European Review for Medical and Pharmacological Sciences 2019; 23: 4333-4340

MicroRNA-488 regulates diabetic nephropathy via TGF-β1 pathway

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Abstract. – OBJECTIVE: The aim of this study was to clarify the biological roles of microRNA-488 and transforming growth factor β1 (TGF-β1) pathway in the occurrence and progression of diabetic nephropathy (DN).

MATERIALS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the expressions of microRNA-488, fibrinogen factors coll, collV, and fibronectin (FN) in Human mesangial cells (HMCs) with high-glucose or low-glucose treatment. After transfection of microRNA-488 mimics or inhibitor, expression levels of coll, collV, and FN in HMCs were determined by qRT-PCR and Western blot. Their expressions in HMC cells treated with different doses of TGF-β1 at different time points were also detected. Finally, we evaluated the potential influence of microRNA-488 on TGF-β1-induced fibrosis of HMC cells by qRT-PCR.

RESULTS: Compared with low-glucose treatment, the expression of microRNA-488 markedly increased in HMCs treated with high-glucose, as well as coll, collV, and FN. Overexpression of microRNA-488 remarkably upregulated mRNA and protein levels of coll, collV, and FN, whereas microRNA-488 knockdown downregulated their levels. Expression levels of microRNA-488, coll, collV, and FN gradually upregulated with the increase of TGF-β1 dose and treatment duration.

CONCLUSIONS: MicroRNA-488 regulates the development of diabetic nephropathy-induced fibrosis by TGF-β1 pathway.

Key Words
MicroRNA-488, TGF-β1 pathway, Diabetic renal fibrosis, Fibrotic protein factor.

Introduction

Diabetes mellitus (DM) is a systemic, chronic disease caused by relative or absolute hyposecretion of insulin in the body. It gradually leads to metabolic disturbances of carbohydrate, fat, and protein, followed by disorders of vitamins, water, and electrolytes, as well as imbalanced oxidation. Both genetic factors and environmental factors are believed to be involved in the pathogenesis of DM. Diabetic nephropathy (DN) is one of the most serious complications of DM. About 40% of DM patients develop DN, and it is the leading cause of end-stage renal disease[1]. In the early stage of DN, pathological manifestations include glomerular and tubular basement membrane thickening, as well as mesangial enlargement. With the aggravation of DN, glomerular sclerosis and renal interstitialization are observed[2]. Renal interstitial fibrosis is a common performance for the development from various chronic kidney diseases to chronic renal failure[3]. The main pathological manifestations are increased renal interstitial fibroblasts and excessive accumulation of extracellular matrix (ECM)[4]. To date, the molecular mechanism of DN, however, has not been fully understood. Therefore, researches on the molecular mechanism of DN will contribute to develop efficient approaches for its prevention and treatment.

Transforming growth factor β1 (TGF-β1) is the most important factor responsible for fibrosis, which participates in the regulation of cell proliferation and ECM formation[5]. TGF-β1 is a key factor in the induction of DN, mainly involved in the regulation of glomerular interstitial sediment synthesis and decomposition[6]. The upregulation of TGF-β1 activates its downstream genes and fibrosis-related factors, including fibrotic factors, inflammatory factors, angiotensin II, and endothelin-1. The TGF-β1 pathway is crucial in the development of renal interstitial fibrosis.

MiRNA is a non-coding, single-stranded RNA of approximately 22 bases in length. It inhibits gene translation to protein through complementarily binding to the 3'untranslated region (3'UTR) of the target gene and finally silences its expression[7]. As an endogenous RNA, miRNA maintains conservative and stable in mammals. At present, multiple miRNAs have been identified to participate in the pathogenesis of DN[8]. MiR-192 deficiency may promote the occurrence of fi-

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brosis in DN[9]. MiR-21 has a protective effect on DN-induced mesangial cell proliferation in db/db mice[10]. Downregulation of miR-29 family members directly targets multiple collagens, causing collagen deposition in renal tubular epithelial cells, mesangial cells, and podocytes induced by TGF-β[11,12].

