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miR-206 Inhibits Cell Proliferation and Extracellular Matrix Accumulation by Targeting Hypoxia-Inducible Factor 1-alpha (HIF-1 α) in Mesangial Cells Treated with High Glucose

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The goal of this study was to investigate the expression of miR-206 in human glomerular mesangial cells (hMCs) treated by exposure to high glucose (HG) levels, to assess the influence of miR-206 on the proliferation and extracellular matrix (ECM) deposition of hMCs, and to investigate the potential mechanisms of action.


Material/Methods: The level of miR-206 was detected by RT-qPCR. MTT assay and colony formation assay were used to assess hMCs cell proliferation ability. Western blotting was carried out to measure the expression of related proteins. Bioinformatics software (<http://www.targetscan.org>) was used to predict the potential target genes of miR-206, and dual-luciferase reporter assay was used to confirm this prediction.

Results: Our results suggest that the level of miR-206 was downregulated in HG-treated hMCs. Cell proliferation was promoted in HG-induced hMCs, while this phenomenon was significantly reversed with miR-206 mimics. miR-206 mimics significantly enhanced p21 expression and decreased cyclin D1 and CDK2 expressions, but the opposite was found in HG-induced hMCs. Moreover, the level of ECM proteins was notably increased in hMCs treated with HG, which was also significantly reversed by miR-206 mimics. miR-206 inhibitor had the opposite effects. Furthermore, HIF-1 α was found to be a direct target of miR-206, and was negatively regulated by miR-206 in hMCs. miR-206 can target HIF-1 α to modulate cell proliferation and ECM accumulation.

Conclusions: Collectively, our results suggest that miR-206 plays a vital role in HG-treated hMCs through inhibiting cell proliferation and ECM accumulation, partly via targeting HIF-1 α .

MeSH Keywords: **Diabetic Nephropathies • Extracellular Matrix • Mesangial Cells • MicroRNAs**

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Background

Diabetic nephropathy (DN), one of the major causes of diabetic microvascular complications, is also a leading cause of chronic renal failure in diabetic patients worldwide [1,2]. The pathological changes of DN include mesangial cell abnormalities and excessive accumulation of extracellular matrix (ECM). Various studies have revealed that proliferation of mesangial cells is critical in the initiation and progression of DN [3]. In HG condition, glomerular mesangial cells (MCs) become dysfunctional, causing a relative imbalance between ECM protein secretion and degradation, and many ECM proteins accumulate in the mesangial area and basement membrane area. This leads to pathological changes in glomerular morphology, structure, and function, and ultimately leads to the occurrence of glomerulosclerosis. In recent years, a variety of factors have been identified to play important roles in DN development, but the underlying mechanisms remain largely unclear.

MicroRNAs (miRNAs), a class of single-stranded noncoding RNAs (~22 nucleotides), can post-transcriptionally regulate gene expression through promoting mRNA degradation and repressing their translation via incompletely binding to the 3' untranslated regions (UTR) of the targeted mRNAs [4–6]. Numerous reports have indicated that abnormal expression of miRNAs is involved in the development of DN [7]. miR-30c was found to be overexpressed in kidneys of db/db mice, and both miR-30c and miR-26a played a protective role against DN by suppressing epithelial-to-mesenchymal transition [8,9]. In high-glucose (HG)-induced podocytes, an upregulation of miR-218 was detected, and miR-218 promoted cell apoptosis by inhibiting heme oxygenase-1 and activating p38-MAPK [10]. These studies indicated a rationale for developing miRNA therapeutics to treat DN. An assay of miRNA and mRNA expression profiling on renal biopsy sections was performed by qPCR and microarrays, showing that miR-206 was downregulated in progressive cases and contributed to chronic kidney disease progression via regulating mRNAs involved in renal homeostasis [11]. Previously, we found that the level of miR-206 was obviously reduced in renal artery tissue in diabetic and hyperlipidemic rats, and it plays an important role in hyperglucose- and hyperlipid-induced hyperreactivity [12]. As miR-206 is involved in both kidney and diabetic diseases, we speculated that miR-206 was also involved in DN.

The goal of this study was to investigate the level of miR-206 in HG-treated human glomerular mesangial cells (hMCs), to assess the influence of miR-206 on the proliferation of hMCs and the extracellular matrix deposition, and to explore its possible mechanisms.

Material and Methods

Cell culture

Human glomerular mesangial cells, HEK293T, and HK-2 cells were purchased from American Type Culture Collection (Manassas, VA, USA). All cells were grown in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing high glucose (HG, 25 mM D-glucose) or normal glucose (NG, 5.5 mM D-glucose), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (Sigma, Poole, United Kingdom). Cells were incubated at 37°C with 5% CO₂.

