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Long non-coding RNA NEAT1 inhibits high glucose-induced EMT and renal fibrogenesis in Human Embryonic Kidney 293 cells via regulating miR-204/SOX4 axis

Mohammad Ahmad Wadaan[1*], Mohammad Ahmad[2], Israa Hussein Hamzah[3*]

[1] Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia
[2] Department of Medical Surgical Nursing, College of Nursing, King Saud University, P.O. Box 642, Riyadh-11421, Saudi Arabia
[3] Biology Department, College of Science, Mustansiriyah University, Iraq

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ABSTRACT

Diabetic nephropathy (DN) is a severe complication of diabetes that increases mortality worldwide. Long non-coding RNAs (lncRNAs) have been investigated in DN, but the precise molecular mechanism is unclear. The research aimed to study the role of lncRNA NEAT1 in DN using an in vitro model, with the goal of uncovering its potential function and molecular mechanism in the development of DN. High glucose was applied to HEK 293 cells in order to create the DN model. The expression levels of NEAT1, miR-204, and SOX4 were assessed using RT-qPCR, along with the mRNA expression of EMT-related biomarkers and fibrosis markers such as α-SMA, E-cadherin, Vimentin, Fibronectin, and Col IV in HEK 293 cells. The interaction between NEAT1, miR-204, and SOX4 was predicted using Starbase 2.0 and confirmed through dual luciferase reporter assay. In HEK 293 cells treated with high glucose, NEAT1, and SOX4 expression were down-regulated, while miR-204 expression increased in a concentration-dependent manner. NEAT1 activation in HEK 293 cells prevented high glucose-induced fibrogenesis and EMT. NEAT1 directly targeted miR-204, and its inhibitory effects on EMT and fibrogenesis were restored by miR-204 overexpression. NEAT1 also regulated high glucose-induced EMT and fibrogenesis through its influence on miR-204 and SOX4. In conclusion, the miR-204/SOX4 axis is a prospective therapeutic target for the treatment of DN since lncRNA NEAT1 inhibited high glucose-induced EMT and fibrogenesis by controlling it in DN.

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Introduction

The most serious consequence of diabetes mellitus, diabetic nephropathy (DN), causes end-stage renal failure (1). DN is characterized by various pathological features, including the accumulation of extracellular matrix (ECM), tubulointerstitial fibrosis (TIF), basement membrane thicknessening, glomerulosclerosis, and renal tubal epithelial-mesenchymal transition (EMT) (2–4). The excessive deposition of ECM proteins such as laminin, proteoglycan, fibronectin, and collagen contributes to renal fibrogenesis (5). Despite the substantial investigation, the specific underlying process of DN remains unknown, highlighting the importance of effective treatment targets.

EMT is essential for the malfunction of podocytes, the onset of renal fibrosis, tubular atrophy, and the production of myofibroblasts (6,7). EMT is a process in which polarized epithelial cells undergo phenotypic changes, resulting in the loss of adhesion properties. As a consequence, these cells acquire a mesenchymal phenotype characterized by increased invasiveness (8). This procedure comprises a rise in mesenchymal-related proteins and a fall in epithelial-related proteins (9). Given the significant role of EMT in stimulating and progressing renal fibrosis in DN, it is essential to explore the underlying mechanisms to better understand the progression of this condition.

Non-coding RNAs of the kind known as long non-coding RNAs (lncRNAs) include more than 200 nucleotides but do not encode proteins (10). Numerous studies have shown that lncRNAs are essential for many biological functions and human illnesses, including metabolic problems and numerous cancers (11). Additionally, previous research has shown that lncRNAs are important in the control of fibrosis and proliferation in DN (12). One specific lncRNA, NEAT1, is recognized as being an essential member of the paraspeckle structure (13,14) and has been implicated in several serious diseases (15). Additionally, NEAT1 has been associated with lactation and mammary gland development (16). However, the specific molecular mechanisms through which NEAT1 influences EMT and renal fibrosis in DN are still unknown. In this research, we developed a laboratory model of DN using HEK 293 cells and examined the impact of NEAT1 on the process of EMT and fibrosis induced by high glucose levels. Furthermore, we aimed to unravel the molecular mechanism underlying NEAT1's involvement in the pathogenesis of DN.