Downregulation of miR-29a induced by high-glucose upregulates the expression of type IV collagen in human renal tubular epithelial cells HK-2[13]. It is believed that miRNA serves as an effector of TGF-β1 and high-glucose, which regulates glomerular sclerosis and tubulointerstitial fibrosis by influencing the synthesis of renal ECM. Meanwhile, miRNA could also affect the structure and function of glomeruli, induce cell fusion and apoptosis, thus leading to the leakage of a large number of proteins. Therefore, it is necessary to elucidate the potential roles of miRNAs in DN. MicroRNA-488 locates at 1q25.2 and has an exon. A related study found that microRNA-488 expresses in brain tissue. It is associated with central polyphagia and obesity and is downregulated in prostate cancer and gastrointestinal stromal tumor. It can protect against osteoarthritic cartilage, suggesting that microRNA-488 may be involved in the regulation of metabolic diseases, immune diseases, and tumors[14-19]. However, the relationship between microRNA-488 and DN has not been reported yet.

This study aims to investigate the role and potential mechanism of microRNA-488 and TGF-β1 in DN. Our results provide a new target for the treatment of DN.

Materials and Methods

Cell Culture

Human mesangial cells (HMCs) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in high-glucose (25 mmol/L) or low-glucose (5.5 mmol/L) Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and placed in a 5% CO_2, 37°C incubator. Cell growth was daily observed, and the medium was replaced every 1-2 days. Cell passage was performed until 80% of confluence.

Cell Transfection

Cells were plated one day prior to the transfection. A mixture of microRNA-488 mimics/inhibitor and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was prepared and added in each well with about 50% of cell confluence. After cell culture for 4-6 h, fresh medium was replaced for the subsequent experiments. MicroRNA-488 mimics were composed of RNA duplexes with the following sequence: 5'-UUG AAAGGCUAUUUCUUGGUC-3'. The sequences of the microRNA-488 inhibitors were as follows: 5'-GACCAAGAAAUAGCCUUUCAA-3' and 5'-CUAUCAAUCGGCGGAUCCUAU-3'. The oligonucleotide sequences used in the experiments were provided by GenePharma (Shanghai, China).

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

We harvested 2×10^5 cells to extract the total RNA using RNAiso Reagent and reversely transcribed into complementary deoxyribose nucleic acid (cDNA). The extracted cDNA was diluted to 500 μL to mix with primers and SYBR Green nucleic acid fluorescent dyes (TaKaRa, Otsu, Shiga, Japan). The relative expression levels of microRNA-488, coII, coIIV, and FN were calculated by $2^{-\Delta\Delta CT}$ method. Primer sequences were as follows: GAPDH (forward): 5'-CGGAGTCAACGGATTTGGTCGTAT-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (reverse): 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'; MicroRNA-488 (forward): 5'-GACGCACCCTTCCTGTCCTTTG-3'; MicroRNA-488 (reverse): 5'-ACAAAGCCGCTGCACACACAA-3'; coII (forward): 5'-ACATCCCACCAATCACCTGC-3'; coII (reverse): 5'-CGTCATCGCACAACACCTTG-3'; coIIV (forward): 5'-GGCCAGAAAGGAGAGATGGG-3'; coIIV (reverse): 5'-ATCAACAGATGGGGTGCCTG-3'; FN (forward): 5'-CTGGAACCGGGAACCGAAT-3'; FN (reverse): 5'-AGAGCTTCTTGTCCTGTCTTTTTC-3'; TGF-β1 (forward): 5'-GACCGCAACAACGCAATCTATGAC-3'; TGF-β1 (reverse): 5'-TGCTCCACAGTTGACTTGAATCTCTG-3'.

Western Blot

Total protein was extracted using the cell lysate (1 mL RIPA (radioimmunoprecipitation assay) +10 μL PMSF (phenylmethylsulfonyl fluoride) +10 μL aprotinin) (Beyotime, Shanghai, China) on ice for 30 min. The protein sample was quantified by bicinchoninic acid (BCA; Pierce, Rockford, IL, USA), separated by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), and blocked with 5% skim milk.

Membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by enhanced chemiluminescence (ECL).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was utilized for the statistical analysis. The quantitative data were represented as mean ± standard deviation (x–±s). The measurement data were analyzed by the *t*-test. $p<0.05$ was considered statistically significant.