Cell transfection

miR-206 mimic, miR-206 inhibitor, miRNA mimics negative control, and inhibitor negative control were obtained from Applied Biological Materials, Inc. (Richmond, BC, Canada). hMCs (5×10⁴ cells/well) were seeded into 6-well plates and cultured under standard conditions. After 24 h, the cells were transfected with miR-206 mimics, mimics control, miR-206 inhibitors, or inhibitor control using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. After 6 h, the culture medium was replaced with RPMI-1640 containing 30 mM glucose (HG) or 5.5 mM glucose (NG), and cells were incubated for 48 h. The efficiency of transfection was detected by RT-qPCR.

MTT assay

MTT assay was used to assay cell proliferation. Briefly, 48 h after cell transfection, cells (1.0×10⁴ cells/well) were seeded into 96-well culture plates and cultured for 24 h, 48 h, or 72 h, then MTT agent was added according to the manufacturer's protocol. The absorption value was determined at 570 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Each experiment was repeated at least 3 times.

Colony formation assay

After transfection, hMCs (1.0×10³ cells/well) were plated in 6-well plates in triplicate. Medium was changed every 3 days and cells were cultured for 1 week. Then, the plates were stained with 0.5% crystal violet in 4% paraformaldehyde for 5 min for counting the number of colonies. Each experiment was repeated at least 3 times.

Western blot analysis

After 48-h treatment, the protein levels of cyclin D1, CDK2, p21, Fibronectin, type IV collagen, Laminin, and HIF-1α were detected by Western blotting. Briefly, the proteins were extracted from

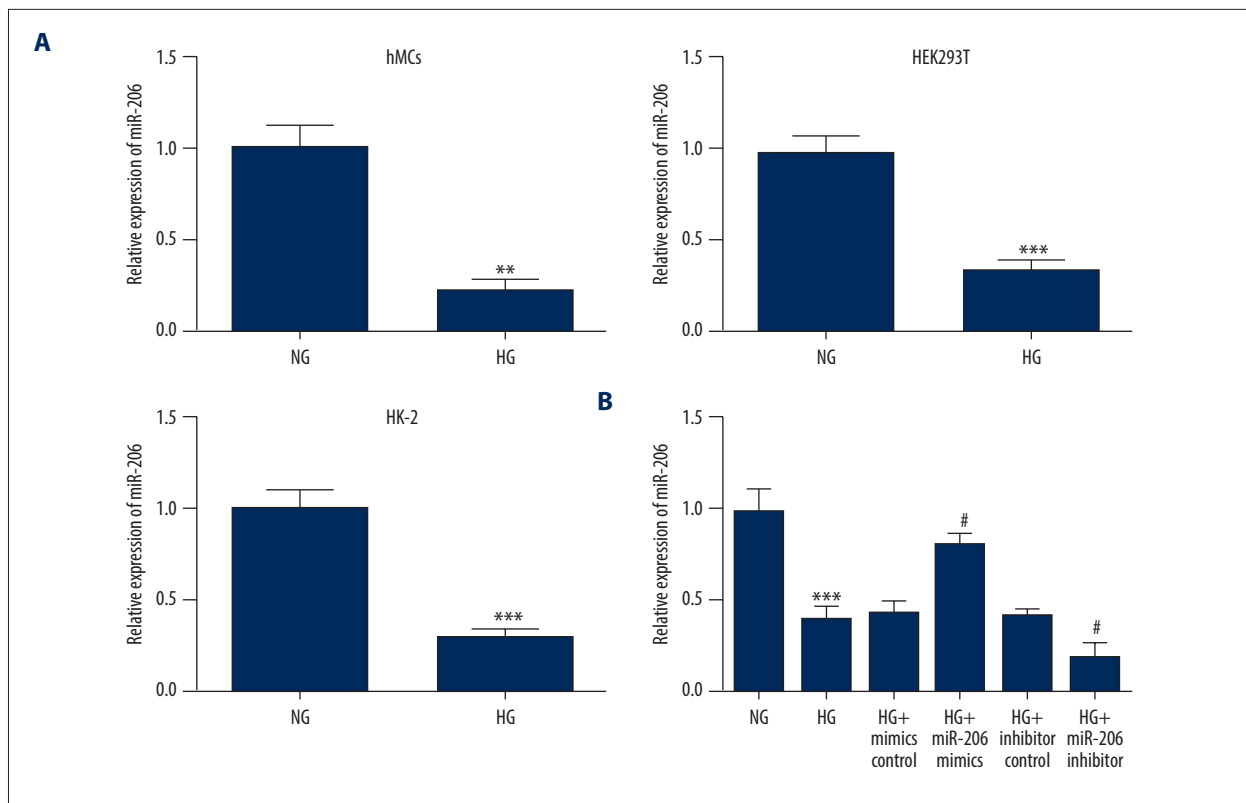


Figure 1. miR-206 was downregulated in hMCs under high-glucose condition. **(A)** hMCs, HEK293T, and HK-2 cells were treated with high glucose (HG, 25 mM D-glucose) or normal glucose (NG, 5 mM D-glucose), and then the level of miR-206 was detected using qRT-PCR. Data are expressed as the mean \pm SD. **(B)** hMCs were transfected with miR-206 mimics, mimics control, miR-206 inhibitors, or inhibitor control, and the transfection efficiency was detected using qRT-PCR. Data are expressed as the mean \pm SD. *** $p < 0.001$ vs. NG; #, ### $p < 0.01, 0.001$ vs. HG.