Materials and Methods

Cell culture

HEK 293 cells were purchased from American Type Culture Collection (ATCC, VA, USA) and cultured in a DMEM (Sigma Aldrich, MA, USA) containing 10% fetal

* Corresponding author. Email: Israaalkadmy.micro@gmail.com; wadaan@ksu.edu.sa

bovine serum (FBS; Biocampre, CA, USA) with 5% CO_2 at 37 ^{0}C. HEK 293 cells were treated with high glucose (HG, 30 mM glucose) and normal glucose (NG, 5.5 mM glucose) for 12, 24, and 48 hrs.

Cell transfection

The following vectors and oligonucleotides were used: NEAT1 overexpression vector, empty vector (pcDNA), miR204 mimic, mimic negative control (miR-NC), si-SOX4 and siRNA negative control (siNC). All the vectors were obtained from RiboBio, Guangzhou, China). Lipofectamine 2000 was used to transfect these vectors into HEK 293 cells (Thermo Fisher, New Jersey, USA) according to the instructions of the manufacturer.

RT-qPCR

Total RNA from HEK 293 cells was extracted using the Trizol reagent (Thermo Fisher, New Jersey, USA). High Capacity cDNA Reverse Transcription kit (GIAGEN, Hilden, Germany) was used to reverse transcribe RNA into cDNA. Finally, SYBR Premix Ex Taq II (TakaraBio, Goteburg, Sweden) was used to measure the expression levels of mRNA. GAPDH and U6 were taken as standardized references. A $2^{-\Delta\Delta Ct}$ method was used to calculate the results of PCR. The primers used are given in Table 1.

HEK 293 cells were obtained from the American Type Culture Collection (ATCC, VA, USA) and grown in DMEM (Sigma Aldrich, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biocampre, CA, USA) at 37°C with 5% CO2. The cells were exposed to high glucose (30 mM glucose) and normal glucose (5.5 mM glucose) for 12, 24, and 48 hours.

Cell Transfection The following vectors and oligonucleotides were utilized: NEAT1 overexpression vector, empty vector (pcDNA), miR204 mimic, mimic negative control (miR-NC), si-SOX4, and siRNA negative control (siNC). All vectors were obtained from RiboBio, Guangzhou, China. Lipofectamine 2000 (Thermo Fisher, New Jersey, USA) was used in accordance with the manufacturer's instructions to transfect these vectors into HEK 293 cells.

Utilizing the Trizol reagent, total RNA for RT-qPCR was obtained from HEK 293 cells (Thermo Fisher, New Jersey, USA). The RNA was converted into cDNA using the High Capacity cDNA Reverse Transcription kit (QIAGEN, Hilden, Germany). The mRNA expression levels were quantified using the SYBR Premix Ex Taq II method (TakaraBio, Goteburg, Sweden). GAPDH and U6 were used as reference genes for normalization. The $2^{-\Delta\Delta Ct}$ method was utilized to analyze and determine the PCR results. Table 1 listed the primers that were utilized.

Dual-luciferase reporter essay

Fragments of NEAT1 (NEAT1-WT) or the 3' UTR of SOX4 (SOX4-WT) containing predicted binding sites for miR-204, along with mutant fragments (NEAT1-MUT or SOX4-MUT), were inserted into pgGL3 plasmids (Biocompare, A, USA). HEK 293 cells were transfected with recombinant vectors and either miR-204 or miR-NC using Lipofectamine 2000 (Thermo Fisher, New Jersey, USA) as per the manufacturer's guidelines. The luciferase activity was assessed using the Dual-Luciferase reporter assay system (Biovision, CA, USA).