Results

MicroRNA-488 Expression Increased With High-Glucose Treatment

To explore the potential role of microRNA-488 in DN, human mesangial cells HMCs were induced with high-glucose (25 mmol/L) and low-glucose (5.5 mmol/L), respectively. As qRT-PCR data revealed, microRNA-488 was highly expressed in high-glucose group relative to low-glucose group (Figure 1A). Besides, mRNA levels of fibrinogen factors coII, coIIV, and FN markedly upregulated in HMCs with high-glucose treatment (Figure 1B). Their protein levels were identically increased compared with low-glucose group (Figure 1C). These results suggested a potential involvement of microRNA-488 in the fibrosis of HMCs.

MicroRNA-488 Regulated Expression Levels of coII, coIIV, and FN

To further explore the effects of microRNA-488 on fibronectin factors coII, coIIV, and FN, HMCs were transfected with microRNA-488 mimics or microRNA-488 inhibitor. Their transfection efficacy was first verified by qRT-PCR (Figure 2A, 2D). Both mRNA and protein levels of fibrinogen

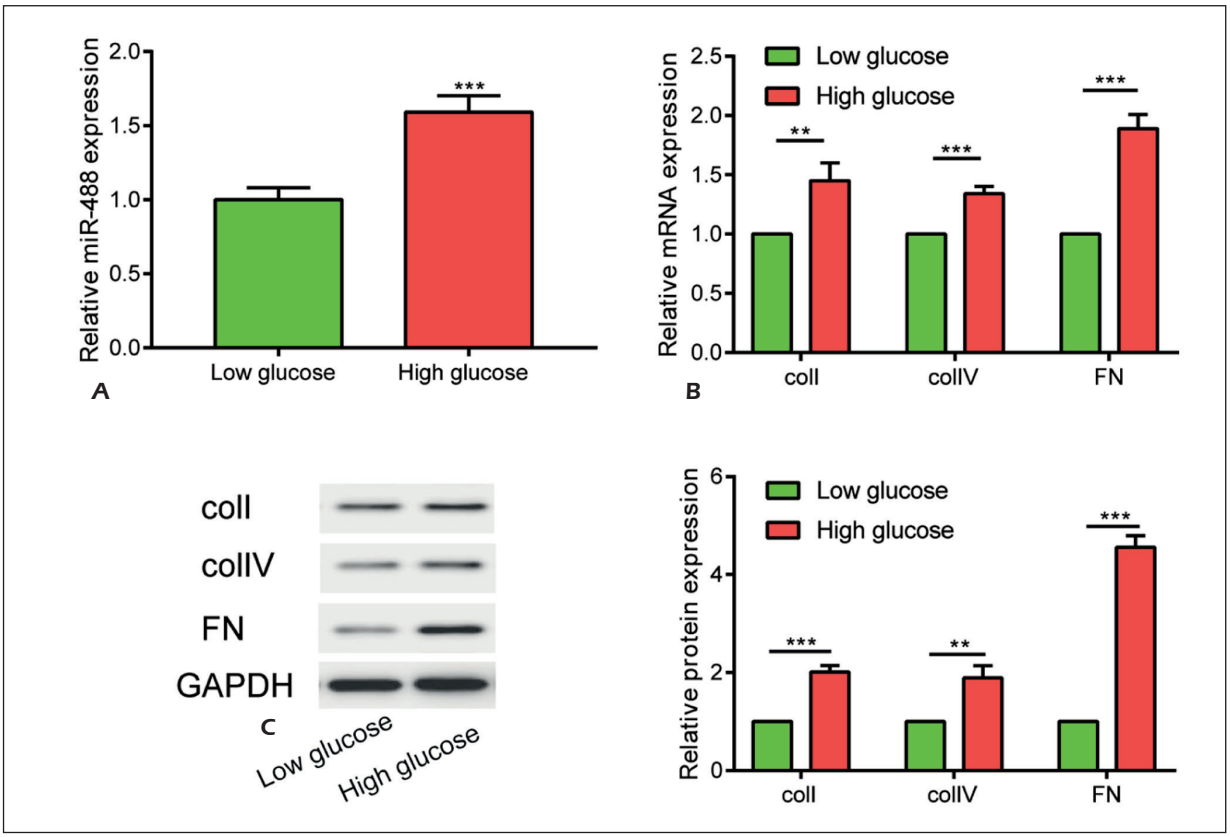

Figure 1. MiR-488 expression increased with high-glucose treatment. **A**, QRT-PCR data revealed that miR-488 was highly expressed in high-glucose group (25 mmol/L) respect to low-glucose group (5.5 mmol/L). **B**, The mRNA levels of fibrinogen factors coII, coIIV, and FN markedly upregulated in HMCs with high-glucose treatment than low-glucose group. **C**, The protein levels of fibrinogen factors coII, coIIV, and FN markedly upregulated in HMCs with high-glucose treatment than low-glucose group. $^{**}p<0.01$, $^{***}p<0.001$.