hMCs using lysis buffer, and the protein concentration was determined using a BCA assay (Thermo Fisher Scientific, Inc.). Equal amounts of protein samples were subjected to 12% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes. Then, membranes were blocked with 5% non-fat milk for 2 h and then incubated overnight at 4°C with primary antibodies against cyclin D1, CDK2, p21, Fibronectin, type IV collagen, Laminin, and HIF-1 α , followed by incubation with horseradish-peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, CA) for 2 h. Protein bands were visualized using an enhanced chemiluminescence detection system (Super Signal West Dura Extended Duration Substrate; Pierce Chemical). GAPDH was used to normalize the signal intensity of each protein. Each analysis was repeated at least 3 times.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted from hMCs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed to cDNA using the PrimeScript reverse transcription reagent kit (Takara Biotechnology Co., Dalian, China). RT-PCR was

performed using the TaqMan Universal PCR Master Mix kit (Thermo Fisher Scientific, Inc.). U6 served as an internal control. The $2^{-\Delta\Delta C_t}$ method was performed for relative gene expression quantification [13]. Each analysis was repeated at least 3 times.

Dual-luciferase reporter assay

Bioinformatics software (<http://www.targetscan.org>) was performed to predict the target gene of miR-206, and the prediction showed that HIF-1 α was a potential target of miR-206. To test our prediction, HIF-1 α -3'UTR-wild-type (WT) or HIF-1 α -3'UTR-mutant were transiently transfected into hMCs, as well as miR-206 mimics or control using Lipofectamine 2000 following the manufacturer's protocol. After 48 h, luciferase activity was calculated using the dual-luciferase reporter assay (Promega, USA). All assays were repeated at least 3 times.

Statistical analysis

Data were analyzed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) and are expressed as the mean \pm standard deviation (SD). Differences between groups were compared by *t* test