Analysis of the data

All collected data were subjected to analysis using SPSS 19.0 (IBM, IL, USA) and presented as mean ± standard deviation (SD). Statistical analysis was carried out using GraphPad Prism 7.0 (GraphPad Prism Software, CA, USA). Student's t-test and one-way analysis of variance (ANOVA) were employed to compare groups, depending on whether there were two or more groups, respectively. Statistical significance was defined as a p-value less than 0.05.

Results

The inhibitory effect of NEAT1 overexpression on HG-induced epithelial-

epithelial-mesenchymal transition (EMT) and renal

Table 1. Primer sequences and other study-related details.

Primers		Sequence (5'→3')
NEAT1	Forward	TGGCTAGCTCAGGGCTTCAG
NEAT1	Backward	TCTCCTTGCCAAGCTTCCTTC
SOX4	Forward	TTCAGCAACCAGCATTCGAG
SOX4	Backward	GCCTCTCGCAGAGGAGTTAT
miR-204	Forward	AGGCAUAGGAUGACAAAGGGAA
miR-204	Backward	AGGCAUAGGAUGACAAAA
α-SMA	Forward	ACGTGGAGCTGTACCAGAAA
α-SMA	Backward	GCAGTGTGTTATCCCTGCTG
E-cadherin	Forward	ACGCATTGCCACATACACTC
E-cadherin	Backward	AGAGGTTCCTGGAAGAGCAC
Vimentin	Forward	CCCTCACCTGTGAAGTGGAT
Vimentin	Backward	TGACGAGCCATTTCCTCCTT
Fibronectin	Forward	GGTACAGGGTGACCTACTCG
Fibronectin	Backward	GGGCTGGCTCTCCATATCAT
Col IV	Forward	CTGGGTAGCCCTGACTGTAG
Col IV	Backward	CCAGAGTTGAGGCAGGAAGT
β-actin	Forward	ACCCAGAAGACTGTGGATGG
β-actin	Backward	TCAGCTCAGGGATGACCTTG

fibrogenesis were investigated in HEK 293 cells. The relative expressions of NEAT1 and miR-204 were assessed using RT-qPCR, revealing that HG treatment led to a significant reduction in NEAT1 expression and an increase in miR-204 expression in a time-dependent manner (Figure 1A-B). In order to determine how NEAT1 affects the development of diabetic nephropathy, HEK 293 cells were transfected with either pcDNA or NEAT1, then stimulated for 48 hours with normal and high glucose levels. Results from RT-qPCR showed that NEAT1 expression was significantly higher in the NEAT1 group than in the pcDNA group (Figure 1C). In addition, the mRNA expression of mesenchymal markers associated with EMT, such as -SMA and vimentin, was increased whereas that of the epithelial marker E-cadherin was decreased in the HG group when compared to the NG group. Conversely, overexpression of NEAT1 in HG-treated HEK 293 cells resulted in the opposite effect on mRNA expression (Figure 1D). Additionally, the mRNA levels of fibrosis markers, including Fibronectin and Col IV, were increased in HG-induced HEK 293 cells compared to the NG group. However, the overexpression of NEAT1 significantly reduced the mRNA expressions of these fibrosis markers (Figure 1E). Collectively, these results indicate that the increased expression of NEAT1 suppresses EMT and fibrogenesis induced by HG in HEK 293 cells.

