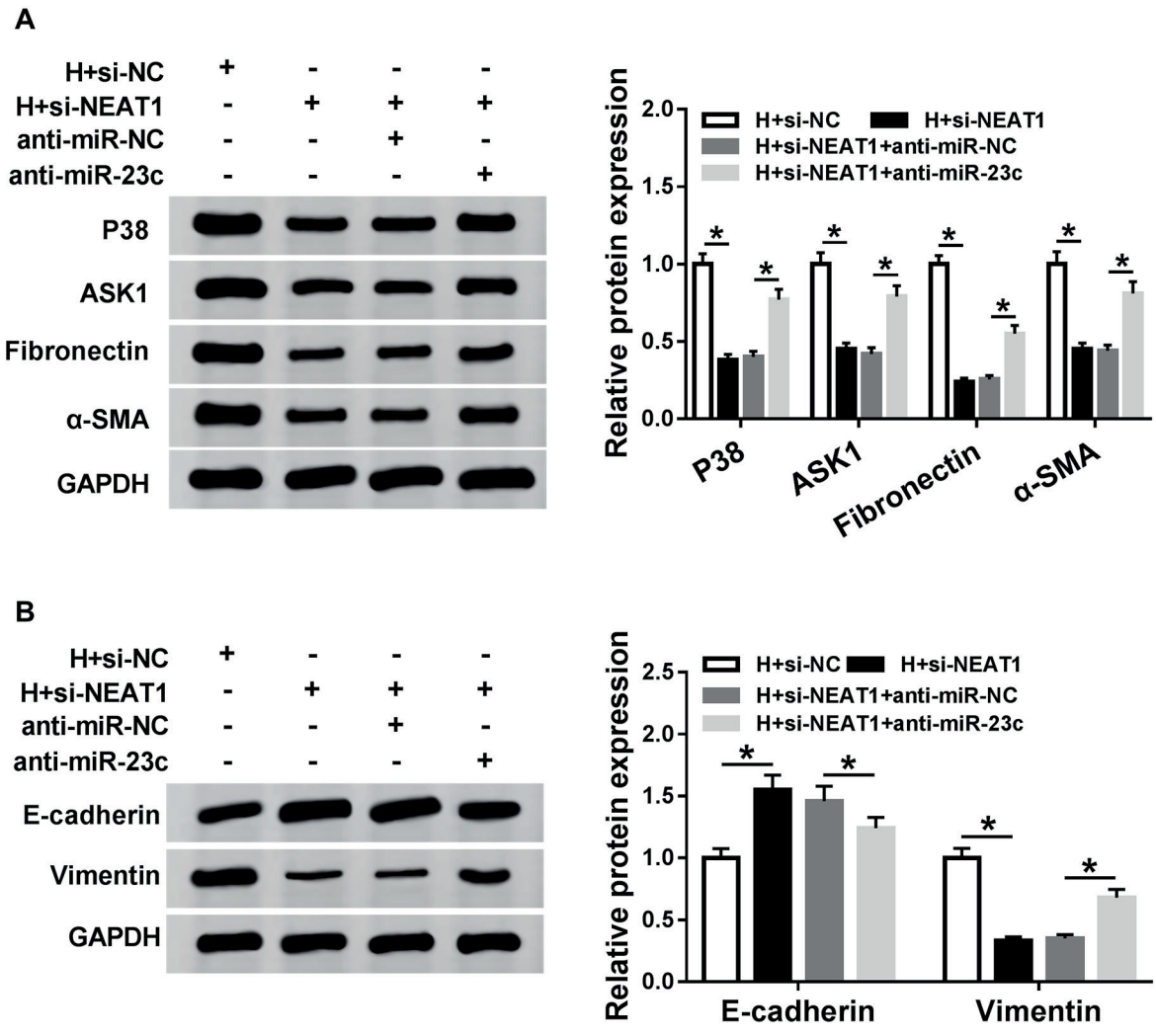

Figure 6. NEAT1 facilitates fibrosis and EMT of MMCs by serving as a competing endogenous RNA (ceRNA) of miR-23c. MMCs were treated with high concentration glucose (H) + si-NC transfection, H + NEAT1 interference, H + NEAT1 interference + anti-miR-NC transfection and H + NEAT1 interference + miR-23c inhibition. **(A)** The enrichment of P38, ASK1, Fibronectin and α-SMA was measured in the above MMCs by Western blot. **(B)** The expression of E-cadherin and Vimentin was detected in the above MMCs by Western blot. *p<0.05.

with si-NC, si-NEAT1, si-NEAT1 and anti-miR-NC or anti-miR-23c. MTT assay was performed to detect the proliferation of MMCs in four different groups. NEAT1 intervention inhibited cell proliferation, and the addition of anti-miR-23c counteracted the suppressive impact of NEAT1 depletion on cell proliferation. Meanwhile, the transfection of miR-23c inhibitor alleviated the suppressive effect of NEAT1 knockdown on cell fibrosis and EMT. Besides, NEAT1 interference promoted cell apoptosis, and the co-transfection of anti-miR-23c reversed the promoting effect caused by NEAT1 inhibition on cell apoptosis.

ERBB2IP has been reported to be a target of miR-23c in HCC[27,28]. We will investigate whether ERBB2IP bind to miR-23c in DN and whether NEAT1/ miR-23c axis functions through ERBB2IP in the future research.

Conclusions

Collectively, lncRNA NEAT1 promoted proliferation, fibrosis and EMT while impeded apoptosis of MMCs via sponging miR-23c. These findings suggested that NEAT1 might function as an effective marker and underlying therapeutic target for DN therapy.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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