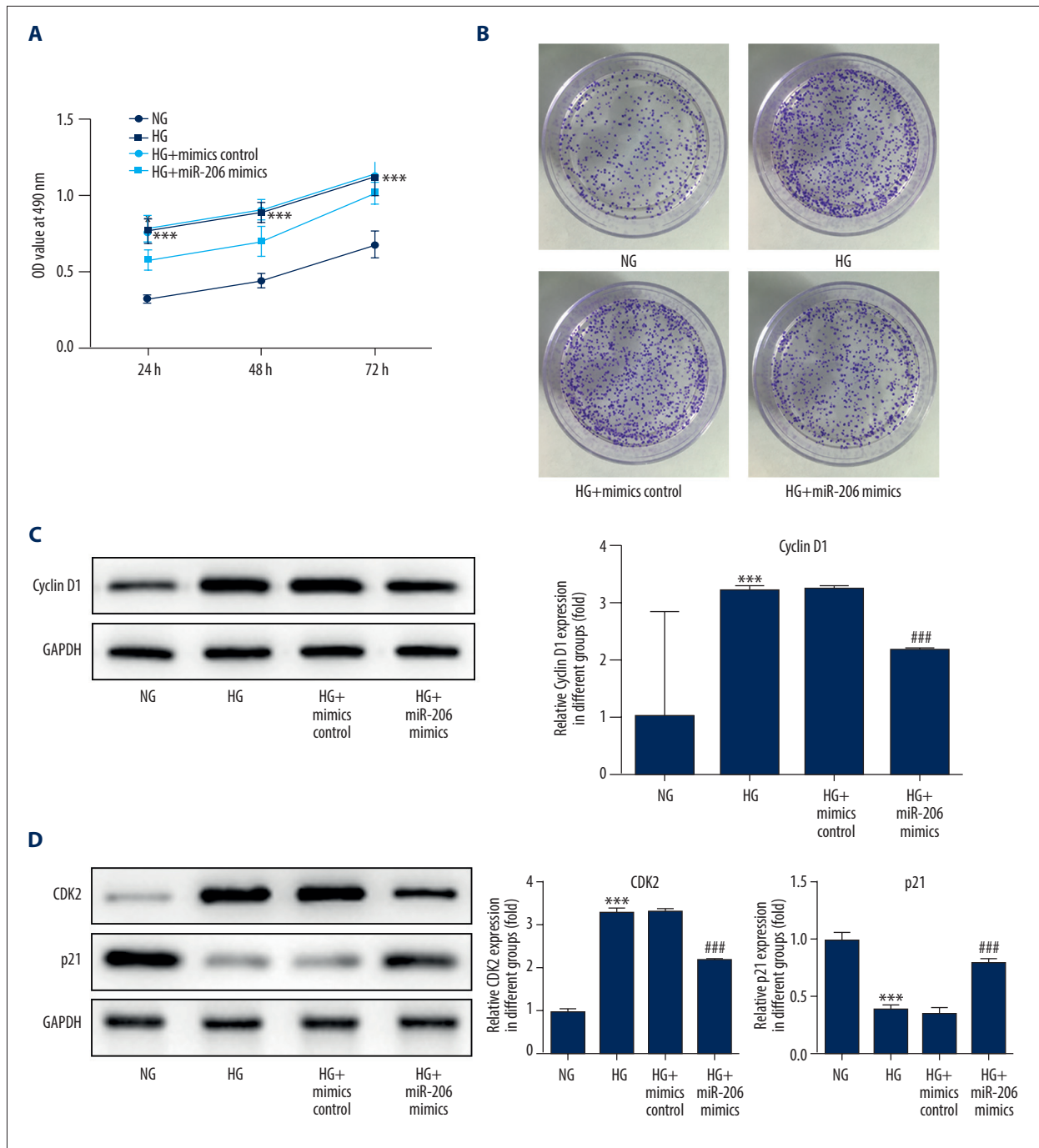


Figure 2. MiR-206 inhibits the proliferation ability of hMCs. After treatment, cell proliferation ability of hMCs was detected using MTT assay (A) and colony formation assay (B). The expression of the proliferation-related proteins, cyclin D1, CDK2, and p21 were analyzed by Western blot (C, D). Data are expressed as the mean±SD. *** $p < 0.001$ vs. NG. #, ### $p < 0.05, 0.001$ vs. HG.