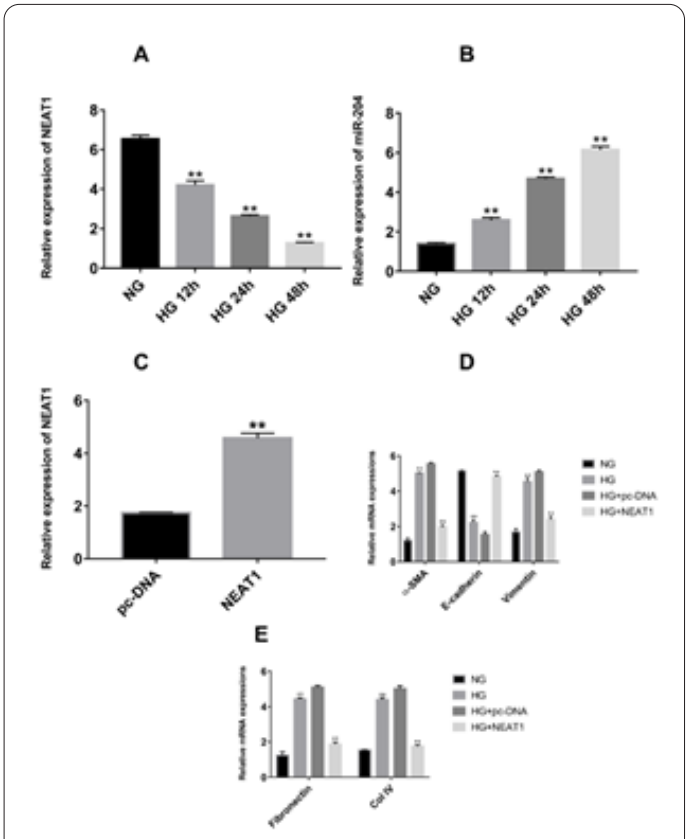

Figure 1. The inhibitory effect of NEAT1 overexpression on HG-induced EMT and renal fibrogenesis in HEK 293 cells (A-B). RT-qPCR was used to determine the relative expression levels of NEAT1 and miR-204 in HEK 293 cells subjected to various concentrations of normal glucose and high glucose (C). NEAT1 expression was evaluated in HEK 293 cells that were transfected with either pcDNA or NEAT1 (D). RT-qPCR analysis was performed to evaluate the relative mRNA expression levels of EMT-related biomarkers (α-SMA, E-cadherin, and Vimentin) and fibrosis markers (Fibronectin and Col IV) in HEK 293 cells transfected with pcDNA or NEAT1.

NEAT1 exerted regulatory control over HG-induced fibrogenesis and EMT in HEK 293 cells by targeting miR-204.

Through the use of dual luciferase reporter assays and Starbase 2.0, the putative binding targets of NEAT1 were identified. Our findings demonstrated that miR-204 dramatically decreased the luciferase activity of NEAT1-WT, but NEAT1-MUT showed no such impact (Figure 2A-B). Furthermore, the expression of miR-204 was significantly elevated in HG-induced HEK 293 cells compared to the NG group. However, the upregulation of NEAT1 suppressed the expression of miR-204 in comparison to the HG+pcDNA group (Figure 2C). Furthermore, we examined the mRNA expression levels of EMT and fibrosis-related markers in HG-treated HEK 293 cells transfected with pcDNA, NEAT1, NEAT1+miR-NC, and NEAT1+miR-204, using RT-qPCR. Our findings revealed that the mRNA levels of α-SMA and vimentin were significantly reduced, while the expression of E-cadherin was markedly increased in HG-treated HEK 293 cells. However, these changes in mRNA expression were restored by the upregulation of miR-204 (Figure 2D). Furthermore, the increased expression of NEAT1 led to a significant decrease in the expression of fibrosis-related markers such as Fibronectin and Col IV. Conversely, the upregulation of miR-NC reversed the effects of changes in mRNA expressions in HEK 293 cells (Figure 2E). The inhibitory effects of NEAT1 overexpression on EMT and fibrogenesis in HEK 293 cells are reversed by the overexpression of miR-204, according to our data, demonstrating that miR-204 is