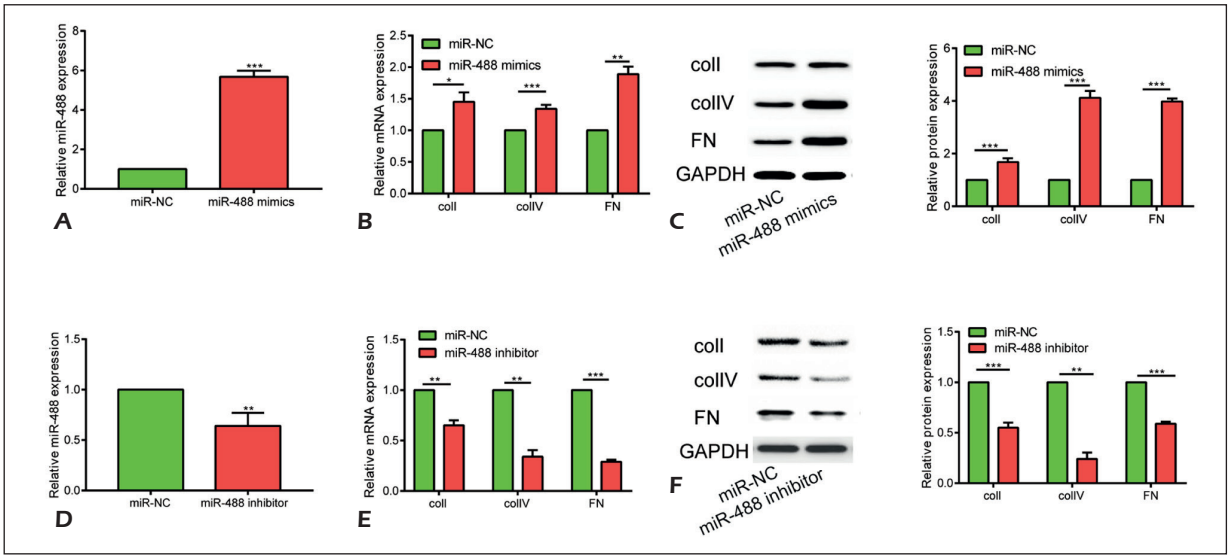

Figure 2. MiR-488 regulated expression levels of coII, coIIV, and FN. **A**, Transfection efficacy of miR-488 mimics in HMCs. **B**, QRT-PCR data revealed that mRNA levels of coII, coIIV, and FN increased in HMCs overexpressing miR-488. **C**, Western blot analyses showed that protein levels of coII, coIIV, and FN increased in HMCs overexpressing miR-488. **D**, Transfection efficacy of miR-488 inhibitor in HMCs. **E**, QRT-PCR data revealed that mRNA levels of coII, coIIV, and FN decreased in HMCs with miR-488 knockdown. **F**, Western blot analyses showed that protein levels of coII, coIIV, and FN decreased in HMCs with miR-488 knockdown. $^{**}p<0.01$, $^{***}p<0.001$.

factors coII, coIIV, and FN increased in HMCs overexpressing microRNA-488 (Figure 2B, 2C). Conversely, expression levels of coII, coIIV, and FN markedly decreased in HMCs transfected with microRNA-488 inhibitor (Figure 2E, 2F). We may conclude that microRNA-488 positively regulated fibrogenic factors coII, coIIV, and FN.