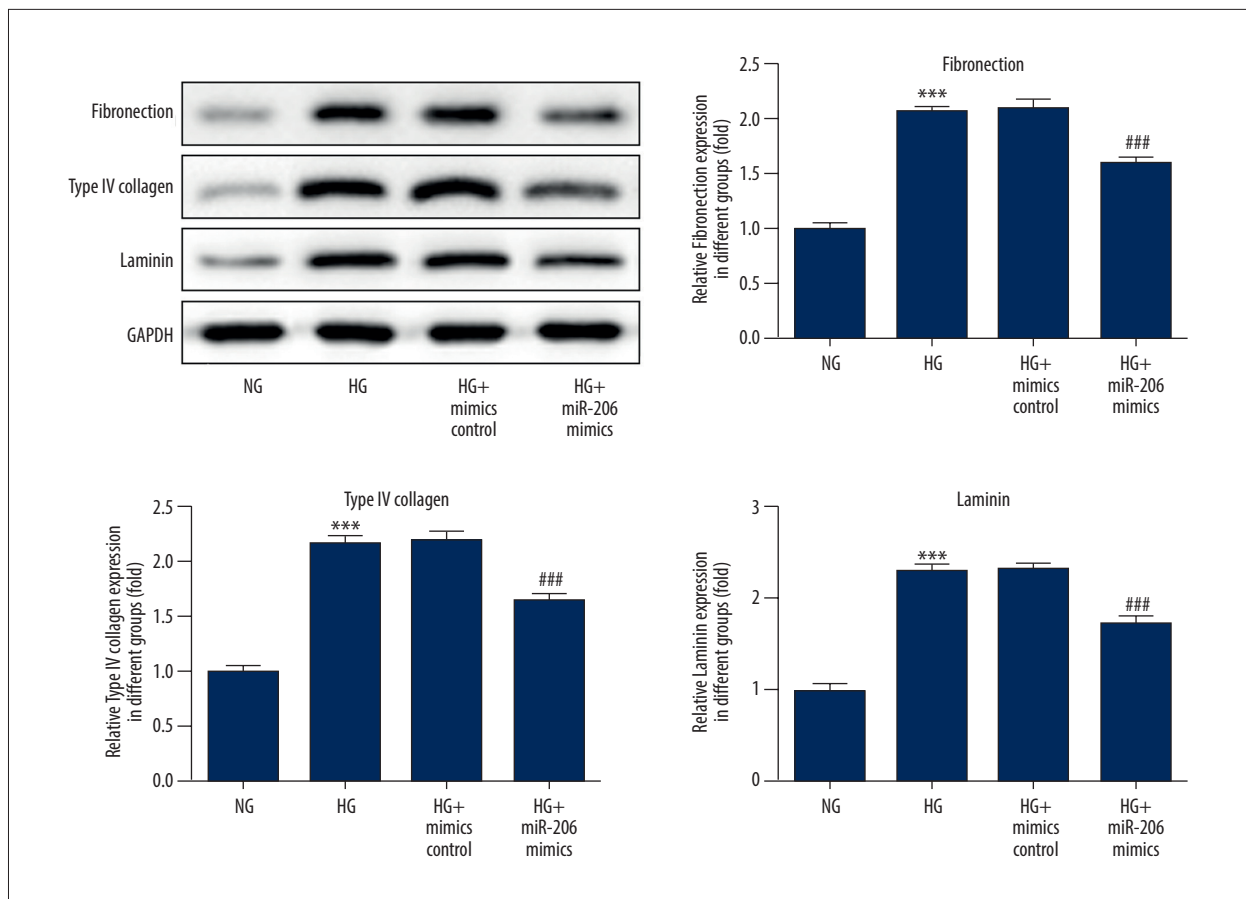


Figure 3. MiR-206 inhibits extracellular matrix accumulation in hMCs. After treatment, the protein expressions of fibronectin, type IV collagen, and laminin were detected using Western blotting and quantified. Data were expressed as the mean \pm SD. *** $p < 0.001$ vs. NG; ### $p < 0.001$ vs. HG.

or one-way ANOVA followed by Tukey's test. Differences were considered as statistically significant at $p < 0.05$.

Results

miR-206 was decreased in hMCs under high-glucose condition

To investigate the role of miR-206 in DN, we first measured the level of miR-206 in HEK293T, HK-2 cells, and hMCs under high-glucose condition. The results showed that the level of miR-206 significantly decreased in cells treated with high glucose compared to the negative control group (NG) (Figure 1A). The level of miR-206 decreased most in hMCs, so we chose hMCs to perform subsequent experiments.

We transfected hMCs with miR-206 mimics, mimics control, miR-206 inhibitors, or inhibitor control, and the efficiency of transfection was detected using qRT-PCR. Compared with HG-induced cells, miR-206 mimics markedly enhanced

miR-206 level, while miR-206 inhibitor decreased miR-206 level (Figure 1B).

miR-206 mimic inhibited hMCs proliferation

First, MTT assay and colony formation assay were carried out to detect the effect of miR-206 on the growth of hMCs. MTT assay showed that compared with NG, HG significantly promoted cell proliferation, while miR-206 mimic obviously inhibited the proliferation ability of hMCs (Figure 2A). Moreover, the colony formation assay demonstrated that the colony formation ability was notably increased when hMCs were treated with HG but was suppressed in cells transfected with miR-206 mimics (Figure 2B).

In addition, some proliferation-related protein expressions were detected to verify the proliferation ability change of hMCs. As shown in Figure 2C, 2D, we assessed the influence of HG and miR-206 on the expression of cyclin D1, CDK2, and p21, which reflected the cell proliferation ability. Our results showed that expressions of cyclin D1 and CDK2 were increased and the