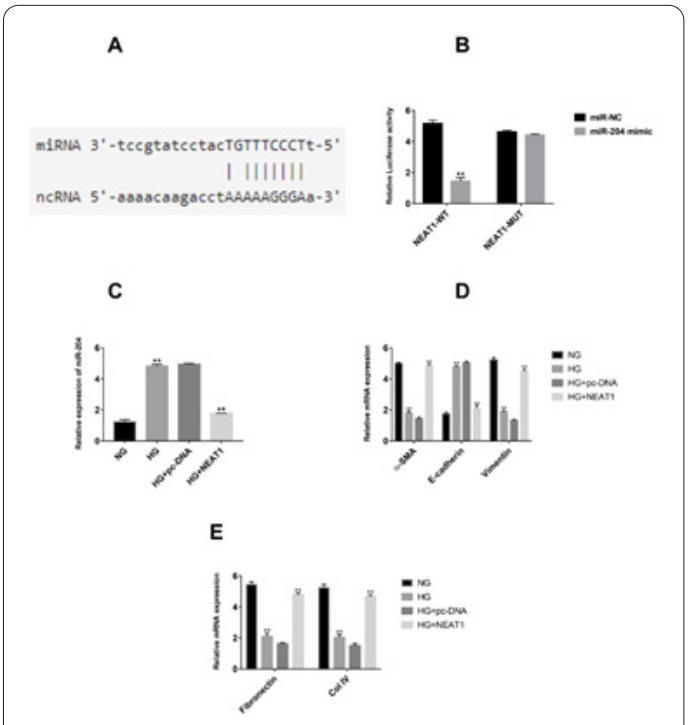

Figure 2. NEAT1 regulated the process of fibrogenesis and EMT induced by HG in HEK 293 cells through its interaction with miR-204: A-B) The interaction between NEAT1 and miR-204 was assessed using Starbase 2.0 software and confirmed with a dual luciferase reporter assay. C) The expression levels of miR-204 were measured using RT-qPCR in HEK 293 cells treated with various interventions. D-E) The mRNA expression levels of EMT-related markers (α-SMA, E-cadherin, and Vimentin) and fibrosis markers (Fibronectin and Col IV) were determined through RT-qPCR analysis in HEK 293 cells exposed to different treatment conditions.

a clear target of NEAT1.

In HEK 293 cells, miR-204 targeted and regulated SOX4

Through the utilization of bioinformatics software 2.0, SOX4 was predicted and identified as a possible target of miR-204 in HEK 293 cells. (Figure 3A) To validate the interaction between miR-204 and SOX4, a luciferase reporter assay was performed. The results demonstrated that miR-204 noticeably diminished the luciferase activity of SOX4-WT. Conversely, there was no alteration in the luciferase activity of SOX4-MUT. (Figure 3B) By using RT-qPCR, it was also shown that HG treatment of HEK 293 cells resulted in a time-dependent, substantial reduction in SOX4 mRNA expression. (Figure 3C) Moreover, when HEK 293 cells were transfected with miR-NC (negative control) and miR-204, the mRNA expression of SOX4 was significantly reduced in the miR-204 mimic group compared to the miR-NC group. This observation suggests that miR-204 overexpression leads to a noticeable decrease in the expression of SOX4 in HEK 293 cells. (Figure 3D) These findings show that in HEK 293 cells, miR-204 specifically targets SOX4 and adversely controls its expression.