TGF-β1 Induction Promoted Fibrosis of HMC Cells

Furthermore, HMCs were induced with different doses of TGF-β1 at different time points. We aimed to elucidate the relationship between TGF-β1 and HMCs fibrosis. QRT-PCR results showed that the mRNA level of microRNA-488 in HMCs gradually upregulated with the increased TGF-β1 dose and prolonged induction time (Figure 3A). MicroRNA-488 expression in HMCs reached the peak at 48 h with 30 mg/mL TGF-β1 treatment. Similarly, expression levels of colI, colIV, and FN in HMCs also increased with the increase of TGF-β1 dose and prolongation of induction time, presenting their peaks in 30 mg/mL TGF-β1 group at 48 h (Figure 3B-3D). Western blot analyses revealed upregulation of colI, colIV, and FN in TGF-β1-induced HMCs, especially at 48 h with 30 mg/mL TGF-β1 treatment as well (Figure 3E). The above data demonstrated that TGF-β1 induced fibrosis in HMCs, which was the strongest at 48 h with 30 mg/mL. In the subsequent experiments, HMCs were induced with 30 mg/mL TGF-β1 for 48 h.

TGF-β1 Induced Expressions of coII, coIIV, and FN by microRNA-488

To investigate the effect of microRNA-488 on TGF-β1-induced HMCs fibrosis, we compared the increased fold in microRNA-488 expression after HMCs overexpressing microRNA-488 were treated with or without TGF-β1. Transfection of microRNA-488 mimics upregulated microRNA-488 expression in HMCs regardless of TGF-β1 induction, but it was much more pronounced in those with TGF-β1 induction (Figure 4A). It is suggested that TGF-β1 exerted a promotive role in microRNA-488 expression. Besides, microRNA-488 also upregulated TGF-β1 expression in HMCs (Figure 4B). TGF-β1 induction remarkably reduced the upregulation folds of coII, coIIV, and FN in HMCs overexpressing microRNA-488 at both mRNA and protein levels (Figure 4C-4F). It is believed that the regulation of TGF-β1 in fibrosis of HMCs required the involvement of microRNA-488.