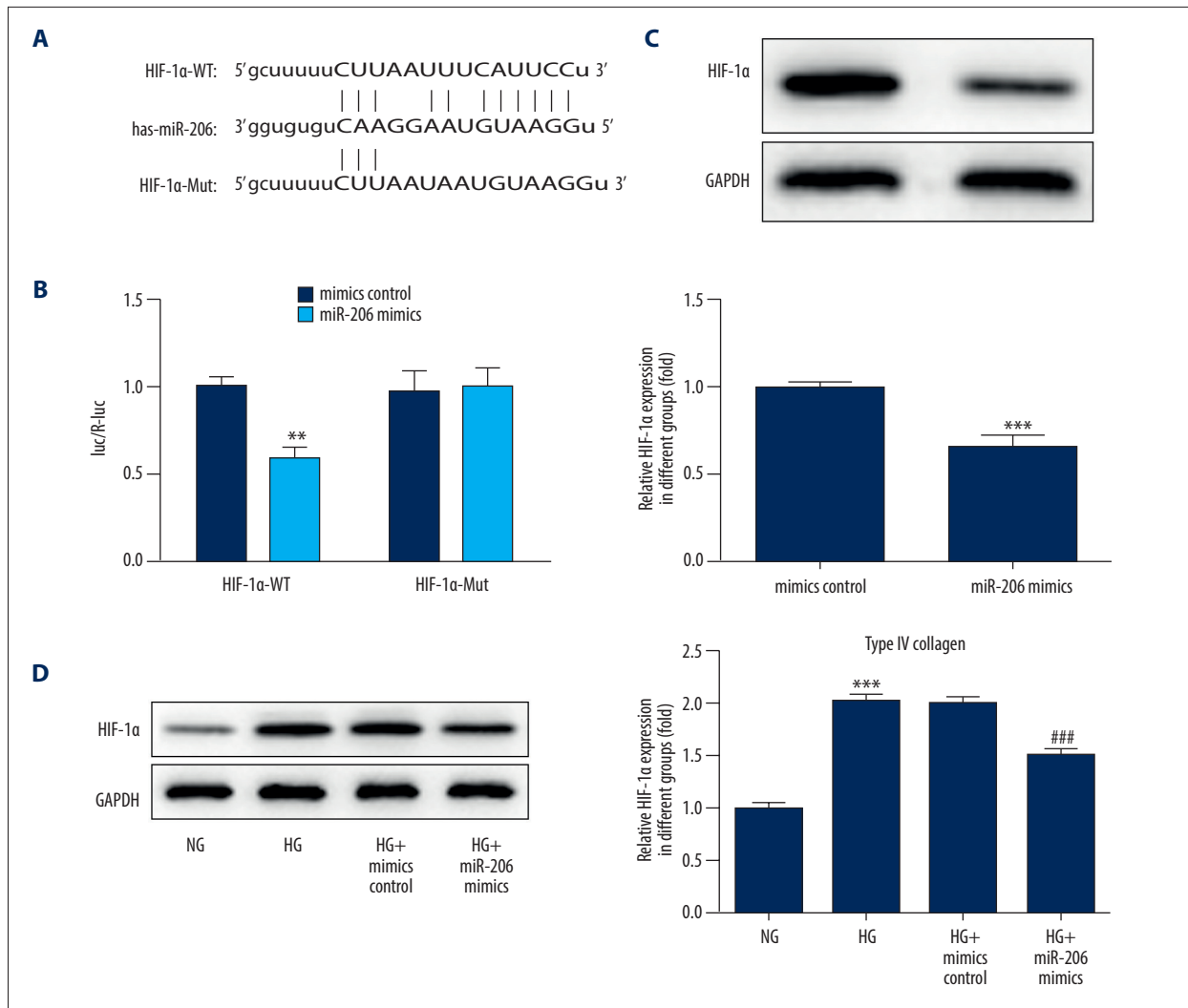


Figure 4. HIF-1 α is a direct target of miR-206. (A) Interaction between miR-206 and 3'UTR of HIF-1 α was predicted by TargetScan; (B) Dual-luciferase reporter assay showed the putative complementary sites within miR-206 with HIF-1 α . WT: wild-type HIF-1 α 3' UTR; MUT: HIF-1 α 3' UTR with a mutation in the miR-206 binding site; UTR, untranslated region. Data are expressed as the mean \pm SD. * $p < 0.05$ vs. control group. (C) Protein levels of HIF-1 α when miR-206 was overexpressed in hMCs were detected using Western blot assay. (D) Protein levels of HIF-1 α in different groups were detected using Western blot assay. Data are expressed as the mean \pm SD. *** $p < 0.001$ vs. NG; ### $p < 0.001$ vs. HG.

expression of p21 was decreased under HG treatment but this was reversed when cells were transfected with miR-206 mimics. Taken together, these results indicated that miR-206 mimics significantly inhibited the growth ability of hMCs, but this was promoted by HG.

miR-206 prevented the ECM accumulation of hMCs

To investigate the influence of miR-206 on ECM deposition of hMCs, we assessed levels of the ECM proteins fibronectin, type IV collagen, and laminin. As shown in Figure 3, fibronectin, type IV collagen, and laminin levels were notably increased in HG, and decreased in hMCs transfected with miR-206

mimics, indicating that high expression of miR-206 prevents ECM accumulation.

HIF-1 α is a target of miR-206

Finally, to explore the underlying mechanism of the effect of miR-206 on hMCs, bioinformatics software (<http://www.targetscan.org>) was used to predict the target gene of miR-206 showing that HIF-1 α was a potential target of miR-206. To test our prediction, dual-luciferase reporter assay was applied, showing that HIF-1 α was a direct target of miR-206, and it was negatively regulated by miR-206 (Figure 4A–4C).