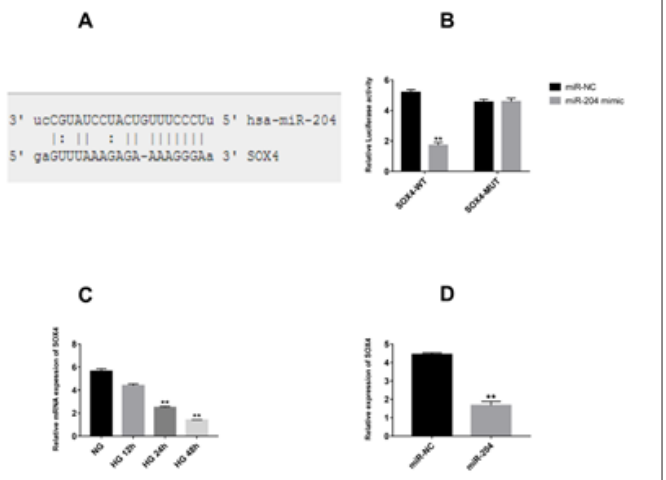

Figure 3. Targeting of SOX4 by miR-204 in HEK 293 cells: A-B) Utilizing Starbase 2.0 software and a dual luciferase reporter assay, the interaction between SOX4 and miR-204 was predicted and confirmed, respectively. C) The mRNA levels of miR-204 were assessed in HEK 293 cells subjected to normal and high glucose treatments. D) The mRNA expression of miR-204 was measured in HEK 293 cells transfected with miR-NC and miR-204, respectively.

NEAT1 inhibited HG-induced fibrogenesis and EMT by targeting SOX4 in HEK 293 cells

To investigate the interplay between NEAT1, miR-204, and SOX4, HEK 293 cells were transfected with pcDNA, NEAT1, NEAT1+miR-NC, and NEAT1+miR-204. Our findings demonstrated that the mRNA expression of SOX4 was significantly upregulated by NEAT1 overexpression, whereas transfection with miR-204 resulted in the opposite effect in HEK 293 cells (Figure 4A). Additionally, HG-treated HEK 293 cells were transfected with pcDNA, NEAT1, NEAT1+si-NC, and NEAT1+si-SOX4, and the mRNA levels of EMT and fibrosis-related markers were examined using RT-qPCR. The findings indicated that the increased expression of NEAT1 led to a notable decrease in the mRNA levels of EMT induced by HG. However, when HEK 293 cells were transfected with si-SOX4, the mRNA levels were restored. (Figure 4B). Furthermore, in the HG+NEAT1 group, the expression levels of fibrosis-related proteins exhibited a significant decrease compared to the HG+pcDNA group. However, this effect was reversed when SOX4 was down-regulated, indicating that the reduction in fibrosis-related protein expression caused by NEAT1 overexpression was dependent on the inhibition of SOX4. (Figure 4C). These results imply that NEAT1 controls SOX4 expression through miR-204 targeting and blocks HG-induced EMT and fibrogenesis by specifically targeting SOX4 in HEK 293 cells.

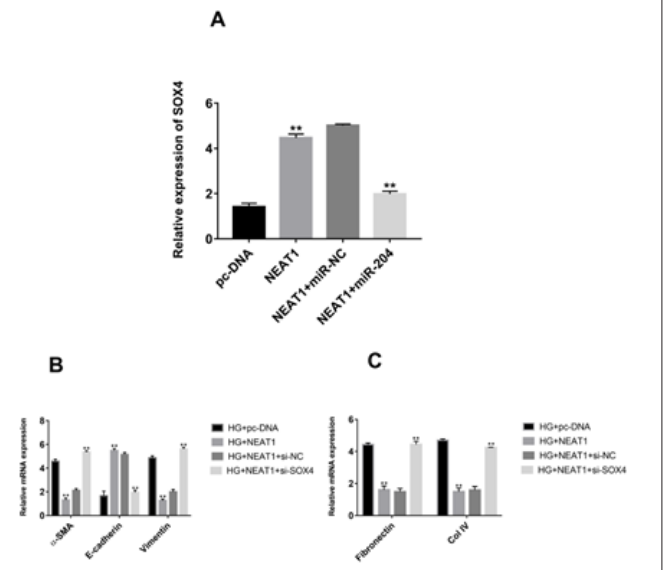

Figure 4. NEAT1 inhibited HG-induced fibrogenesis and EMT by targeting SOX4 in HEK 293 cells: A) HEK 293 cells were subjected to transfection with different constructs, including pcDNA, NEAT1, NEAT1+miR-NC, and NEAT1+miR-204. The mRNA expression level of SOX4 was subsequently assessed using RT-qPCR, B-C) The relative mRNA expression of EMT-related biomarkers (α-SMA, E-cadherin and Vimentin) and fibrosis markers (Fibronectin and Col IV) in HEK 293 cells after transfection with pcDNA, NEAT1, NEAT1+siNC, NEAT1+siSOX4 were determined by using RT-qPCR. $P < 0.05$