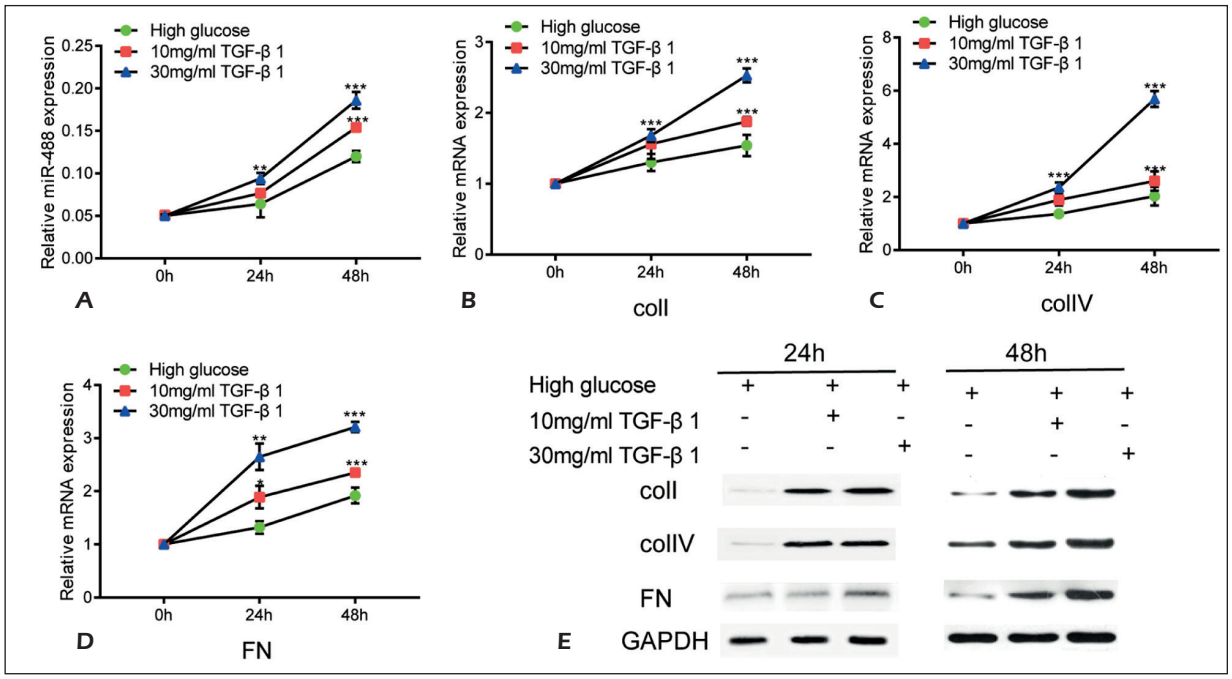

Figure 3. TGF-β1 induction promoted fibrosis of HMC cells. **A**, QRT-PCR results showed that the mRNA level of miR-488 in HMCs gradually upregulated with the increase of TGF-β1 dose and prolongation of induction time. **B**-**D**, The mRNA levels of colI, colIV, and FN in HMCs increased with the increase of TGF-β1 dose and prolongation of induction time. **E**, The protein levels of colI, colIV, and FN in HMCs increased with the increase of TGF-β1 dose and prolongation of induction time. **$p<0.01$, ***$p<0.001$.

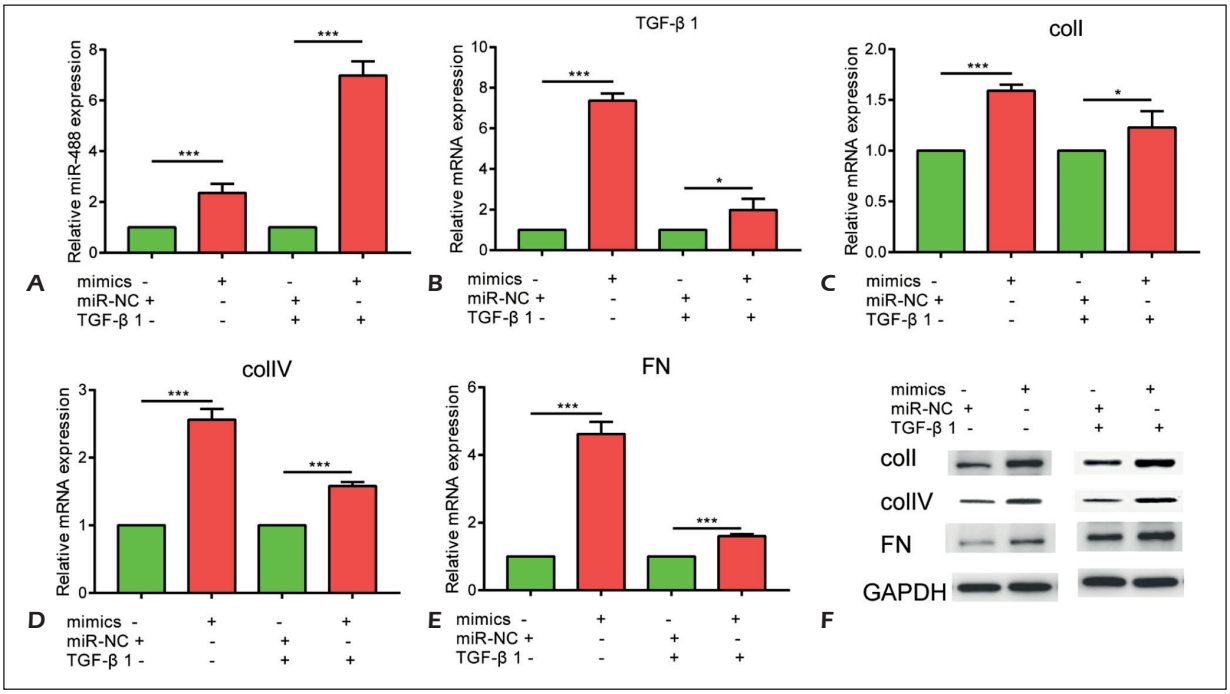

Figure 4. TGF-β1 induced expressions of colI, colIV, and FN by miR-488. **A**, Transfection of miR-488 mimics upregulated miR-488 expression in HMCs regardless of TGF-β1 induction, but it was much more pronounced in those with TGF-β1 induction. **B**, Transfection of miR-488 mimics upregulated TGF-β1 expression in HMCs regardless of TGF-β1 induction. **C**-**E**, Transfection of miR-488 mimics upregulated mRNA levels of colI, colIV, and FN in HMCs regardless of TGF-β1 induction. **F**, Transfection of miR-488 mimics upregulated protein levels of colI, colIV, and FN in HMCs regardless of TGF-β1 induction. *$p<0.05$, ***$p<0.001$.