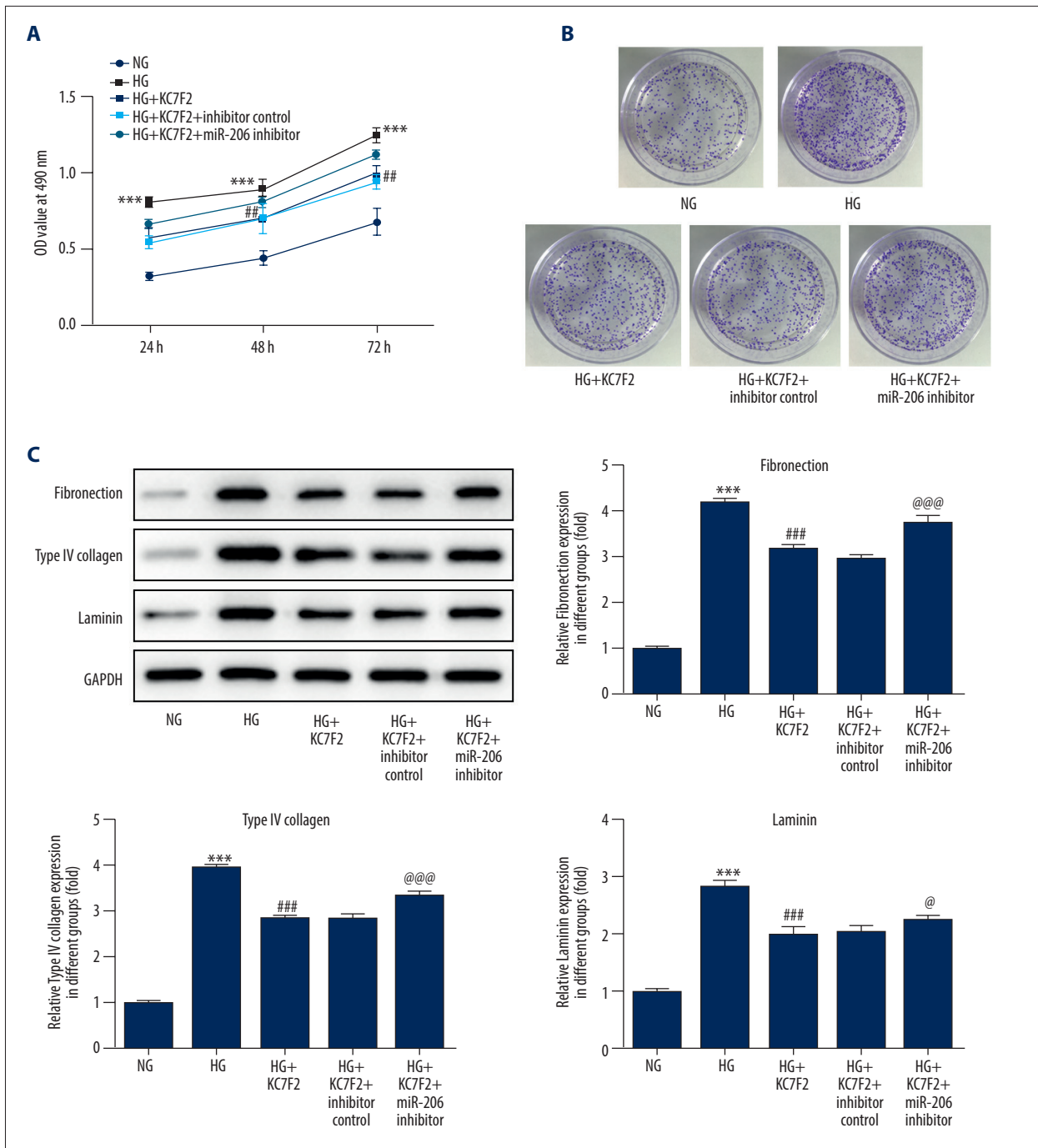


Figure 5. MiR-206 may function via regulating HIF-1 α . After hMCs were treated with high glucose, cells were treated with KC7F2 (an inhibitor of HIF-1 α) and transfected with miR-206 inhibitor. Cell proliferation was determined by MTT assay (A) and colony formation assay (B). Protein expressions of fibronectin, type IV collagen, and laminin were detected using Western blotting (C). Data are expressed as the mean \pm SD. *** p<0.001 vs. NG; #, ##, ### p<0.05, 0.01, 0.001 vs. HG; @, @@@ p<0.05, 0.001 vs. HG+KC7F2.

Since HIF-1 α has been demonstrated to be a target of miR-206, Western blot analysis was performed to detect the expression of HIF-1 α in each group. As shown in Figure 4D, the expression of HIF-1 α was significantly upregulated under HG treatment,

and this condition was inhibited when the HG-treated hMCs were transfected with miR-206 mimics. These results suggest that HIF-1 α is a target of miR-206 and is negative regulated by miR-206.