Discussion

Although significant research has been conducted on the treatment of DN, the prognosis for DN patients remains poor. Previous research has shown how crucial EMT is to the onset of tubulointerstitial fibrosis in DN (17). Nevertheless, the pathophysiology of DN still has not been fully explained. NEAT1, a long non-coding RNA, has been shown to play a crucial role in the regulation of various diseases, including cancer, by protecting cells from apoptosis (18, 19, 20). Another study reported up-regulation of NEAT1 in sepsis-induced acute kidney injury (21). In our investigation, HG treatment of HEK 293 cells resulted in an increase in the mRNA levels of EMT markers and fibrosis-related markers, whereas diabetic nephropathy was associated with a decrease in NEAT1 expression. Furthermore, overexpression of NEAT1 inhibited HG-induced EMT and fibrogenesis in HEK 293 cells.

Small non-coding RNAs known as microRNAs (miRNAs) are essential for controlling gene expression. They have been linked to the pathophysiology of several renal disorders in addition to kidney growth and homeostasis

(22). Among these miRNAs, miR-204 has been shown to be down-regulated in kidney biopsies from chronic kidney disease patients (23) and in kidney allografts with interstitial and tubular fibrosis (24). In order to control gene expression, long non-coding RNAs (lncRNAs) have been widely explored for their capacity to attach to miRNAs in a competitive manner (25). The Starbase software 2.0 predicted that miR-204 could be a target of NEAT1. Prior research has indicated that IL6R amplifies the expression of miR-204 in an in vitro model involving renal tubular epithelial cells (26). Our findings demonstrated that miR-204 expression was increased in HG-treated HEK 293 cells, consistent with previous reports. Furthermore, NEAT1 acted as a sponge for miR-204, regulating EMT and fibrogenesis.

The mRNA targets MEIS1, HOXA4, RUNX2, and SIRT1 have all been recognized as significant regulators of typical cell development (27). Among these targets, the well-known sex-determinating gene SOX4 is present, and it is distinguished by a conserved region in the HMG DNA-binding domain (28). It has been demonstrated that SOX4 expression is elevated in a number of malignancies, including ovarian, lung, brain, breast, and salivary gland cancer (29). Additionally, SOX4 has been identified as a direct target of TGF-β1 and is involved in the regulation of TGF-β1-induced EMT in various human cells (30). Recent studies have demonstrated that miR-204 directly targets SOX4, reducing migration, cell proliferation, and invasion in renal cell carcinoma cell lines (31). In our research, we discovered that miR-204 has a direct impact on SOX4, effectively reducing its expression in HEK 293 cells treated with HG. Furthermore, NEAT1 influenced the expression of SOX4 by acting as a sponge for miR-204. This inhibition of miR-204 by NEAT1 resulted in the suppression of HG-induced EMT and fibrogenesis in HEK 293 cells by increasing the levels of SOX4 expression.

In conclusion, the study revealed that NEAT1 plays a crucial role in regulating the expression of SOX4 through its interaction with miR-204. Moreover, NEAT1 was found to effectively counteract the adverse effects of HG by modulating the NEAT1/miR-204/SOX4 pathway, resulting in improved EMT and decreased fibrogenesis. These findings indicate the potential of targeting this pathway as a therapeutic strategy for managing diabetic nephropathy.

Interest conflict

The authors declare no conflicts of interest.

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