Discussion

DN is an important chronic microvascular complication of DM, which is the leading cause of end-stage renal disease. The main pathological manifestations are glomerular basement membrane, thickening of renal tubular basement membrane, progressive accumulation of ECM in the glomerular mesangial area, and glomerular interstitial fibrosis. Without sufficient treatment, DN eventually leads to glomerular sclerosis, proteinuria, and even renal failure. At present, DN animal models are commonly established by surgery, drug induction, and genetic engineering. Unfortunately, the basic pathological features of glomeruli in DN could not be well simulated. The most commonly used procedure for establishing DN animal model is STZ induction, which could not rule out the non-specific nephrotoxicity. Spontaneous and genetic engineering approaches for establishing DN animal models could well simulate the pathogenesis of DN. However, these approaches have not been widely applied due to the rare resources and high expenses[20]. In this study, HMCs induced by high-glucose showed upregulated mRNA and protein levels of coII, coIIV, and FN. Advantages of simple culture, high reproducibility, low experimental cost, and short experimental duration all lay the foundations for subsequent experiments.

TGF-β is an important member of the transforming growth factor superfamily (TGF-s) and is produced by a variety of cells. It is composed of various related proteins, such as activin and bone morphogenetic protein. TGF-β is currently recognized as one of the strongest fibrotic factors with five isomers, and mammals have three forms, namely TGF-β1, TGF-β2, and TGF-β3[21]. Each isomer has a certain tissue specificity in its distribution. TGF-β1 is mostly expressed in the kidney, which is also abundantly distributed in the glomeruli and renal tubules, participating in the pathological process of renal fibrosis. TGF-β1 can induce cell hypertrophy in the kidney through autocrine and paracrine pathways, promote the synthesis of ECM components such as ECM I, II, III, IIV, and FN. It also inhibits the expressions of matrix metalloproteinases and upregulates metalloproteinase inhibitors, thus hindering the degradation of EMC. In this investigation, we examined the expression levels of fibrogenic protein factors in high-glucose-induced HMCs stimulated with different doses of TGF-β1 at different time points. The expressions of fibrogenic protein factors increased in a time- and dose-dependent manner. In particular, the fibrotic phenomenon of HMCs was the most pronounced at 48 h with 30 mg/mL TGF-β1 treatment.

As a key member of various biological processes, miRNAs are involved in stem cell differentiation, tumorigenesis, cellular behaviors, stress response, and individual development. A growing number of studies have shown that some specifically expressed miRNAs in kidney tissue may be involved in the pathophysiological mechanisms of DN[20,22], such as miR-196[23], miR-192[24], miR-186[25], miR-146[26], etc. MicroRNA-488 is a tumor-associated miRNA, which is abnormally expressed in tumors. For example, microRNA-488 inhibits expressions of estrogen receptors in prostate cancer cells[19]. MicroRNA-488 serves as a tumor-suppressor gene in gastric cancer[27]. MicroRNA-488 inhibits cell proliferation and cisplatin sensitivity in non-small cell lung cancer by activating eif3a-mediated NER pathway[28]. MicroRNA-488 inhibits proliferation and invasion of hepatocellular carcinoma cells by targeting ADAM9 and lncRNA HULC[29]. Growth and metastasis of renal cell carcinoma cells are suppressed by microRNA-488 via targeting HMGN5[30]. Through targeting aquaporin3, microRNA-488 inhibits proliferation, invasion, and interstitial transformation of osteosarcoma cells[31].

In the present study, microRNA-488 expression was upregulated in HMCs induced with high-glucose. Furthermore, we observed the regulatory effect of microRNA-488 on DN-induced fibrotic degree. The transfection of microRNA-488 mimics in HMCs upregulated both mRNA and protein levels of fibrinogen factors coII, coIIV, and FN. Conversely, transfection of microRNA-488 inhibitor downregulated expressions of these fibrinogen factors.

Conclusions

We demonstrated that microRNA-488 regulates the development of diabetic nephropathy-induced fibrosis by TGF-β1 pathway, which may be a novel therapeutic target for diabetic nephropathy.

Competing interests

The authors declare that they have no competing interests.

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