Inhibition of HIF-1 α suppressed cell proliferation and ECM accumulation

To further understand the underlying mechanism of miR-206, KC7F2, an inhibitor of HIF-1 α , was used in this study. As shown in Figure 5A, MTT assay indicated that KC7F2 inhibited cell proliferation, which was promoted by HG, and the inhibition function of KC7F2 was reversed by miR-206 inhibitor, and the same was found in colony formation assay (Figure 5B). KC7F2 also decreased the protein expression of fibronectin, type IV collagen, and laminin, which was increased under HG condition, but miR-206 inhibitor increased the expression of fibronectin, type IV collagen, and laminin, indicating that miR-206 regulates ECM accumulation through HIF-1 α (Figure 5C). Taken together, our results show that inhibition of HIF-1 α significantly suppresses cell proliferation and ECM accumulation, and miR-206 exerts its role by regulating HIF-1 α .

Discussion

In the present study, we determined that miR-206 was down-regulated in HG-induced hMCs, and miR-206 inhibited the proliferation and ECM accumulation in hMCs through directly targeting HIF-1 α . We revealed that miR-206 might serve as a potential therapeutic target for treatment of DN.

Previous reports suggested that miR-206 plays critical roles in tumors. In prostate cancer, miR-206 negatively regulates proliferation and migration and arrested cell cycle of prostate cancer cells by directly regulating the expression of CXCL11 [14]. miR-206 is involved in the inhibition of proliferation and invasion in cervical cancer cells induced by lncRNA UCA1 down-regulation [15]. Pan et al. suggested that miR-206 overexpression prevents osteosarcoma cell proliferation, migration, and invasion and promotes apoptosis through targeting ANXA2 by blocking the AKT signaling pathway [16]. Wang et al. reported that miR-206 inhibits human cervical cancer cell proliferation, migration, and invasion by targeting BAG3 [17]. Pang et al. found that miR-206 inhibits cell growth by targeting CDK9 in hepatocellular carcinoma [18]. A large body of evidence shows that miRNAs have important roles in the development of DN [19,20]. Recent studies suggested that miR-206 regulates the activation TGF- β signaling [21], which is involved in DN, and promotes the ECM deposition in a variety of renal cells in diabetes [22,23]. Our study found the level of miR-206 was decreased in HG-treated hMCs. Various studies have shown that mesangial cell proliferation is crucial in the occurrence and development of DN, and mesangial cell abnormalities and excessive accumulation of ECM are pathological changes of DN. Indeed, our findings indicated that miR-206 suppresses cell proliferation and prevents the ECM deposition of hMCs.

We also found that HIF-1 α is a novel target of miR-206 in hyperglycemic conditions. Using the miRNA target prediction algorithm (TargetScan), we found a highly conserved binding site for miR-206 in the 3'-UTR region of HIF-1 α . We concluded that miR-206 directly targets the 3'-UTR of HIF-1 α and inhibits the expression of HIF-1 α in HG-treated hMCs. Consistent with the observation that ANXA2, BAG3, and CDK9 are also direct targets of miR-206 [18–20], it is clear that miR-206 exerts its role through regulating various targets.

HIF-1 α , a type of HIF-1, is a heterodimeric transcription factor. Growing evidence shows that HIF-1 α is involved in the pathogenesis of various kidney diseases. However, whether the activation of HIF-1 α worsens DN still remains controversial. On one hand, the activation of HIF-1 α can alleviate DN. Bohuslavova et al. found that, in a mouse model of STZ-induced diabetes, HIF-1 α deficiency accelerated pathological changes in the early stage of DN, and HIF-1 α deficiency especially affects podocyte survival in the early phase of DN, resulting in diabetic glomerular injury [24]. Nordquist et al. showed that HIF-1 α activation prevented diabetes-induced alterations in oxygen metabolism, mitochondrial respiration leakage, and kidney function, and reduced proteinuria and tubulointerstitial damage. On the other hand, activation of HIF-1 α can worsen DN. Enhanced expression of HIF-1 α was found in the glomeruli of diabetic mice and type 2 diabetes mellitus rats [25,26], in agreement with our finding that HIF-1 α was upregulated in HG-treated hMCs. Lin et al. showed that HIF-1 α was significantly higher in db/db mice and in HG-exposed HEI-OC₁ cells, and experiments have demonstrated that HIF-1 α plays a crucial role in diabetes-related hearing impairment, both *in vivo* and *in vitro* [27]. Sagar et al. showed that HIF-1 α was overexpressed in DN, and was connected with renal interstitial fibrosis and serum creatinine values [28]. Thus, the high expression of HIF-1 α might be a risk factor for DN, and inhibiting the expression of HIF-1 α might help to improve DN. In our study, inhibition of HIF-1 α significantly suppressed cell proliferation and ECM accumulation in HG-treated hMCs, and miR-206 was found to have a crucial role in DN via regulating HIF-1 α .

Conclusions

We found that miR-206 plays a protective role in diabetic nephropathy through inhibition of hMCs proliferation and ECM accumulation by targeting HIF-1 α . miR-206 may be useful as a novel therapeutic target in the treatment of DN.

Conflict of interest

None.

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